Surface-enhanced Raman Scattering as an Approach to Monitor Lysosomal Function

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Abstract

Lysosomes are organelles of great importance for degradation processes of membranes and proteins in cells, and play a crucial role in cellular homeostasis. They can also serve as a potential target for cancer therapy. Monitoring lysosomes that accumulate lipids with sufficient temporal and spatial resolution still represents a considerable challenge. This thesis focuses on the development of surface-enhanced Raman scattering (SERS) as a method to monitor intact lysosomes, in particular regarding the influence of drug molecules that interfere with lipid metabolism. In order to evaluate the potential of SERS for studying lysosomal biology in live cells, the interactions between tricyclic antidepressants and acid sphingomyelinase (ASM) were studied. First, model systems were investigated. In particular, the interactions between the antidepressant molecules desipramine, imipramine, and amitriptyline, and gold and silver nanoparticles were characterized by SERS and surface-enhanced hyper-Raman scattering. The combined one- and two-photon approach showed that molecules interact with the surface of gold nanoparticles via their ring moiety and the methylaminopropyl side chain. As a model system of the lipid-rich environment in the lysosomes, composites of liposome and gold nanoparticles were prepared and studied by SERS and cryogenic electron microscopy. The SERS spectra are characteristic of the vesicles' lipid composition, and the data identify the lipids' polar head as the main contact site with the nanoparticles. The interactions between the antidepressants and the lysosomes were studied in the fibroblast cell line 3T3 by SERS and complementary methods. Apart from the collection of SERS spectra from the live cells, the cellular ultrastructure was characterized by transmission electron microscopy and cryo X-ray tomography. The ultrastructural data show an accumulation of lipids in the lysosomes, induced by the inhibition of ASM by the antidepressant molecules. In agreement with the SERS spectra

obtained from the model systems, the SERS spectra of live cells show signatures of both, the antidepressants and the lipids. In order to reveal the differences in the lysosomal composition between treated and non-treated cells, a random forest approach was used. Moreover, SERS was used to study the lipid distribution in Leishmania-infected macrophages that are known to accumulate lipids due to the presence of the parasite. The spectra were found to be very characteristic of the infected cells, and strong contributions from phospholipids and sterols are detected. The results show that SERS can be used to investigate lipid composition in live cells of different cell types. Multifunctional drug delivery systems can be envisioned that carry drug molecules, and at the same time permit to monitor the influence of the drug in the cells by SERS. As a new methodological development, the random forest analysis of SERS spectra from the cells shows that machine learning approaches can be useful for a better understanding of such data from complex biological systems.

Zusammenfassung

Lysosomen sind Organellen, die eine Schlüsselrolle bei der zellulären Homöostase einnehmen und ebenso von großer Bedeutung für Abbauprozesse von Membranen und Proteinen in Zellen sind. Lysosomen können auch als mögliche Ansatzstellen für Krebstherapien dienen. Die Beobachtung von Lipid-Anreicherungen in Lysosomen mit ausreichender Zeit- und Ortsauflösung stellt heutzutage immer noch eine Herausforderung dar. In dieser Dissertation soll die oberflächenverstärkte Raman-Streuung (engl. Surface-enhanced Raman Scattering, SERS) als Methode zur Untersuchung intakter Lysosomen etabliert werden. Ein besonderes Augenmerk liegt hierbei auf dem Einfluss von aktiven Substanzen auf den zellulären Lipidmetabolismus. Zur Bewertung von SERS als potentiell Hilfmittel bei der Untersuchung der lysosomen-assoziierten Prozesse in Zellen wurde die Wechselwirkung von saurer Sphingomyelinase (engl. acid sphingomyelinase, ASM) mit trizyklischen Antidepressiva betrachtet. Der erste Schritt bestand hierbei in der Nutzung von Modellsystemen. Insbesondere wurden die Wechselwirkungen zwischen den Antidepressiva Desipramin, Imipramin und Amitriptylin, und Gold- und Silbernanopartikeln durch SERS und oberflächenverstärkte Hyper-Raman-Streuung charakterisiert. Die Kombination eines solchen. Ein- und Zweiphotonen-Ansatzes zeigte, dass die Moleküle sowohl über die Ringeinheit als auch über die Methylaminopropyl-Seitenkette mit der Oberfläche der Goldnanopartikel interagieren. Mischungen von Liposomen und Goldnanopartikel wurden mit SERS und Kryoelektronenmikroskopien untersucht und dienten als Modellsystem der lipidreichen Umgebung in Lysosomen. Die SERS-Spektren sind charakteristisch für die Lipidzusammensetzung der Vesikel, und die Daten identifizieren die polare Kopfgruppe der Lipide als Hauptkontaktstelle mit den Nanopartikeln. Die Wechselwirkungen zwischen

den Antidepressiva und den Lysosomen wurden in der Fibroblasten-Zelllinie 3T3 durch SERS und komplementäre Methoden untersucht. Neben der Aufnahme von SERS-Spektren in lebenden Zellen wurde die zelluläre Ultrastruktur durch Transmissionselektronenmikroskopie und Kryo-Röntgentomographie charakterisiert. Die Daten zeigen eine Ansammlung von Lipiden in den Lysosomen, induziert durch die Hemmung der ASM durch die Antidepressiva. In Übereinstimmung mit den aus den Modellsystemen erhaltenen SERS-Spektren weisen SERS-Spektren lebender Zellen Signaturen sowohl der Antidepressiva als auch der Lipide auf. Um die Unterschiede in der lysosomalen Zusammensetzung zwischen behandelten und nicht behandelten Zellen aufzudecken, wurde ein Random-Forest-Ansatz verwendet. Darüber hinaus wurde die Lipidverteilung in Leishmania-infizierten Makrophagen, von denen bekannt ist, dass die Anwesenheit der Parasiten zu Lipidanreicherung führt, mit Hilfe von SERS untersucht. Die Spektren erwiesen sich als sehr charakteristisch für die infizierten Zellen, und es wurden starke Beiträge von Lipiden und Sterolen nachgewiesen. Die Ergebnisse zeigen, dass SERS besonders geeignet ist, um die Lipidzusammensetzung in lebenden Zellen verschiedener Zelltypen zu untersuchen. Man kann sich nun multifunktionale Drug-Delivery-Systeme vorstellen, welche Wirkstoffmoleküle transportieren und gleichzeitig die Überwachung der Wirkung der Medikamente in den Zellen durch SERS ermöglichen. Als neue methodologische Entwicklung zeigt die Random-Forest-Analyse von SERS-Spektren aus den Zellen, dass machine learning-Verfahren nützlich sein, um solche Daten aus komplexen biologischen Systemen besser zu verstehen.

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Table of Contents

Abstract	i
Zusammenfassung	iii
Acknowledgments	v
Table of Contents	vii
Chapter 1	1
Motivation and goals of the thesis	1
Chapter 2	5
Introduction and state-of-the-art	5
2.1 Lysosomes	5
2.1.1 Lysosomal storage diseases	6
2.1.2 Acid sphingomyelinase	7
2.2 Tricyclic antidepressants as functional inhibitors of acid sphingomyelinase	8
2.3 Raman scattering and its application to bioanalysis	12
2.4 Surface-enhanced Raman scattering (SERS)	14
2.5 Gold nanoparticles in cells	16
2.6 SERS analysis of cells	18
Chapter 3	21
Materials and Methods	21
3.1 Materials	21
3.2 Gold nanoparticles synthesis and characterization	22
3.3 SERS and SEHRS experiments with antidepressants in solution	22
3.4 Preparation of liposome-nanoparticle composites and their characterization	23
3.5 Experiments with antidepressants treated cells	24
3.6 Random forest analysis of SERS spectra	27
3.7 Experiments with Leishmania infected macrophages	30
Chapter 4	33

Interactions of tricyclic antidepressants with gold and silver nanoparticles	33
4.1 SEHRS and SERS spectra of TCAs.....	35
4.2 Difference in interactions with gold and silver nanoparticles.....	45
4.3. In vitro spectra in biological experiments	48
Chapter 5	55
Interactions between lipids and gold nanoparticles	55
5.1 Interactions between gold nanoparticles and lipid bilayers	56
5.2 Negative staining transmission electron microscopy of the liposome-nanoparticles composites	58
5.3 Cryogenic electron microscopy of liposome-nanoparticles	58
5.4 Optimization of the conditions for the SERS experiments	62
5.6 SERS spectra of the liposome-nanoparticle composites.....	64
5.7 Influence of the liposome composition on the interaction with the gold nanoparticles.....	68
5.8 Negatively charged liposomes and gold nanoparticles	71
5.9 Interaction of desipramine with lipid bilayers in the presence of nanoparticles	72
Chapter 6.....	75
6.1 Cells treated with antidepressants prior to treatment with gold nanoparticles.....	77
6.1.1 Nanoparticles aggregation and lipid accumulation in cells influenced by antidepressants ..	77
6.1.2 SERS spectra.....	82
6.1.3 Analysis of SERS spectra by random forest algorithm and chemical imaging	89
6.2 Cells treated with gold nanoparticles prior to treatment with antidepressants.....	96
6.2.1 Morphology of the nanoaggregates.....	96
6.2.2 SERS spectra.....	97
6.2.3 Analysis of SERS spectra by random forest algorithm and chemical imaging	102
6.3 Cells treated with gold nanoparticles and antidepressants simultaneously.....	109
6.3.1 Influence of the antidepressants on the aggregation of the nanoparticles.....	109
6.3.2 SERS spectra.....	112
6.3.3 Analysis of SERS spectra by random forest algorithm and chemical imaging	117
6.4 Summary	122
Chapter 7	123
SERS characterization of Leishmania infected cells	123
7.1 Leishmania parasite	124
7.2 SERS spectra from Leishmania infected cells	125
7.3 SERS mapping of the biochemistry in live cells.....	131
7.4 PCA analysis of spectra from different cellular compartments	135
Chapter 8.....	139
Summary and outlook	139
Bibliography	145

List of Figures	165
List of Tables.....	171
List of Abbreviations.....	173
Declaration	175

Chapter 1

Motivation and goals of the thesis

Lysosomes are digestive organelles found in many cell types. They were originally discovered by Christian de Duve in 1955 [1, 2], for which he won the Nobel Prize in Physiology or Medicine in 1974. Cellular lysosomes are important for degradation processes of membranes and proteins, playing a crucial role in cellular homeostasis [1, 2]. In addition, lysosomal permeation is a key hallmark in lysosome-triggered cell death [3-7]. Important enzymes such as the acid sphingomyelinase (ASM) are crucial for balancing between lysosome permeation and stability, and are thus potential targets in cancer therapy. In some disease, ASM has dramatically reduced activity, e.g., in lysosomal storage diseases such as Neimann-Pick disease [8-10]. Lysosomal storage diseases impair lysosomal function and can lead to potential cell death. Lysosomal storage diseases can also be induced also by external stimuli. It has been proposed that tricyclic antidepressants enrich in lysosomes and detach ASM from membranes [11]. ASM interacts with bis(monoacylglycero)phosphate (BMP), a specific lipid of the intralysosomal membrane that serves as its docking site. In the presence of antidepressants, the interaction between ASM and BMP is diminished, and ASM is rendered to rapid proteolytic degradation. This process has been confirmed by a vast body of evidence outside of cells [12-21]. However,

this process has never been confirmed in living cells, and this thesis proposes an approach to study enzyme action in living cells in cell cultures.

Monitoring lysosomal storage diseases in complex cellular environment with sufficient temporal and spatial resolution still represents a key challenge. In particular, monitoring events that take place at the water-membrane interfaces. These events are usually monitored by *ex situ* methods like HPLC-MS with a significant loss of information. Contrary to that, fluorescence-based assays are mostly used as *in situ* methods [22]. Fluorescence-based methods have answered many questions successfully. However, they require the use of fluorescently modified molecules. The use of fluorescently labeled molecules is a highly debated issue, especially in the study of lipid-related events [23]. Thus, sensitive methods capable of providing information about cellular events without extensive labeling are needed. Here, vibrational spectroscopy, which provides information about molecular structure and molecular composition in biological systems will be used to study molecular composition of lysosomes.

Vibrational spectroscopy, in particular, Raman spectroscopy, offers the possibility of probing the molecular events in living cells with minimal or no labeling [24]. To increase the sensitivity of Raman spectroscopy, surface-enhanced Raman scattering (SERS) with gold nanoparticles is used. SERS makes use of dramatically increased Raman signals in the proximity of gold nanostructures that, in principle, enables detection of single molecules [25, 26]. Gold nanoparticles can reach the cellular interior by endocytosis, since they are taken up from the culture medium into endosomal vesicles. Therefore, SERS can specifically report on the biochemical environment of the gold nanoparticle probes in the endo-lysosomal system. Also in this thesis, the gold nanoparticles will serve as intracellular nanosensors in the lysosomes. Previous work suggests that gold nanoparticles within lysosomes can attach to membrane surface, depending on their surface properties [27]. Due to the strong distance dependence of SERS, the method should be perfectly suited to study topology-related processes on intra-lysosomal membranes.

So far, SERS has been used as a sensitive tool to characterize the endo-lysosomal system. Specifically, SERS nanoprobe are used for determination of pH in different vesicles of endo-lysosomal system [28], and to follow trafficking of nanoparticles in the endo-lysosomal pathway [29, 30]. Moreover, SERS nanoprobe can detect metabolically important molecular species such as adenosine monophosphate [31] or lipid granules [32].

These findings prove the capability of SERS to study interaction in the endo-lysosomal system.

This thesis has the goal to study the capability of SERS to monitor enzymes involved in lipid metabolism in lysosomes of live cells. In order to understand the SERS spectra of cells, SERS experiments using model systems and individual components, such as antidepressants and lipids, were performed. In particular, interactions with lipids were studied with vesicle models and a model of *Leishmania*-infected cells: where lipids are highly abundant in some compartments. The data obtained from the model systems were combined with an analysis of the data from the live cells. The latter included the application of multivariate statistics, chemical imaging and machine learning. As will be shown by the results, SERS has the potential to monitor the inhibition of ASM and to characterize the impact of TCAs on lipid accumulation. The combination of TCAs carried by gold nanoparticles opens new perspectives in drug delivery. The interactions of the gold nanoparticles and TCAs with the cellular ultrastructure were studied by transmission electron microscopy (TEM) and cryo X-ray tomography (cryo-XRT). In a more general sense, the project contributes to the development of SERS as a tool for cell biology and understanding of basic processes in live cells.

This thesis consists of eight chapters. Chapter 2 introduces lysosomes and lysosomal storage diseases, explains the role of ASM. It also summarizes the state-of-the-art in the application of SERS as a method of probing cells. In Chapter 3, details of experiments performed in this thesis are described. The results of this thesis are shown in Chapters 4-7. Chapters 4 and 5 discuss the model systems needed to understand the complex interaction of antidepressants, lipid membranes, and gold nanoparticles in cells. In Chapter 4, the interaction between tricyclic antidepressants (TCAs) and gold or silver nanoparticles are discussed using data from SERS and surface-enhanced hyper-Raman scattering (SEHRS). From these data, the orientation of the molecules at the surface of the nanoparticles is inferred, and data of experiments relevant studies in the cells are reported. Chapter 5 presents the results of the SERS and cryogenic electron microscopy study of lipid vesicles and gold nanoparticles. The interaction between lipid membranes and nanoparticles is discussed, and the new insights into the interaction with citrate stabilized gold nanoparticles are shown. In Chapter 6, the SERS, TEM, and cryo-XRT studies of antidepressants treated cells were performed. Complex SERS data were analyzed using the Random Forest (RF) algorithm,

and the results were combined with the SERS data from the model systems. The evaluated data suggest that the impact on lysosomal biochemistry can be monitored by SERS. Chapter 7 shows the application of SERS to the analysis of Leishmania-infected cells that accumulate large amount of lipids.

Chapter 2

Introduction and state-of-the-art

2.1 Lysosomes

Lysosomes are acidic compartments containing many hydrolytic enzymes that are key players in the breakdown of macromolecules [2]. Moreover, lysosomes take part in many other processes, such as apoptosis [7], autophagy [33], cell membrane repair [34], cell signaling [35], and in cancer therapy [5]. Products of macromolecule degradation are further reused in the cell homeostasis. Thus, lysosomes are recognized as cell stomach.

Lysosomes vary greatly in their size, shape, and morphology, but they are always surrounded by a double membrane [2]. The outer leaflet of the membrane is called glycocalyx and protects the rest of the cell from the hydrolases contained in the lysosome [36, 37]. Glycocalyx is composed of the carbohydrate part of lysosomal integral membrane proteins and lysosomal-associated membrane proteins. These proteins enable hydrolases to reach their targets, thus preventing the cleavage of the membrane.

One of the main roles of lysosomes is the degradation of molecules taken up by endocytosis. The endocytic pathway is a system of organelles that are specialized in the uptake, sorting, and degradation of the extracellular material. Endocytic vesicles and early endosomes constitute the early endocytic pathway. The vesicles taking up material fuse with early endosomes. The late endocytic pathway is represented by late endosomes, and they fuse

with the lysosomes and hybrid organelles – multivesicular bodies [38]. During endosomal maturation, pH in the vesicles decreases from around 7.2 in the early endosomes to below 5 in the lysosomes [39].

The composition of the lysosomal membrane is different compared to the cell membrane. The biggest difference is reflected in very low cholesterol level in the lysosomal membrane compared to the cell membrane. Lysosomes contain lipids that are specific to the lysosomal membrane. In particular, bis(monoacylglycero)phosphate (BMP) is an anionic lipid found in the inner lysosomal membrane [40, 41]. BMP is negatively charged even at the low pH values expected in lysosomes, and the amount of BMP drastically increases from early endosome to lysosome, mostly due to cells autophagy. BMP has a considerably longer lifetime compared to other lipids in lysosomes because of its unusual configuration. It is essential for stabilization of the enzymes involved in lipid degradation, e.g., GM1- β -galactosidase, b-hexosaminidases A, and acid sphingomyelinase (ASM), and thus required for the degradation of the sphingolipids in the lysosomes.

2.1.1 Lysosomal storage diseases

Defects or irregularities in the functioning of lysosomal enzymes leads to the pathological states called lysosomal storage diseases [42]. Diseases can be classified according to the substance that is accumulated in the lysosomes, e.g., sphingolipidosis or phospholipidosis. Most severe diseases are connected with the accumulation of sphingolipids. The reason for the accumulation lies in the catabolism of (glyco)-sphingolipids that is sequential, which means if one of the enzymes in the cascade does not work it influences whole lipid metabolism thus inducing accumulation.

Most lysosomal storage diseases are caused by defects in the lysosomal proteins. However, non-lysosomal proteins can also be the cause for lysosomal storage diseases, e.g., mucopolipidosis II/III or Niemann–Pick disease type C1. In some storage diseases such as Niemann–Pick disease type C1, several substances are accumulated. Niemann–Pick disease is a rare lipid storage disease caused by the hereditary mutation of acid sphingomyelinase (ASM) [43]. Large amounts of sphingomyelin are accumulated in the lysosomes due to improper function of ASM.

It has been shown that some drug molecules induce lipidosis as well [44, 45] and they are commonly called cationic amphiphilic drugs (CADs). Characteristic properties of these drugs are high lipophilicity and one or more hydrophobic rings and a hydrophilic tail with a polarizable amine group. Due to their lipophilicity, at the physiological pH these drugs permeate cell membrane. Once they are protonated inside the lysosome their accumulation starts, causing perturbation of lysosomal function. As a consequence, lysosomal degradation of lipids is disrupted, and lipids accumulate in the lysosomes leading to drug-induced lipidosis. Myelin figures are a clear sign of lipidosis and they consist of lipid lamellae that alternate with layers of water [44].

2.1.2 Acid sphingomyelinase

ASM is a hydrolytic enzyme that exists in lysosomal and secreted form. ASM catalyzes the degradation of sphingomyelin to phosphorylcholine and ceramide (Figure 2.1.). ASM is a highly conserved and multi-domain protein, featuring the N terminal saposin domain, a proline-rich linker, an ill-defined C terminal, and a metallo-dependent phosphatase catalytic domain [46]. The lysosomal form is membrane-bound and attached to the inner leaflet of the lysosomal membrane by electrostatic interactions. The optimal pH is around 5, and the enzyme is Zn^{2+} dependent.

Figure 2.1. The substrate for the acid sphingomyelinase is sphingomyelin, a lipid found in cellular membranes. Upon enzymatic reaction, sphingomyelin is split into two products: ceramide and phosphorylcholine.

At first, the interest in ASM was limited to studies of Niemann-Pick disease [47]. After the discovery of ASM's role in several different processes and diseases, research interest in this enzyme increased. ASM was reported to play a role in cell signaling and many different diseases, e.g., Wilson disease [48], cystic fibrosis [49], atherosclerosis, acute lung injury [50], and lung emphysema [51]. Recently, a potential role of ASM in cancer therapy was described [52]. The influence of ASM on apoptosis and cell signaling is attributed to the effect of ASM on the local changes in sphingomyelin, ceramide, and cholesterol content. As ceramide is considered as a second messenger in transduction of signals in the cell membrane [53], disruption of its metabolism leads to apoptosis, further showing the importance of ASM in cellular homeostasis [54].

Despite the physiological and pharmaceutical importance of ASM, the 3D structure of the enzyme was solved only in 2016 [55, 56]. Gorelik et al. showed that the crystallized ASM in the presence of lipids rearranges the saposin domain and reveals lipid molecules bound within this part [56]. In line with previously shown results that the positively charged part of ASM interacts with the negatively charged lysosomal membrane [21], mutation of Lys120 leads to a decrease of ASM activity [55, 56]. This further proves that electrostatic interactions play a role in ASM binding to the lysosomal membrane. Moreover, substrate hydrolysis and binding were studied, as well as mapping of mutations that occur in NPD [55, 56]. These data are valuable in further studies of NPD and in inhibitor development for the implication of ASM in other diseases and as a target for potential cancer therapy.

2.2 Tricyclic antidepressants as a functional inhibitors of acid sphingomyelinase

The inhibition of ASM can be achieved in two different mechanisms: direct and indirect. The direct inhibitor is a molecule similar to a substrate for an enzyme and several direct inhibitors of ASM were developed and investigated [11]. Inhibitor of an enzyme is also a molecule that decreases the function of an enzyme without direct inhibition. This is important class of ASM inhibitors, and they are called indirect “functional” inhibitors of ASM (FIASMA) [57].

Since the 1970s, it has been shown that tricyclic antidepressants induce inhibition of ASM [13]. Investigation of the ASM inhibitors shows that the inhibitors are weak organic bases that have high lipophilicity. They usually consist of hydrophobic ring core and a side chain

with a cationic amine group. The amine groups are protonated under the physiological conditions in the lysosomes. Studying of the potential ASM inhibitors showed that not only high pK_a and $\log P$ are important characteristics of the inhibitors, but the steric hindrance of the positively charged nitrogen presents itself an important property [58]. Most of the functional inhibitors of ASM are previously well-described drugs that have been in clinical use for a long time. They include many different therapeutics substances used to treat several different diseases, e.g., infections, depressions disorders, and allergies [57].

An important class of functional inhibitors of ASM are tricyclic antidepressants (TCAs). TCAs are used to treat major depression disorders. Upon administration, TCA molecules bind to the membrane and are metabolized in the liver. TCAs are no longer considered as a primary treatment in depressive disorders due to the many side effects, e.g., poor tolerance by patients, required doses for successful treatment are close to toxic dose, etc [14]. Due to their highly hydrophobic structure with a cationic amine group, TCA accumulates in the lysosomes of the cells in high concentrations. The interaction between desipramine and ASM was, for the first time, reported in 1981 [12]. Albous et al. reported a decrease in the ASM activity in the presence of desipramine in cultured murine neuroblastoma cells and human fibroblasts [12]. Several studies lead to a rather detailed mechanism about the interaction between antidepressants and ASM [19, 59, 60]. It is suggested that upon administration in the cells, antidepressants pass cell membrane by diffusion due to their high lipophilicity, although they also enter in cells by endocytosis. Antidepressants end up in the cells' lysosomes, where they are protonated and are not able to diffuse back from the lysosomes and thus are enriched in these organelles [61]. Accumulation of antidepressants leads to proteolytic degradation of ASM. The proposed mechanism of the degradation is as follows: positively charged antidepressants interact with negatively charged BMP in lysosomal membrane. BMP binds ASM to the inner leaflet of lysosomal membrane and it is necessary for its proper function. The antidepressants diminish interaction between BMP and ASM leading to detachment of the enzyme from the membrane (Figure 2.2.), that further leads to cleavage of ASM by proteases in the lysosomal compartment [19].

