Bachelor Assignment
Faculty of Science and Technology (TNW)

Detection and analysis of neutrophilic granulocytes on opsonized gold islets with Raman spectroscopy

Author: G.P.M. Boerboom
Supervisors: Prof. Leon Terstappen, Dr. Cees Otto, Dr. Séverine le Gac
Date: August 30, 2013
Study: Bsc Advanced Technology

UNIVERSITEIT TWENTE.

MCBP

PREFACE

This bachelor thesis is my completion of Advanced Technology at the University of Twente. I performed the thesis at the Medical Cell BioPhysics group. I gained a lot of experience and knowledge during my time at this group.

I would like to thank Leon Terstappen for the opportunity to do my thesis here. I thank Cees Otto for his supervision and advise, Aufried Lenferink for his help with the Raman spectroscope, Eline Vos for the assistance in the lab, Barbara Liszka for helping me out with the SEM and Eddy de Weerd for his help with the AFM.

At last I would like to thank Eline, Linda, Marianne, Martijn, Merlin, Redmar, Robbert, Simone and Tom for the great times we had in the student room, during the triathlon, the barbecues and in the TAP.

Guido Boerboom
July, 2013

ABSTRACT

Neutrophil granulocytes are important leukocytes in the innate immune system. They are the most abundant white blood cells and are capable of phagocytosis. They can engulf pathogens like bacteria, and digest them via an respiratory burst. This respiratory burst is activated by fusion of the phagosome with lysosomes and includes the enzyme myeloperoxidase and reactive oxygen species from the NADPH oxidase complex.

These neutrophils can be imaged with Raman spectroscopy. This technique is based on molecular vibrations. A laser beam radiates on a focus point on the cell. The molecules in this focus point can scatter the photons with the same or a different frequency than the incident beam. Changes in the frequency are caused by the molecular vibrations of the molecules. Each kind of molecule causes a very specific shift in wavelength. By measuring the full spectra in a raster of focus points it is possible to visualize composition of a cell. When a Raman spectrum is measured in the near vicinity of a metal with free electrons, called surface plasmons, the Raman signal is immensely enhanced.

The neutrophils are capable of phagocytosis, and an investigation is done to see if it is possible to use this mechanism to adhere the cells to a surface coated with gold islets.

Glass and CaF_2 substrates are vapor deposited with a 3 nm thick layer of gold. The absorption spectra of both substrates showed different absorption peaks, which indicate differences in the morphology of the gold. These differences are measured with SEM and AFM and showed that on CaF_2 the gold formed islets of 40 nm in size. The glass substrates showed no differences in grain size between empty glass and vapor deposited gold on glass.

NGs are isolated from donor blood using a dextran solution, which promotes erythrocytes rouleaux formation, a Ficoll-Paque centrifugation, which separates cells based on their density, and a hypotonic lysis of remaining red blood cells. The cells are added to substrates, which are opsonized in FBS, immersed in a phagocytosis buffer. After fixation with formaldehyde the cells should be ready for measuring with Raman spectroscopy. Unfortunately the cells did not adhere to the surface via the phagocytosis mechanism.

Further investigation of the possibility to adhere NGs to a gold coated surface might include different distributions of the gold islets. Also a opsonization with human serum instead of FBS might be considered.

CONTENTS

LIST OF ABBREVATIONS

AFM	Atomic Force Microscope
FBS	Fetal Bovine Serum
HCA	Hierarchical Cluster Analysis
IgG	Immunoglobuline
MPO	Myeloperoxidase
NGs	Neutrophilic granulocytes
PBS	Phosphate Buffered Saline
QCM	Quartz Crystal Microbalance
ROS	Reactive Oxygen Species
RT	Room temperature
SERS	Surface Enhanced Raman Spectroscopy
SEM	Scanning Electron Microscope

INTRODUCTION

Neutrophil granulocytes

Neutrophil granulocytes (NGs) are the most common and important type of white blood cells. NGs stem from the hematopoietic stem cell, as shown in figure 1, and make up 60-70% of all leukocytes in peripheral blood.[1] Because of their abundance and capability to phagocytize pathogens, NGs are very important in the innate immune system.

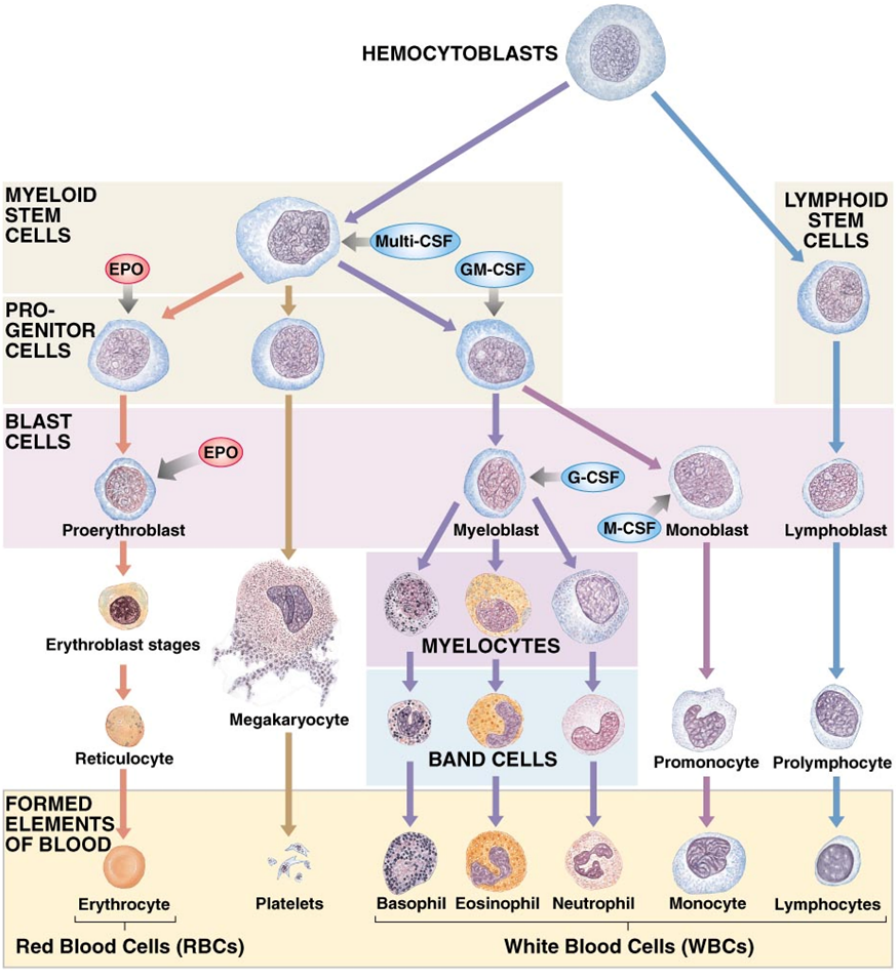


Figure 1: Differentiation of hematopoietic stem cell. [3]

NGs are approximately 10-20 μm in diameter and are characterized by a multilobed nucleus with two to five lobes. This multilobed nucleus gives the cell more flexibility which is needed for amoeboid movement to chase pathogens, this is called chemotaxis. In the cytoplasm of NGs a small Golgi apparatus can be found, few mitochondria and ribosomes and no endoplasmic reticulum. There are two types of granules in NGs, primary or azurophilic which are myeloperoxidase (MPO) positive granules containing hydrolases, MPO and lysozyme, and secondary granules, which are MPO negative containing lactoferin and lysozyme. These granules are formed during the differentiation of myeloid stem cells into neutrophils and play a role in phagocytosis.

Before phagocytosis the NG will first migrate to the pathogen by chemotaxis. It follows the chemi-

cal gradient of stimuli released by the pathogen, like lipopolysacharide, glycolipids or methylated DNA. The NG can detect these chemicals with 7-transmembrane heterotrimeric G-protein coupled receptors. Phagocytosis consists of four major steps: receptor-ligand binding, pseudopodextention, internalization and lysosomal fusion. The first contact between the NG and a pathogen will be the binding of FC receptors to antibodies connected to the pathogen, like IgG. This activates the NG. The cell membrane will deform by forming pseudopods and invaginate the pathogen. When totally engulfed in the cell, this vesicle is called a phagosome. Since neutrophils are extremely efficient, this engulfing can take place in 20 seconds. [15] The newly formed phagosome has no antimicrobial activity, therefore it will undergo major functional changes. Surface receptors and adhesion molecules change, lysosomal fusion can lead to injection with enzymes which digest the pathogen. The granules with MPO is one of the most common fusion in NGs. MPO is the most abundant enzyme in NGs. MPO is able to form a wide variety of reactive oxygen species (ROS) with high potential for tissue, bacterial or fungi damage.[8] Furthermore cytochrome b_{558}, which is located at the membrane of secondary granules, plays an important role. When cyt. b_{558} is translocated to the phagosomal membrane it is able to produce superoxide anions via the NADPH oxidase complex.

Raman spectroscopy

Raman spectroscopy is an analytical method which gives structural information about materials. When laser light interacts with a material, a small fraction of the light is scattered. This scattered light can have the same frequency as the laser light, called Rayleigh scattering, or a shifted frequency called Raman scattering. A Raman spectrum gives a unique fingerprint of a material, because the frequency shifts are dependent on molecular vibrations which are unique for all molecules. This effect was first described by C.V. Raman for which he won the Nobel prize in 1930. In biology Raman spectroscopy can provide structural insight without interfering on site, because it is label free.

Aim of the research

The aim of this research project is to detect and analyze single NGs on opsonized gold islets with Raman spectroscopy. It will be investigated if NGs are capable of adhering to a surface of gold islets via the mechanism of phagocytosis. If NGs can adhere to gold islets this can create new opportunities in the characterization of these cells with surface enhanced Raman spectroscopy. Frustrated phagocytosis is when a NG tries to engulf a particle that is too big to be engulfed. The toxic reagents will then be released extracellularly, which in vivo can cause tissue damage. This happens in many autoimmune diseases.

Research strategy An investigation of the substrates will be done. What are the differences between glass and CaF_2 substrates and how does this effect the morphology of the gold deposited on it. The substrates will be opsonized by incubation in fetal bovine serum. NGs will be isolated from peripheral donor blood using a dextran solution, Ficoll-paque centrifugation and hypotonic lysis of red blood cells. The NGs will be deposited on the opsonized substrates and incubated in a phagocytosis buffer and afterwards fixated with formaldehyde. With confocal Raman imaging an analysis will be done of empty substrates, substrates with gold, opsonized substrates and opsonized substrates with NGs.

THEORY

Raman Spectroscopy

The energy of laser light can be absorbed by a molecule and induce a dipole moment in the molecule. This dipole moment depends on the ability of the molecule to deform such as (anti)symmetric stretching and bending of the molecule. The molecule can be in its ground state and store the light's energy in the dipole moment and emit light at a lower frequency. This is called Stokes Raman scattering. When the molecule is in an excited state and transfers the vibrational energy to the emitted light, the light will have a higher frequency and is called anti-Stokes Raman scattering. The molecular energy levels in Figure 2 illustrate the Rayleigh, Stokes Raman and anti-Stokes Raman scattering. Here v_0 is the incident frequency, and v_R and v_k the frequency of the Raman scattered light and molecular vibrations.

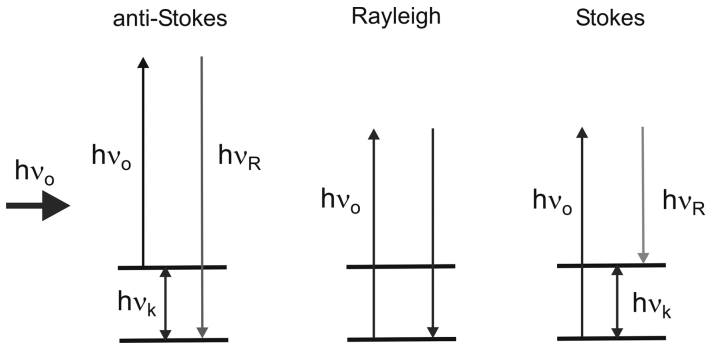


Figure 2: Molecular energy level diagrams illustrating light scattering. [4]

The wavenumber of a Raman shift for a specific vibrational mode is determined by the strength of the bond and mass of the atoms. These shifts are measured as the difference in wavenumbers between Raman scattered and laser light and expressed in cm^{-1}:

$$\Delta w = (\frac{1}{\lambda_0} - \frac{1}{\lambda_R}) \tag{1}$$

where λ_0 is the incident wavelength and λ_R the scattered wavelength. Typical shifts in a fingerprint region are between 500 cm^{-1} and 1800 cm^{-1}. [13]

Raman spectroscopy can be used as a microscopic analysis tool. In hyperspectral imaging the Raman spectra are measured from usually 32x32 or 64x64 pixels. Images can be created showing presence and position of specific components as proteins and DNA. This method is very specific and minimally invasive because it is label-free.

In a Raman spectrometer, as schematically represented in figure 3 the emitted light from a krypton laser goes through a band pass filter to get a clean emission line. With a mirror the light goes to a dichroic beam splitter which reflects the wavelength of the laser, but transmits the Raman scattering. A scanning mirror can reflect the laser beam to change the focus point by raster-scanning. A water immersion objective focuses the laser light onto a sample and collects the Raman scattering. This scattering goes back to the scanning mirror, trough the dichroic beam splitter and trough a long pass filter to eliminate the remaining laser light. With a confocal pinhole the out of focus light is removed, before a CCD detector collects the Raman spectra of the measured sample. The spectral data acquired can be used as a fingerprint to detect the presence and distribution of specific chemical compounds in a cell.

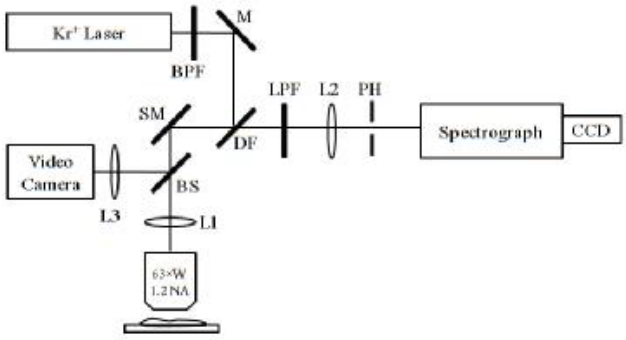


Figure 3: Schematic representation of Raman microscope. [13]

Consider the interaction of a molecule with an electromagnetic wave, e.q. laser beam, with electric field vector oscillating with frequency v_0

$$\vec{E} = \vec{E}_0 cos(2\pi v_0 t) \tag{2}$$

The oscillating field induces an electric dipole in the molecule

$$\vec{\mu}_{ind} = \tilde{\alpha}(v) \cdot \vec{E}_0 cos(2\pi v_0 t) \tag{3}$$

where $\tilde{\alpha}$ is the polarisability which describes response of the electron distribution in molecules which oscillate with the normal mode frequency v_k

$$\tilde{\alpha} = \tilde{\alpha}_0(v_0) + \left(\frac{\delta\tilde{\alpha}}{\delta Q_k}\right)_0 cos(2\pi v_k t) \tag{4}$$

Equations (3) and (4) can be combined to obtain

$$\vec{\mu}_{ind} = \vec{E}_0 \left[\tilde{\alpha}_0(v_0) + \left(\frac{\delta\tilde{\alpha}}{\delta Q_k}\right)_0 cos(2\pi v_k t)\right] cos(2\pi v_0 t) \tag{5}$$

Which can be expressed as

$$\vec{\mu}_{ind} = \vec{E}_0 \left[\tilde{\alpha}_0 cos(2\pi v_0 t + \left(\frac{\delta\tilde{\alpha}}{\delta Q_k}\right)_0 Q_k cos[2\pi(v_0 + v_k)t] + \left(\frac{\delta\tilde{\alpha}}{\delta Q_k}\right)_0 Q_k cos[2\pi(v_0 - v_k)t]\right] \tag{6}$$

Here it can be seen that in the first term corresponds with Rayleigh scattering, same frequency as incident, and the second and third term with anti-Stokes respectively Stokes Raman scattering.

Surface Enhanced Raman Scattering Molecules on rough surfaces of certain metals, as gold, may experience an enhancement of the Raman scattering. Small metal particles with respect to the wavelength of the incident light can align the collective vibrations of the free electrons of the metal, called surface plasmons. The incident electric field $\vec{E}_0(v_0)$ induces an oscillating electric dipole in the small particles which cause an additional electric field normal to the surface with frequency v_0. The enhancement is dependent on the dielectrical properties and the refractive index of the surrounding. The total electric field then becomes

$$\vec{E}_{tot}(v_0) = \vec{E}_0(v_0) + \vec{E}_{ind}(v_0) \tag{7}$$

The enhancement of the field is thus

$$F_E(v_0) = \frac{|\vec{E}_0(v_0) + \vec{E_{ind}}(v_0)|}{|\vec{E}_0(v_0)|} = |1 + 2g_0| \tag{8}$$

Where g_0 is related to the dielectric properties of the metal

$$g_0 = \frac{\tilde{\epsilon_r}(v_0) - 1}{\tilde{\epsilon_r}(v_0) + 2} \tag{9}$$

Where $\tilde{\epsilon_r}(v_0)$ is the frequency dependent dielectric constant divided by the diffraction index

$$\tilde{\epsilon_r}(v_0) = \frac{\epsilon_{re}(v_0) + i\epsilon_{im}(v_0)}{n_{solv}^2} \tag{10}$$

Molecules close to this surface will be excited with an enhanced field and thus have more Raman scattering. It also works the other way around, because the electric field caused by Raman scattered light of normal mode k with frequency $v_0 \pm v_k$ also induces a secondary electric field with frequency $v_0 \pm v_k$ and enhances the Raman signal. The total enhancement factor is

$$F_{SER}(v_0 \pm v_k) = [(1 + 2g_0)(1 + 2g_{RA})]^2 \tag{11}$$

Thus an enhancement of the incident beam by a factor 10 already gives a enhancement of 10^4 in Raman intensity[4].

MATERIALS AND METHODS

Substrates

The substrates used are 20 mm diameter CaF_2 slides and 1 inch D263 T eco Thin Glass. These substrates are plasma cleaned and vapor deposited at Ssens with gold at a speed of 0.05 nm/sec. Measurements with a QCM showed that the gold layer is 3,050 nm thick. The substrates are opsonized by incubation in fetal bovine serum for 1,5 hour at 37° Celsius

NG isolation

Blood is collected from the ECTM Donor Service at the University of Twente. This blood is mixed with 3% dextran/saline solution and incubated for 20 minutes at RT to extract the leukocyte-rich plasma from the red blood cells. Cells are separated from the plasma by centrifugation. Ficoll-paque solution is used to extract the NGs and remaining red blood cells. The remaining red blood cells are lysed twice by adding a hypotonic solution (0.2% NaCl) and separated by centrifugation. The protocol used can be found in Appendix I

Raman microspectroscopy and imaging

All Raman measurements were done using the home built confocal Raman microspectrometer from MCBP previously described [18]. A Kr-ion laser (Coherent, Innova 90-K) with a wavelength of 647.1 nm was used as incident laser beam with 35mW power. For Raman imaging the full spectrum (width $\sim$ 4000 cm^{-1}) was recorded for each position in raster scanning. Images of single cells were created after the measurement by using the vibrational bands of interest. The protocol used can be found in Appendix II. All spectral data were corrected for setup response. Raman images were constructed using intensity as a function of position. Hierarchical cluster analysis (HCA) is performed to visualize regions with high similarities in spectral data.

UV-vis absorption

A Shimadzu UV-2450 spectrophotometer is used to measure the absorption bands of the gold layer on the substrates. Absorption can be measured in a wavelength range of 190 $\sim$ 960 nm with a resolution of 0.1 nm. The absorption bands give structural information of a sample by matching the energy difference of two vibrational energy levels.

Scanning Electron Microscope

A Metrology JEOL JSM 5610 SEM is used to analyze the substrate surfaces. By irradiating a surface sample with an electron beam of 5kV at a working distance of 10 mm, the sample will emit secondary electrons, which can be observed by scanning the electron probe over the surface. This makes it possible to construct an image of the surface.