Several studies were done in order to confirm the accumulation of TCAs in the lysosomes and their inhibition of ASM [61-64]. In particular, accumulation in the lysosomes was studied using deuterium-labeled desipramine in human fibroblast cells [62]. Accumulation of desipramine is confirmed in normal lysosomes, while in lysosomes with increased pH,

desipramine does not accumulate, confirming the importance of the lysosomal pH in the accumulation of TCAs [62]. Besides, a detachment of ASM by desipramine was studying using a liposomal membrane that contained 20 mol % BMP [59]. A membrane with bound ASM was deposited on a chip for surface plasmon resonance measurements. A detachment of ASM from the liposomal membrane was monitored upon addition of antidepressant desipramine and as a control negatively charged hexanoic acid was used [59]. Desipramine led to the detachment of ASM from the liposomal membrane, while the negatively charged hexanoic acid did not lead to the release of the enzyme [59].

Figure 2.2. Schematic representation of the interaction between antidepressants and acid sphingomyelinase (ASM) in lysosomes. In figure uptake of desipramine by cell and its enrichment in lysosomes is depicted.

As a consequence of the ASM inhibition by TCAs, accumulation of sphingomyelin (SM) in lysosomes occurs. The SM accumulation was confirmed in the lysosomes isolated from the hippocampus of wild-type mice treated for two weeks with amitriptyline [16]. The time for the accumulation in live animals is longer compared to cell lines. The formation of so-called myelin figures, caused by the accumulation of SM in the cells is shown even only after 24 h treatment [44]. As another example, amitriptyline has been used for treating cystic fibrosis and currently is in the third phase of clinical trials [65]. In course of cystic

fibrosis, ceramide is accumulated in the bronchial epithelial cells. Using amitriptyline leads to ASM inhibition and shows a beneficial effect on patients with cystic fibrosis [65].

It has been suggested that ASM supports the stability of the lysosomal membrane in cancer cells thus preventing lysosomal membrane permeabilization [60]. Inhibition of ASM activity should in principle lead to the death of cancer cells. Petersen et al. showed that ASM could be used successfully as a potential target for cancer treatment [52]. Functional inhibitors of ASM showed higher activity in cancer cells compared to normal cells [52]. The treatment with the ASM inhibitors led to a decrease in the tumor growth in animal models and enhanced the influence of chemotherapeutics [52]. The cancer cells were more susceptible to the treatment with chemotherapeutics when cells were treated with an inhibitor of ASM [52]. Nevertheless, the mechanism of action here is not completely understood. Studying the role of TCAs and other inhibitors of ASM can provide insight into the closely related lipid homeostasis in the lysosomes and the lysosomal cell death in the cancer cells.

2.3 Raman scattering and its application to bioanalysis

Monitoring chemical events in a complex environment such as cells remains a key challenge for bimolecular research. Several methods based on fluorescence were developed to monitor the dynamics of cellular events. These methods have been successful in many studies despite the fact that fluorescence suffers from several major drawbacks, e.g., need for bulky label dyes, photobleaching, limitation in the excitation wavelength. Thus, the need for a better-suited method for analysis of the molecular events in the living cells is constantly present.

For investigation of molecular events in the complex cell environment, e.g., interaction of drug molecules with cell, vibrational spectroscopy methods are by far more suitable than fluorescence [66]. Vibrational spectroscopy techniques offer several advantages over fluorescence in cellular environment. First, vibrational spectra show high molecular specificity providing detailed information about molecules in the complex environment. Second, many molecules can be identified simultaneously using the narrow bands in vibrational spectrum. Finally, unlike fluorescence, vibrational spectroscopy does not require molecule labeling. Several vibrational spectroscopy methods are used in cellular research, in particular, Raman scattering or infrared absorption (IR). Raman spectroscopy is potentially non-invasive and non-destructive. Infrared absorption has also been used for studying biological samples [67, 68], although IR suffers from the strong absorption of water. Since, Raman based methods do not suffer from strong water signals they are ideal for biological and biomedical applications.

The Raman effect was discovered independently by Raman and Krishnan [69, 70] and Landsberg and Mandelstam [71]. In 1930, Raman won the Nobel prize "for his work on the scattering of light and for the discovery of the effect named after him." Raman scattering is an inelastic process which occurs during the interaction of the incident light with a molecule. The incident light interacts with the molecules, resulting in the change of energy of the photon that is subsequently scattered compared to the incident photon (Figure 2.3.). The photon losses or gains energy, depending on whether the molecule is encountered in the ground or excited vibrational state. Change in energy corresponds to the energy of the molecular vibration. The detection of the Raman photons results in a spectrum with narrow bands that correspond to the specific vibrational modes of a molecule. Raman cross-section depends on the change of polarizability of the molecule, during the molecular vibration.

The polarizability is a measure of interaction between an electron cloud of a molecule and the incident light.

Figure 2.3. Schematic representation of Stokes Raman scattering (A), anti-Stokes Raman scattering (B), and hyper-Raman scattering (C). Only non-resonant processes are displayed. ν_0 represents the initial state for Stokes and hyper-Raman scattering, while ν_1 is initial state for anti-Stokes scattering. Dashed line represents virtual excited state.

Figure 2.3. shows the possible outcomes during the Raman process:

- (i) the emitted photon has lower energy than the incident photon, which is known as the Stokes scattering, and
- (ii) the emitted photon has higher energy than the incident photon, which is known as the anti-Stokes scattering.

Stokes and anti-Stokes frequency shifts form a symmetric pattern, while Stokes shift exhibits stronger peaks. The anti-Stokes scattering is weaker due to the small number of molecules in the higher energy state that is governed by Boltzmann distribution.

It is also possible that two photons interact simultaneously with the molecules (Figure 2.3. C) in Stokes Raman scattering at an energy shifted with respect to the double of excitation energy. This process is termed hyper-Raman scattering and has a very low cross-section.

A typical Raman experiment requires a laser, a monochromator, and a detector. Laser wavelengths used in the Raman experiments range from deep-UV to near-IR, covering a wide spectral range. Excitation in the near-IR, i.e., the use of low-energy photons, helps to avoid photodegradation of biological samples. Moreover, autofluorescence of biological material is weak when exciting in the near-IR region.

Since its discovery, Raman scattering has evolved into a sensitive (bio)analytical method. Raman spectrum contains the molecular fingerprint of the sample, allowing the identification of molecules in the complex matrices such as cells. Sensitive to molecular

conformation and environmental changes, Raman spectroscopy has been used for monitoring different cellular processes [72, 73]. Moreover, subcellular organelles were also studied by means of Raman spectroscopy [72, 74, 75].

The main drawback of Raman spectroscopy in a biological and biomedical analysis is an inherently low signal. Several developments were made to increase the Raman output by employing different methods, such as surface-enhanced Raman scattering (SERS), surface enhanced resonance Raman scattering (SERRS).

2.4 Surface-enhanced Raman scattering (SERS)

High enhancement of the Raman scattering by metal surfaces is known as surface-enhanced Raman scattering. Fleischmann recorded the first SERS spectrum in 1974, by observing high-intensity Raman scattering of pyridine on the surface of silver electrode [76]. A few years later, SERS effect was described by Jeanmarie and Van Duyne [77] and Albrecht and Creighton [78]. By use of noble metals the Raman signal can be enhanced by several orders of magnitude [79]. When light interacts with the surface of metal nanoparticles from gold, silver, or copper, it leads to the collective oscillation of the conductive electrons. In the nanoparticles, so-called localized surface plasmon resonance (LSPR) occurs when the frequency of the light matches the oscillating frequency of the conducting electrons. As a consequence of the LSPR, the high local fields of the induced dipole enhances the electromagnetic field of incident light, as well as the field of the Raman light (Figure 2.4). The major contribution comes from the electromagnetic enhancement. In addition, chemical enhancement takes place, when the analyte molecule interacts with the metal surface, e.g., through charge-transfer resonance [80]. The SERS effect is distance dependent, and the signal is enhanced if the molecules reside in the close proximity of nanoparticles. The strong enhancement in SERS, in principle, enables the detection of individual molecules [25]. Extremely high local field enhancements were observed on aggregates formed by silver or gold nanoparticles [81].

Figure 2.4. Schematic representation of the surface-enhanced Raman scattering process. Molecules are positioned in the close proximity of gold nanoparticles. Laser light interacts with plasmonic nanoparticles and enhances incident light (ν_L) and Raman light (ν_{RS}).

SERS has become an attractive bioanalytical tool due to the ultrahigh sensitivity and structural characterization of the molecules. Moreover, the narrow bands in the vibrational fingerprint offer possibilities for multiplexing [82, 83], including the use of a label [84], but also to study intrinsic cellular molecules, which made SERS a very useful tool in living cell analysis [85, 86]. Offering high sensitivity and probing in very small volumes, SERS has become the method of choice for sensitive cellular analysis, as well as in many analytical and catalytic processes.

Due to the high sensitivity of the SERS probes, spectra often show multiple chemical events that occur in the proximity of gold nanoparticles. Such a feature is both, the biggest advantage of SERS and also a potential drawback in a complicated environment such as cells. Many reactions and processes occur in the cells at the same time, and all of them can potentially be detected if they occur in the vicinity of the nanoparticles. This leads to complex SERS spectra that might be difficult for interpretation, also many biomolecules have a similar Raman signature, which yields an overlap in the spectra. To overcome these obstacles and to fully exploit the wealth of information in the SERS spectra multivariate statistics can be applied. Specifically, SERS data obtained from living cells are often analyzed using principal component analysis (PCA) for purposes of identification and classification of cancer cells, bacteria [87-89], viruses [90], or pollen species [91]. PCA was also used in combination with the linear discriminant analysis (LDA), which offers the

possibility for classification of SERS spectra of different cells and an improved differentiation between cancerous and healthy cells [92]. This combination also has been successfully applied to distinguish between different vesicles of the endocytic pathway, [93] and for the analysis of autophagy [94]. Artificial neural networks, as a machine learning approach, can be used for SERS data of samples that exhibit higher complexity, e.g., identification of aqueous pollen extracts [95]. Recently, also a random forest analysis was applied in the interpretation of SERS spectra [96]. In this thesis, SERS data obtained from lysosomes of living cells will be analyzed by random forest algorithm.

2.5 Gold nanoparticles in cells

When a SERS substrate, e.g., gold nanoparticles, gets into contact with a biological environment such as a cells, the complex interactions occur [97]. In order to use gold nanoparticles as optical probes for biological investigations, the interactions between particles and cells have to be characterized. Thus far, a lot of effort has been invested in understanding nano-bio interfaces [27, 84, 98-102]. As soon as nanoparticles are introduced into cell culture media for delivery, they are surrounded by biomolecules, and a so-called corona is formed [103]. The main components of the corona formed around the particles are proteins [103, 104] and they remain on the surface of the nanoparticles during the SERS measurements and most probably contribute to the SERS signal [105]. The interactions between proteins and the nanoparticles are complex and depend on many parameters, e.g., material and surface properties of the nanoparticles, the medium for their delivery, type of the cell, and the biological processes in the cells [100, 106]. Properties of the nanoparticles together with the protein corona determine the uptake mechanism into cells [107]. This enables designing specific nanoprobe that allows targeting of the different organelles in the cell [108].

Figure 2.5. Endocytic uptake of metal nanoparticles by a cell. (A) Schematic representation of endocytosis. (B-C) TEM micrographs of 3T3 fibroblast cells incubated with gold nanoparticles. Nanoparticles are observed in the close proximity of the cell surface (B), and the invagination of the cell membrane is observed (C). The scale bars: (B) 500 nm and (C) 100 nm.

In the majority of the reported SERS applications in cultured cells, gold nanoparticles are taken up from the culture medium through endocytosis. Endocytosis is a process by which a cell transfers different species, such as nutrients and artificial nanoparticles, from cell culture media into the cellular interior [109, 110]. Endocytosis is also involved in the cells signaling. During the endocytosis, nanoparticles approach the cell surface, membrane invaginates and form a vesicle inside the cell that contains the nanoparticles (Figure 2.5.). The endocytic pathway consists of different compartments. First is the early endosome, where sorting of the vesicles' content occurs and the nanoparticles proceed further towards the lysosomes [110, 111]. On the way to the lysosome, the content of the early endosomes is fused with multivesicular bodies, which are fastly acidified to a pH value of 5.5, and then reaches the late endosome. In the stage of the late endosome, the endosomal content, together with the nanoparticles is fused with the lysosome. As previously discussed, lysosomes are complex organelles in charge of the degradation of biomolecules. Figure 2.5. B and C show TEM images of nanoparticles of a size of 30 nm approaching the surface of a cell (B) and the invagination of the cell membrane (C).

Localization of the nanoparticles allows to study the processes occurring in the lysosomes, and the obtained SERS spectra provide a wealth of chemical information at a high level of

sensitivity. Therefore, the SERS studies of cellular lysosomes can provide an important insight into the biochemistry of lysosomes and the influence of different drug molecules.

2.6 SERS analysis of cells

SERS has the unique advantage of being able to identify biomolecules in the close proximity of the nanoparticles in the cellular environment without molecule labeling [25, 84]. SERS signal is confined to the small volume around the nanoparticles and largely depends on their local surrounding, thus enabling detection of the changing cellular environment. The morphology and aggregation of the nanoparticles play an important role in SERS enhancement. Therefore, it is useful to confirm the localization and morphology of nanoparticles in the cells. This can be achieved by means of TEM and cryo X-ray tomography (cryo-XRT) [27, 112].

Over the last decade, SERS has been used as a sensitive tool to characterize the interaction of nanomaterials with the molecules inside animal cells [27, 112-114], to gain better insight into endosomal transport [29], and to study interaction of drugs with nanostructures and cells [115]. Generally, two approaches are possible, where either modified or labeled nanoparticles are delivered to cells or where nanoparticles without any label molecule are used. In principle, nanoparticles are a convenient platform for surface modification in order to achieve different results inside the cells. Nanoparticles can be functionalized by different ligands, such as antibodies [116], nuclear targeting sequence [117], or pH reporters and they are commonly used in cell investigations. In the latter example, a molecule serves as pH sensor, e.g., *para*-mercaptobenzoic acid (*p*MBA) that has a pH sensitive Raman spectrum. Gold nanoparticles coated with *p*MBA were employed for pH sensing inside endosomes by SERS and surface-enhanced hyper-Raman scattering (SEHRS), further useful in distinguishing between different stages in the endocytic pathway [28].

Label-free detection and study of biomolecules are important in understanding processes in healthy cells and diseases. The obtained SERS signal reports on the cellular environment. Thus, direct probing of intrinsic molecules in cells, including the state of a cell under the influence of different stimuli is possible. SERS is now commonly used for studying bioorganic molecules such as proteins, amino acids [118], nucleobases [119, 120] outside as well as in the living cell [121]. SERS spectra can be used to identify and classify different cells or to differentiate between cancerous and healthy cells [122-125]. Often SERS is used

to probe events in the endocytic pathway like autophagy [94], and apoptosis [126, 127]. Tracking single nanoparticle in the endocytic pathway is possible by combining SERS and dark field microscopy [29]. The SERS spectra provide information on the molecules that the particle encounters on the way through the endocytic pathway [29]. Furthermore, differences in the SERS signal in the stage of the endosome and the lysosome were studied and attributed to the degradation of molecules inside the lysosome and a rising acidity [93].

SERS can be employed to study the interaction of nanoparticles with different cell types [112, 114]. The studies showed that the influence of nanoparticle concentration, of aggregation state, and of culture media on the interactions of nanoparticles and cells is significant. The nanoparticle localization and morphology were further studied using cryo-XRT, which showed the localization of nanoparticles in vesicles of endo-lysosomal system [27]. Moreover, nanoparticles in the cells were quantified by laser ablation ion coupled plasma mass spectrometry [128]. Localization and quantification of the nanoparticles inside the cells are related to the SERS signals, which allows a comprehensive characterization and understanding of nanoparticles-cell interaction.

Among other possibilities, SERS reveals time-dependent changes in cells. Dynamic, time-resolved SERS spectra can provide data about different processes that occur in the cellular environment, as a consequence of drug exposure or influence of different external stimuli. For example, SERS was used to follow hyperoxia induced by exposure to a high amount of oxygen [129]. Hyperoxia induces the formation of the reactive oxygen species that further induce the oxidation of lipids and amino acids, and degradation of nucleic acid, that is then reflected in the SERS spectra. Based on the combined SERS spectra with proteomics and fluorescence data, it was proposed that hyperoxia induces the acidification of the cells, and significant changes in Raman spectra were seen with a prolonged exposure to oxygen [129]. Furthermore, DNA damage induced by reactive oxygen species was confirmed by SERS [130]. These studies demonstrate the possibility of SERS to study changes in cells.

As the SERS signals are sensitive to small changes in the cellular environment, SERS has been proposed to investigate the uptake and intracellular fate of drug molecules in cells. The molecular characterization of drug activity in cells is an important parameter for determining the efficacy of the drug molecules. Specifically, SERS can be used to monitor changes upon delivery of drug molecules to cells, or as a method to follow a drug inside a cell [131, 132].

The first SERS study to monitor drug interactions in cells was for the anticancer drug doxorubicin, in the early 1990s [133]. The investigation of the influence of doxorubicin on cells by SERS continued in other studies, including combination with fluorescence [134]. SERS nanoprobe were used for the simultaneous delivery and monitoring of doxorubicin [135]. In another study, SERS revealed the binding sites of the doxorubicin analog mitoxantrone, on the plasma membrane [115]. Several probes were created to follow the interactions of different drugs in different organelles by creating nanoprobe with targeting sequences for organelles [136]. In particular, a nuclear targeting sequence was coupled with gold nanostars and used to monitor changes induced by the presence of cisplatin (cis-Pt), fluorouracil (5-FU), and camptothecin (CAMP) [137]. Cys-Pt is an anticancer drug that binds to DNA and leads to programmed cell death. 5-FU binds for thymidylate synthase, while CAMP interacts with topoisomerase I. The SERS spectra show predominate changes in the modes connected to these interacting species, confirming the mechanism of interaction in living cell [137]. In a different study, gold nanoparticles were delivered to cells prior to the delivery of the drug molecules [138]. Namely, 6-mercaptopurine and methimazole were delivered, and the diffusion of the drug molecules into the cells was monitored. Long-term tracking of single drugs and their mixture was achieved [138]. Also normal Raman spectroscopy has been used in the chemical imaging of metal–carbonyl complexes in cells [139]. Gold nanoparticles are often considered as a potential vehicles for drug delivery. It was shown that combining drug delivery and optical properties of gold nanoparticles, it is possible to simultaneous deliver and detect the influence of the drug in the living cells by using SERS [140]. For example, drug delivery of the liposomes with deuterated lipids and proteins that circumvent endocytosis was studied. All mentioned studies demonstrated the possibility of SERS to follow changes induced in cells upon administration of different drug molecules.

In this thesis, the role and action of TCAs will be studied inside the lysosomes by SERS. Of particular interest are interaction of drugs with nanoparticles and its target – ASM. Gold nanoparticles will be delivered to the lysosomes via endocytosis where the interactions of tricyclic antidepressants and ASM are known to take place. Studying the inhibition of ASM in the lysosomes may reveal the molecular mechanism of TCAs interactions with ASM.

Chapter 3

Materials and Methods

3.1 Materials

Desipramine hydrochloride ($\geq 98\%$, powder), imipramine hydrochloride ($\geq 99\%$, powder), amitriptyline hydrochloride ($\geq 98\%$, powder), gold(III) chloride hydrate (99.995%), silver (I) nitrate ($\geq 99\%$), sodium hydroxide ($\geq 98\%$), sodium chloride ($\geq 99.5\%$), chloroform, methanol, hydrochloric acid, sphingomyelin ($\geq 95\%$), L- α -Phosphatidylcholine ($\geq 99\%$), 3-sn-Phosphatidic acid sodium salt from egg yolk lecithin ($\geq 98\%$), ascorbic acid were purchased from Sigma-Aldrich. Trisodium citrate dihydrate ($\geq 99\%$) was obtained from Th. Geyer. Cholesterol was purchased from Avanti Polar Lipids, Alabaster, AL, USA. 3T3 mouse fibroblast cells were purchased from DSMZ, Barunschweig, Germany. Fetal calf serum (FCS) and Dulbecco's Modified Eagle Medium (DMEM), cell shield, PBS buffer, PBS buffer without Ca and Mg salts, trypsin/EDTA solution, DMSO, ethanol were purchased from Biochrom. All solutions were prepared using Milli-Q water ($0.055 \mu\text{S cm}^{-1}$) using PURELAB Plus water purification systems from ELGA.

3.2 Gold nanoparticles synthesis and characterization

Gold nanoparticles were obtained by reduction of gold (III) chloride hydrate with sodium citrate according to the protocols reported in ref. [141]. Briefly, solution of gold (III) chloride hydrate was added in Millipore water at 84 °C followed by addition of sodium citrate solution. Reaction was allowed to occur for the next 30 minutes at 80 °C with stirring.

The average size of the nanoparticles was determined by transmission electron microscopy to be 30 nm (see Figure 4.2.). From the known size of the nanoparticles, and assuming that the tetrachloroauric acid used in the nanoparticle synthesis was completely reduced, the final nanoparticle concentration was estimated according to the following equation:

$$C = \frac{n_{\text{Au}} \cdot M_{\text{Au}}}{V_{\text{AuNP}} \cdot \rho \cdot V_{\text{sol}} \cdot N_A}$$

Where n_{Au} is the amount of gold(III) used for synthesis, M_{Au} is the molar mass of gold, V_{AuNP} is the volume of the nanoparticles determined from the TEM images, ρ the density of the gold, V_{sol} the volume of the solution, and N_A is Avogadro's constant. The nanoparticle concentration was 4×10^{10} M.

The SERS enhancement factor of the gold nanoparticles was determined as described previously in ref. [142], using crystal violet (10^{-6} M) as a test molecule nanoparticles. The estimated enhancement factor is 4×10^6 .

3.3 SERS and SEHRS experiments with antidepressants in solution

One-photon excitation at 532 nm was generated as a second harmonic signal from a picosecond 1064 nm laser and the applied intensity was 1.2×10^{10} W cm⁻². Excitation at 785 nm was provided by a CW laser with an excitation intensity of 2.0×10^5 W cm⁻². SERS spectra were accumulated for durations of 1–5 s. All spectra were frequency calibrated using a spectrum of toluene for 532 nm and a mixture of toluene-acetonitrile (1:1) for 785 nm.

Surface-enhanced hyper-Raman (SEHRS) spectra were obtained at 1064 nm with an excitation intensity of 2.1×10^{11} W cm⁻² using a mode-locked laser producing 7 ps pulses

with a repetition rate of 76 MHz. The sample solution was placed in a microcontainer, and the excitation light was focused on the samples through a microscope objective. SEHRS spectra were accumulated for 5 min. The Raman light was collected in backscattering geometry and detected by a liquid nitrogen-cooled CCD (Horiba, Munich, Germany). Spectral resolution was 3–6 cm^{-1} in the full spectral range.

SEHRS spectra were background corrected using a protocol provided in ref [143]. Most spectral analyses were carried out on averaged spectra, averages were separately calculated from 100 SERS spectra and 30 SEHRS spectra.