Atomic Force Microscope

A Pacific Nanotechnology AFM is used to analyze the substrate surfaces. By scanning the surface with a very thin needle mounted on a cantilever while remaining a constant force between the probe and the sample and monitoring the motion of the cantilever a three dimensional profile can be constructed of the surface.

RESULTS

UV-Vis absorption

The absorption spectra of the gold nanolayers on the substrate slides are measured with a UV Vis spectrophotometer. Empty slides of CaF_2 and glass are first measured with a reference of air. The offset of the curves in figures 4 and 5 can be assigned to reflection instead of absorption. For CaF_2 absorption can be seen at wavelengths smaller than 250 nm. For glass the absorption can be seen at wavelengths smaller than 400 nm as expected for D263 glass [5]. The absorption of the eight CaF_2 slides are measured with an empty CaF_2 slide as reference. The ten glass slides are measured with an empty glass slide as reference. The heavy fluctuations at lower wavelengths for the glass slides is because the signal is divided by the reference signal. Since both signals are absorbing heavily, it is a division of zero by zero which results in high fluctuations. The coated slides differ in two aspects, namely the wavelength and the bandwidth of the peak. For glass the peak is at 600.5 nm and for CaF_2 the peak is at 536.5 nm. Both values deviate from the absorption value of 550.5 nm found in literature [2]. The bandwidth for the CaF_2 substrates is almost twice as big as the bandwith for glass. These deviations can indicate differences in morphology of the gold, like different sizes or heights of the small aggregates. Research by Gupta showed that annealing of thin gold layers at different temperatures resulted in different shapes of islets. Also a relation between the contact angle with the substrate and the islets was related to absorption in the UV/vis spectra. A peak at 536.5 nm can indicate an island shape of 115°, while a peak at 600.5 nm might relate to an angle lower then 90° [10]. However the substrates used are not annealed at a high temperature and may thus have different shapes. A further analysis with a Scanning Electron Microscope might give more information about the morphology of the islets.

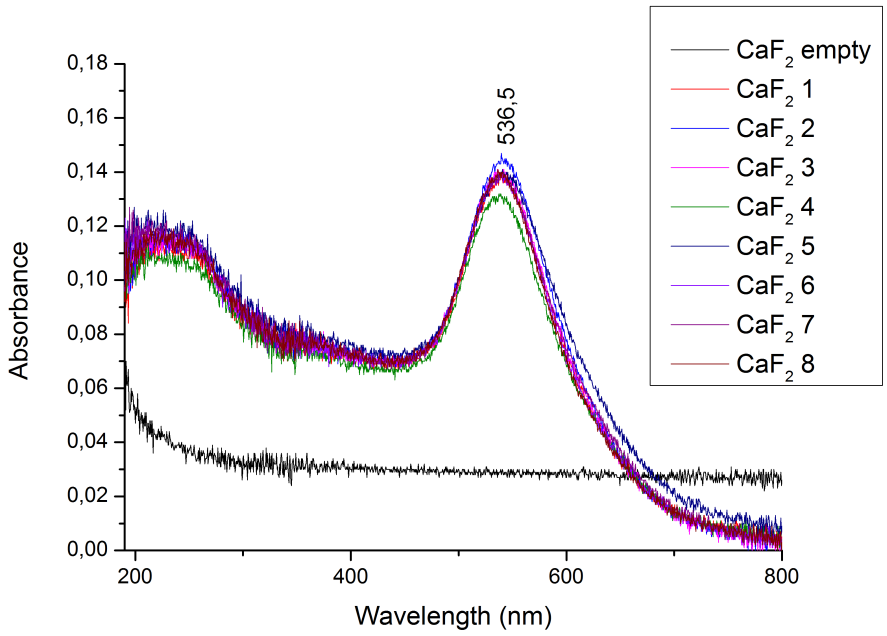


Figure 4: UV vis absorption of CaF_2 with air as reference and gold coated CaF_2 with empty CaF_2 as reference.

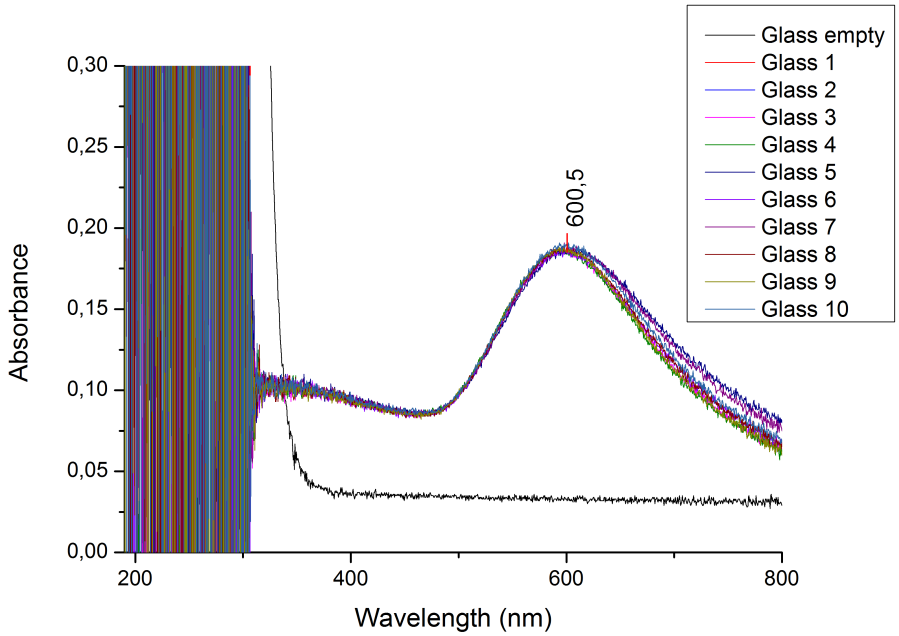
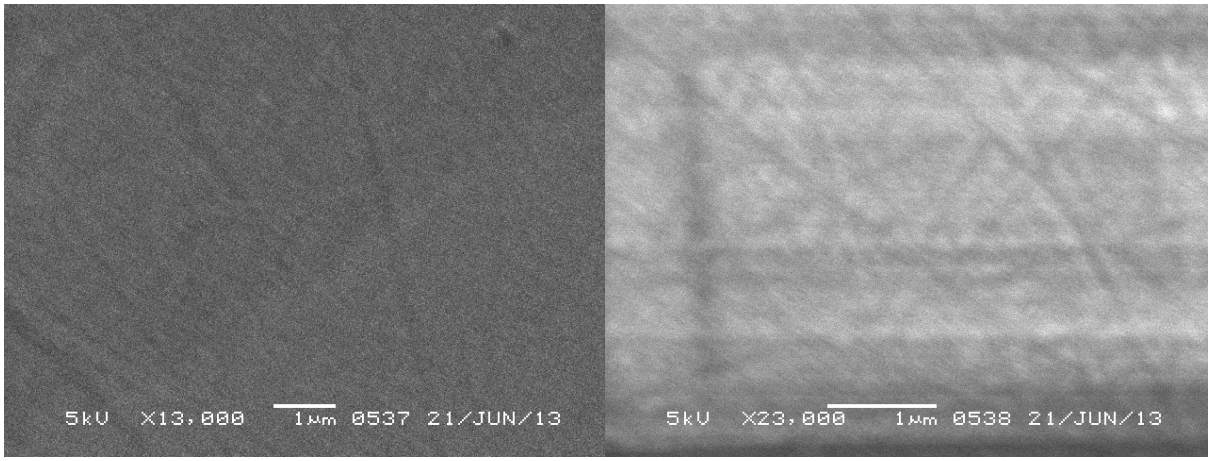


Figure 5: UV vis absorption of empty glass with air as reference and gold coated glass with empty glass as reference

Scanning Electron Microscopy

Images acquired with SEM on CaF_2, figure 6, did not show any indication of gold islets. Some faint lines were the best result we could get, but no outlines of gold islets were to be found. These lines are small scratches on the surface. On glass substrate it was not possible to get any images without destroying the sample. The electron beam bombards hydrocarbon molecules in the vicinity of the sample and deposits them on the surface. This causes the image to be blurred because it is covered with the deposited hydrocarbons.[9] The reason why there are no gold islets visible on the surface of the CaF_2 might be because the islets are very small. The lateral resolution of the SEM is 3,5 nm and should thus be able to detect the islets. The thickness of the gold layer is 3,050 nm and it might be a problem to focus on the surface. Another explanation might be that the gold layer is flat and has no islets on them. This might be further investigated with an AFM.



(a) (b)

Figure 6: Surface of gold on CaF_2 measured with SEM at a magnification of a) 13000x and b) 23000x using secondary electron imaging

AFM

Measuring the surface with AFM did result in the images in figure 7. In (a) a CaF_2 with gold is measured. It can clearly be seen that there are small aggregates on the surface. The small islets are 41 $\pm$ 6 nm in diameter with a height of 10.6 $\pm$ 2.5 nm. In (b) a clean CaF_2 substrate is measured and no clear grains can be seen, but lines are visible. These lines are small scratches which were not removed during the polishing of the slides, or made afterwards. In (c) and (d) the glass substrate with and without gold can be seen. Here also some lines are visible from scratches. Also some pits can be found in the surface. The grain sizes are for both slides 25 $\pm$ 5 nm in diameter with a height of 3.2 $\pm$ 1.0 nm respectively 3.1 $\pm$ 0.8 nm. This can either indicate that the islets are as big as the grain size of glass, or that the gold has uniformly coated the surface and the detected grains have the shape of the glass beneath.

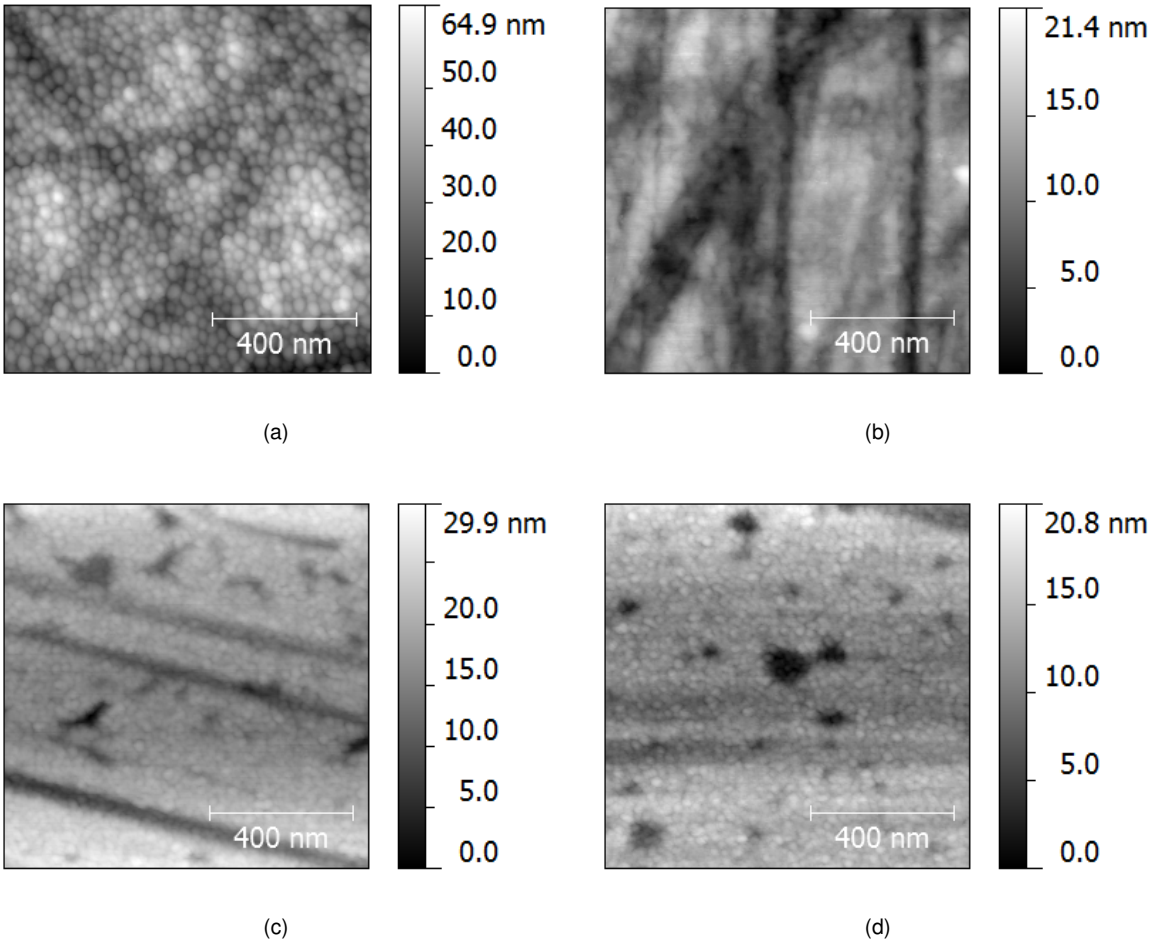


Figure 7: AFM images from (a) CaF_2 with gold, (b) CaF_2 without gold, (c) glass with gold and (d) glass without gold.

Raman spectroscopy

During measurements on the samples it is possible that there will be interference in the spectra of substrate materials used such as the glass or CaF_2 slides. To know how these materials could influence the spectra, these materials were measured with Raman spectroscopy. Figure 8 shows that CaF_2 is very silent in this spectra except for one peak at 322.0 cm^{-1} which is typical for CaF_2 [17]. The glass slide has a spectrum with specific peaks around 111.5 cm^{-1}, 476.5 cm^{-1}, 917.8 cm^{-1} and 1072.4 cm^{-1} similar to previously documented raman spectra of certain silicates [20]. At higher wavenumbers the signal slowly increases. Also some small fluctuation in the signal can be found at higher wavenumbers. These fluctuations are caused by artifacts in the correction data due to drifting of the signal from the CCD. Hence also a small negative peak in the signal at 2329 cm^{-1} can be found as an overcompensation for the presence of nitrogen in air [12]. However this is mostly outside of the fingerprint area and will thus not interfere with measurements on biological material.

Another aspect of interest is the SERS-effect of the vapor-deposited nanolayer of gold. The Raman spectra of a CaF_2 slide with gold is measured and compared with a CaF_2 without gold. In this signal in figure 9 an enhancement of a signal in CaF_2 is found at 460 cm^{-1} and a broad band with its peak at 891 cm^{-1}. This band is in the range of aliphatic chain vibrations[22]. The small negative peak at 2329 cm^{-1} can be assigned to an overcorrection for nitrogen present in the air [12].

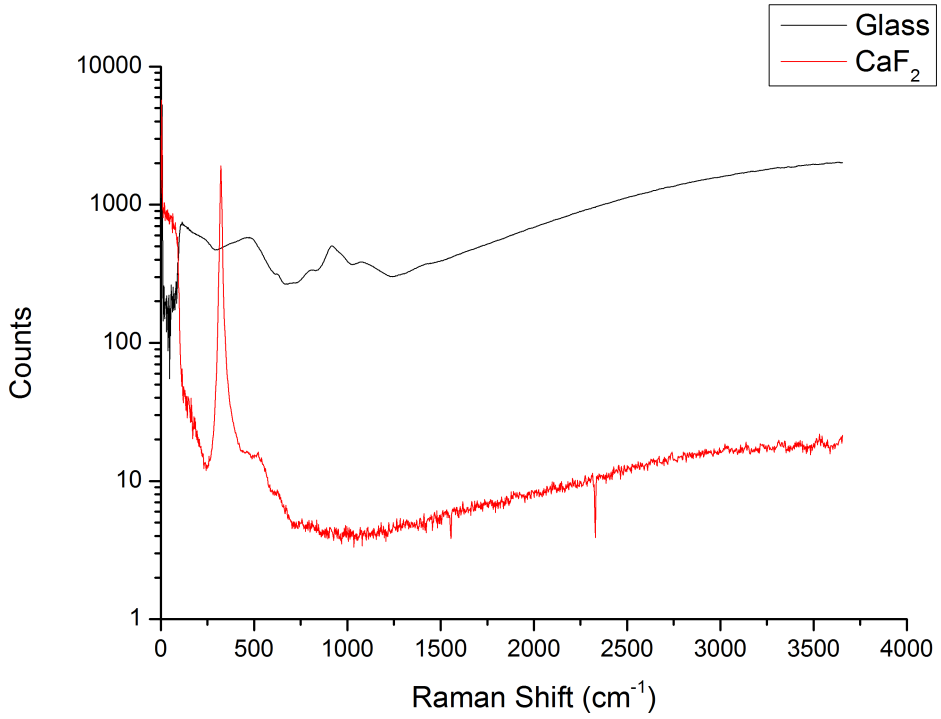


Figure 8: Raman spectra of expected background signal from CaF_2 and glass, average of 1024 spectra, 35mW laser power, 0.25s exposure time, readout rate 50kHz, gain 4x.

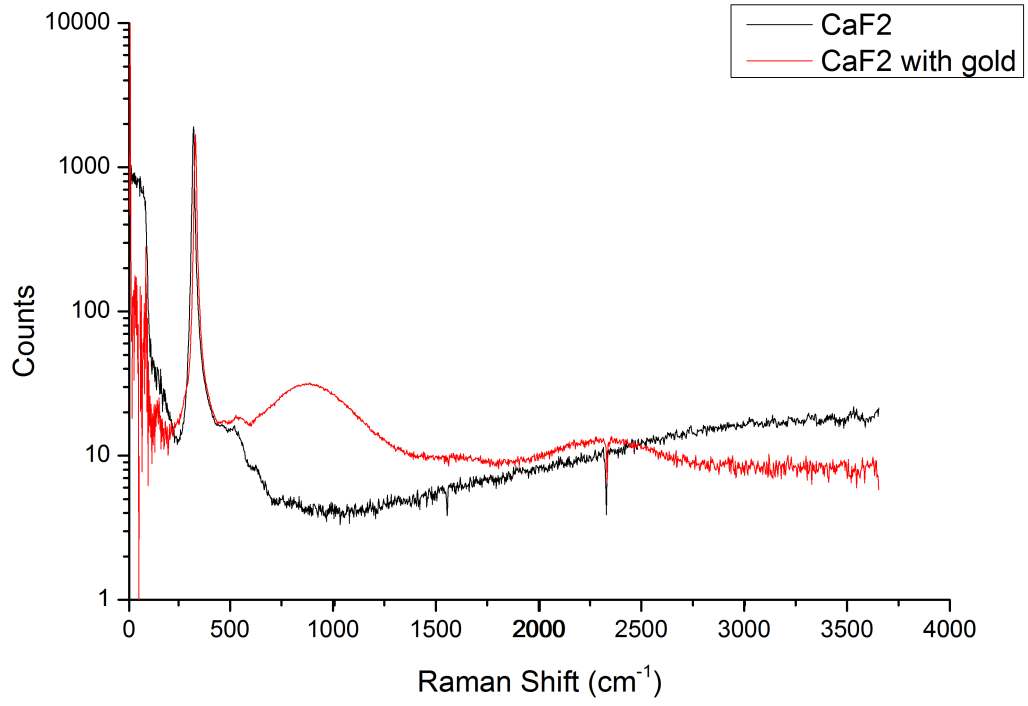


Figure 9: SERS effect on CaF_2, average of 1024 spectra, 35mW laser power, 0.25s exposure time, readout rate 50kHz, gain 4x.

In the spectrum of the SERS-signal of glass in figure 10 two bands can be found at 1355 cm^{-1} and 1570 cm^{-1}. These bands do not imply enhancements of impurities in the glass, but correspond with a broad disorder or D-band and E_{2g}-band of amorphous carbon[21][11], which could have come onto the substrates from the air. Also a band at 2868 cm^{-1} is found which is a combination of both D and E_{2g} bands[21].