3.4 Preparation of liposome-nanoparticle composites and their characterization

Thin layer hydration

Phosphatidylcholine (PC), sphingomyelin (SM), cholesterol (CH), phosphatidic acid (PA) were dissolved in a chloroform/methanol (1:1) mixture at a concentration of 2 mg/mL. After preparation of the lipid mixture, the solvent was removed under a stream of argon. The dried lipids were rehydrated with an aqueous solution of gold nanoparticles. The mixture was freeze-thawed six or more times in liquid nitrogen and at 37 °C. After the freeze-thaw cycles, the lipids were shortly sonicated and extruded through a polycarbonate membrane (Whatman® Nuclepore™ Track-Etched Membranes, pore size 0.2 μm) using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA).

Reverse phase evaporation

Phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol (CH), and phosphatidic acid (PA) were dissolved in a chloroform/methanol (1:1) mixture at a concentration of 2 mg/mL. The lipids in organic solution were mixed with the nanoparticles in aqueous solution. After mixing, the sample was sonicated until a stable emulsion is formed. In the next step, the organic solvent was removed using a rotary evaporator. At the end, the liposome-nanoparticle composites were extruded through a polycarbonate membrane (Whatman® Nuclepore™ Track-Etched Membranes, pore size 0.2 μm) using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA).

Cryogenic electron microscopy

Cryogenic electron microscopy (cryo-EM) was carried out on a JEOL JEM-2100 transmission electron microscope (JEOL GmbH, Eching, Germany). Cryo-EM specimens were prepared as follows: a 4 μL drop of the sample dispersion was deposited on a lacey carbon-coated copper TEM grid (200 mesh, Electron Microscopy Sciences, Hatfield, PA) and then plunge-frozen in liquid ethane at its freezing point with an FEI vitrobot Mark IV (conditions: 4 $^{\circ}\text{C}$ and 95% humidity). Vitrified grids were either directly transferred to the microscope cryo transfer holder (Gatan 914, Gatan, Munich, Germany) or stored in liquid nitrogen. Imaging was carried out at temperatures around 90 K. The TEM was operated at an acceleration voltage of 200 kV and a defocus of the objective lens of about 1.5–2 μm was used to increase the contrast. Cryo-EM micrographs were recorded at a number of magnifications with a bottom-mounted 4*4k CMOS camera (TemCam-F416, TVIPS, Gauting, Germany). The total electron dose in each micrograph was kept below 20 $\text{e}^{-}/\text{\AA}^2$. These measurements were done in collaboration with Dr. Zdravko Kochovski and Prof. Dr. Yan Lu from Helmholtz-Zentrum Berlin für Materialien und Energie and University of Potsdam.

SERS measurements of liposome-nanoparticle composites

Surface-enhanced Raman (SERS) spectra were excited at 633 nm with an excitation intensity of $3 \times 10^4 \text{ W cm}^{-2}$. The sample was placed in a microcontainer, and the excitation light was focused on the samples through a 60 $\times$ water immersion objective. The Raman light was collected in backscattering geometry and detected by a liquid nitrogen-cooled CCD (Horiba, Munich, Germany). SERS spectra were acquired for durations of 1 s to 5 s. The spectra were frequency-calibrated using a spectrum of a toluene-acetonitrile mixture and background corrected using the method provided in ref. [143]. Spectral analysis was carried out on averaged spectra; averages were calculated from 100 SERS spectra each.

3.5 Experiments with antidepressants treated cells

Subculturing protocol

Cell lines were stored in a cryogenic container under liquid nitrogen (boiling point -195.79°C). Cells were removed from the cryogenic container, unfrozen and centrifuged to remove the rests of the freezing medium. Then, cells were grown as monolayers in a cell

culture flask (75 cm²) in DMEM supplemented with 10 % FBS and 1 % of Zell Sheild. After 2-3 days, cells were subcultured in the following manner: cell culture medium was removed and cells were washed with PBS buffer without Ca²⁺/Mg²⁺. After removing of PBS, 1 mL of trypsin/EDTA solution was added to the cell culture in the flask until cells were detached from the surface of the flask (5-10 min). Detachment can be observed under a microscope. Following the detachment, cells were centrifuged at 4 °C, 2000 rpm for 5 minutes. The supernatant was removed and cells were resuspended in 5 mL of culture medium. Further, cells were counted and seeded in 75 cm² flask at a density of 2.5×10^3 - 4.0×10^3 cells/cm².

Preparation of cells for TEM imaging

Transmission electron microscopy (TEM) represents a suitable method for studying localization of inorganic nanoparticles in cells [144, 145]. TEM provides resolution in nanometer range with good contrast, allowing studying individual cellular organelles and induced changes [146]. Sample preparation has several main steps: chemical fixation of cells, embedding, staining, and slicing. TEM offers the opportunity to study cellular ultrastructure at great resolution, nevertheless TEM is impaired by several drawbacks. Sample preparation procedure is very demanding, cells need to be fixed and sliced in order to be imaged [145]. TEM imaging of the samples was done in collaboration with Petra Schrade and Prof. Dr. Sebastian Bachmann from Charité – Universitätsmedizin Berlin.

In these experiments, cells were fixed using 2.5% glutaraldehyde in cacodylate buffer. Post-fixation was done with 1% OsO₄, 0.8% K₄(Fe (CN)₆) in 0.1 M cacodylate buffer. The fixed cell suspension was embedded in agarose. Further steps included drainage by ascending alcohol series starting at 50%, 70%, 80%, 95% (each 2×10 -12 min) and 3×15 min in absolute alcohol. Samples were further immersed in propylene oxide (SERVA) as an intermediate medium (2×15 min). Further immersion was done with a mixture of propylene oxide and Epon in the ratio: 2:1 for 1 hour, 1:1 for 1 hour, and 1:2 for 1 hour, following by overnight immersion with pure Epon (SERVA). Samples were placed in encapsulants, filled with fresh Epon, and polymerization was carried out at 60 °C for 48 hours. The encapsulating agent was removed, and the Epon blocks were trimmed. Semi-thin sections, 0.5 to 1 µm thick, were prepared on Microtome RM 2065 from Leica, Bensheim. Sections were further stained with Richardson staining solution (methylene blue / azure). After selecting the area of interest, the rest of the tissue was removed and ultrathin

sections, 70 nm thick, were made on the Ultracut S, Leica. Sections were deposited on copper grids and stained with uranyl acetate and lead citrate according to Reynolds. Images of the sections were obtained using the EM 906 electron microscope (Zeiss, Oberkochen).

Preparation of cells for cryo X-ray tomography

For X-ray microscopy, cells were grown as a monolayer on Formvar-coated grids. Cells were grown under standard cell conditions for 24 h. After 24 h, cells were incubated with gold nanoparticles and/or gold nanoparticles/desipramine for 6 and 24 hours in cell culture medium. For vitrification, grids with cells were washed with PBS buffer. Excess of PBS buffer was blotted with filter paper, followed by snap-freezing on a plunge freezer using liquid ethane.

X-ray microscopy was performed at beamline U41-XM equipped with a cryo-stage at the electron storage ring BESSY II (Helmholtz-Zentrum Berlin für Materialien und Energie, Germany) in collaboration with research group X-Ray Microscopy lead by Gerd Schneider. Projection images of vitrified cells were obtained at a tilt angle of 0°. Tilt series were acquired from individual cells at different angles in increments of 1° at a pixel size of 9.8 nm (25 nm zone plate objective). Depending on the sample thickness, the exposure time was adjusted for each tilt angle series varying between 1 and 4 s.

Preparation of cells for SERS experiments

Cells were seeded on the sterilized glass slide (1.5 × 1.5 cm) in 6 well plates at a density of 2.5×10^2 - 4.0×10^2 cells/cm². Nanoparticles were delivered to cells from the cell culture medium. Nanoparticles were mixed with the culture medium (DMEM with 10 % FBS) in 1:9 molar ratio. Nanoparticles were incubated for several different time periods.

Incubation of antidepressants, separately and together with gold nanoparticles was also done in the cell culture medium. Solutions of desipramine hydrochloride, imipramine hydrochloride, and amitriptyline hydrochloride were prepared using sterilized MilliQ water in concentration 1×10^{-5} M and added to cell culture medium. When incubated with gold nanoparticles together, antidepressants were mixed with gold nanoparticles prior to mixing with culture medium. After different incubation times, the culture medium was removed and cells were thoroughly washed with PBS buffer and SERS spectra were obtained.

SERS spectra of 3T3 cells were obtained while the cells were adherent on glass slides. Cells were thoroughly washed with PBS buffer before the measurements and SERS spectra were obtained in PBS buffer. The Raman setup was equipped with a CCD detector and a diode laser operating at 785 nm. Use of a 60 $\times$ water immersion objective (Olympus, Hamburg, Germany) resulted in a laser spot size of 1 μm . Spectra were recorded from 300 cm^{-1} to 1900 cm^{-1} at a resolution of $\sim 5\text{--}8 \text{ cm}^{-1}$ in raster scans on the sample, with an acquisition time of 1 s per spectrum. The spectra were frequency calibrated using a spectrum of a toluene-acetonitrile mixture (1:1).

3.6 Random forest analysis of SERS spectra

Random forest (RF) is an ensemble machine learning method that can be used for classification and regression [147] and that is not prone to overfitting. One additional application of RF is the determination of important variables based on variable importance (VIMP) that is based on permuting variables and calculating the prediction error [147]. Therefore, variables are ranked depending on their impact on prediction performance and different approaches have been developed to select important variables according to VIMP. However, VIMP is biased by several variable characteristics and, hence, different methods like minimal depth (MD)[148] have been developed that determine variable importance independent from permutations and prediction error. MD determines variable importance based on the position of the variable in the decision tree, and it is based on the structure of the decision trees of random forests [148]. Based on the minimal depth of unimportant variables a threshold can be defined to select important variables based on minimal depth. [148]. Seifert et. al. combined the idea of MD with the concept of surrogate variables, that was originally developed to cope with missing values in the data [147]. This new approach is called surrogate minimal depth (SMD) and can be applied to select variables based on their mutual causality, and hence includes variable relations in the selection process [149]. Another application of this method that is utilized here is that the relationship between primary split variables and surrogate variables can be analyzed as a proxy for variable relations. For chosen variables, the parameter mean adjusted agreement is determined for each of the other variables based on the decision trees. This parameter can take values between 1 and 0. A value of 1 means total correspondence of the variables in the random forest, and hence that these variable are highly related, while a value of 0 means no relation between the variables. Similar to the selection of important variables based on MD and

SMD also a threshold for the selection of related variables can be determined based on the random selection of unrelated variables [149].

In this work MD to select important variables and SMD to select variables that are related to these important variables is utilized. However, in a first RF analysis (mtry = 31, ntree = 10000, min.node.size = 1) the the SERS spectra from cell mapping data on spectra that contain information (non-empty) from spectra that do not contain information (empty) were separated. Prior to separation between empty and non-empty spectra, SERS spectra were despiked, interpolated in the range from -1900 cm^{-1} to -351.2 cm^{-1} with a distance of 1.6 cm^{-1} and vector-normalized. RF was first trained on empty spectra and spectra with signals that were manually selected and then used for the separation of the data. The result of the separation is shown in Table 3.1.

Table 3.1. Results of random forest analysis separation of spectra that do not carry spectral information from the cells (empty) and spectra that carry spectral information (non-empty).

Experiment	Ami		Des		Imi	
	Non-empty	Empty	Non-empty	Empty	Non-empty	Empty
1	886	928	875	605	897	926
2	232	2058	205	2799	321	1484
3	1736	555	1556	565	897	822

In the first experiment (Table 3.1, first column), cells were incubated with antidepressants (Ami, Imi, or Des) for 24 h prior to incubation with gold nanoparticles for 6 h. Control sample for the respective experiment were cells incubated with nanoparticles for 6 h. The second experiment (Table 3.1, second column) were cells incubated with the nanoparticles for 6 h prior to incubation with antidepressants for 24 h. The control sample were cells incubated with nanoparticles for 6 h and grown for 24 h in the culture medium. In the third experiment (Table 3.1, third column), cells were incubated simultaneously with antidepressants and gold nanoparticles for 24 h. The control for this experiments were cells that are incubated with nanoparticles for 24 h.

For the classification between treated and non-treated cells, a the training set containing of 150 randomly selected SERS spectra of treated cells and 150 randomly selected spectra of control cells was used. The additional samples were used to determine the classification quality: For the cells treated with amitriptyline, 583 drug spectra and 129 control spectra

were correctly classified, while 153 and 13 spectra, respectively were wrongly classified. Hence, sensitivity and specificity of the classification were 0.7921196 and 0.9084507, respectively. Cells treated with desipramine show a sensitivity of 0.7804552 and a specificity 0.8873239, while in the imipramine treated cells sensitivity is 0.7875862, and specificity 0.8239437.

After the classification of the spectra, important variables are selected using MD. Important variables will be presented and discussed in Chapter 6 of the thesis. The important variables will be assigned to the bands from SERS spectra. Subsequently, SMD was applied to identify variables that are related to these important variables. As an example for the selection of related variables, Figure 3.1. is shown. The related variables of the band at 816 cm^{-1} from cells treated with desipramine for 24 h prior to treatment with nanoparticles for 6 h are above the threshold that is represented by the line. As can be seen, according to the SMD results the band at 816 cm^{-1} is related to several other bands, e.g., bands at 492, 650, 1520, and 1595 cm^{-1} .

Figure 3.1. The variables related to the band at 816 cm^{-1} , selected using the surrogate minimal depth (SMD) approach. Threshold is marked with the line.

The results of SMD analysis and detailed discussion are presented in Chapter 6. The random forest analysis and variable selection was done in collaboration with Dr. Stephan Seifert from Kiel University.

3.7 Experiments with *Leishmania* infected macrophages

Growth and differentiation of *L. Mexicana*

All experiments with infected macrophage cells were done in collaboration at Robert Koch-institute, Berlin, Germany by Dr. Geo Semini in accordance with ethical standards.

A clone of *L. mexicana mexicana* (MNYC/BZ/62/M379) expressing *DsRed* [150] was cultured in semi-defined culture medium (SDM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Thermo Fisher Scientific, Germany), 0.1 mM adenine, 1 µg/ml biotin, 5 µg/ml hemin, and 2 µg/ml biopterin (all from Sigma-Aldrich, Germany). Promastigotes were incubated at 25.5 °C and 5 % CO₂. Differentiation of *L. mexicana* promastigotes to amastigotes in axenic culture was carried out as described previously [151]. Briefly, stationary promastigotes were diluted 1 to 10 in Schneider's *Drosophila* medium (Sigma-Aldrich) supplemented with 20 % heat-inactivated fetal bovine serum (Gibco Thermo Fisher Scientific, Germany), MES [2-(n-morpholino) ethanesulfonic acid] adjusted to pH 5.5, and were incubated for 1 week at 33 °C until complete differentiation into amastigotes was terminated.

Infection of bone marrow-derived macrophages and incubation with gold nanoparticles

All animal procedures were licensed and carried out according to the local authorities (I C113 - T0263/17). Bone marrow was flushed from femura of BL/6 mice purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained in a conventional animal facility. Macrophages were differentiated from bone marrow of 6-8 weeks old mice as described [152], and infected with axenic *L. mexicana::DsRed* amastigotes in DMEM (Capricorn Scientific, Germany) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco Thermo Fisher Scientific, Germany) and 1 % M-CSF at a multiplicity of infection of 5. For transmission electron microscopy analyses, bone marrow derived macrophages (BMDMs) seeded on polymer 35 mm µ-dish (Ibidi, Germany) were first infected with amastigotes for 2 h and, after washing out parasites in excess, further incubated with gold nanoparticles (1:10 v/v or 1:5 v/v dilution in culture medium) at 33 °C and 5 % CO₂ until fixation. For SERS analyses, BMDMs were infected with amastigotes in the presence of gold nanoparticles (1:10 v/v dilution in culture medium) for 2 h and, after

removing parasites in excess, infected BMDMs were incubated in culture medium containing nanoparticles (1:10 v/v) for 4 days at 33 °C and 5 % CO₂ until analysis at the Raman microscope.

Thin section transmission electron microscopy

Infected bone marrow derived macrophages (BMDMs) containing gold nanoparticles were fixed in situ, at the bottom of the μ -dish, with 1 % formaldehyde (produced from paraformaldehyde by heating to 70 °C for 30 min) and 2.5% (v/v) glutaraldehyde in 0.05 M HEPES buffer (pH 7.2) for 20 min at room temperature. Cells were washed twice with 0.05 M HEPES for 10 min at RT and fixation was completed by adding a 1 % osmium tetroxide solution for 60 min in the dark. The fixative was washed out twice in ddH₂O and cells were dehydrated in an ethanol series (70 %, 80 %, 90 %, 100 %, for 15 min each) and by three incubation steps in 100 % acetone (for 15 min each). Infiltration with resin was done in mixtures of acetone and Epon (3:1, 1:1, and 1:3) at RT for 1 h each, followed by incubation with pure Epon at RT overnight. After replacing the Epon with fresh Epon, polymerization was carried out at 60 °C for 6 days.

For microscopic analysis, a small region (usually 5 x 5 mm) of the embedded cell layer was extracted and used for ultramicrotomy. Resin blocs were trimmed with a milling machine (Leica EM RAPID; Leica Microsystems) and ultrathin sections (70–80 nm) were produced using an ultramicrotome (Leica EM UC7; Leica Microsystems).

SERS experiments

SERS spectra of the infected macrophage cells were obtained while the cells were adherent on glass slides. Cells were thoroughly washed with PBS buffer before the measurements and SERS spectra were obtained in DMEM without phenol red. Before the experiment, the regions of the parasitophorous vacuole or other regions in the cell were identified visually in the microscope, and the position of the measured map was selected. The Raman setup was equipped with a CCD detector and a diode laser operating at 785 nm. Use of a 60 $\times$ water immersion objective (Olympus, Hamburg, Germany) resulted in a laser spot size of 1 μ m. Spectra were recorded from 300 cm⁻¹ to 1900 cm⁻¹ at a resolution of $\sim$ 5–8 cm⁻¹ in raster scans on the sample, with a step size of 1 μ m and using an acquisition time of 1 s per spectrum. The spectra were frequency calibrated using a spectrum of a toluene-acetonitrile mixture.

Data analysis

Analyses were performed using MatLab (The MathWorks, Inc., Ismaning, Germany) and CytoSpec (CytoSpec, Inc., Berlin, Germany). Data pre-processing included the elimination of spikes, interpolation, vector-normalization, and calculation of first derivatives. For chemical mapping, intensities were determined from the original spectra after baseline correction. Colours were assigned to relative intensity values for each spectral band in each map and overlaid with the bright field images. The principal component analysis was carried out with vector-normalized first derivatives using the information over the whole recorded spectral range (300 cm^{-1} to 1900 cm^{-1}).

Chapter 4

Interactions of tricyclic antidepressants with gold and silver nanoparticles

Tricyclic antidepressants (TCA) are an important group of drugs. Initially being used for the clinical treatment of major depression disorders, TCA have been suggested as potential treatments for a variety of other disease like, neuropathic pain disorders [153], or irritable bowel syndrome [154]. Additionally, TCA molecules have anti-inflammatory, anti-microbial,[155] and anti-tumoral activity [156]. The molecules were shown to have an influence on lipid metabolism [64], specifically, TCA reduce the activity of the acid sphingomyelinase and acid ceramidase enzymes in the lysosomes [17, 157]. A destabilization of the lysosomal membranes by tricyclic antidepressants and thus lysosomes has been proposed as potential target for cancer therapy [17, 52, 60, 158]. However, details about the molecular interaction of the TCA molecules with the biological environment and different bioorganic molecules must be elucidated. In this chapter, interactions between

TCA and gold and silver nanoparticles will be investigated, with aim to study influence of TCA molecules in live cells by SERS. This chapter was published and reprinted with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society. [159]

Nanoparticles and antidepressants

In order to investigate the interactions of the antidepressants, three molecules amitriptyline (Ami), desipramine (Des), and imipramine (Imi) were used (Figure 4.1.). One- and two-photon excited surface enhanced Raman scattering, that is, SERS and SEHRS, were employed to study their interactions with gold and silver nanoparticles.

Figure 4.1. Structure and labeling of antidepressant molecules, imipramine (Imi), desipramine (Des) and amitriptyline (Ami). Figure reprinted with permission from ref. Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

Both, gold and silver nanostructures were synthesized using citrate as reducing agent and stabilizer. Transmission electron micrographs of the nanostructures used in the experiments are shown in Figure 4.2, together with UV-Vis absorbance spectra of nanoparticles alone and in the presence of the TCA molecules. The silver nanoparticles have a size of 110 nm (Figure 4.2. A). Two different samples of citrate stabilized gold nanoparticles were used, one with a mean diameter of 30 nm (Figure 4.2. B), the other with a diameter of 60 nm (Figure 4.2. C). Figure 4.2 D shows the absorbance of the plasmonic nanoparticles, while panel E shows absorbance in the presence of one of the TCA molecules, desipramine. Prior to the addition of the TCA molecules, the citrate-reduced silver and gold nanoparticles exhibit the typical absorbance maxima around 420 nm for silver, and 530 nm and 542 nm for the two different batches of gold nanoparticles, respectively. After the addition of the

TCA molecules, all nanoparticles indicate absorbance by a broad, extended plasmon resonance (Figure 4.2 panel E). A change in color is also observed by eye, when the TCA molecules are added.

Figure 4.2. Transmission electron micrographs of (A) the gold nanoparticles with average size 30 nm (Au30) (B) gold nanoparticles with average size 60 nm (Au60), and (C) silver nanoparticles (Ag). (D) UV-Vis absorbance spectra of the AgNP (black line), Au30 (red line), and Au60 (blue line) and (E) with addition of Des. Figure adapted with permission from ref. Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

4.1 SEHRS and SERS spectra of TCAs

The SEHRS spectra of Ami, Des, and Imi (Figure 4.1.) were excited at a wavelength of 1064 nm (Figure 4.3. panel A), and corresponding one-photon excited SERS spectra using 532 nm excitation were measured (Figure 4.3. panel B). The SEHRS signals with silver and gold nanoparticles are on the same order of magnitude, this is in accordance with the main contribution of the SEHRS enhancement being discussed to result from the local fields of nanoaggregate structures [31, 160], whose formation is indicated in the absorbance spectra (Figure 4.2. panel E). So far, relatively few reports on SEHRS spectra using gold nanoparticles are known [161, 162]. In the case of the system studied here SEHRS and SERS spectra obtained with gold nanostructures are of particular interest due to the biocompatibility of the gold and further investigation of antidepressants interactions in cells.

Both one- and two-photon excited spectra with the two types of nanoparticles are characteristic of the three TCA molecules, and the SERS spectra on silver nanoparticles obtained here (Figure 4.3. panel B, second, fourth, and bottom trace) are in good agreement with those reported previously [163]. At first glance the spectral differences between spectra on silver and gold appear greater than the differences between the three molecules. Nevertheless, as will be shown, the spectra of the molecules display significant qualitative differences, depending on their structure and specific interaction with a particular kind of nanoparticle (cf. every second spectrum in Figure 4.3. panel A and Figure 4.3. panel B). Band assignments for the spectra of Ami, Des, and Imi are provided in Table 4.1., Table 4.2., and Table 4.3., respectively. After evaluating common spectral features for all three molecules first, some further specifics in the SEHRS spectrum of each TCA are discussed in a separate paragraph.

Figure 4.3. (A) SEHRS and (B) SERS spectra of the antidepressants imipramine, desipramine and amitriptyline obtained with silver and gold nanostructures. Concentration of the TCA molecules: 9×10^{-5} M for Au and 9×10^{-4} M for Ag nanostructures. (A) Excitation wavelength: 1064 nm, intensity: 2.1×10^{11} W cm⁻², acquisition time: 5 min. (B) Excitation wavelength: 532 nm, laser intensity, 1.2×10^{10} W cm⁻², acquisition time 5 s. Scale bars: (A) SEHRS, 0.1 cps, (B) SERS 50 cps. Figure reproduced with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

Table 4.1. Raman shift values in the SEHRS and SERS spectra of amitriptyline, and assignment to vibrations of the amitriptyline molecule, based on refs [127, 163-165]. Table reproduced with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968.