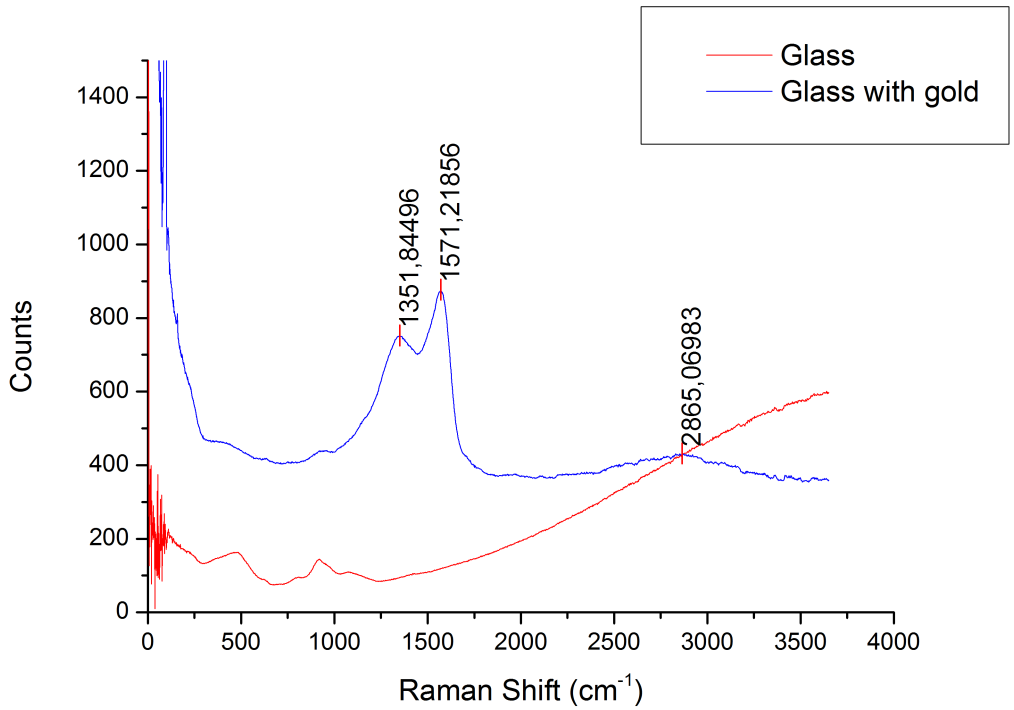


Figure 10: SERS effect on glass, average of 1024 spectra, 35mW laser power, 0.25s exposure time, readout rate 50kHz, gain 4x.

The gold coated substrates are opsonized by incubation in FBS for 2 hours at 38°C. Proteins from the FBS, like immunoglobulin and albumin, will attach to the surface of the substrates. To detect these molecules with Raman spectroscopy, different laser intensities are tested because in figure 11 it can be seen that too high laser power will result in amorphous carbon formation. The spectrum of CaF_2 at 9 mW laser power does not clearly show the carbon associated peaks at 1355 cm^{-1} and 1570 cm^{-1}. When zooming in on this spectrum, 12, a small peak can be seen at 233 cm^{-1} from the CaF_2. This peak is lower then previously seen in figure 9 because a lower laser power is used. Also peaks at 883 for ρCH_2 protein assignment [23], 1005 cm^{-1} and 1587 cm^{-1} for phenylalanine [23] [16], 1411 cm^{-1} for υ_s COO^- [23] indicate that there are proteins adhered to the surface and the opsonization was successful.

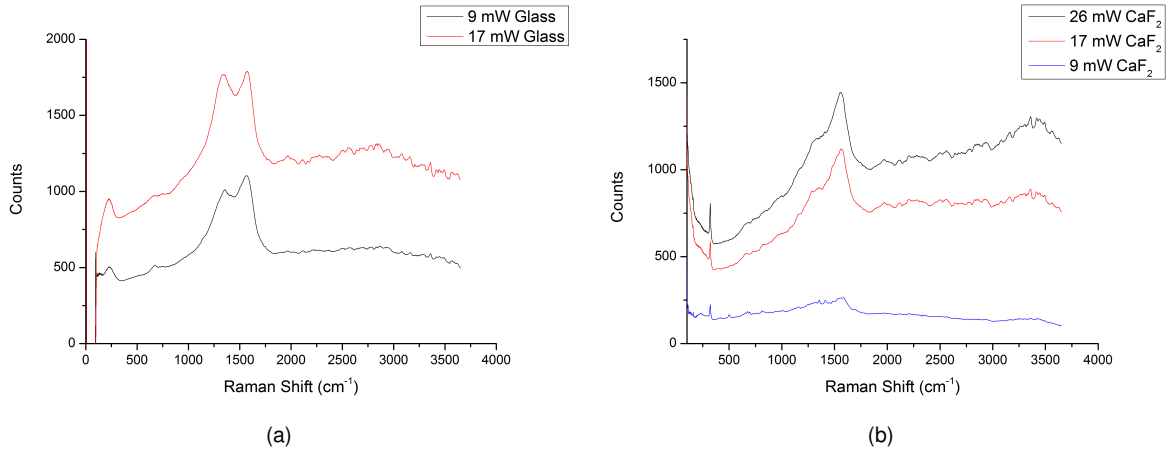


Figure 11: Raman spectra of opsonized, gold coated a) glass and b) CaF_2 substrates at different laser intensities. Spectra are average of 2048 spectra, 0.25s exposure time, readout rate 50kHz, gain 4x.

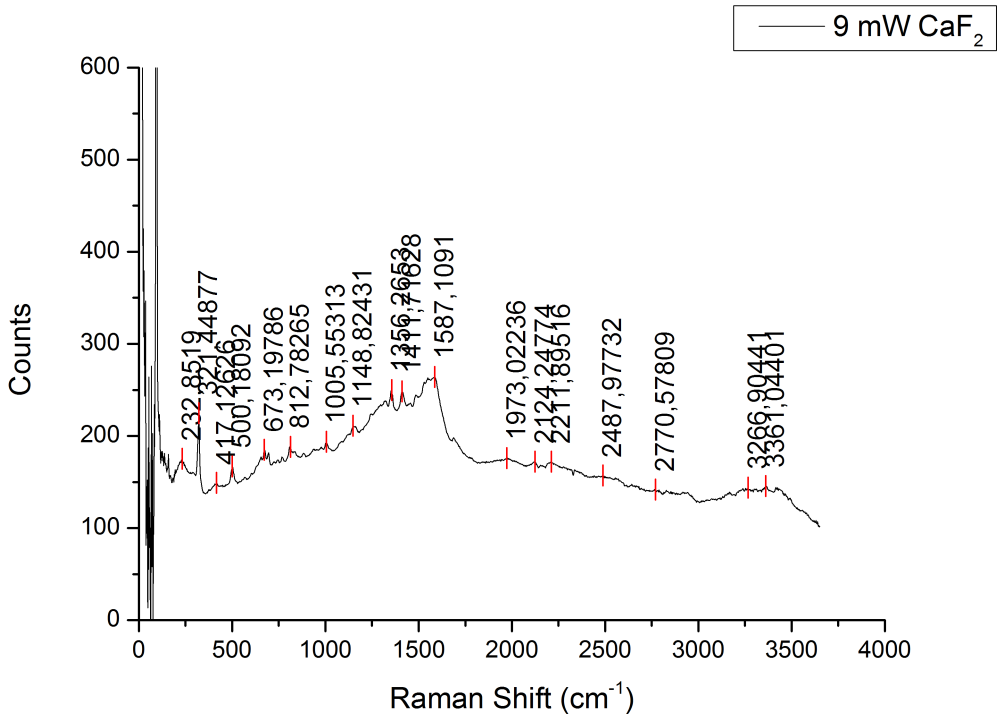
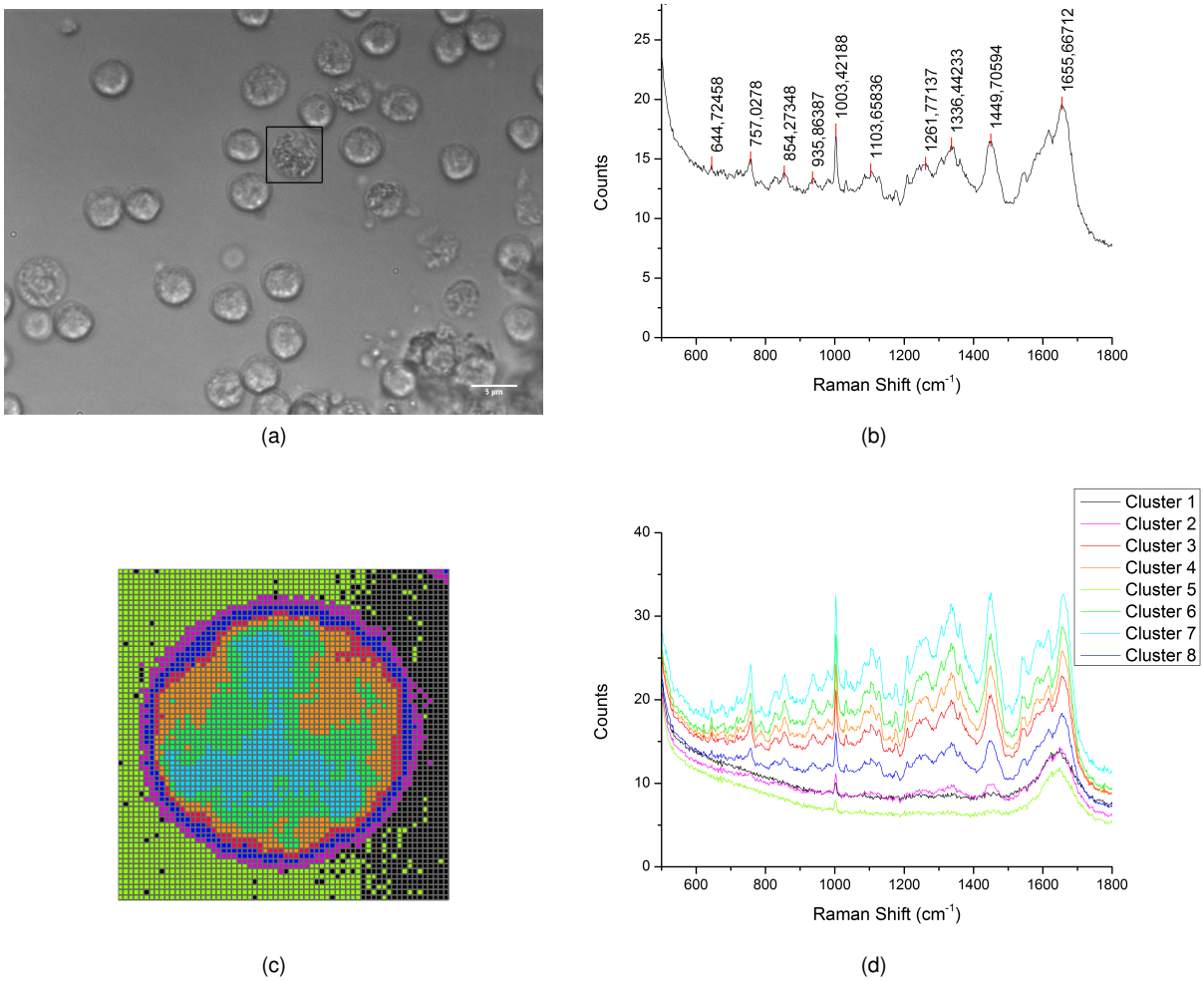


Figure 12: Peak analysis of Raman spectrum from opsonized gold coated CaF_2 at 9 mW laser power. Spectrum is average of 2048 spectra, 0.25s exposure time, readout rate 50kHz, gain 4x.