Raman shift / cm ⁻¹		assignments
SEHRS	SERS	
1623	1614	i-p bend (CC/RI,RII)
1590	1590	str (CC/RI,II), def (CH/RI,II)
1575	1556	str (CC/RI,II)
1487	1486	bend (CH ₂)
1464	1462	bend (CH ₂ /aliph), str (RI)
-	1438	bend (CH ₂ /aliph), str (RI)
-	1428	i-p bend (NC/Me)
-	1378	i-p bend (CH ₂)
1360	1363	rock (CH ₂ /aliph), str (CC/RIII), def CH ₃
-	1335	bend (CH ₂ /aliph)
1295	1290	str (CC/RIII, bridge bond)
1280	1273	i-p bend (CH/RI,II), bend (CH ₂ /aliph), str (RI)
-	1221	i-p bend (RI,II)
1206	1207	str (CC/RIII), i-p bend (RI,II), rock (CH/RII), str (CC/RI,II)
1177	1170	i-p bend (CH ₂)
1161	-	rock (CH/ RI,II)
-	1159	str (CC/RIII), rock (CH/RI), i-p bend (RI,II), str (CC/RI,II)
-	1055	str (CC/RI), rock (CH/RI)
1039	1040	str(CC/aliph), rock (CH/RII)
1018	-	str (CC/RI,II)
-	973	str (CC/RIII), rock (CH ₂ /RIII), i-p bend (RI)
-	870	o-o-p bend (CH/RI,II)
782	791	twist (RI,II), wag (N2H), i-p bend (RI,II), str (CC/RIII)
-	846	i-p bend (CH ₂)
-	812	o-o-p bend (CH/RI,II)
775	774	twist (RI,II), wag (N2H), i-p bend (RI,II), str (CC/RIII)
724	710	twist (RI,RII)
-	685	i-p bend (RI,II), str (CC/RIII)
633	635	def (RI,II)
-	620	twist (RI,II), i-p bend (RIII)
585	585	twist (RI,II,III)
-	562	i-p bend (RI,II,III) ¹
533	533	i-p bend (RII), twist (RI,II), str (CC/RIII), i-p bend (CC/RIII)
-	485	in-plane bend (RI,II)
-	445	twist (RI,II,III)

str, stretching; def, deformation, bend, bending; sciss, scissoring;
wag,wagging; rock, rocking; twist, twisting, i-p bend, in-plane
bending, o-o-p bend, out-of-plane bending

Table 4.2. Raman shift values in the SEHRS and SERS spectra of desipramine, and assignment to vibrations of the desipramine molecule, based on refs.[127, 163-165]. Table reproduced with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968.

Raman shift [cm ⁻¹]		assignments
SEHRS	SERS	
1630	-	str (CC/RII)
-	1597	i-p bend (CC/RI,RII)
1575	1576	str (CC/RI), str (CC/RII)
1487	-	bend (CH ₂)
1475	1472	def (CH ₂) ³ , def (CH ₃)
-	1440	sciss (CH ₂ /aliph)
1386	1363	symm def (CH ₃),
1329	1335	i-p bend (CH ₂ /aliph)
1296	1295	str (CC/RIII, bridge bond)
1280	-	i-p bend (CH ₂)
1232	1229	str (NC/RIII), rock (CH/RI), twist (CH ₂ /RIII)
1206	1207	str (CC/RIII), i-p bend (RI,II), str (CC/RI,II)
1155	1152	rock (CH/RI,II)
1107	-	bend (CH/RI,II)
1055	1056	str (CC/RI), rock (CH/RI)
1039	1040	str (CC/aliph), str (CC/RII)
1018	-	str (CC/RI,II)
-	973	str (CC/RIII), rock (CH ₂ /RIII)
-	865	o-o-p bend (CH/RI)
-	846	bend (CH ₂)
	812	o-o-p bend (CH/RI,II)
-	791	bend (CH ₂), bend (RI)
-	774	twist (RI,II), wag (N2H), i-p bend (RI,II), str (CC/RIII)
765	-	def CH (RI,II)
-	685	i-p bend (RI,II), str (CC/RIII), twist (RI,II)
677	673	i-p bend (RI,II)
622	620	twist (RI,II), i-p bend (RIII)
585	585	twist (RI,II,III)
566	562	i-p bend (RI,II,III), wag (N1C/aliph)
-	538	i-p bend (CH ₂), twist (RI)
-	496	twist (RI,II,III), i-p bend (RI,II), wag (N1C/aliph)

str, stretching; def, deformation, bend, bending; sciss, scissoring; wag, wagging; rock, rocking; twist, twisting, i-p bend, in-plane bending, o-o-p bend, out-of-plane bending

Table 4.3. Raman shift values in the SEHRS and SERS spectra of imipramine, and assignment to vibrations of the imipramine molecule, based on refs. [127, 163-165]. Table reproduced with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968.

Raman shift [cm ⁻¹]		assignments
SEHRS	SERS	
-	1590	i-p bend (CC/RI,RII)
1575	1576	str (RI,II)
-	1556	str (RI,II)
1487	-	bend (CH ₂)
1475	1472	def (CH ₂) ³ , def (CH ₃)
-	1440	i-p bend (Me),sciss (CH ₂ /aliph)
1386	1363	symm def (CH ₃),
1333	1329	i-p bend (CH ₂ /aliph)
1296	1295	str(CC/RIII, bridge bond)
1284	-	i-p bend(CH ₂)
1232	1229	str (NC/RIII), rock (CH ₂ /aliph), twist (CH ₂ /aliph), str (NC/aliph)
-	1221	i-p bend (RI,II)
1206	1202	str (CC/RIII), i- p bend (RI,II)
1177	1167	i-p bend (CH ₂)
1155	1156	rock (CH/RI,II)
1055	-	str (CC/RI), str (NC/aliph)
1039	1040	str (CC/aliph), sciss (CH ₂ /aliph), i-p bend(NC/Me)
-	1030	str(CC/aliph), sciss(CH ₂ /aliph), i-p bend(NC/Me)
-	973	str (CC/RIII), rock (CH ₂ /RIII)
-	861	o-o-p bend (CH/RI), i-p bend (CH ₂)
-	846	i-p bend (CH ₂)
-	812	o-o-p bend(CH/RI,II)
-	787	o-o-p bend(CH/RI), bend(CH ₂)
765		twist (RI,II), wag(N2H), i-p bend (RI,II), str (CC/RIII)
-	685	i-p bend (RI,II), str (CC/RIII), twist (RI,II)
677	673	i-p bend (RI,II)
622	620	twist (RI,II), i-p bend (RIII)
585	585	twist (RI,II,III)
566	562	i-p bend (RI,II,III), wag (N1C)
-	538	i-p bend (CH ₂), twist (RI)
-	496	i-p bend (CH ₂ /aliph),rock (NC)

str, stretching; def, deformation, bend, bending; sciss, scissoring; wag, wagging; rock, rocking; twist, twisting, i-p bend, in-plane bending, o-o-p bend, out-of-plane bending

The respective SEHRS and SERS spectrum differs greatly for each molecule and type of nanoparticles (compare each line in the combined Figure 4.3. A and B), over the whole spectral range. Many of the bands are present in both the one-photon and the two photon excited case, although with varied relative intensities. This similarity of the SEHRS and the SERS spectra is expected, since the interaction of the molecules with the nanoparticles' surface lowers their symmetry further, specifically of the ring moieties. As examples, contributions by the C-C stretching modes, together with C-H deformation vibrations of the rings are found in most examples, as indicated by the bands around 1206 cm^{-1} , e.g. in the spectra obtained with the silver nanoparticles (second, fourth, and bottom trace in Figures 4.3. A and B), and at $\sim 1160\text{ cm}^{-1}$ (all spectra in Figure 4.3. A and B). Similarly, a C-C stretching vibration of the seven-membered ring at $\sim 1295\text{ cm}^{-1}$ is found both in SEHRS and SERS spectra, when the molecules interact with a silver surface. Furthermore, C-C stretching from the aliphatic tail at $\sim 1040\text{ cm}^{-1}$ appears in both, SERS and SEHRS spectra, with very similar relative intensity, except in SEHRS of Des and Imi on gold nanostructures.

Even though the SEHRS spectra share many of the bands with the SERS spectra, they are very different with respect to many of them as well. A large number of modes in the fingerprint region of the spectra occur either in the SEHRS or in the SERS spectrum, or have very different relative intensities in both spectra, illustrating the complementarity of the spectral information. The fact that some vibrations contribute to the SEHRS spectrum of a molecule when interacting with e.g., gold nanoparticles, but to the SERS spectrum of the same molecule only with silver nanoparticles, evidences the very sensitive probing of local surface interaction by both methods, and indicates the strong influence that the interaction with the nanoparticles exerts on molecular symmetry.

An obvious difference between SERS and SEHRS spectra common to all three TCA molecules is the absence of the intense signal of ring deformation mode at 685 cm^{-1} in the SEHRS spectra on silver nanoparticles. Similarly, on gold nanoparticles, the very intense bands at 846 and 812 cm^{-1} (Figure 4.3. B) that is assigned to N-C deformation vibrations of the methyl-aminopropyl side chain, are absent from the SEHRS spectra. In the normal Raman spectra, these bands, specifically the former two, are also very pronounced. In similarity to the SEHRS spectra, the bands are absent in IR spectra, as was reported for the case of Des [165]. While most of the SERS spectra display a band of a breathing vibration

of the dibenzazepine ring at 973 cm^{-1} [163], probably of a six-membered ring, this band is not found in any of the SEHRS spectra. This observation is in accord with the absence of bands of this and similar modes in the SEHRS spectra of other organic molecules in the previous studies [118, 119].

Vice versa, a strong band at 765 cm^{-1} is particularly enhanced in SEHRS of Des and Imi, but absent from their SERS spectra (Figure 4.3. A and B, red and black traces). It is not seen in the normal Raman spectra (data not shown) either, but makes a very intense contribution to the IR spectrum of Des. There, the band is assigned to a deformation vibration of the $-\text{CH}$ groups in the aromatic rings. Furthermore, the SEHRS spectra, specifically on silver nanoparticles, show a strong signal at 585 cm^{-1} , which is neither visible in the SERS data of Figure 4.3. B nor in the normal Raman spectra. The IR spectrum, however, clearly reveals a band at 585 cm^{-1} . It is possible that an in-plane bending vibration of dibenzazepine ring is probed by SEHRS here, which would be specifically enhanced upon an interaction of the ring moieties with the silver surfaces that was predicted in ref. [163]. The SEHRS spectra, further, are all characterized by an intense band at 1487 cm^{-1} , which is assigned to a deformation mode of CH_2 group(s) of the aliphatic chain, observed at this frequency also in IR spectra [165] (Figure 4.3. A, first, third and fifth trace). In the SERS spectra in this frequency range, other deformation modes of CH_2 groups at lower frequency, from both the seven-membered ring and the aliphatic chain dominate this spectral region, with contributions around $\sim 1462\text{ cm}^{-1}$ and $\sim 1440\text{ cm}^{-1}$ (Figure 4.3. B, first, third and fifth trace) [163, 165]. The high similarity of the SEHRS spectra with IR data is in accord with previous observations in SEHRS experiments with other organic molecules [118, 119, 166-169], and with predictions according to the selection rules for the hyper Raman process that can probe IR active or even silent vibrational modes.

SEHRS spectrum of amitriptyline

Figure 4.3. A shows the SEHRS spectra of Ami obtained with gold and silver nanostructures, respectively (blue traces in Figure 4.3. A). For comparison, the one-photon excited SERS spectra of the identical samples are shown in Figure 4.3. B (blue traces). The assignments of the bands are given in Table 4.1. As stated above, the spectra show intense contributions from vibrations of the dibenzazepine ring of the molecule, and also from the aliphatic chain. The dominating band at 1487 cm^{-1} in the SEHRS spectra of the two CH_2 groups of the aliphatic chain is complemented by smaller contributions that originate from

other vibrations of the chain. Furthermore, an intense band at 1206 cm^{-1} in the spectrum obtained on the gold nanoparticles, assigned to the C–C stretching vibration in the seven-membered ring is observed (Figure 4.3. A, first spectrum). The relative intensity of this C–C stretching vibration with respect to other C–C stretching is similar to that of the mode at 1623 cm^{-1} and for example also is higher on gold nanoparticles than on silver. Another SEHRS signal that is assigned to the dibenzazepine ring is found at 782 cm^{-1} , it has a higher frequency than the CH deformation of the phenyl rings in Imi and Des discussed above, and a stronger contribution on silver nanoparticles. Its position suggests a contribution from a C–C stretching mode of the seven-membered ring, which in Ami is lacking a nitrogen atom, hence providing an additional C–C bond compared to Des and Imi. In addition to the bands at 560 cm^{-1} and 585 cm^{-1} , another in-plane mode of the phenyl rings and the seven-membered ring is present at 533 cm^{-1} in the Ami SEHRS spectrum on silver nanoparticles, confirming the previous observation that the interaction of the ring system with silver nanoparticles differs from that of Des and Imi [163].

SEHRS spectra of desipramine and imipramine

The SEHRS spectra of desipramine (Des, red traces) and imipramine (Imi, black traces) obtained with gold and silver nanostructures are shown in Figure 4.3. A. Figure 4.3. B contains the corresponding SERS spectra excited at 532 nm. Compared to the spectra of Ami, the SEHRS spectra of Des and Imi share many characteristics, specifically regarding their interaction with the silver and the gold nanoparticles. The band assignments of the SEHRS and SERS spectra of Des and Imi are listed in Table 4.2. and Table 4.3., respectively.

The presence of a nitrogen atom in the seven-membered ring of both molecules leads to several vibrational modes of the N–C bonds, specifically the stretching vibration at 1232 cm^{-1} , which is quite pronounced in all SEHRS spectra but in the SERS spectra only clearly visible when Des and Imi interact with silver nanostructures. Similarly, the C–C bridge bond vibration of the ring at 1295 cm^{-1} is only weak in the SERS spectra on silver nanostructures, but very strong in all the SEHRS spectra (compare Figure 4.3. A, last four spectra with Figure 4.3. B, fourth and sixth trace). As very specific feature of the SEHRS spectra, the –CH deformation vibration of the phenyl rings at 765 cm^{-1} gives strong signals. While the breathing vibrational mode at 973 cm^{-1} is absent from the SEHRS spectra,

different in-plane bending modes of the phenyl rings at 1206 cm^{-1} and at 622 cm^{-1} are very pronounced in the SEHRS spectra.

Also, the signals in the methyl-aminopropyl side chains of the molecules differ from those known for Ami. In addition to the intense signal at 1487 cm^{-1} of CH_2 groups, vibrational modes that are assigned to the methyl groups are visible, for example, the CH_3 symmetric deformation vibration at 1386 cm^{-1} , and a contribution at 1475 cm^{-1} , assigned to deformation vibrations of both CH_2 and CH_3 groups. Both bands have higher intensity in the SEHRS spectra obtained with gold nanostructures. In contrast, in the SERS spectra, the CH_3/CH_2 deformation at 1363 cm^{-1} is relatively weak and only found on the gold nanoparticles, pointing towards an interaction of the methyl-aminopropyl side chain with the gold surface. An important contribution to the band at 1363 cm^{-1} coming from the methyl groups is inferred from the presence of the band also in the SERS spectra of Ami (blue traces in Figure 4.3. B), where much less signal from CH_2 can be expected, but where two CH_3 groups are terminating the chain. Furthermore, a band at 1325 cm^{-1} in Des and at 1333 cm^{-1} in Imi, assigned to a bending vibration of the CH_2 groups in the aliphatic chain is prominent in the spectra obtained with gold nanoparticles (Figure 4.3. A, third and fifth spectrum). In the SERS spectra measured under these conditions, a signal at 1335 cm^{-1} that is assigned to this vibration is found as well (Figure 4.3. B, third and fifth spectrum). The different contributions of the C–H deformation modes in the alkyl chains in the SEHRS and SERS spectra indicate very clearly the presence of different kinds of specific interactions of the CH_2 groups with the nanoparticle surfaces.

The SEHRS spectra show several smaller differences between Imi and Des, for example, the band at 1177 cm^{-1} in the Imi spectrum, and the band at 1155 cm^{-1} in the Des spectrum. In both molecules, it is assigned to the rocking of the CH from the phenyl rings [163], and this difference being pronounced on the silver nanoparticles, underpins the conclusion on a strong interaction of the ring system with the silver surface. Also, C–C stretching modes of the phenyl ring around 1107 cm^{-1} and at 1055 cm^{-1} display slightly different contributions. On the gold nanoparticles, only very weak or no bands at this position are present.

4.2 Difference in interactions with gold and silver nanoparticles

Considering the high sensitivity of the SEHRS and the SERS spectra with respect to molecular interaction and surface environmental changes, the great differences that become evident during the discussion of the SEHRS and SERS spectra obtained on gold and silver nanoparticles must be the result of a varied interaction of the molecules with the surfaces of both kinds of nanoparticles. In the SERS data, the appearance and relative intensity of a certain band can provide information on the orientation of the molecules [170-172]. Specifically, the vibrational modes of the aromatic rings give this information based on the relative intensity of in-plane and out-of-plane vibrations [172, 173]. In SEHRS, relying on other selection rules, changes in adsorbate orientation can lead to much more pronounced changes in the spectra than in SERS [174-176], and the possibility to probe other adsorbate species and/ or interaction sites on the same surface [118, 177] can add a wealth of information. Due to the complementarity of the SEHRS and SERS spectra, both are used together to discuss the differences in the interaction of the TCA molecules. Apart from the excitation of SERS spectra at 532 nm, Figure 4.4. also shows SERS spectra that were obtained with an excitation wavelength of 785 nm on both metal nanostructures.

Figure 4.4. SERS spectra of the antidepressants Imi, Des and Ami obtained using sgold (A) and silver (B) nanostructures. Excitation, 785 nm; laser intensity, $2.0 \times 10^5 \text{ W cm}^{-2}$; acquisition time, 1 s; concentration, $9 \times 10^{-7} \text{ M}$ (gold), $9 \times 10^{-5} \text{ M}$ (silver); scale bars: 40 cps (gold), 4 cps (silver). Figure adapted with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

On the silver surface, the SEHRS spectra of all molecules contain pronounced signals assigned to several vibrational modes of the ring systems, specifically the in-plane bending modes of the phenyl rings at 622 cm^{-1} , 585 cm^{-1} , and 566 cm^{-1} (see Figure 4.3. B, second, fourth, and sixth trace). Their higher intensity on the silver nanostructures suggests an

interaction of the rings with the surface. On the silver nanoparticles, the strong band at 765 cm^{-1} of the -CH deformation of the phenyl rings shows a lower ratio with respect to the in-plane deformation modes than on the gold nanoparticles (compare respective red and black spectra in Figure 4.3. A). In the case of Ami, this is less pronounced, and a C–C stretching of the seven-membered ring appears at 782 cm^{-1} . All SERS spectra measured with silver nanoparticles, those excited at 532 nm (Figure 4.3. B) as well as those obtained at an excitation wavelength of 785 nm (Figure 4.4. B), support the observations made in the SEHRS spectra, which point at an interaction of the ring system with the silver surface. As examples, the breathing and strong C–C stretching modes of the ring at 973 cm^{-1} and $\sim 1040\text{ cm}^{-1}$, respectively, are enhanced, and the band at 1207 cm^{-1} of one of the dibenzazepine C–C stretching modes is quite pronounced. The SEHRS spectra and also the SERS data observed here confirm the interaction of the molecules with the silver surface that was reported in SERS spectra previously [163]. The larger enhancement of the in-plane modes of the ring system than of the out-of-plane modes in our SERS spectra obtained with the silver nanostructures is in agreement with a proposed tilted orientation [163]. It has been suggested that one of the phenyl rings of Des is closer to the nanoparticle surface, while Imi interacts through the whole ring system [163].

Even though some in-plane vibrational modes of the rings, such as the band at 560 cm^{-1} , are strongly enhanced in the SERS spectra obtained with gold nanoparticles (see e.g., Figure 4.4. A), these data indicate in general significant increases in the enhancement of some out-of-plane vibrations, together with additional vibrations from the aliphatic chain. The former becomes evident specifically from the SERS data obtained at 532 nm (Figure 4.3. B) and also at 785 nm (Figure 4.4. A). As an example, SERS spectra excited at 785 nm display pronounced signals at $\sim 760\text{ cm}^{-1}$ and $\sim 790\text{ cm}^{-1}$, assigned to the out-of-plane deformation of the phenyl rings with a contribution of wagging of the amino group in the aliphatic tail [163] (Figure 4.4. A). Furthermore, enhancement of the band of the phenyl rings twisting at $\sim 620\text{ cm}^{-1}$ [163] is accordance with the strengthening of out-of-plane modes in the flat orientation of the molecules.

The strong contributions from the aliphatic chain when the TCA molecules interact with the gold surface are very obvious in the SEHRS spectra (Figure 4.3 A). This is suggested by the wealth of deformation modes of CH_2 and CH_3 groups, including those at 1487 cm^{-1} , 1475 cm^{-1} , 1386 cm^{-1} and also $\sim 1330\text{ cm}^{-1}$. Interaction of all molecules with the gold

surfaces via the methyl-aminopropyl side chain is visible in the SERS spectra as well. The SERS spectra of all TCA molecules on gold contribute information on the N–C deformation vibrations of the methyl-aminopropyl side chain at 846 cm^{-1} and 812 cm^{-1} (Figure 4.3. B), as well as on vibrational modes of the CH_2 groups other than those found in SEHRS, at 1462 cm^{-1} , $\sim 1440\text{ cm}^{-1}$, 1363 cm^{-1} , and also at 1335 cm^{-1} and 1030 cm^{-1} (Figure 4.3. B and Figure 4.4. A). According to the presented data, the Des and Imi molecules interact with the gold nanostructure with both, the ring system and aliphatic tail. Due to the rigidity of the Ami molecule that is caused by the $\text{C}=\text{C}$ double bond in the aliphatic chain, the spectra show an interaction of the dibenzazepine system with the gold surface different from Des and Imi, as evidenced, e.g., by the relative intensities of the C–C modes at 1206 cm^{-1} and 1623 cm^{-1} (first traces in Figure 4.3. A). An interaction with the surface via the phenyl rings would force the molecules into a tilted position. This is supported by the presence of both, out-of-plane and in-plane modes in the spectrum [171]. In such a tilted orientation, an additional interaction through the amino group in the aliphatic chain can take place.

Both SEHRS and SERS data indicate that the molecular orientation on the surface compared to the interaction with the silver is very different. The decrease in the intensity of the in-plane vibration of the ring system in the spectra on the gold nanostructures suggests a change in the orientation from tilted to the flat in Des and Imi.[173] Furthermore, the increase in the intensity of the mode from aliphatic tail suggests closeness of this part of the molecule to the nanoparticles' surface. The different interaction of the gold surface with the ring moiety and the important function of the alkyl chain in the interaction could render part of the ring structure easier accessible for interaction with potential biological targets, for example with lipid membranes [178].

4.3. In vitro spectra in biological experiments

Nevertheless, the possibility to use NIR excitation to obtain both one-and two-photon SERS spectra of the drug molecules employing biocompatible gold nanoparticles is much more attractive with potential applications in live cells or tissues in mind. As was shown already in the discussion of Figure 4.4., SERS spectra can be measured with the gold nanostructures also at an excitation wavelength of 785 nm , and all further experiments that occur in a Raman microspectroscopic setup, enabling the selection and mapping of live cells [179] will be done under these conditions. Using these excitation conditions, experiments are

performed using gold nanoparticles in environments that are relevant for experiments with cell cultures.

An important step is to assess the stability of the nanoparticles and the spectral signatures in the biological conditions and interaction with components of delivery media. Components present in cell culture growth medium, used for delivery of the nanoparticles-antidepressant system, have an effect on the cellular uptake and further behavior of nanoparticles in cells [99]. Upon entering the cell culture medium, and later inside the cellular environment nanoparticles are usually coated with proteins present in the surroundings that become visible in the SERS spectra [31, 114, 180]. To understand whether components from the cell culture media may influence nanoparticle-drug interaction, spectra were obtained in the presence of a typical cell culture medium Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS), which is an important constituent of cell culture media.

Figure 4.5. UV-Vis absorbance spectra of the Au30 (black line), Au30 with the addition of desipramine (red line), and Au30 with addition of desipramine and DMEM-FCS (blue line). Figure reprinted with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

UV-Vis spectra of the drugs adsorbed on gold nanoparticles were obtained in the cell culture medium, Figure 4.5. shows the example of desipramine. As discussed, Des causes an aggregation of the nanoparticles (Figure 4.2. E). After addition of the DMEM- FCS to the nanoparticles-drug system, a slight blue shift of the extended plasmon band is visible,

indicating interaction of the molecules from the culture medium with the nanoaggregates surface.

Figure 4.6. SERS spectra of the antidepressants desipramine obtained using gold nanostructures without the DMEM-FCS and in the presence of DMEM-FCS. Excitation wavelength: 785 nm; excitation intensity: $2.0 \times 10^5 \text{ W cm}^{-2}$; acquisition time: 1 s, Concentration of desipramine: $9 \times 10^{-7} \text{ M}$. Scale bars: 50 cps. The bands highlighted in red show slight intensity variations and are discussed in the text). Figure reprinted with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

The SERS spectrum of Des in the presence and absence of DMEM-FCS is shown in Figure 4.6. SERS spectrum in presence of DMEM-FCS does not exhibit great changes. Nevertheless, some smaller changes are noted. The relative intensity of the bands at 760 cm^{-1} and 791 cm^{-1} is changed. The slight decrease of the signal assigned to the twisting of the phenyl ring coupled with wagging of NH in aliphatic chain at 791 cm^{-1} could indicate a slightly more upright position in the presence of potentially co-adsorbed components from the cell culture medium. Furthermore, the relative intensity in the band at 1538 cm^{-1} decreases in the presence of DMEM-FCS. Nevertheless, neither the main vibrational signature of desipramine including relative intensities, nor the overall enhancement is influenced in the presence of the culture medium. Considering the strong affinity that some

of the components of DMEM-FCS have towards the surface of gold nanoparticles [181, 182], it can be concluded that interaction between the TCA molecules and nanoparticles is very stable. Furthermore, the spectra were measured at several concentrations, ranging from 10^{-4} M to 10^{-7} M, all yielding very similar spectral fingerprints. It is estimated that full coverage of the nanoparticle's surface is achieved around 10^{-6} M concentration.

Figure 4.7. SERS spectra of the antidepressant desipramine obtained using gold nanostructures at pH 3.4, pH 4.4, and pH 6.0. Excitation wavelength: 785 nm; excitation intensity: $2.0 \times 10^5 \text{ W cm}^{-2}$; acquisition time: 1 s, Concentration of desipramine: 9×10^{-7} M. Scale bars: 30 cps. The bands highlighted in red show slight intensity variations and are discussed in the text). Figure reprinted with permission from Živanovic *et al.*, *J. Phys. Chem. C* 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.

In biological surroundings, nanoparticle-based drug delivery systems can encounter very different pH values, depending on the route of delivery and uptake into tissues and cells. In Figure 4.7., the SERS spectra of Des at different pH values are shown. The characteristic spectrum is not influenced, nevertheless small changes in some relative intensities occur.

The band at 535 cm^{-1} decreases when pH decreases from pH 6 to pH 3.4. At the same time, the in-plane bending mode of the dibenzazepine ring at 585 cm^{-1} intensifies (compare top trace in Figure 4.7. with middle and bottom trace), while the ring twisting mode at 710 cm^{-1} / 703 cm^{-1} becomes weaker. Changes in these vibrational modes indicate a slight re-orientation of the ring systems with respect to the gold nanoparticle surface, when pH is lowered. Similarly, the band at 772 cm^{-1} , assigned to ring deformation modes of the phenyl rings and C-C stretching of the seven-membered ring disappears, maybe pointing towards a more tilted orientation when pH decreases. Additionally, smaller influences on bands at 1534 cm^{-1} and 1570 cm^{-1} can be seen. Since the TCA are molecules with very high pKa value (~ 9.5), we do not expect that the change in pH values has a direct influence on the protonation/deprotonation of molecules themselves but rather indirectly on the interaction by protonation and deprotonation of the stabilizing species, and interaction with the citrate molecules on the nanoparticle surfaces.

The robustness of the spectral signature of nanoparticle-antidepressant system to changing pH suggests that studies inside the endosomal system, where pH lowers significantly over time, are feasible. Previously reported pH values of the early endosomes are around 6, and during the process of maturation pH becomes more acidic and reaches as low as 4 in the lysosome [183]. Furthermore, several pathologies are associated with very low pH values in the tissues [184].

The fact that the TCA-gold nanostructure assemblies are so stable could inspire new kinds of multifunctional nanocarriers for TCA molecules. In general, nanoparticle based drug carriers can offer many advantages for targeted delivery including optical imaging and monitoring possibilities. Our results here show that the characterization of the interaction of a gold-TCA drug delivery system would in principle be feasible. The function of SERS and SEHRS therein would lie in a sensitive probing of the molecules attached to the surface, and possibly also the cellular environment and its response to the delivered drug [84]. Obtained SERS and SEHRS spectra here, especially under the biological conditions, may be used for further investigation of nanoparticle based drug delivery platforms for tricyclic antidepressants. As revealed from the SEHRS and SERS spectra obtained on biocompatible gold nanostructures, in all three molecules, the methyl-aminopropyl side chain plays a very important role in the interaction with the gold, along with parts of the ring system. This is very different from the interaction with the silver nanostructures, where ring system mainly

interacts with nanoparticles. The NIR excited SERS spectra of the TCA-gold nanoparticles are greatly invariant with respect to changes in TCA concentration and size of the biocompatible gold nanostructures. They show remarkable stability in the presence of cell culture media and upon decrease of pH in the typical ranges of pH values in late endosomal structures. These findings suggest SERS as a tool for monitoring and characterization of interactions of antidepressants in cells.

Chapter 5

Interactions between lipids and gold nanoparticles

Acid sphingomyelinase (ASM) binds to the inner leaflet of the lysosomal membrane. To study the interaction between ASM and the lysosomal membrane and its inhibition by the tricyclic antidepressants, a liposomal assay has been developed [59]. In this thesis, the existing assay was modified to meet the requirements of the SERS experiments. Gold nanoparticles, which serve as optical probes, were introduced into the liposomes. Owing to the optical properties of the nanoparticles, SERS can be exploited for probing lipid interaction with the nanoparticles, as well as the interaction between lipid components and other molecules such as antidepressants. Thus, SERS will be used to report on the liposome components interacting with the gold nanostructures and the antidepressants. In this chapter, the interaction between gold nanoparticles and liposomes prepared under several different conditions will be discussed. The influence of the liposomal composition and the nanoparticles will be investigated, and conditions for studying the interaction of antidepressants and lipid bilayers in the presence of nanoparticles will be optimized. Part of this chapter is published and reprinted with permission from *Živanović et al. J. Phys.*

Chem. Lett. 2018, 9, 23, 6767-6772 [185]. Copyright 2018 American Chemical Society, and from V. Živanović, J. Kneipp, *Nano-bio interactions as characterized by SERS: The interaction of liposomes with gold nanostructures is highly dependent on lipid composition and charge*, *Proc. SPIE 10894, Plasmonics in Biology and Medicine XVI*, 1089404, 2019 [186].

5.1 Interactions between gold nanoparticles and lipid bilayers

When interacting with the liposomes, depending on the size and surface properties of nanoparticles and also on the properties of the liposomes different outcomes can be foreseen. In Figure 5.1. several possible interactions between the nanoparticles and liposomes are shown. First, gold nanoparticles can be incorporated into the aqueous cavity of the liposome or be attached to the surface of the liposome. Second, depending on the size and hydrophobicity, gold nanoparticles can be incorporated in the hydrophobic part of the liposome. Third, nanoparticles can be wrapped by lipid molecules, which behave similarly to surfactants.

Figure 5.1. Schematic representation of possible interactions of gold nanoparticles and lipid membranes

The interactions between lipid membranes and colloidal particles depend on many factors, e.g., the surface charge of particles and liposomes, the size of the vesicles and particles, membrane composition, and surface composition of the particles [187]. Many theoretical studies described the interaction between colloidal particles and lipid membranes. On the other hand, only several experimental studies describing these interactions were conducted [188, 189].

For the purpose of these experiments, gold nanoparticles-liposome composites were prepared using two different methods, thin layer hydration and reverse phase evaporation (Figure 5.2.). Briefly, lipids were dissolved in a methanol/chloroform mixture, the organic

solvents were evaporated, and the lipids were hydrated with the nanoparticle solution, freeze-thawed and extruded. For the reverse phase evaporation method, lipids in an organic phase were mixed with the nanoparticles in aqueous solution. A rotary evaporator was used to slowly evaporate the organic phase. After evaporation, the liposomes and nanoparticles were extruded (for detailed protocol see Materials and Methods chapter).

Thin layer hydration

Reverse phase evaporation

Figure 5.2. Schematic representation of gold nanoparticles-liposome composites preparation using thin layer hydration and reverse phase separation methods. Figure reprinted with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772 [185]. Copyright 2018 American Chemical Society.

To determine the interaction between the nanoparticles and the liposomes, the samples were studied by the negative staining transmission electron microscopy (TEM) and cryogenic electron microscopy (cryo-EM). TEM was done in collaboration with Sören Selve from Technical University Berlin, ZELMI, and cryo-EM measurements were done in collaboration with Dr. Zdravko Kochovski and Prof. Dr. Yan Lu from Helmholtz-Zentrum Berlin and University of Potsdam.

5.2 Negative staining transmission electron microscopy of the liposome-nanoparticles composites

TEM can be employed for evaluation of the morphology of the liposome-nanoparticle composites. Gold nanoparticles are easily visible in the TEM micrographs, however, to be able to visualize the liposomes, they were embedded in an electron-dense material to gain contrast [190]. Phosphotungstic acid (PTA) is used as the most widespread anionic stain for liposome and lipoproteins [191]. Staining of the liposomes occurs when PTA with multiple negative charges binds to the positively charged choline group from phospholipids [191]. Figure 5.3. shows TEM micrographs of the liposome-nanoparticle composites prepared using the original solution of nanoparticles and liposomes composed of 10 mol % sphingomyelin (SM), 20 mol % cholesterol (CH), and 70 mol % phosphatidylcholine (PC). The TEM images revealed that the nanoparticles form small aggregates. However, the spatial arrangement of nanoparticles and liposomes is not fully conclusive. The data suggest that the nanoparticles are associated with the liposomes.

Figure 5.3. TEM micrographs of liposomes and citrate stabilized gold nanoparticles ($C_{AuNP} = 8 \times 10^{-10}$ M) prepared using the thin layer hydration method. Liposome composition: 10 mol % SM, 20 mol % CH, and 70 mol % PC. Total lipid concentration: 2 mM. Scale bars: 100 nm.

The obtained results should be taken with caution because of drying of the sample during preparation. After deposition on the grid, the samples were dried in air. Drying of the samples can cause artifacts and change in the morphology of the liposome-nanoparticle composites. It has been shown that liposomes possess a completely different morphology after drying [192]. The staining agent also can influence the morphology of the liposome-nanoparticle composites.

5.3 Cryogenic electron microscopy of liposome-nanoparticles

To overcome artifacts of the TEM imaging and get a better insight into the structure of the liposome-nanoparticle composites cryo-EM was employed [193, 194]. Cryo-EM reveals

the morphology of the sample that is closest to that of the native state [195]. By plunge-freezing in liquid ethane at its freezing point, the sample is fixed in its current state due to the fast vitrification process. In contrast to TEM, dehydration of the sample is prevented by preparation in a controlled environment, 95 % humidity and at 4 °C. While using the TEM, structures are embedded in the stain and only the surface of the sample and contours are observed, cryo-EM allows visualization of the whole sample [193]. Cryo-EM allows the observation of the differences between hollow and solids objects. The cryo-EM measurements show the spatial arrangement of the nanoparticles and liposomes, and the lipid structures with respect to each other.

The cryo-EM images of preparations of a ternary mixture of phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol (CH), shown to be a good model for a typical cell membrane's outer leaflet [196] are displayed in Figures 5.4. The sample prepared with the original nanoparticle solution by thin layer hydration is shown in Figures 5.4. A-C. Figures 5.4. D-E display a preparation by reverse phase evaporation using the same lipid composition and nanoparticles.

Figure 5.4. Cryo-EM micrographs of liposomes and citrate stabilized gold nanoparticles ($C_{AuNP} = 8 \times 10^{-10}$ M) prepared using the thin layer hydration method (A)-(C) and reverse phase evaporation method (D)-(F). Liposome composition: 10 mol % SM, 20 mol % CH, and 70 mol % PC. Total lipid concentration: 2 mM. Scale bars: 100 nm. Figure reprinted with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772. Copyright 2018 American Chemical Society.

As clearly visible in all panels, the lipids are organized around individual gold nanoparticles or small nanoparticle aggregates in a scrambled, non-bilayer fashion. All nanoparticles are surrounded with the scrambled lipids, and some of those nanoparticles are associated with intact liposomes without nanoparticles.

As a control, samples were prepared by mixing gold nanoparticles with already formed liposomes, shown in Figure 5.5. In this case, the bilayer structure of the liposomes interacting with gold nanoparticles is preserved, and nanoparticles are associated with the surface of the liposomes. Some of the particles interact with more than one liposome (Figure 5.5. C).

Figure 5.5. Cryo-EM micrographs of a mixture of liposomes and citrate stabilized gold nanoparticles prepared in situ using thin layer hydration. Liposome composition: 10 mol % SM, 20 mol % CH, and 70 mol % PC. Total lipid concentration: 2mM. Scale bar: 100 nm. Figure reprinted with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772. Copyright 2018 American Chemical Society.

Further optimization of the experiments included diluting the solution of gold nanoparticles, while total lipid concentration was kept constant (2 mM). Nanoparticles were diluted from their original concentration to 1×10^{-10} M. In order to investigate a potential influence of the amount of citrate on the nanoparticle-lipid interaction, an excess of citrate ions from the synthesis [197], was removed, and these nanoparticles were used in the preparations. In this case, only a citrate layer adsorbed directly on the nanoparticles surface

remains [198]. The change in the concentration of citrate does not affect the surface potential but induces changes in ionic strength and pH, shown in Table 5.1.

Table 5.1. Concentration, zeta potential, and pH of nanoparticles used in the experiments. Table was reproduced with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772. Copyright 2018 American Chemical Society.

Concentration (M)	Solution	Zeta potential (mV)	pH
8×10^{-10}	excess citrate	29.8	3.78
8×10^{-10}	no excess citrate	29.1	4.77
1×10^{-10}	excess citrate	33.8	3.84
1×10^{-10}	no excess citrate	34.7	4.83

Figure 5.6. Cryo-EM micrographs of the samples composed of liposomes and citrate stabilized gold nanoparticles. Liposomes are composed of (A)-(B) PC, (C)-(D) PC/CH, (E)-(F) PC/SM. Total lipid concentration: 2 mM. Scale bars: 100 nm. Figure reprinted with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772. Copyright 2017 American Chemical Society.

Cryo-EM micrographs were obtained from intact liposomes of different lipid composition produced in situ in the absence of excess citrate in the gold nanoparticle solution by thin layer hydration. The vesicles were composed of SM, PC, a combination of both, and mixtures of the respective lipids with cholesterol. In all samples, unilamellar and/or multilamellar vesicles are formed regardless of the lipid composition, and most of the nanoparticles and their aggregates are adsorbed on the vesicular structures (Figure 5.6). Figure 5.6. A shows a larger multivesicular structure that encloses a nanoparticle, in agreement with the theoretical consideration that nanoparticles may be enclosed by larger,

but not smaller vesicles [199]. Cryo-EM shows with unprecedented clarity that some of the nanoparticles interact with more than one liposome (Figure 5.5. C and Figure 5.6. E) and thereby prevent vesicular fusion by holding the vesicles in place, providing ultrastructural evidence that lipid vesicles can be stabilized by gold nanoparticles [200, 201].

In contrast, when excess citrate is removed before the preparation by thin layer hydration, the vesicles' structure is preserved, and the nanoparticles are adsorbed on the surface of the liposomes (Figure 5.6.). Since the surface charge of the nanoparticles does not vary when citrate concentration in the solution is changed, the fact that the bilayer membrane is destroyed upon interaction with the nanoparticles at high citrate concentration (Figure 5.3.) suggests that the difference in ionic strength and pH (Table 5.1.) must be important. Both can act by influencing the electrostatic forces that are known to play a key role in the interaction of lipids with the citrate stabilized gold nanoparticles [202-204]. Further forces are known to act, including van der Waals, hydrophobic, and hydration forces [202]. Several of them can be altered by subtle changes in charge, e.g., by changed citrate concentration [205, 206] or surface modification of the nanoparticles [189]. The cryo-EM data in Figure 5.3. provide direct ultrastructural evidence that a high citrate concentration disturbs the proposed equilibrium between adhesion forces and the elastic force of the liposomes-nanoparticles interaction [199], and thereby the balance between the wrapping of the nanoparticles by lipids and their adsorption on the intact liposomes.

5.4 Optimization of the conditions for the SERS experiments

Due to the fast decay of the local fields around the nanoparticles, surface-enhanced Raman scattering exclusively provides information about molecules in close proximity to the nanoparticles. The SERS spectra give a wealth of molecular information about the species interacting with the surface of gold nanoparticles. For a long time, Raman scattering has been used for studying orientation and conformation of lipid molecules in the liposomes [207-209]. Here, the possibilities of SERS will be exploited to study surface interaction of liposomes and nanoparticles at the nm-size distance from the nanoparticles.

Figure 5.7. Representative SERS spectra of liposome-gold nanoparticles composites prepared using reverse phase evaporation method (black trace) and thin layer hydration method (blue trace). Used excitation wavelengths are 633 nm (A) and 785 nm (B). Liposome composition: 10 mol % SM, 20 mol % CH, and 70 mol % PC. Total lipid concentration: 2mM. $c_{\text{AuNP}} = 8 \times 10^{-10}$ M. Scale bar: 50 cps. Figure adapted with permissions from V. Živanović, J. Kneipp, *Nano-bio interactions as characterized by SERS: The interaction of liposomes with gold nanostructures is highly dependent on lipid composition and charge*, *Proc. SPIE 10894, Plasmonics in Biology and Medicine XVI*, 1089404 (7 March 2019); <https://doi.org/10.1117/12.2508584>.

Figure 5.7. shows SERS spectra of liposome-gold nanoparticle composites excited at 633 nm (A) and 785 nm (B). The liposomes are composed of 10 mol % SM, 20 mol % CH, and 70 mol % PC. The liposome-nanoparticles composites were prepared using both thin layer hydration and reverse phase evaporation method. As evidenced by the micrographs in Figure 5.3., both methods yield similar non-bilayer structure around the nanoparticles. However, the SERS spectra show slightly different features.

In general, Raman spectra of lipid molecules show very characteristic vibrations of the hydrocarbon chains [210]. The vibrations of hydrocarbon chain are observed in the regions between 1500 and 1300 cm^{-1} assigned to deformation of CH_2 and CH_3 groups and 1050-1200 cm^{-1} , assigned to C–C stretching vibrations. The vibrational modes of the polar heads of the phospholipids are expected in the region between 500 cm^{-1} and 900 cm^{-1} .

The SERS spectra of the liposome-nanoparticle composites excited at 785 nm mainly show features from C–C stretching and CH_2/CH_3 deformation vibrations. Detailed assignments are shown in Table 5.2. The band at 1086 cm^{-1} , assigned to C–C stretching, indicates disordered hydrocarbon chains [209]. In the CH_2/CH_3 deformation region the band at 1440 cm^{-1} can be seen. The band at 1440 cm^{-1} is distinctive in the Raman spectra of lipid molecules, and is assigned to CH_2 deformations [211]. The band at around 1550 cm^{-1} is

assigned to the C–N stretching in the polar head [210]. The SERS spectra excited at 633 nm provide several additional bands of the polar head.

Excitation at longer wavelengths, e.g., at 785 nm is frequently used for Raman experiments with cell samples due to low sample damage and relatively low background. Excitation at shorter wavelengths leads to an increase in signal intensity, but also a higher background. SERS spectra excited at 633 nm were background corrected as described in Section 3.4. In the SERS experiments reported in this Chapter, excitation using a wavelength of 633 nm will be used.

5.6 SERS spectra of the liposome-nanoparticle composites

SERS spectra, collected from all sample solutions, provide comprehensive details on the interactions between the lipids and the nanoparticles in the different preparations. SERS selectively probes the structure and interaction of the lipid molecules with the gold nanoparticles. Trace A in Figure 5.8. is a spectrum of the gold nanoparticles and the unordered ternary lipid mixture (as shown in Figure 5.3.). Figure 5.8. B shows the spectrum of the control liposomes with gold nanoparticles (compare Supporting Information Figure 5.4.). Spectrum C of Figure 5.8. was obtained from an in situ liposome preparation after removal of excess citrate from the nanoparticle solution (compare Figure 5.5.). Comparing spectrum 5.8. A of the unordered lipid coating (see Figure 5.8. A) with the control sample containing intact liposomes and gold nanoparticles (Figure 5.8. B), the latter displays several more sharp bands. The SERS spectrum of the control sample is similar to the spectrum of intact liposomes that are formed when no excess citrate is present (Figure 5.8. C).

Figure 5.8. Representative SERS spectra of (A) liposome and citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 8 \times 10^{-10}$ M) prepared using the thin layer hydration method, (B) mixture of liposomes with gold nanoparticles, (C) liposome and citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 1 \times 10^{-10}$ M), after removal of excess citrate ions. Liposome composition: 10 mol % SM, 20 mol % CH, and 70 mol % PC. Total lipid concentration: 2 mM. Excitation wavelength: 633 nm, intensity: 3×10^4 W cm $^{-2}$, acquisition time: 15 s. Scale bar: 20 cps. Figure adapted with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772. Copyright 2018 American Chemical Society.

In all samples, the spectral region from 500 cm $^{-1}$ to ~ 900 cm $^{-1}$, which mainly represents the vibrational modes of the lipid head groups, contains several signals, indicating an interaction of the lipid molecules with the nanostructures via the head groups. In all three samples, the band at ~ 720 cm $^{-1}$ can be assigned to the symmetric C–N stretching vibration of the N $^+$ (CH $_3$) $_3$ part of the choline group [207, 210, 212] (Table 5.2.). Additionally, all spectra show a signal at a Raman shift of ~ 1020 cm $^{-1}$, a vibration assigned to the C–N symmetric stretch of the lipid heads [213]. The very sharp bands in the region from 500 cm $^{-1}$ to ~ 900 cm $^{-1}$ in the spectrum of the liposomes prepared in the absence of excess citrate (Figure 5.8. C) suggest a preferred interaction of choline with the gold nanoaggregates at lower citrate concentration, as most of them are assigned to vibrations of the choline group [207]. This is in agreement with the high affinity of the nitrogen from the choline group to the surface of gold nanoparticles [189, 203]. The frequencies of the totally symmetric C–N stretching and the symmetric C–N stretching band in Figure 5.8. A at ~ 900 cm $^{-1}$ and ~ 760 cm $^{-1}$, respectively, indicate that the choline heads are in trans conformation [207] while they interact with the gold nanoaggregates. This result verifies the proposed interaction of the positively charged nitrogen of the choline group with the negatively charged nanoparticle surface leading to a surface reconstruction and conformational changes of membranes observed previously [189]. Upon contact, the nitrogen can tilt [189], which would induce the observed trans conformation of choline.