Cells

Before detecting phagocytosing neutrophils on gold islets, an investigation of the resting and phagocytosing conditions of neutrophils fixed with poly-L-lysine on CaF_2 is done. Based on the morphology seen with a white light microscope, some cells were selected to investigate with Raman spectroscopy. In figure 13(b) the average Raman spectrum of the cell in figure 13(a) can be found. There are specific peaks that indicate proteins (644,757,854,936,1003,1103 cm^{-1}), nucleotides (1262 cm^{-1}) and lipids (1449, 1655 cm^{-1})[23]. However this was not the signal we expected for a neutrophil. The specific peaks at 757, 1003, 1104, 1261, 1336, 1449 and 1655 cm^{-1} are very characteristic for lymphocytes [7]. In (c) a hierarchical cluster analysis (HCA) is done. This separates clusters with corresponding spectra. The spectra are shown in (d). Cluster 6 would suggest the nuclei of the cell because of its shape. The spectrum of cluster 6 confirms this because it has peaks at 1179, 1336 and 1662 cm^{-1} which indicate the presence of DNA[23]. In (a) also other cells can be found. Some look similar to the measured cells and are most probably also lymphocytes. The other cells could be neutrophils, but were not measured.



(a) (b)

(c) (d)

Figure 13: Measured lymphocyte on CaF_2 with poly-L-lysine. (a) shows a white light image, (b) the average of 4096 spectra of a raster scan, (c) is a HCA image and (d) shows the corresponding HCA spectra. Raster scan (64x64 pixels) of 6.4x6.4μm, 0.1s exposure time, readout rate 50kHz, gain 4x.

Also lymphocytes with small clusters in them were found, see 14. It was expected that these might be polystyrene beads, but the corresponding Raman, spectra did not match with the distinctive peaks

at 1001, 1031 and 1602 cm^{-1} of polystyrene [6]. In the HCA-image in (c) three bodies were visible. In (d) it can be seen that these bodies have three very clear peaks at 1003, 1153 and 1510 cm^{-1}. These peaks correspond with carotenoids present in gall bodies in lymphocytes [14].

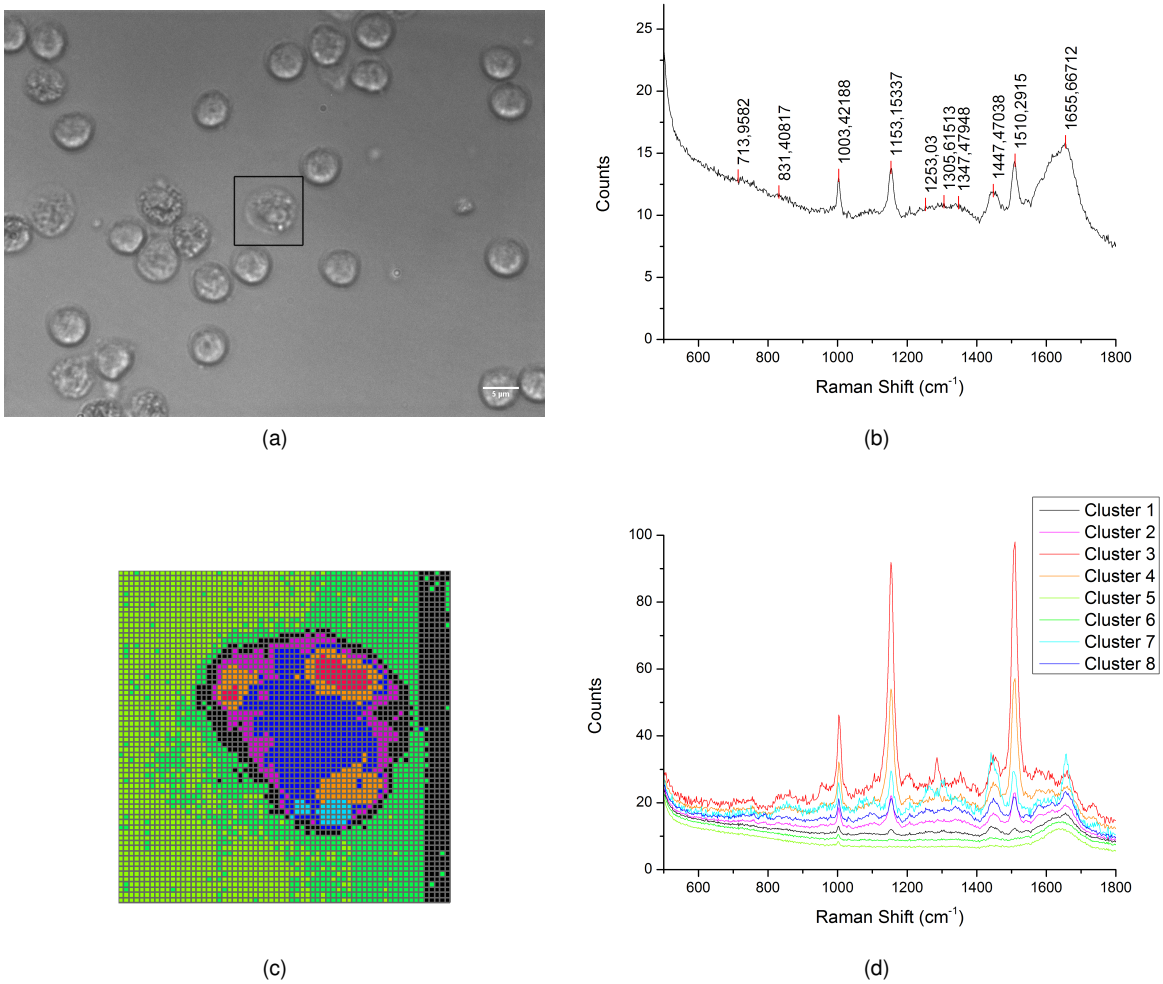
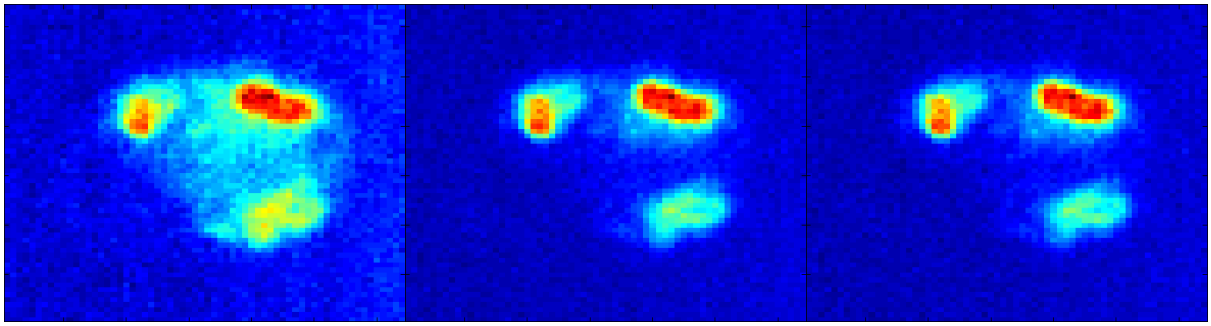


Figure 14: Measured lymphocyte with gall bodies on CaF_2 with poly-L-lysine. (a) shows a white light image, (b) the average of 4096 spectra of a raster scan, (c) is a HCA image and (d) shows the corresponding HCA spectra. Raster scan (64x64 pixels) of 6.4x6.4μm, 0.1s exposure time, readout rate 50kHz, gain 4x.

The distribution of these carotenoids can also be visualized by spectral imaging. The intensities of the three peaks is plotted against the position in the cell in figure 15.



(a) (b) (c)

Figure 15: Spectral images at (a) 1003, (b) 1153 and (c) 1510 cm^{-1} show the distribution of the carotenoids in gall bodies in lymphocytes

Glass and CaF_2 were opsonized by incubation in FBS. Neutrophils were isolated and fixated on the opsonized substrates via the protocol described in Appendix A. After the fixation step it was expected that some neutrophils would have attached themselves to the surface of the substrates. When looking at the substrates with a microscope, nearly no cells were to be found. The few cells which were to be found were not attached to the surface but loose in the PBS solution. Attempts to measure these cells with Raman spectroscopy did not result in usable data, because the cells were slowly drifting away and out of focus during the measurements. The reason why there are no cells attached to the surface might be that the cells were unable to phagocytize and attach to the surface because it was too big. Another reason can be that the binding to the surface was too weak and that the washing steps were too rough and the cells got washed away from the surface.

DISCUSSION AND CONCLUSIONS

Glass en CaF_2 substrates are vapor deposited with gold. These gold coated substrates are investigated with UV-Vis photospectroscopy and showed that there were different absorption peaks for each substrate. These peaks are an indication that there are differences in the structure of the gold layer on the different substrates. A further investigation with SEM did not give a clear insight in the structure of the gold, but AFM showed differences. The CaF_2 substrates had small aggregates of around 40nm in diameter. The small aggregates on glass substrates had a diameter of around 25nm, which is the same as the roughness of the glass.

With Raman spectroscopy the spectra of empty substrates and gold coated substrates are measured. For the CaF_2 substrates with gold there was a broad band extra visible in comparison with empty CaF_2. This band can be assigned to aliphatic chain vibrations. The glass substrates with gold showed a heavy signal for amorphous carbon, which even at lower laser intensities was still visible.

After opsonisation of the substrates it was possible to detect peaks in the Raman spectrum which correspond with proteins. Which indicates that the opsonisation was successful . Raman measurements of isolated cells showed that the isolation procedure did not only isolate neutrophils but also other leukocytes such as lymphocytes.

Attaching the isolated cells to the gold coated substrates via phagocytosis did not work. The cells might not be able to attach to a surface bigger then themselves, or the binding is not strong enough.

RECOMMENDATIONS

Further investigation of the adherence of NGs on gold islets might contain different distribution of gold islets. If the islets are more separated, it might be easier for the cells to extend their pseudopods around the islets. Also different sizes of islets can have an effect.

Treating the surface with poly-L-lysine will improve the attaching of the neutrophils to the surface, but it becomes very difficult to see if the neutrophils then are phagocytizing the gold islets, or are only attached because of the poly-L-lysine.

Neutrophils are known to mediate phagocytisis after a specific receptor recognizes a certain lipid, sugar or protein. If one could attach these lipids, sugars or proteins only to the gold islets and not to the whole surface, then the phagocytosis take place more directed to the gold islets and might be more effective.

The opsonization process might possibly be slightly improved by incubating in fresh human serum instead of FBS. However FBS is commonly used as an opsonization product and should do the trick.

REFERENCES

[1] David Male, Jonathan Brostoff, David B Roth, Ivan Roitt. *Immunology*. Elsevier, Philadephia, USA, 2006.

[2] Y. Fleger and M. Rosenbluh. Surface plasmons and surface enhanced raman spectra of aggregated and alloyed gold-silver nanoparticles. *Research Letters in Optics*, 2009, 2009.

[3] Frederic H. Martini; Edwin F. Bartholomew. *Essentials of anatomy and physiology*. Benjamin Cummings, San Fransisco, USA, 2010.

[4] Friedrich Siebert and Peter Hildebrandt. *Vibrational Spectroscopy in Life Science*. Wiley-VCH, Weinheim, 2008.

[5] Przisions Glas & Optik GmbH. Optical transmission spectrum of d263, June 2013. URL `http://www.pgo-online.com/intl/katalog/curves/D263_kurve.html`.

[6] McCreery Research Group. Raman standard spectra, 2013. URL `http://www.chem.ualberta.ca/~mccreery/ramanmaterials.html`.

[7] M. Kozicki A. Weseucha-Birczyska-G. Biesiada T. Mach J. Czepiel, M. Birczyska and A. Garlicki. Raman micro-spectroscopy of human lymphocyte. *N.A.*, N.A., "2012".

[8] J.Arnhold. Free radicals - friends or foes? properties, functions, and secretion of human myeloperoxidase. *Biochemistry*, 69:4–9, 2004.

[9] JEOL. Sem q&a, June 2013. URL `http://www.medicine.mcgill.ca/femr/SEM%20Sample%20Prep%20JEOL.pdf`.

[10] G. Gupta; D. Tanaka1; Y. Ito; D. Shibata; M. Shimojo; K. Furuya; K. Mitsui; K. Kajikawa. Absorption spectroscopy of gold nanoisland films: optical and structural characterization. *Nanotechnology*, 20:9, 2009.

[11] Un Jeong Kim. Vibrational Modes and Thermal Transformations of Purified Single Walled Carbon Nanotubes. Master's thesis, The Pennsylvania State University, the USA, 2006.

[12] A. Lofthus and P. Krupenie. The spectrum of molecular nitrogen. *Journal of Physical and Chemical Reference Data*, 6:153–241, 1977.

[13] C. Otto and V. V. Pully. Hyperspectral raman microscopy of the living cell. *Applications of Raman Spectroscopy to Biology*, 5:148–173, 2012.

[14] K.J. de Grauw N.M. Sijtsema L.L.J. van der Maas J. Greve C. Otto C.G. Figdor R.B. Ramanauskaite, G.M.J. Segers-Nolten. Carotenoid levels in human lymphocytes, measured by raman microspectroscopy. *Pure and applied chemistry*, 69:2131–2134, "1997".

[15] Pontus Nordenfelt; Hans Tapper. Phagosome dynamics during phagocytosis by neutrophils. *Journal of Leukocyte Biology*, 90:271–284, 2011.

[16] R. P. Kengne-Momo; Ph. Daniel; F. Lagarde; Y. L. Jeyachandran; J. F. Pilard; M. J. Durand-Thouand; G. Thouand. Protein interactions investigated by the raman spectroscopy for biosensor applications. *International Journal of Spectroscopy*, 2012:7, 2012.

[17] Arends J. Tsuda H. Detection and quantification of calcium fluoride using micro-raman spectroscopy. *Caries Research*, 27:249–257, 1993.

[18] H. J. van Manen; A. Lenferink; and C. Otto. Noninvasive imaging of protein metabolic labeling in single human cells using stable isotopes and raman microscopy. *Analytical Chemistry*, 80:9576–9582, "2008".

[19] N. M. Sijtsema; C. Otto; G. M. J. Segers-Nolten; A. J. Verhoeven; and J. Greve. Resonance raman microspectroscopy of myeloperoxidase and cytochrome b558 in human neutrophilic granulocytes. *Biophysical Journal*, 74:3250–3255, 1998.

[20] David G. Minser. William B. White. Raman spectra and structure of natural glasses. *Journal of Non-Crystalline Solids*, 67:45–59, 1984.

[21] Richard L. McCreery Yan Wang, Daniel C. Alsmeyer. Raman spectroscopy of carbon materials: Structural basis of observed spectra. *Chemistry of Materials*, 2:557–563, 1990.

[22] HORIBA Jobin Yvon. Raman spectroscopy for analysis and monitoring, June 2013. URL `http://www.horiba.com/fileadmin/uploads/Scientific/Documents/Raman/bands.pdf`.

[23] Ihtesham U. Rehman Zanyar Movasaghi, Shazza Rehman. Raman spectroscopy of biological tissues. *Applied Spectroscopy Reviews*, 42:493–541, 2007.

APPENDIX A

Granulocyte Isolation Procedure Based Upon:

ISOLATION OF NEUTROPHILS FROM PERIPHERAL BLOOD (using Ficoll), ELINE VOS

(references: *"Robert A. Clark, William M. Nauseef. Isolation and Functional Analysis of Neutrophils. Current Protocols in Immunology Unit 7.23"."Yazdanbakhsh M, Eosinophils do respond to fMLP. Blood. 1987 Aug;70*(**??**)*:379-83")*
Blood: 9 ml EDTA

Solutions (see calculations below) **:**

1. NaCl 9% (500 ml)(Stock)
2. 0.9% (50 ml) RT
3. 0.2% (20 ml) (ice-cold)
4. 1.6% (20 ml) (ice-cold)

1. Dextran/saline solution: 3% dextran T-500 (pharmacia) in 0.90% NaCl, (RT).

1. Ficoll-Hypaque solution

Phagocytosis buffer (1 x) 500 ml :

1. PBS/ 1mM CaCl2/ 0.5 mM MgSO4 / 5mM glucose / 1% BSA (RT)

Isolation Procedure:

Granulocytes have to be purified from the leukocytes of 9 mL blood, see "Current Protocols in Immunology Unit 7.23".

Granulocytes: "Current Protocols in Immunology", isolation:

1. Mix blood with an equal volume of 3% dextran/saline solution.
2. Incubate in upright position, RT, 20 min (+-20 min to see a clean interface between plasma and RBC). *(Dextran promotes erythrocytes rouleaux formation, which results in differential sedimentation of RBC and the formation of a leukocyte-rich, RBC-poor plasma layer above the sedimented RBC).*
3. Aspirate and save the leukocyte-rich plasma (upper layer) using a pipette and rubber bulb. If necessary, aspirate a little amount of RBC to completely recover the white cells layer.
4. Pellet cells from the plasma by centrifuging (10 min, 250 x g, 5°C).
5. Aspirate and discard supernatant.

This cell pellet still contains many RBC. The proportion of neutrophils versus other leukocytes is increased in comparison with blood, but many lymphocytes and monocytes remain.
Removing mononuclear cells by Ficoll-Hypaque centrifugation:

1. Immediately resuspend cell pellet in a volume of HBSS equal to the starting volume of blood (9 ml: 4,5 + 4,5 ml in two tubes).
2. Put 2 ml Ficoll-Hypaque solution in a 15 ml tube (2x).
3. Layer above the Ficoll-Hypaque the cell suspension using a pipet or a syringe fitted with a long blunt-end needle. Take care to preserve a sharp interface between the Ficoll solution and the cell suspension.
4. Centrifuge 40 min, 400 x g, no brake, 20 C. *(The 20 C temperature is important for achieving the best cell separation.)*
5. Aspirate the top (saline) layer as well as the Ficoll-Hypaque layer, leaving the neutrophil/RBC pellet.

Remove RBC (hypotonic lysis): "Current Protocols in Immunology Unit 7.23".

1. Immediately resuspend each leukocytes/RBC pellet in 4.5 mL of cold 0.2% NaCl for exactly 30 sec.
2. Restore isotonicity by adding 4.5 mL ice-cold 1.6% NaCl.
3. Centrifuge (6 min, 250 x g, 5°C if possible). Discard supernatant.
4. Repeat this steps until the cell pellets appear relatively free of RBC (a total of two time should be sufficient).
5. Resuspend cells in 1 ml HBSS, and combine tubes.
6. Determine cell concentration using Luna cell counter or count chamber Brker-Turk.
7. Adjust cell concentration (around 2 x 10^6 cells/ml).

Sample preparation and phagocytosis experiment for Raman measurement (use a multi-well plate):

Opsonization

1. Incubate with ? mL 10% fetal bovine serum (FBS) for 1.5 h at 37°C.
2. Wash the disc with PBS twice.
3. Put the disc in 1 ml phagocytosis buffer
4. Add neutrophils onto the disc, approximately 300 l with a concentration of 2 x 10^6/ml.
5. Incubate 20 min for 37°C.
6. Wash the disc three times with 1x PBS.