Interaction with the lipid heads in all samples is further evidenced by signals from the phosphate groups and ester bonds. The band at 801 cm^{-1} , assigned to the stretching vibration of the phosphate group [214], is visible in the spectra of the two liposome samples prepared in situ in the presence of nanoparticles (Figure 5.8. A and 5.8. C). The band of the asymmetric stretching vibration of the PO_3^{2-} group at 1004 cm^{-1} [214] is observed at low citrate concentration (Figure 5.8. C), as well as in the control sample that contains intact liposomes (Figure 5.8. B). The strong bands at 1160 cm^{-1} and 1184 cm^{-1} (Figure 5.8. C) are assigned to vibrational modes of ester bonds [215, 216]. The pronounced enhancement of these signals at low citrate concentration (Figure 5.8. C), in particular, those from the phosphate group, evidences a strong interaction via the head groups. Also, recent studies by fluorescence spectroscopy suggest that the phosphate group can be involved in the interaction with the nanoparticle surface [217]. In intact liposomes, the heads are naturally exposed to the aqueous environment, and the increasing amount of water molecules that build up around the choline groups can allow the phosphate groups to approach the surface of the nanoparticles [217].

The SERS spectra in Figure 5.8. also give information about the conformation of the acyl chains of the lipids. In the C–C stretching region, a broad band at 1086 cm^{-1} is present, indicating their predominant gauche conformation in all samples [209]. The C–C stretching band at 1146 cm^{-1} [218] points towards a more ordered state of the lipid chains when the nanoparticles are added to pre-prepared liposomes in the control sample (Figure 5.8. B). All samples present with bands $\sim 1380\text{ cm}^{-1}$, 1423 cm^{-1} , and 1453 cm^{-1} that are assigned to deformation modes of the CH_2 groups [209, 219]. The varying enhancement of the bands in the different preparations indicates a varied conformation of the lipid chains [209] in the intact liposome preparation at low citrate concentration and in the control (compare Figure 5.8. C and 5.8. B, respectively), and also in the case of the sample with disordered lipid chains (Figure 5.8. A).

Table 5.2. Raman Shifts and Assignments of Important Bands in the SERS spectra of liposome-gold nanoparticles composites. Assignments Based on Refs: [164, 210, 211, 214, 220-222].

Raman shift [cm^{-1}]	Assignments
1556	C–N, C–C stretching
1537/1544	CH ₂ scissoring
1479/1483	CH ₂ /CH ₃ deformation
1453	CH ₂ deformation
1423	CH ₂ deformation
1363/75/81	CH ₂ deformation
1298	CH ₂ twisting
1281	CH ₂ twisting
1270	=C–H deformation
1256	PO ₂ [−] asymmetric stretching
1184	Ester linking group (O=C–O–C)
1160	Ester linking group (O=C–O–C)
1151	C–N stretching
1146	C–C stretching
1130	C–C stretching, trans conformation
1087	C–C stretching, gauche conformation
1073	C–C stretching, trans conformation
1018	C–N ⁺ symmetric stretching
1002	PO ₃ ^{2−} asymmetric stretching
989	PO ₃ ^{2−} stretching
917	C–N symmetric stretching
902	C–N stretching
867	C–N symmetric stretching
816	PO ₂ [−] stretching
801	PO ₂ [−] stretching
759	C–N symmetric stretching
720	C–N symmetric stretching
704	C–H from the ring of cholesterol
681	O=C–O deformation

5.7 Influence of the liposome composition on the interaction with the gold nanoparticles

As could be observed, the influence of the state of nanoparticles on the liposome-nanoparticle composites is substantial. However, the influence of the lipid membranes composition has to be investigated. Furthermore, the possibility of SERS to follow the change in the liposomal composition should be evaluated. To that end, data from intact liposomes of different lipid composition produced in situ in the absence of excess citrate in the gold nanoparticle solution by thin layer hydration were obtained. The vesicles were composed of SM, PC, a combination of both, and mixtures of the respective lipids with cholesterol. Cholesterol, as an important component of many cellular membranes, was added to the membranes.

The SERS spectra of liposomes composed of phosphatidylcholine (Figure 5.9. A), sphingomyelin (Figure 5.9. B), and their mixture (Figure 5.9. C) are shown together with a spectrum of the respective liposomes that in addition contain cholesterol. The spectra of phosphatidylcholine liposomes and those that also contain cholesterol show very similar features (Figure 5.9. A). When cholesterol is present, the main differences are found in the regions containing contributions from vibrations of the lipid chains. The bands at 1074 cm^{-1} and 1127 cm^{-1} belong to C–C stretching vibrations and indicate that the acyl chains are mainly in *trans* conformation [223] in the absence of cholesterol (Figure 5.9. A, bottom trace). In the presence of cholesterol, they disappear (Figure 5.9. A, top trace). In accord with such a modified conformation when cholesterol is present, the band associated with deformation vibrations of the CH_2 groups at 1281 cm^{-1} is shifted to higher frequency [224], and its relative intensity decreases (Figure 5.9. A, top trace). All changes in the spectra suggest the presence of a less ordered state of the acyl chains [211, 221] upon addition of cholesterol due to its interaction by the hydrophobic ring system [225, 226]. Moreover, a broad and intense band at 1151 cm^{-1} with contributions from the ester bond vibration (Figure 5.9. A, top trace) indicates hydrogen bonding of the OH group of cholesterol with the polar head [225], which in turn alters the contact of the lipid head group with the gold nanoparticles.

Figure 5.9. Representative SERS spectra of citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 1 \times 10^{-10}$ M) and liposomes composed of: (A) PC and PC/CH, (B) SM and SM/CH, (C) SM/PC and SM/PC/CH. Total lipid concentration: 2 mM. Excitation wavelength: 633 nm, intensity: 3×10^4 W cm⁻², acquisition time: 5 -15 s. Scale bars: 20 cps. Figure reprinted with permission from Živanović *et al. J. Phys. Chem. Lett.* 2018, 9, 23, 6767-6772. Copyright 2018 American Chemical Society.

From the SERS spectra of liposomes containing sphingomyelin we can infer on a varied interaction with the gold nanoparticles, compared to the other mixtures (Figure 5.9. B). As

an important hallmark, the characteristic C–C stretching bands of acyl chain vibrations around 1070 cm^{-1} and 1130 cm^{-1} of *trans*, and around 1090 cm^{-1} of *gauche* oriented molecules, respectively, are missing (compare Figure 5.9. B bottom trace to Figure 5.9. C). The strongest signal in the spectrum of sphingomyelin is the band at 1018 cm^{-1} (Figure 5.9.B bottom trace), assigned to a C–N⁺ symmetric stretching vibration of the polar head groups. It decreases in intensity when cholesterol is present in the liposome (compare upper and lower trace in Figure 5.9. B). This clearly shows a weakened interaction and change of the orientation of the polar head groups in the presence of cholesterol. In accord with this, signals assigned to different vibrational modes of ester group at 1160 cm^{-1} and 1184 cm^{-1} decrease in the presence of cholesterol, indicating that contacts of the ester group are absent. The band at 1087 cm^{-1} (Figure 5.9. B, top trace) appears, suggesting *gauche* orientation of the alkyl chains, which is brought about by insertion of the rigid ring system of cholesterol into lipid bilayers (Figure 5.9. B, top trace). The different interaction of sphingomyelin liposomes with the gold nanoparticles is very likely caused by the presence of the amide bond in sphingomyelin. Moreover, the amide bond also causes a different interaction with cholesterol, by the interaction of its nitrogen with cholesterol's OH group [226]. The addition of cholesterol to sphingomyelin-containing vesicles has a different effect on the interaction between vesicles and nanoparticles than in phosphatidylcholine liposomes.

Mixtures of cholesterol in a lipid bilayer represent a highly complex system, especially with multicomponent bilayer membranes. In sphingomyelin / phosphatidylcholine liposomes lacking cholesterol (Figure 5.9. C, bottom trace), the two C–C stretching bands that are indicative of a *trans* conformation of the acyl chains at 1128 cm^{-1} and at 1073 cm^{-1} are present. Together with the CH₂ deformation signals at 1441 and 1256 cm^{-1} , this spectrum indicates highly ordered acyl chains [211, 224]. The high degree of order of the acyl chains evidenced by the SERS spectra confirms the indirect observation made for other lipids that gold nanoparticles may induce the formation of a gel phase at the site of adsorption in the liposome [205]. In the ternary mixture, an intensity decrease at 1128 cm^{-1} and increase at 1086 cm^{-1} (Figure 5.9. C, top trace) indicates a transition *trans* to the *gauche* conformation of the acyl chains when cholesterol interacts, in agreement with the data reported for the interaction phosphatidylcholine and sphingomyelin vesicles in their respective mixtures with cholesterol above.

5.8 Negatively charged liposomes and gold nanoparticles

The inner leaflet of the lysosomal membrane differs from many other membranes in the cell by the content of negatively charged lipid bis-(monoacylglycero)-phosphate (BMP) [10]. It has been shown that BMP is an important component in the interaction of desipramine with the lipid bilayers [59]. The hypothesis is that the positively charged nitrogen from desipramine interacts with negatively charged phosphate groups from BMP [59]. To be able to follow the interaction between lipid bilayers with negatively charged lipids and desipramine using SERS, liposome-nanoparticles composites that contain negatively charged lipids were prepared and characterized. The SERS spectra of negatively charged liposomes and gold nanoparticles are shown in Figure 5.10. In this particular case, instead of BMP, phosphatidic acid (PA) was used. Phosphatidic acid is phospholipid that has similar properties as BMP and carries negative charge at physiological pH [227]. In Figure 5.10, the SERS spectra of negatively charged liposomes and gold nanoparticles are shown. The lipid composition of the negatively charged liposomes is 10 mol % PA, 10 mol % SM 20 mol % CH, and 60 mol % PC (black trace in Figure 5.10.) and 20 mol % PA, 10 mol % SM 20 mol % CH, and 50 mol % PC (blue trace in Figure 5.10.).

Figure 5.10. Representative SERS spectra of citrate-stabilized gold nanoparticles ($c_{\text{AuNP}} = 1 \times 10^{-10}$ M) and liposomes composed of (A) 10 mol % PA, 10 mol % SM 20 mol % CH, 60 mol % PC, and (B) 20 mol % PA, 10 mol % SM 20 mol % CH, 50 mol % PC. Total lipid concentration: 2mM. Excitation wavelength: 633 nm, intensity: 3×10^4 W cm⁻², acquisition time: 5-15s. Scale bar: 50 cps. Figure reprinted with permission from V. Živanović, J. Kneipp, *Nano-bio interactions as characterized by SERS: The interaction of liposomes with gold nanostructures is highly dependent on lipid composition and charge*, *Proc. SPIE 10894, Plasmonics in Biology and Medicine XVI*, 1089404 (7 March 2019); <https://doi.org/10.1117/12.2508584>.

The SERS spectra of the liposomes containing 10 and 20 mol % of PA show several differences. These are, in particular, observed in the bands at around 1140 cm^{-1} , 1185 cm^{-1} , and 1575 cm^{-1} . The band at 1185 cm^{-1} is assigned to the vibration of the ester group of the polar head [210] (Table 5.1.), while 1575 cm^{-1} is assigned to the C–N stretching of the choline group [207]. These are vibrations of the polar head and they decrease in relative intensity (Figure 5.10.). These changes suggest that the increase in the amount of the PA in the liposomes diminishes the interaction between the polar head of the liposomes and the gold nanoparticles. If the negative charge of the liposomal surface increases, it is likely that it leads to repulsion between the liposomes and the negatively charged surface of the gold nanoparticles. Also, it is suggested that the presence of PA in the liposomes induces a high curvature in the membranes [228], and it may additionally influence the interaction with the nanoparticles, which is reflected in the SERS spectra. This is an indication that a changed interactions between the polar heads and gold nanoparticles induced changes in the acyl chain as well. The strong band at 1140 cm^{-1} , assigned to the C–C stretching of the alkyl chain, is completely diminished in the SERS spectra of the liposomes containing a higher amount of PA.

When compared to the SERS spectra of the ternary mixture that does not contain PA (see Figure 5.8.), the SERS spectrum of liposomes containing 20 mol % PA highly resembles the spectrum of the scrambled lipid molecules. In section 5.6. was concluded that the charge plays an important role in the interaction between the liposomes and gold nanoparticles, and that an excess of the citrate ions that are negatively charged can induce scrambling of the liposomes interacting with the nanoparticles (Figure 5.8.). The spectra in Figure 5.10. show that a negative charge introduced with PA leads to similar interaction between the liposomes and the nanoparticles, regardless of the origin of the negative charge.

5.9 Interaction of desipramine with lipid bilayers in the presence of nanoparticles

Studying the interaction of desipramine and lipid bilayers, in this case, can be approached in two different ways. The first is to add desipramine to already prepared liposome-nanoparticles composites and to monitor the changes induced upon addition of desipramine. Alternatively, the second approach would be addition of desipramine to gold nanoparticles before incorporation into liposome-nanoparticles composites, and to observe changes in the interaction after the addition of lipids.

Addition of desipramine solution to the liposome-nanoparticles composites, even after longer incubation, did not lead to observable changes in the SERS spectra (data not shown). In contrast, mixing of desipramine with nanoparticles before the incorporation into liposome-nanoparticle composites yielded the SERS as shown in Figure 5.11.

Figure 5.11. Representative SERS spectra of liposome-gold nanoparticles composites (blue trace) and liposomes-desipramine-gold nanoparticles composite. Liposomes were composed of 10 mol % PA, 10 mol % SM 20 mol % CH, and 60 mol % PC. Total lipid concentration: 2 mM. Excitation wavelength: 633 nm, intensity: $3 \times 10^4 \text{ W cm}^{-2}$, acquisition time: 5 -15 s. Scale bars: black trace, 500 cps, blue trace, 50 cps.

The coating of nanoparticles with desipramine prior to mixing with liposomes leads to the changes in the spectral signature of the liposomes. In the presence of desipramine, the changes in the spectra are mainly related to the bands assigned to modes of hydrocarbon chains. Specifically, the band at 1140 cm^{-1} decreases in relative intensity, and the band at 1077 cm^{-1} is almost completely diminished. As previously stated, these two bands are usually an indication of ordered chains in the *trans* conformation. Moreover, a significant change can be seen in the band at 1440 cm^{-1} and in general in the region from 1380 cm^{-1} to 1570 cm^{-1} . This region is mainly assigned to different deformation of CH_2 and CH_3 groups [209]. The ring moiety of desipramine intercalates between the acyl chains, leading to a disordering of the chains, which is reflected in the SERS spectra. Vibrational signature of polar heads shows relatively small changes pointing out that, the presence of desipramine influences mostly acyl chains. The obtained results confirm that TCAs have a strong affinity toward lipid molecules. In a study using confocal Raman spectroscopy, it was shown also, that TCAs also induce disordering of lipid bilayers [178].

Interestingly, the SERS signature of the desipramine is diminished in the presence of lipids, while in the presence of culture medium changes in SERS spectra of desipramine are minor (see Figure 4.6.). The data in Chapter 4, have demonstrated that the interaction between gold nanoparticles and desipramine is not influenced by the presence of proteins in cell culture media. In contrast, the presence of the liposomes diminishes the interaction between gold nanoparticles and desipramine, suggesting a strong interaction between the antidepressants and the lipids.

The data shown in this chapter demonstrate the possibility to study the molecular structure of lipid vesicles with gold nanoparticles directly at the site of interaction by SERS. Evidenced by the structural information from SERS and cryo-EM, the gold nanoparticles change the structure of liposomes upon interaction with the lipids in the presence of a high amount of citrate in the solution, while at lower citrate concentration as well as in control samples comprising mixtures of pre-prepared liposomes with gold nanoparticles, the nanoparticles are adsorbed on the liposomal surface. The SERS spectra are characteristic of the vesicles' lipid composition and vary when cholesterol is present, indicating an altered interaction of the liposome with the surface of the gold nanoparticles. In intact vesicles, the data identify the polar head region as main contact site with the nanoparticles. Due to a different interaction of the head groups of phosphatidylcholine and sphingomyelin both with the gold nanoparticles and with cholesterol, the presence of cholesterol has different effects on the contacts of the vesicles with the nanoparticles. Moreover, liposomes containing negatively charged lipids exhibit a similar type of interaction to the liposome-gold nanoparticles composites prepared in the presence of high citrate concentration. It is shown that the interaction between the desipramine and lipid bilayers occurs and that desipramine interacts with lipids with high affinity.

Chapter 6

Influence of tricyclic antidepressants on lysosomal function

The interaction between tricyclic antidepressants (TCAs) and cells can be characterized by their accumulation in the cells' lysosomes, the detachment and degradation of acid sphingomyelinase (ASM), and finally, by the accumulation of sphingomyelin and other lipids in the lysosomes [13]. The influence of antidepressants on the lysosomal biochemistry is significant [13]. Localization of the gold nanoparticles in the vesicles of endo-lysosomal system represents an opportunity to use surface-enhanced Raman scattering (SERS) to follow the changes in the lysosomes caused by interactions with antidepressants. To that end, a set of experiments was performed in order to optimize SERS as a method to monitor changes in the lysosomal function. In Chapters 4 and 5, SERS spectra of the key players of ASM inhibition in cells, antidepressants, and lipids, are presented, and their interactions with the nanoparticles are discussed. Here, the cells are incubated with both, TCAs, desipramine (Des), imipramine (Imi) and amitriptyline (Ami), and gold nanoparticles. Part of this chapter is published and reprinted with permission from

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For the optimization of SERS as a method for monitoring the lysosomal function the following aspects must be considered:

- (i) Should the TCA molecules be administered to the cells before, after or together with gold nanoparticles?
- (ii) What is the optimal incubation time with the TCA molecules in order to induce a lipid accumulation that can be detected by SERS?

As know from previous experiments, depending on the time and the incubation conditions nanoparticles can be found in all vesicles of the endo-lysosomal system, while the TCAs are expected to be mainly entrapped in the lysosomes [12, 13]. In order to determine the most efficient way of detecting the interaction and influence of TCA molecules in the cells by SERS, three different experiments were preformed with a different sequence of incubation. A schematic representation of the performed cell incubation experiments is shown in Figure 6.1. In the first experiment, cells were first incubated with antidepressants for 24 h and then with gold nanoparticles for 6 h (Figure 6.1. A). The second experiment consisted of cells incubated first with nanoparticles for 6 h and then with antidepressants for 24 h (Figure 6.1. B). In the third experiment, the cells were incubated simultaneously with antidepressants and gold nanoparticles for 24 h (Figure 6.1. C). The controls consisted of cells incubated with nanoparticles for the same time period as in the respective experiments, and replacing the culture medium containing TCAs with standard culture medium. Before all SERS measurements, cells were washed with buffer, ensuring that obtained SERS signal originates from the inside of the cells.

Figure 6.1. Schematic representation of performed SERS experiments in the cells. (A) Cells were incubated with TCA molecules for 24 h prior to the incubation with gold nanoparticles for 6h. (B) Cells were incubated with gold nanoparticles for 6h prior to the incubation with TCA molecules for 24 h. (C) Cells were incubated with TCA molecules and gold nanoparticles for 24 h simultaneously. Figure reprinted with permission from Živanovic *et al.*, *ACS Nano* 2019, 13, 8, 9363-9375. Copyright 2019 American Chemical Society.

Based on previous research in our group, the optimal concentration of gold nanoparticles for SERS cell measurements was established to be 0.4 nM [128, 180]. Additionally, previous studies suggest that inhibition of ASM by different TCA molecules occurs in the concentration range of 1-10 μM TCA [52]. Therefore concentration for all three TCA molecules was chosen to be 10 μM .

6.1 Cells treated with antidepressants prior to treatment with gold nanoparticles

6.1.1 Nanoparticles aggregation and lipid accumulation in cells influenced by antidepressants

Fibroblast cells of the cell line 3T3 were grown in monolayers and incubated with TCA molecules for 24 h and 6 h with gold nanoparticles. In order to confirm the localization of gold nanoparticles and the overall state of the cells, they were chemically fixed, embedded in resin and sectioned for TEM imaging (for detailed protocol see 3.5 Materials and Method). All TEM images presented in this Chapter were obtained in collaboration with Petra Schrade and Prof. Dr. Sebastian Bachmann from Charité Universitätsmedizin, Berlin.

In Figure 6.2. and 6.3. TEM micrographs of fibroblast cells incubated with desipramine for 24 h and gold nanoparticles for 6 h are shown.

Figure 6.2. TEM micrographs of 3T3 fibroblast cells incubated with desipramine for 24 h prior to the incubation with gold nanoparticles for 6 h showing vesicle with a significant amount of lipids accumulated and associated with single or aggregated gold nanoparticles. The arrowhead in B indicates gold nanoparticle. Scale bar: 100 nm.

Figure 6.3. TEM micrographs of 3T3 fibroblast cells incubated with desipramine for 24 h prior to the incubation with gold nanoparticles for 6 h showing vesicles with significant myelin accumulation and associated with gold nanoparticles (A, C, D) and endolysosomal vesicle with aggregated gold nanoparticles (B). Arrowheads indicate gold nanoparticles (not all nanoparticles are labeled). Scale bar: 200 nm.

The TEM micrographs show a variety of the vesicles that contain gold nanoparticles. For example, vesicles that contain a single membrane (Figure 6.2. C and Figure 6.3. B), and vesicles that contain multilamellar membranes (Figure 6.2. A, B, D and Figure 6.3. A, C, D). Vesicles that contain multilamellar membranes are an indication of an accumulation of the lipids and are called myelin figures. This is in accordance with previously published results [44].

As evidenced by the TEM images, the nanoparticles are associated with vesicles that show significant lipid accumulation due to the exposure to the TCA molecules (Figure 6.2. C and D). The presence of the gold nanoparticles in the close proximity of the myelin figures suggests that probing of the lysosomal changes is in principle possible by means of SERS. However, gold nanoparticles are also located in the vesicles that do not accumulate lipids (Figure 6.3. B).

With careful examination of the vesicles' membranes and content, it is possible to differentiate between vesicles in different stages of the endo-lysosomal pathway. For example, late endosomes have mainly a round to oval shape and a size between 250 and 1000 nm [230]. On the other hand, lysosomes appear as spherical organelles, with an electron-dense structure full of proteins and lipids, similar in size to late endosomes. Lysosomes usually have a lamellar membrane and tubules that can be visualized [230]. Based on Figures 6.2. and 6.3. shown here and other of the acquired TEM micrographs, the vesicles observed in this experiment range from early endosome to lysosome. This outcome were expected due to the continuous exposure to the nanoparticles right before fixation of the cells. The nanoparticles are taken up over the course of 6 h, and while some of them are already located in the lysosomes, others may have been taken up just before fixation.

From TEM micrographs, two important conclusions can be drawn:

- (i) A significant accumulation of lipids is observed in the cells after 24 h of exposure to desipramine.
- (ii) The nanoparticles are predominantly associated with vesicles where a significant accumulation of lipids occurs

Figure 6.4. TEM micrographs of 3T3 fibroblast cells incubated with gold nanoparticles for 6 h showing localization of the gold nanoparticles. Scale bars in: A, C, D, E, F 500 nm, B 1 μ m.

TEM images of the control sample are shown in Figure 6.4. There, the nanoparticles are found as single particles or in small aggregates in different types of vesicles. The nanoparticle aggregates contain mostly between two and four single particles, which differs from desipramine treated cells, where aggregates are larger (compare Figure 6.3. and 6.4.) Electron-dense vesicles that can be identified as lysosomes with gold nanoparticles are observed (Figure 6.4. E). Moreover, a significantly smaller accumulation of lipids is observed in the control samples, and only very few vesicles show multilamellar membranes, in contrast to the treated cells.

Cryo X-ray tomography (cryo-XRT) offers 3D imaging of cells in a nearly native state [231]. By means of cryo-XRT, whole cells can be imaged without the need for chemical fixation and sectioning. It has been shown that cryo-XRT can be used for studying the localization of nanoparticles and their mechanism of uptake [112]. In this experiment, the fibroblast cells previously exposed to desipramine and nanoparticles are vitrified using liquid ethane cooled at the temperature of liquid nitrogen. Despite the benefits, resolution of cryo-XRT measurements is lower compared to TEM measurements. However, the two imaging methods can be combined for correlative analysis. While TEM images show localization and aggregation state of nanoparticles in a thin section of chemically fixed cells, cryo-XRT shows localization of nanoparticles in the 3D volume.

Figure 6.5. X-ray microscopic image (A) and a slice of tomographic reconstructions (B) of a vitrified 3T3 fibroblast cells after incubation with desipramine for 24 h and nanoparticles for 6 h in the culture medium. Arrowheads are pointing to the particles that are enclosed in vesicular structures. All images were acquired with a 25 nm zone plate (9.8 nm pixel size). Scale bars: 1 μm . Abbreviations: NM, nuclear membrane.

X-ray microscopic image (Figure 6.5. A) and a slice of a tomographic reconstructions (Figure 6.5. B) of a vitrified 3T3 fibroblast cells after incubation with desipramine for 24 h and nanoparticles for 6 h shows many dark vesicles, some associated with gold nanoparticles. All tomographic reconstruction presented in this thesis were done by Dr. Daniela Drescher, Humboldt-Universität zu Berlin. These vesicles that appear darker in the images and tomographic reconstruction are carbon- or nitrogen-rich organelles [232]. In TEM images of the same samples, these vesicles were identified as myelin figures. As Figure 6.5. indicates, the nanoparticles and their aggregates are associated with the membrane of these vesicles.

Figure 6.6. Projection images (A, B, C) and a slice of tomographic reconstructions (D) of a vitrified 3T3 fibroblast cells after incubation with gold nanoparticles for 6 h in cell culture medium. Arrowheads are pointing to the particles that are enclosed in vesicular structures. All images were acquired with a 25 nm zone plate (9.8 nm pixel size). Scale bars: 1 μm . Abbreviations: N, nucleus; NM, nuclear membrane.

In the images of the control sample (Figure 6.6.), smaller aggregates of nanoparticles enclosed in vesicles as well as single nanoparticles are clearly seen. In the slice of the reconstructed tomogram (Figure 6.6. D) the cellular ultrastructure is visible, including the nuclear membrane and mitochondria, and many vesicles. The results from TEM and cryo X-ray imaging are in a good agreement with each other.

6.1.2 SERS spectra

The observed SERS spectra from the cell mapping experiments reflect the biochemistry and dynamics of the endo-lysosomal vesicles. Vesicles of the endocytic pathway are very different and very dynamic structures, and the observed SERS spectra indicate the molecular changes in the environment of the nanoparticles [28, 229, 233]. As antidepressants have a significant effect on the lysosomal biochemistry and induce lipid accumulation, as seen in Figure 6.2. and 6.3., changes in the SERS spectra are expected. In Figure 6.7. the average SERS spectra of five desipramine treated cells and four control cells

are shown. Together with the average of the spectra (Figure 6.7. A and B), examples of single spectra are shown for comparison (Figure 6.7. C and D). Figure 6.8. displays SERS spectra of imipramine and amitriptyline treated cells, also here averages (Figure 6.7. A and C) and single spectra (Figure 6.7. B and D).

Figure 6.7. Average of SERS spectra of the cells treated with Des for 24 h, prior to the incubation of gold nanoparticles for 6h (A) and control cells exposed to gold nanoparticles for 6 h (B). Example SERS spectra from treated (C) and control cells (D). Excitation wavelength: 785 nm, acquisition time per spectrum: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar in: (A) and (B) 100 cps; (C) and (D) 20 cps.

Figure 6.8. Averages (A) and examples (B) of SERS spectra of the cells treated with Imi for 24 h, prior to the incubation of gold nanoparticles for 6h. Averages (C) and examples (D) of SERS spectra of cells treated with Ami for 24 h, prior to incubation of gold nanoparticles for 6h. Excitation wavelength: 785 nm, acquisition time per spectrum: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bars: 100 cps.

The average of the SERS spectra of different cells under the respective conditions exhibit a high degree of similarity (Figure 6.7. and Figure 6.8), indicating very low cell-to-cell variability. Moreover, the average SERS spectra of cells treated with different antidepressants exhibit a high degree of similarity (see Figures 6.7. A, and 6.8. A and C), but significantly differ compared to average spectra of control cells (Figure 6.7. B). This further suggests that the biomolecular composition of lysosomes of treated cells is different from that in control cells. The three antidepressants have a similar effect on cells and activity of ASM in the lysosomes thus the similarities in SERS spectra are expected.

Comparing the SERS spectra of cells treated with TCA molecules and control samples the following differences are observed. Several bands around 1000 cm^{-1} contribute to the SERS spectra of treated cells more than to the spectra of control cells. These bands are mainly assigned to the phenylalanine and tyrosine amino acids [222]. All band assignments are given in Table 6.1. This difference suggests that more proteins and/or protein fragments are present in the treated cells. The band ratio between the bands at 1310 cm^{-1} and 1350 cm^{-1} changes in favor of 1310 cm^{-1} in treated cells. The band at 1310 cm^{-1} is assigned to the CH_3/CH_2 twisting mode of lipids, while the band at around 1350 cm^{-1} is assigned to CH deformation mainly from proteins [222]. The appearance of the modes assigned to lipid molecules in the SERS spectra suggests an abundance of lipids in the endo-lysosomal vesicles. In addition to the lipid band at 1310 cm^{-1} , the band at 415 cm^{-1} , assigned to sterols [74] appears in the SERS spectra of the treated cells. Moreover, in the single SERS spectra (Figure 6.7. C) fingerprints similar to the lipid spectra of the vesicle models, discussed in Chapter 5, are seen, pointing to a lipid-rich environment. For example, Figure 6.7. C, bottom trace, shows a spectrum from a desipramine treated cells with bands at 660 cm^{-1} , 1078 cm^{-1} , 1450 cm^{-1} and 1560 cm^{-1} , all of which are assigned to the different vibrational modes of lipids.

The most prominent band in the spectra of treated cells is a band at around 1130 cm^{-1} , while in the spectra of control cells these are several bands between 1300 and 1460 cm^{-1} . The band at around 1130 cm^{-1} is assigned to C–C stretching of long lipid chains [211], again proving a strong contribution of the lipid-related vibrational modes in the SERS spectra of treated cells. On the contrary, the strong deformation mode of the CH_2 group of lipid molecules around 1440 cm^{-1} decrease in relative intensity in the spectra of the treated cells. Spectra also exhibit differences in the band around 1220 cm^{-1} assigned to proteins. Treated

and non-treated cells also share many bands that are assigned to protein vibrations, e.g., bands at around 500, 650 and 830 cm^{-1} .

Even though the high concentration of TCA molecules is expected in the cells' lysosomes after treatment for 24 h, the characteristic SERS signature of the respective TCA molecules as a whole signature, as observed in the SERS spectra shown in Chapter 4 (Figure 4.4.) was not found in spectra of the antidepressants treated cells. However, several strong vibrations characteristic of the rings of the TCA molecules can be identified, in particular bands at 780 cm^{-1} , 1030 cm^{-1} (e.g., Figure 6.7 A, and 6.8. A and B). Possibly due to the hydrophobic nature of TCA molecules and their interaction with the lysosomal membrane, the interaction with the gold nanoparticles is diminished, and only some vibrational modes can be observed. For example, the band at around 1030 cm^{-1} is the strongest band in the SERS spectra of TCA molecules outside the cells (see Figure 4.4.), and it is assigned to the C–C stretching vibrations of the aliphatic part of the molecule [159].

Table 6.1. Raman shifts and tentative assignments of important bands in the SERS spectra from antidepressants treated and control cells. Assignments based on Refs: [74, 112, 164, 210, 222, 223, 234, 235]. Table was reproduced with permission from Živanović *et al. ACS Nano* 2019, 13, 8, 9363-9375 [229]. Copyright 2019 American Chemical Society.

Raman shift /cm ⁻¹	Tentative assignments	Raman shift /cm ⁻¹	Tentative assignments
410	Phosphoinositol, CH ₂ bending	1503	-
420	Sterols, CH ₂ bending in the ring	1514	Proteins, N-H deformation
429	Cholesterol	1550	Trp, C=C stretching
505	Proteins, S-S stretching	1560	Trp, Amide II
641 / 651	Amino acids, C-S stretching	1572	Proteins, Amide II
713 / 728	Sterols, ring deformation	1602	Ergosterol, C=C str
750	Trp	1618	Trp
806	RNA, O-P-O stretching	1630	Lipids, C=C stretching
835	Tyr, ring breathing	1669	Cholesterol ester
893	Backbone CC skeletal	1674	Cholesterol
920	Proteins - proline	1696	Amide I
990	Phosphorylated proteins, P-O-P stretching	1681	Amide I
1030	Tyr, in plane ring deformation	1700	Amino acids, aspartic and glutamic
1086	Phospholipids, C-C stretching	1725-1754	Lipids
1130	Proteins, Tyr, C-C stretching		
1140	Lipids, C-C stretching		
1155	C-C, C-N stretching in proteins		
1160	-		
1174	Proteins, Tyr		
1188	asymmetric stretching of phosphate		
1211	Tyr, Phe, C-C ₆ H ₅ stretching		
1220	Amide III		
1230	asymmetric stretching of phosphate		
1252	Amide III		
1263-69	Lipids, fatty acids		
1278	Lipids and proteins, PO ₄ ³⁻ stretching and amide III		
1305	Proteins and lipids, CH ₂ /CH ₃ deformation		
1322	Proteins, CH ₂ , CH ₃ deformation		
1337 / 1344	Carbohydrates, C-O-H		
1336	Proteins		
1370	Lipids, CH ₂ deformation		
1381	Lipids, CH ₃ deformation		
1418	Amino acids, CO ₂ ⁻ stretching		
1440	Lipids, CH ₂ deformation		
1452	Lipids, proteins, carbohydrates, CH ₂ deformation		
1460	Tyr		
1465	Lipids		
1482	Amide II		
1495	Amino acids		

Averages of SERS spectra are convenient for representation and discussion of big data sets. However, some information might be lost compared to using single SERS spectra, e.g., spectral patterns of low occurrence in large data sets may average out. To be able to get a full overview of the bands in the SERS spectra, the frequency of the occurrence of bands was analyzed in single spectra. The frequency of occurrence was calculated as the number of times a band appears in all SERS spectra divided by the total number of the SERS spectra in one experiment. The frequency of occurrence for different bands is shown in Figure 6.9. Bands that can be assigned to proteins and antidepressants are shown in Figure 6.9. A and to lipids in Figure 6.9. B for easier comparison.

Figure 6.9. Frequency of occurrence of most prominent Raman bands in the SERS spectra of the cells treated 24 h with TCA molecules prior to 6 h incubation with gold nanoparticles. (A) Bands assigned to the antidepressants and proteins, and (B) bands assigned to lipids.

From the frequency of band occurrence, one can see a clear trend that shows a higher count of the bands in treated cells compared to control cells (Figure 6.9.). This is in accordance with a higher number of particles and vesicles being present in the treated cells as was seen in TEM micrographs (Figure 6.2. and 6.3.). The biomolecular composition in the lysosomes is different when antidepressants are present. Higher frequency of occurrence in treated cells suggest that more protein fragments and amino acids are present due to the inhibition of ASM, e.g., indicated by bands at 830 cm⁻¹, 1220 cm⁻¹, and more lipids are accumulated

(bands shown in Figure 6.9. B). The endo-lysosomal system of treated cells shows single spectra that are more similar between themselves than the control cells regarding the frequency of occurrence. The biggest differences in the frequency of occurrence are found in bands that are also different in the average SERS spectra (Figures 6.7. and 6.8.). Those bands are at around 420 cm^{-1} , 1000 cm^{-1} , 1030 cm^{-1} , 1130 cm^{-1} , 1220 cm^{-1} , 1270 cm^{-1} , and 1310 cm^{-1} . In contrast to the decrease in intensity of the band at around 1440 cm^{-1} in treated cells, its frequency of occurrence is higher than in control cells. This can be caused by a different interaction of the gold nanoparticles with the lipids in the presence of TCAs, and/or different lipid structure and the composition in the myelin figures. In general, it can be concluded that presence of antidepressants causes changes in the lysosomes, which is reflected in the SERS spectra of treated cells.

6.1.3 Analysis of SERS spectra by random forest algorithm and chemical imaging

Random forest (RF) analysis is used here for classification of SERS spectra of treated and control cells and for the selection of variables that are important for classification, i.e., bands. The so-called minimal depth (MD) method [147] is used for selection of the variables, and the important variables are determined based on the calculated threshold. The variables that are selected as important are shown in Table 6.2. Each variable is assigned to a specific vibration of the molecules, and their tentative assignment is shown in Table 6.1.

RF analysis reveals different bands that are relevant for the classification of treated and control samples, despite the high similarities in the average of SERS spectra of cells treated with different antidepressants. The bands in Table 6.2. are colored according to their assignment, bands that are most probably assigned to the proteins [222] are colored in green, to the lipid molecules [210] in yellow, and to the antidepressant molecules [159] are in red. The variables that are selected as the important variables are in agreement with the bands that frequently appear in SERS spectra of cells. Moreover, some of the bands appear as strong modes in the SERS spectra of treated cells (Figure 6.6. and 6.7.).

Table 6.2. Important bands selected based on minimal depth analysis of the SERS spectra of cells treated with TCA for 24 h, prior to 6 h incubation with gold nanoparticles. With red color are marked variables (bands) assigned to antidepressants, with yellow to lipids and with green to proteins.

TCA	Proteins					Lipids					Antidepressants			
Ami	505	746	833	1130		1018	1061	1138	1381	1458	961	1028	1537	
Des	505	818	1125	1505	1610	1013		1149		1457	780	871	1034	1538
Imi	505	1125	1487	1548	1607	1014		1151		1459			1032	1532

Important bands in the classification of cells treated with different antidepressants and controls, show significant overlap, e.g., 505 cm^{-1} , 1125 cm^{-1} , 1458 cm^{-1} , 1030 cm^{-1} , 1537 cm^{-1} . However, in amitriptyline treated cells, the lipid bands are represented more, while in desipramine and imipramine treated cells protein related bands are dominant. The most characteristic antidepressants signature is found in the desipramine treated cells with four bands assigned to the desipramine. All treated cells have common bands around 1030 cm^{-1} and 1537 cm^{-1} (last two columns in Table 6.2). The band at 1030 cm^{-1} is the strongest band in the SERS spectrum of antidepressants outside of the cells (see Figure 4.4 in Chapter 4), while the band at 1537 cm^{-1} changes in intensity with change in the pH outside of the cell (Figure 4.6.) [159]. The bands at 505 cm^{-1} and at around 1130 cm^{-1} are very strong bands in the average spectra and have an influence on the separation. Moreover, these bands have a high frequency of occurrence (Figure 6.9). Not all bands that have both, a high count and high intensity have an influence on the classification, e.g., bands at around 1450 cm^{-1} .

In order to relate some of the Raman signals to the lipid-rich environment, SERS spectra of the model system made of nanoparticles and liposomes and SERS spectra from cells were compared. Based on the SERS spectra of liposomes containing only sphingomyelin (SM) or liposomes containing SM and other lipids (shown in Figure 5.9. in Chapter 5), it can be seen that the band at around 1018 cm^{-1} is the strongest band in the spectra and can be assigned to sphingomyelin interacting with gold nanoparticles [185]. This band is selected as an important variable for separation between treated and control cells. As already discussed in Chapter 5, this band is assigned to a C–N stretching vibration of the choline polar heads in lipids and it is very characteristic in SM containing liposomes. This part of the sphingomyelin carries a positively charged nitrogen and has preferable interaction with gold nanoparticles. In addition, the band at 818 cm^{-1} is also assigned to SM liposomes and appears in the vesicles containing only SM (Chapter 5, Figure 5.9.) [185]. This band is identified as an important variable only in the case of desipramine treated cells.

Selection of the sphingomyelin related bands as important variables suggests that the content of sphingomyelin in the vesicles of the treated cells is important for the separation of treated cells and controls.

The important variables selected using the MD method do not take into account correlation between the variables. A surrogate minimal depth (SMD), variable selection method, is applied in order to assess variables relations, thus so-called related variables for each important variable are selected. The variables that show correlation to the important bands are presented in the Tables 6.3., 6.4., and 6.5. The main idea is to analyze related variables to get information about functional group or molecule that distinguish treated and control cells. Each important band is related to several other bands in the spectra. Similarly to the important variables, the related variables are assigned to one of the three classes of the molecules – proteins, lipids, and antidepressants. The relations of the bands assigned to antidepressants is shown in Table 6.3.

Table 6.3. Important bands assigned to antidepressants and their related variables selected based on SMD analysis of SERS spectra of cells treated 24 h with TCA prior to 6 h incubation with gold nanoparticles. With red color are marked variables (bands) are assigned to antidepressants, with yellow to lipids and with green bands to proteins. (Bands without color are unassigned modes).

Ami											
Important bands	Related bands										
961	776	866	880	892	928	1135	1272	1290	1302	1315	1329
1028	652	1128	1138	1208	1219	1309	1609	1624			
1537	515	835	1460	1573	1585	1593					
Des											
Important bands	Related bands										
780	581	589	722	729	882	897	964	1123	1273	1608	1632
870	579	903	938	1125	1270	1306	1325				
1034	641	669	715	746	753	1475	1493	1504			
1538	1446	1460	1488	1573							
Imi											
Important bands	Related bands										
1032	616	655	661	681	754	846	1055	1479	1530		
1532	514	1487	1576	1593	1605						

The common bands selected as important for cells treated with all three molecules are 1030 cm⁻¹ and 1537 cm⁻¹. In desipramine treated cells as important bands at 780 and 870 cm⁻¹ are selected while in amitriptyline treated cells, the band around 960 cm⁻¹ is selected as an important. The band at 780 cm⁻¹ is assigned to different modes of the rings

coupled with wagging of the N–H group from the aliphatic part. This band is related with three other bands from the molecule, 581 cm^{-1} , 882 cm^{-1} , and 960 cm^{-1} , while the band at 870 cm^{-1} is related to the band at around 580 cm^{-1} . All mentioned bands, important and related, are assigned to different ring modes of the molecules (for exact assignment see Tables 4.1., 4.2. and 4.3. in Chapter 4). In addition, the band at 780 cm^{-1} is related to vibrational modes assigned to lipids, 720 cm^{-1} and 1273 cm^{-1} . The band at around 720 cm^{-1} is assigned to the C–N stretching of the choline part of phospholipids, while the band at 1273 cm^{-1} is assigned to =CH deformation of the lipid chain (see Figure 5.9. and Table 5.1.). Relations between the ring modes of the antidepressants and these lipid bands may indicate an interaction between lipids and antidepressants in the cells. In principle, interactions between antidepressants and lysosomal membranes are based on the electrostatic interactions between negatively charged lipids in the inner lysosomal membrane, BMP, and positively charged amino group of antidepressants [59]. However, the ring part of the antidepressants molecules can also interact with the hydrophobic part of the lysosomal membrane [178]. Correlation of bands related to antidepressants and CH_2 deformation (1446 cm^{-1} , 1460 cm^{-1} , 1480 cm^{-1}) and C–C stretching (1055 cm^{-1} , 1135 cm^{-1}) of the hydrophobic part of the membrane suggests that an interaction of such type can occur in cells as well.

In Table 6.4. variables related to the important bands that are assigned to vibrational modes of lipid molecules are shown. Important bands assigned to lipid molecules are mainly related to other lipid bands, particularly in the case of amitriptyline treated cells.

Table 6.4. Important bands assigned to lipids and their related variables selected based on SMD in the cells treated with 24 h TCA prior to 6 h incubation with gold nanoparticles. With red color are marked variables (bands) assigned to antidepressants, with yellow assigned to lipids and with green bands assigned to proteins. (Bands without color are unassigned modes).

Ami											
Important bands	Related bands										
1018	501	653	837	1128	1223	1274	1310	1320	1636		
1061	415	653	828	834	1129	1309	1609	1624			
1138	1316	1341	1661								
1381	1168	1193	1243	1415							
1458	1397	1492	1530	1547	1576						
Des											
Important bands	Related bands										
818	492	518	545	626	650	665	1072	1399	1520	1530	1595
1013	506	834	1010	1127	1271	1312	1486	1504	1530	1608	1632 1614
1130	1274	1316	1698	1651	1681						
1149	1339	1364	1347	1355	1806						
1456	1492	1515	1530	1545							
Imi											
Important bands	Related bands										
1014	501	510	655	837	1125	1315	1480	1495	1532	1541	1748
1151	1315	1328	1340	1355							
1458	1495	1515	1533	1540	1556	1581					
1487	1532	1542	1577	1594	500						

Some important bands are also related to the variables assigned to vibrations of antidepressants (Table 6.4. red markings) or proteins (Table 6.4. green markings). In particular, the vibration of the lipid chains around 1460 cm^{-1} is very often related to the antidepressant band around 1530 cm^{-1} . The relations of lipid bands to each other might suggest that they originate from vesicles with a high local concentration of lipids. Having in mind that the Raman cross sections of lipids are significantly lower compared to that of some amino acids, the occurrence of many lipid bands suggests a high abundance of lipids in the vesicles containing nanoparticles, which is in accordance with TEM images of the desipramine treated cells (Figure 6.2. and 6.3.).

In Table 6.4., the variables related to the important bands that are assigned to vibrational modes of proteins are shown. Here, it can be seen clearly that the bands are related mainly to other vibrational modes of proteins (Table 6.5. green markings). Additionally, some of the bands are related to the bands assigned to lipid molecules (Table 6.5. yellow markings).

Table 6.5. Important bands assigned to proteins and their related variables selected based on SMD in the cells treated with 24 h TCA prior to 6 h incubation with AuNP. With red color are marked variables (bands) assigned to antidepressants, with yellow bands to lipids and with green bands to proteins. (Bands without color are unassigned modes).

Ami									
Important bands	Related bands								
505	415	501	654	837	1128	1271	1311	1640	
746	580	590	599	703	883	1112			
833	415	461	507	654	1128	1531	1598	1641	
Des									
Important bands	Related bands								
505	415	459	452	656	834	1130	1503	1608	1632
1125	909	1272	1316	1297	1680	1650	1702		
1541	1487	1468	1461	1575	1590				
Imi									
Important bands	Related bands								
505	381	373	460	655	836	1127	1620	1637	1478
1125	502	1127	1272	1293	1315	1657	1698	1733	
1548	511	518	645	1487	1497	1593	1605		
1607	376	417	459	510	657	1576	1644	1729	1741

Analysis of the relations between bands shows that all bands assigned to antidepressants are preferably related to lipid vibrational modes, while bands assigned to the lipid molecules are preferably related to the other vibrational modes of lipid molecules. Furthermore, the relation of different lipid modes suggests that the use of SMD method for the selection of related variables is a suitable method for studying patterns and relations in the SERS spectra.

To gain insight into the distribution of molecules represented by the important bands and related variables, some of the bands are mapped and shown in Figure 6.10. Chemical maps of two cells treated with amitriptyline are generated based on bands assigned to the proteins (505 cm^{-1} and 830 cm^{-1} , blue maps), lipids (1130 cm^{-1} and 1458 cm^{-1} , green maps), and antidepressants (960 cm^{-1} and 1030 cm^{-1} , red maps). Colocalization of lipids (green maps) and antidepressants (red maps) is high. However, lipids can be found where antidepressant molecules are not found (compare map at 960 cm^{-1} and map at 1130 cm^{-1}) suggesting that not all lipid content in the cells is necessarily correlated with antidepressants which is also seen in the relations of the bands. Lipids are also present in vesicles of the endo-lysosomal system that are not affected by antidepressants, which is further proven by correlation of the maps showing localization of lipids and proteins. This further suggests that we probe

vesicles of the endo-lysosomal system which are influenced by antidepressants, but also vesicles that are not.