Fixation:

1. Put the disc in a clean well and fill the well with 2% formaldehyde.

2. Incubate 20 min at RT.
3. Wash the disc a couple of times with 1x PBS and put it in a bigger petri dish (5 cm / 5 cm).
4. Measure the cells with Raman (using an immersion objective).

Measurements:

1. Clean glass and CaF_2 discs
2. Opsonized glass and CaF_2 discs
3. Discs with cells

Calculations
NaCl
9.00% NaCl (45.0 g NaCl / 500 ml H_2O) – STOCK SOLUTION – Filter and autoclave
0.900% NaCl Diluting stock solution: (0.900/9.00) x 50.0 ml = 5000 l 9.00% NaCl to 50.0 ml H_2O
0.200% NaCl Diluting stock solution: (0.200/9.00) x 20.0 ml = 444 l 9.00% NaCl to 20.0 ml H_2O
1.60% NaCl Diluting stock solution: (1.60/9.00) x 20.0 ml = 3555 l 9.00% NaCl to 20.0 ml H_2O
Make portions of 4.50 ml per tube.
Dextran: 50.0 ml
3.00% dextran T-500 (1.50 g dextran + 5.00 ml 9.00% NaCl + 45.0 ml H_2O).
PBS-AC:
1x PBS
0.50% BSA
0.38% trisodium citrate

Phagocytosis Buffer (1x) 1000 ml :
= PBS + 1.00 mM $CaCl_2$ + 0.500 mM $MgSO_4$ + 5.00 mM glucose + 1.00% BSA

NaCl (58.44 g/mol) 0.144 M : 8.40 g
KCl (74.55 g/mol) 0.0027 M: 0.200 g
KH_2PO_4 (136.09 g/mol) 0.0147 M: 2.00 g
$Na_2HPO_4x2H_2O$ (156.01 g/mol) 0.065 mM: 10.1 g
$CaCl_2$ (110.98 g/mol) 0.001 M (from 1 M solution): 1.00 ml
$MgSO_4$ (120.37 g/mol) 0.0005 M: 0.06 g
Glucose (180.16 g/mol)0.005 M: 0.90 g
BSA 1.00% : 10.0 g BSA (albumin)
Dissolve in water and add NaOH conc. solution until pH 7.4
Filter through 0.220 m filter and store at +4°C.

APPENDIX B

Appendices*Appendix I: Protocol for Raman spectroscopy and imaging***Manual Sunrise Raman microspectrometer-imaging**
Kr^+ laser – Andor CCD
Contact Aufried Lenferink for help or questions.
Do not touch the components on the table which are marked “Do not adjust”, unless you understand precisely what you are doing.

1. The Kr^+ Laser:

1. (a) Pressing the laser button above to the light switch. This also turns on the laser warning sign outside the lab.
   (b) Turn the key in the laser power supply from “off” to “standby”
   (c) Press the on-button on the interface panel of the laser (which is usually located under the computer monitor stand)
   (d) Allow the laser to properly warm up. This takes approximately 1/2 hour.
   (e) Make sure the laser is operated in “light mode” (laser interface shows LT), if it isn’t, press the “light mode” button to make sure your light output is constant during your experiments.
   (f) Adjust laser power to 35 mW at sample site by placing power meter under microscope objective (Olympus, 40x air) and adjusting the laser power on the laser interface (usually 35mW through this objective corresponds to approximately 79-80mW on the laser interface).

1. The CCD:

   (a) CCD power supply is connected to one of the multiple sockets, switch these on.

1. The computer:

   (a) Also connected to one of the multiple sockets, switch these on, if you didn’t do that already.
   (b) Switch on the computer.
   (c) Log in with your TNW or student ICT account.
   (d) Open the Andor Solis program (the CCD now starts cooling). \

1. Calibration procedure

1. **Toluene calibration (align the setup, scale axis calibration)**

   (a) Check CCD temperature:
      i. Above -70$\circ$C no reliable measurements can be made.
      ii. If temperature has not reached -70$\circ$C, wait until sufficient cooling is established.
   (b) Adjust laser power:
      i. After the laser is warmed up and stable ($>$ 1 hr), usually the power at the sample has changed (little).
      ii. Readjust the laser (control panel) until power at sample site is again 35 mW (approximately 79-80mW on the laser interface)

(c) Prepare toluene sample:

i. Pipette toluene solution in a cup-like microscopy slide. Close with a cover slip.

2. Program settings Acquisition $>$ Set-up Acquisition

Go to the “acquire” tab (first tab) and check the following:

1. (a) i. Read-Out should be in FVB (Full Vertical Binning)
ii. Acquisition mode should be Single
iii. Acquisition time should be 1s
iv. Read-Out rate should be low: 50 kHz
v. Pre-acquisition gain should be Conventional (1x)
vi. Vertical Pixel Shift should be 9.75

1. (a) Focus laser in sample

i. Place the microscopy slide on the sample plateau of the microscope
ii. Adjust plateau position until the sample cup is under the objective
iii. In Andor Solis, start a real time (RT) measurement Carefully bring the plateau towards the objective, keeping track of the Raman signal on the monitor
iv. Once your focus has gone through the glass of the cover slip, you will see the toluene spectrum popping up.
v. Adjust plateau height until baseline is straight
vi. You will know when you have lost the optimum: when moving the focus through the sample, a second glass wall will be hit: the microscopy slide.

* Since default acquisition time is 1 second, you can speed up the focussing by adjusting the acquisition time (acquisition $>$ acquisition setup$>$ acquire). Note that by decreasing the acquisition time, also the maximum amount of counts feasible decreases (next point).

1. (a) Adjust CCD pinhole:

i. Check the intensity of the highest peak in the toluene spectrum (1004 cm^{-1}). The intensity of this peak is at least 28.000 counts per second (1s) in optimal conditions. This is what you should aim for!

* Note that by decreasing the acquisition time, also the maximum amount of counts feasible decreases: if in doubt: record a toluene spectrum with 1s acquisition time to check, before you start fiddling around with the pinhole (next point).

1. (a) i. If intensity is less than 20.000 cps (1s) , adjust the CCD pinhole by tweaking the mm-adjustments in all dimensions (x,y,z) on the box placed before the CCD to reach a maximum yield. Be careful: the adjustments are very sensitive. If this doesn’t help sufficiently, contact Liesbeth (ZH167), Vishnu (ZH166) or Aufried (ZH2..).
ii. Stop the RT measurement.

- Save the file in calibration folder Like: Tol-1s-35mW-G1.spe, save in spe format for further data processing in QCR program

White light spectrum (sensitivity vs wavelength)

1. (a) Block the laser light by closing the shutter
(b) Change the “fixing place” of the objective

- adjust the objective scale at 0.17
- be sure that the objective is situated in stable position

1. (a) Place the white light source under the microscope objective
   (b) Move up the plateau until objective is only +/- 0.5 mn away from white light
   (c) Monitor the focus with the real time function
   (d) Find the maximum by adjusting properly position of the plateau in horizontal plane
   (e) Program settings -Acquisition $>$ Set-up Acquisition

- Go to the “acquire” tab (first tab) and check the following:
-Read-Out should be in FVB (Full Vertical Binning)
-Acquisition mode should be Kinetic (256 of 512 series)
- gain 4
- exposure time – aprox.0.4s (be sure that the signal does not clip to maximum signal)

1. (a) Save the measurement as a “spe”file and save it calibration folder
   (b) Switch off the white light source and start background measurement with the same setting as for white light
   (c) Save measurement as a “spe”file
   (d) Replace the objective to the first “fixing place”

- check scale and right position

Argon calibration (scale axis calibration)

1. block the laser power by closing the shutter
2. switch on argon source
3. take up the mirror in front of the argon source
4. program settings

1. (a) -Acquisition $>$ Set-up Acquisition

- Go to the “acquire” tab (first tab) and check the following:
-Read-Out should be in FVB (Full Vertical Binning)
-Acquisition mode should be single
- gain 4
- about 0.01 s (to avoid the signal clippies)

1. (a) Switch off the argon source move down the mirror
   (b) Store this spe. File in the calibration folder

1. Sample measurements
   (a) Adjust properly settings for your sample

Go to the “acquire” tab and change the following:

1. (a) i. Acquisition mode to Kinetic
   ii. Adjust kinetic series length (the more measurements, the better your average SNR)
   iii. Pre-acquisition gain Conventional from 1x to 4x
   iv. Acquisition time to time of sample measurements *

1. (a) Measure spectra for air with the same setting as for the sample (necessary for data correction)
   (b) Take under consideration:

- possibility of solution evaporation from your sample during time
- possibility of destroying your sample by to high power

1. Shutting down the system:
   (a) Manually close the shutter
   (b) Turn off the laser “off” on control panel
   (c) Turn the key in laser power supply from standby to off
   (d) Shut down the computer
   (e) Switch off all 3 multiple sockets
   (f) Turn on the lab lights
   (g) Turn off the laser warning sign / main laser power supply
   (h) Open the curtains

Measurement of depolarization ratio

1. Place the polarizator in front of the detector
2. Adjust polarization angle 90^0

- find the maximum of intensity of toluene peaks by adjusting x,y,z axis of the confocal pinhole
- measure the water sample
- measure the air with the same settings
- measure the white light intensity (the same way as in “calibration point)

1. Adjust polarization angle 0^0

- find the maximum of intensity of toluene peaks by adjusting x,y,z axis of the confocal pinhole
- measure the air with the same settings
- measure the white light intensity (the same way as in “calibration point)

1. Correction in QCR

- create separately a calibration curves for samples with both polarization angle
- don’t normalize calibration curve to 1!!!!
- find the ratio in an intensity between spectra with polarization angle 0 and 90

1. Find a depolarization ratio R= I_{90}/I_0

Raman imaging

1. Open the programs: AVT smart view, SPM_LV9

- close the laser shutter
- lift up the filter
- close the laser entrance to the microscope
- connect the cable under microscope
- find the light spot on your sample
- find the place which you want to measure
- adjust the best focus
  - back to the measurement settings