Figure 6.10. Distribution of signals related to molecular species characteristic of amitriptyline treated cells, representing lipids (green), proteins (blue), and antidepressants (red) species together with bright field images of two exemplary cells. Chemical images are generated by mapping the intensity of the bands at 505 cm^{-1} of disulfide bond in proteins, 830 cm^{-1} of tyrosine, 960 cm^{-1} and 1030 cm^{-1} assigned to antidepressants, 1130 cm^{-1} of phospholipid alkyl chains, 1458 cm^{-1} to CH_2 deformation in phospholipids, and the full spectral range 300 – 1800 cm^{-1} .

6.2 Cells treated with gold nanoparticles pripr to treatment with antidepressants

Unlike, in the previous section, describing the cells incubated with gold nanoparticles prior to the influence of antidepressants, here, gold nanoparticles were pre-incubated in the fibroblast cells for 6 h and cells then were exposed to TCA in culture media for 24 h (Figure 6.1. B). In this way, it is ensured that no gold nanoparticles were taken up by the cells together with the TCA molecules. SERS spectra were obtained after 24 h long incubation with TCAs.

6.2.1 Morphology of the nanoaggregates

X-ray tomographic data of the fibroblast cells incubated with nanoparticles for 6 h prior to the incubation with the antidepressants for 24 h were obtained after vitrifying the cells. In Figure 6.11. X-ray microscopic images and a tomographic slice are shown. As evidenced by the cryo-XRT data, no single nanoparticles are found in the cells, but small and large nanoaggregates are formed. In the projection images (Figure 6.11. A-C) and the reconstructed tomograms (Figure 6.11. D), all nanoparticles are associated with the vesicular structures that appear darker compared to the rest of the vesicles. As previously discussed, the darker structures in the reconstructed tomograms and projections are related to the accumulation of the lipids in the lysosomes. Similar results are seen in TEM micrographs and cryo-XRT images in the experiment where cells were first exposed to antidepressants and then to nanoparticles (Figure 6.3. and 6.4.). This indicates that TCAs influence lysosomal function independently of the incubation sequence.

Figure 6.11. X-ray microscopic image (A, B, C) and a slice of tomographic reconstructions (D) of a vitrified 3T3 fibroblast cells after incubation with nanoparticles for 6 h and desipramine for 24 h in the culture medium. Arrowheads are pointing to the particles that are enclosed in vesicular structures. All images were acquired with a 25 nm zone plate (9.8 nm pixel size). Scale bars: 1 μm . Abbreviations: NM, nuclear membrane, N, nucleus.

After the continuous exposure for 6 h, the nanoparticles are located in all vesicles of the endo-lysosomal system. Removing the nanoparticles from the culture media during the incubation with the TCA molecules allows the nanoparticles to move along the endo-lysosomal pathway and to be located predominantly in the lysosomes. This offers several benefits, amongst them a lower variation of the SERS signal that occurs due to the localization of nanoparticles in vesicles of different stages of endocytic pathway. More importantly, this incubation sequence allows to monitor mainly the lysosomes that are known to be affected by TCAs. Thus, the SERS spectra of these samples should in principle reflect the biochemistry of the lysosomes influenced by antidepressants.

6.2.2 SERS spectra

Figure 6.12. A and B shows averages of SERS spectra of the desipramine treated cells and control samples, as well as examples of single spectra (Figure 6.12. C and D). The average spectra of the cells treated with imipramine and amitriptyline are shown in Figure 6.13. A and B, as well as examples of single spectra (Figure 6.13. C and D). As discussed before,

in both, treated and control samples, the average SERS spectra from different cells under the same conditions do not vary significantly, suggesting stability of the SERS signal in the cells and low cell-to-cell variability. Although all three antidepressants are known to have a similar influence on lysosomal function, the SERS spectra of amitriptyline and imipramine treated cells are similar (Figure 6.14. A and B), while the averages of the SERS spectra of desipramine treated cells are different (Figure 6.14. A). Differences in the spectra of cells treated with different antidepressants imply stronger influence of individual molecules on the SERS spectra from cells. For example, the spectrum shown in Figure 6.13. C (third trace from the bottom) is highly similar to SERS spectrum of imipramine observed in the model system outside of the cells (see Figure 4.4.).

In contrast to the average spectra of the control sample and samples that are exposed TCA molecules prior to nanoparticles, the spectra here are not dominated only by strong protein modes, but several additional modes appear, e.g., 680, 740, 811, 1202, 1225, 1530 cm^{-1} (Figures 6.12. and 6.13.). Some of them can be assigned to vibrations of antidepressant molecules, e.g., 960 cm^{-1} , the strong contributions at 1030 cm^{-1} , 1202 cm^{-1} , and 1530 cm^{-1} . The others are mainly assigned to the different lipid and protein vibrations (for assignments see Table 6.1.). The strongest bands in the average spectra of the treated cells are in the region between 1300 cm^{-1} to 1440 cm^{-1} . The bands in this region are assigned to different CH, CH₂, CH₃ vibrational modes of lipids and proteins [222]. An increase in intensity in this region compared to the control cells shows that in the lysosomes of the treated cells more lipids and proteins are observed. Moreover, the single spectra of the treated cells (Figure 6.12. C and D and Figure 6.13. C and D) show significant contribution of several important lipid bands, 1090 cm^{-1} (C–C stretching of the acyl chains), 1140 cm^{-1} (C–C stretching of the acyl chains), 1440 cm^{-1} (CH₂ deformation of the acyl chains). Overall, the presence of lipids and antidepressant bands is evidenced in these samples as indicated by the spectra.

Figure 6.12. AverageS of SERS spectra of cells incubated 6 h with gold nanoparticles prior to exposure to desipramine for 24 h (A) and control cells exposed to the gold nanoparticles 6 h and 24 h to standard culture medium (B). ExampleS SERS spectra from treated (C) and control cells (D). Excitation wavelength: 785 nm, acquisition time: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar in: A, B, and C 10 cps; D 50 cps.

Figure 6.13. Averages of the SERS spectra of the cells incubated 6 h with gold nanoparticles prior to exposure to amitriptyline (**A**) and imipramine (**B**) for 24 h. Examples SERS spectra from treated with imipramine (**C**) and cells treated with amitriptyline (**D**). Excitation wavelength: 785 nm, acquisition time: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bars: 50 cps.

In Figure 6.14., frequencies of occurrence of the bands in the SERS spectra of treated and control cells are shown. Bands assigned to proteins and antidepressants (Figure 6.14. A) and bands assigned to lipids (Figure 6.14. B) are shown separately. Control cells show a

clear trend of higher occurrence of almost all bands. Some of the bands in the treated and control cells show a similar occurrence, e.g., 500, 1350 cm^{-1} . A significantly higher count of the bands is notable particularly in the bands assigned to the proteins, e.g., 635, 835, 1005 cm^{-1} . The desipramine treated cells mostly show a higher frequency of occurrence of bands compared to the imipramine and amitriptyline treated cells. This points out strong influence of individual antidepressants on the interaction with nanoparticles and the cells that is also reflected in the average SERS spectra (Figure 6.12 A and Figure 6.13 A and C). The SERS spectra of cells treated with the different antidepressants show more uniformity when cells were first exposed to antidepressants and then to gold nanoparticles compared to reverse sequence.

Figure 6.14. The frequency of the occurrence of most common bands in the SERS spectra of fibroblast cells that are treated 6 h with gold nanoparticles prior to 24 h incubation with TCA molecules. (A) Bands assigned to the antidepressants and proteins, and (B) bands assigned to lipids.

In addition, the vesicles of endocytic pathway other than lysosomes also contribute less to the SERS signal allowing individual difference of TCAs to be more pronounced.

6.2.3 Analysis of SERS spectra by random forest algorithm and chemical imaging

Important bands selected based on minimal depth (MD) analysis of random forest classification of the SERS spectra from treated cells and controls are shown in Table 6.6. The most of the important bands for the separation are assigned to different vibrations of lipid and TCA molecules (red and yellow markings in Table 6.6.). Only a few bands assigned to proteins are selected (red markings in Table 6.6.). Furthermore, in the data of imipramine treated cells, significantly fewer bands are selected as important compared to the desipramine and amitriptyline treated cells (last two rows in Table 6.6.)

Table 6.6. Important bands selected based on minimal depth analysis of the SERS spectra of the cells incubated 6 h with gold nanoparticles prior to treatment with TCAs for 24 h. With red color are marked variables (bands) that are assigned to antidepressants, with yellow to lipids and with green to proteins. (Bands without color are unassigned modes).

Ami										
620	691	783	869	960	1028					
721	1082	1090	1138	1165	1180	1276	1420	540	610	886
640	669	829	853	1340	1607	1618	1253			
Des										
1026	1036	1533								
1277	1325	1380	1432	1437	1458	1468	1477			
750	837	1237	1505	1554						
Imi										
1028										
1093	1087	1098	1111							

Several characteristic bands of the antidepressant molecules are selected as important variables in the classification of the amitriptyline treated cells and controls, while in the case of the desipramine and imipramine treated cells, only few bands are selected. The important bands assigned to antidepressants in the amitriptyline treated cells are assigned to both, ring modes and to the aliphatic side chain modes, while in the case of the desipramine and imipramine bands are assigned only to modes of the ring moiety. This is in agreement with the SERS spectra of antidepressants outside of the cells (Chapter 4, Figure 4.4.), when the interactions of amitriptyline was also discussed and shown to be different compared to imipramine and desipramine [159]. The important bands assigned to antidepressants overlap with the bands that are selected as important in that of cells with

reverse sequence of incubation (see Table 6.2.). There, the important bands of cells treated with different antidepressant molecules were similar. In contrast, here, higher variability of important bands is observed for cells treated with the different molecules. These findings point toward a greater influence of each individual antidepressant molecules on lysosomes and gold nanoparticles. For example, it is known that the activity of antidepressants is dose dependent, and amitriptyline shows higher activity in cells compared to desipramine and imipramine [14, 52]. Interestingly, almost all lipid bands selected as important in the amitriptyline treated cells (Table 6.6. second row), can be found in the SERS spectra of the liposome-nanoparticles model system (Chapter 5, Figure 5.9.) [185]. In the case of desipramine and imipramine treated cells, the important bands assigned to lipids are mainly assigned to the different modes of the hydrophobic tails of the lipid molecules, while in the amitriptyline treated cells, bands of the polar head also are selected, e.g., 610, 720, and 1180 cm^{-1} .

The bands related to those identified as important are selected using the SMD approach are shown in Tables 6.7., 6.8., and 6.9. The Tables are divided accordingly to the assignments of the important band to antidepressants (Table 6.7.), lipid (Table 6.7.), and proteins (Table 6.7.). First, the relations of the bands assigned to antidepressants will be analyzed.

Table 6.7. Important bands assigned to antidepressants and their related variables selected based on SMD in the cells incubated 6 h with gold nanoparticles prior to the treatment with TCA for 24 h. With red color are marked variables (bands) assigned to antidepressants, with yellow to lipids and with green bands to proteins. (Bands without color are unassigned modes).

Ami															
Imp. bands	Related bands														
620	371	429	441	478	522	536	666	672	685						
691	544	570	577	598	609	753	883								
783	568	722	731	879	894	913	1216	1261	1283	1323	1364	1351	1312		
869	716	772	792	924	962	966	1274	1279	1286	1292	1303	1330	1366		
960	715	725	732	737	768	776	875	888	900	931	1368	1373	1381	1426	
1028	842	998	1446	1486	1500	1505	1514	1523	1530	1538					
Des															
Imp. bands	Related bands														
1026	1498	584	663	668	678	688	733	749	771	789	849	868	890	911	970 1259
1036	565	584	672	705	698	722	735	749	767	784	861	900	929	948	970 1267
1533	1205	1432	1455	1479	1494	1563	1593								
Imi															
Imp. bands	Related bands														
1028	510	493	653	838	1128	1135	1623								

In Table 6.7. important bands assigned to antidepressants and their related bands are shown. The bands assigned to the respective antidepressants in amitriptyline and desipramine treated cells are related to the several other characteristic bands of each antidepressant. For example, the important bands of amitriptyline around 870 cm^{-1} and 960 cm^{-1} show strong correlation, which is evidenced by their mutual function as important and related bands (Table 6.7. rows 4 and 5). Other vibrational modes of the antidepressants appear as well, e.g., bands at around 580, 522, 1530 cm^{-1} .

Moreover, important bands assigned to the antidepressants are further related to the variables assigned to the vibrational modes of lipid molecules. Only a few protein bands are selected as related. In accordance with the findings reported in section 6.1, one can conclude that bands assigned to the TCA molecules and lipids are correlated, pointing towards a strong interaction between the lipid membrane and antidepressant molecules in the lysosomes.

Table 6.8. Important bands assigned to lipids and their related variables selected based on SMD in the cells incubated 6 h with gold nanoparticles prior to the treatment with TCAs for 24 h. With red color are marked variables (bands) assigned to antidepressants, with yellow to lipids and with green to proteins. (Bands without color are unassigned modes).

Ami																	
Imp.	Related bands																
721	564	580	768	792	879	890	907	1259	1281	1324							
1082	1162	1184	1191	1548	1564	1575	1173										
1090	1158	1166	1175	1191	1202	1754											
1138	1278	1316	1709	1659													
1165	1220	1238	1284	1373	1703	1687	1774	1799									
1180	1219	1234	1243	1272	1677	1693	1704										
1253	1153	1167	1174	1223	1288	1307	1688	1702									
1276	1127	1137	1225	1235	1300	1322	1686	1704									
1420	1155	1166	1176	1203	1219	1231	1237	1241	1258	1271	1279	1374	1695				
540	585	599	711	749	762												
610	540	585	675	695	715	728	762										
1188	1234	1241	1684	1693	1702	1711											
886	714	731	753	765	780	792	918	924	961	972	1259	1277	1309	1328	1370	1374	1427
1580	1192	1428	1436	1455	1467	1485	1532	1548	1580								
Des																	
Imp	Related bands																
1277	719	780	770	872	892	903	913	924	1130	1144	1223	1306	1679	1689			
1325	568	724	783	794	868	904	915	923	942	1133	1287	1355	1420	1663	1690	1701	1706
1380	781	793	869	896	905	920	925	1177	1250	1279	1336	1351	1410	1690			
1432	566	730	768	780	791	808	1191	1211	1236	1246	1286	1357	1372	1388	1398	1462	1521
1437	730	771	781	797	807	818	1162	1172									
1458	1198	807	816	1190	1509	1521	1532	1547									
1468	810	816	1191	1198	1207	1520	1542	1557									
1477	660	809	818	1505	1546												
Imi																	
Imp	Related bands																
1093	1172	1161	1189	1203	1220	1244											
1087	1170	1191	1415														
1098	1158	1191	1202	1210	1244	1244	1250	1420	1427	1693	1702	1168					
1111	1219	1255	1266	1290	1304	1313	1663	1704	1715								

In Table 6.8. important bands assigned to lipids and their related bands are shown. Most of the important bands are related to other vibrational modes of lipid molecules (yellow marking in Table 6.8.). Unlike the experiment with reverse sequence, where the lipid bands were mostly related to other lipid bands, here one can see that the lipid modes are also related to antidepressants bands and some of the protein bands (see red and green markings

in Table 6.8.). For example, the band at around 721 cm^{-1} , assigned to C–N stretching from the choline group, is related to four different bands assigned to the amitriptyline (Table 6.8. first row). These bands (564 , 580 , 768 , and 792 cm^{-1}) are mainly assigned to different ring modes and wagging of the amino group from the aliphatic chain of amitriptyline. This suggests a correlation of vibrations of the choline part of the lipid bilayers with vibrations of amitriptyline. Moreover, the bands at 540 and 886 cm^{-1} , assigned to cholesterol and choline head group vibrations, are also related to amitriptyline bands. In the case of the desipramine treated cells, the bands around 1325 , 1380 , and 1430 cm^{-1} , assigned to vibrational modes of the alkyl chains of the lipid molecules, are also associated with bands that can be assigned to the antidepressant. Similarly, like in the amitriptyline treated cells, the related bands assigned to antidepressants mainly originate from the different ring modes. In contrast, the bands at 1325 cm^{-1} and 1432 cm^{-1} are related to the band at around 560 cm^{-1} that is assigned to the wagging of N–C from the amino group in the aliphatic chain of desipramine. The bands at 1325 cm^{-1} and 1432 cm^{-1} are assigned to CH_3CH_2 wagging and CH_2 deformation, respectively. These results show that the aliphatic chain of antidepressants is correlated with the aliphatic chains of lipids.

Table 6.9. Important bands assigned to proteins and their related variables selected based on SMD in the cells incubated 6 h with gold nanoparticles followed by treatment with TCA for 24 h. With red color are marked variables (bands) assigned to antidepressants, with yellow to lipids and with green bands to proteins. (Bands without color are unassigned modes).

Ami													
Important	Related bands												
829	504	655	1000	1014	1022	1127	1505	1521	1544	1487			
1607	515	520	548	1461	1484	1523	1539	1561					
1618	389	416	463	490	507	650							
640	357	367	428	441	466	479	660	683					
669	523	535	619	638	701	737	747						
853	701	710	718	739	766	802	881	911	925	962	973	1379	1390
1340	1139	1272	1286	1300	1681	1704							
Des													
Important	Related bands												
837	505	655	1053	1067	1074	1129	1137	1315	1323	1510	1605	1619	1631
1237	1147	1151	1277	1636	1693	1693	1076	1721	1777				
750	542	565	572	579	588	606	679	695	704	861	870	884	915
1505	816	1449	1553	1593	1591								

In Table 6.9. important bands assigned to proteins and their related bands are shown. From Table 6.9. one can see that the bands related to the protein bands can be assigned to different

molecules. This significantly differs from the experiment with reverse sequence where protein bands were mainly related to other protein bands (Table 6.5.).

In order to get a better insight into the distribution of molecules in the cells, different bands selected as important are mapped and showed in the chemical maps in Figure 6.15. Maps generated based on antidepressants are shown in red, based on lipid bands in green, and on protein bands in blue. In all maps, high co-localization of all components is found. The strong correlation between all components shown in the chemical maps is the agreement with the correlation found in the SMD analysis.

Figure 6.15. Distribution of signals related to molecular species characteristic of amitriptyline treated cells, representing lipids (green), proteins (blue), and antidepressants (red) species together with bright field images of two exemplary cells. Chemical images are generated by mapping the intensity of the bands at 620 cm^{-1} , 780 cm^{-1} , 960 cm^{-1} , and 1030 cm^{-1} assigned to antidepressants, 1140 cm^{-1} of phospholipid alkyl chains, 1280 cm^{-1} to CH_2 deformations of phospholipids alkyl chains, 1420 cm^{-1} to CH_2 deformation in phospholipids, and 830 cm^{-1} of tyrosine, 1340 cm^{-1} of CH_2 deformations in proteins, and the full spectral range $300 - 1800\text{ cm}^{-1}$. Adapted in part with permissions from Živanović *et al.* *ACS Nano* 2019, 13, 8, 9363-9375 [229]. Copyright 2019 American Chemical Society.

Based on the analysis of important and related bands it is possible to get an insight into the interactions between antidepressants and the cells. From the related variables, it is clear that there is a correlation between bands assigned to antidepressants and lipids. These correlations indicate possible interaction of antidepressants and lipids inside the cells. Furthermore, the SERS spectra exhibit more bands assigned to antidepressants and lipids when the nanoparticles are mainly contained in the lysosomes, where they are at the site of the interactions of antidepressants.

6.3 Cells treated with gold nanoparticles and antidepressants simultaneously

Here, experiments where fibroblast cells were exposed to gold nanoparticles and antidepressants simultaneously will be discussed. The strong interaction of the antidepressant molecules and the gold nanoparticles have been shown outside of the cell and in cell culture medium (Chapter 4, Figure 4.2.) [159]. Antidepressant molecules cause aggregation of gold nanoparticles, which is observable in the UV-vis spectra shown in Figure 4.2. Moreover, the SERS spectra of antidepressants were found to change only slightly in the presence of cell culture medium (see Figure 4.6.) suggesting that the antidepressants remain on the surface of the gold nanoparticles in the presence of proteins from the cell culture medium. In the results discussed in the following, the concentration of antidepressant molecules is significantly higher compared to the available surface area of the nanoparticles, thus some of the antidepressant molecules could be delivered to the cell separately. The fibroblast cells were exposed for 6 h and 24 h to antidepressant molecules and nanoparticles simultaneously, as described in the beginning of this Chapter.

6.3.1 Influence of the antidepressants on the aggregation of the nanoparticles

Figure 6.16. shows TEM micrographs of cells treated with desipramine and nanoparticles. As evidenced by the TEM images the gold nanoparticles form aggregates of varying size, and no single particles are found. The nanoparticles are already aggregated with antidepressant molecules prior to their delivery to cells (Figure 4.2.) and obtained TEM images suggest that the pre-formed aggregates remain as aggregates in the cellular

environment as well. From the TEM images it can be seen that the appearance of multilamellar vesicles due to the lipid accumulation has started after 6 h exposure to the TCAs (Figure 6.16.). However, the amount of vesicles that show accumulation of the lipids is significantly lower than after 24 h exposure (see Figure 6.2.). Interestingly, gold nanoparticles are associated almost exclusively with the vesicles where that formation of myelin figures started.

Figure 6.16. TEM micrographs of 3T3 fibroblast cells incubated with desipramine and gold nanoparticles simultaneously for 6 h showing nanoparticles localized in different vesicles inside the cells (endosomes, lysosomes) and formation of the myelin figures (A, B). Arrowheads indicate gold nanoparticles (not all nanoparticles are labeled). Scale bars in: (A), 500 nm; (B, C, and D), 200 nm.

Cryo-XRT data of the samples exposed to desipramine and nanoparticles were obtained. Figure 6.17. shows slices of reconstructed tomographic data. There the influence of desipramine on the interaction of the nanoparticle with cell is clearly visible. In the case of the co-incubation (Figure 6.17.), large nanoparticle aggregates are formed that appear to be attached for the endo-lysosomal membrane. Vesicles associated with the gold nanoparticles appear darker in the tomograms. The darker appearance of some of the vesicles is in agreement with the accumulation of lipids caused by the presence of desipramine, that was discussed in the previous sections. Additionally, the formation of multivesicular bodies can be seen in some instances (Figure 6.17. A). Cryo-XRT data are in agreement with the TEM

micrographs shown in Figure 6.11. where the association of gold nanoparticles and vesicles with accumulated lipids is also seen. Besides, the cellular ultrastructure is clearly visible, especially the mitochondria and nuclear membrane.

Figure 6.17. Slices of tomographic reconstructions of a vitrified 3T3 fibroblast cells after incubation with nanoparticles and desipramine simultaneously for 6 h. Arrowheads are pointing to the particles that are enclosed in vesicular structures. All images were acquired with a 25 nm zone plate (9.8 nm pixel size). Scale bars: 1 μ m. Abbreviations: N, nucleus; NM, nuclear membrane.

Figure 6.18. Slices of tomographic reconstructions of a vitrified 3T3 fibroblast cells after incubation with nanoparticles and desipramine simultaneously for 24 h. Arrowheads are pointing to the particles that are enclosed in vesicular structures. All images were acquired with a 25 nm zone plate (9.8 nm pixel size). Scale bars: 1 μm . Abbreviations: N, nucleus; NM, nuclear membrane.

Cryo-XRT data were also obtained from cells exposed to desipramine and gold nanoparticles simultaneously for 24 h and shown in Figure 6.18. Similarly to the 6 h exposure, the nanoparticles are also agglomerated and enclosed in vesicles that appear darker compared to the rest of the cells. The formed aggregates are larger, compared to 6 h exposure and clearly associated with the membrane of the endo-lysosomal vesicles (Figure 6.18. A and B). The cryo-XRT and TEM data suggest that the nanoparticles and desipramine co-localize in the cells, thus providing the optimal conditions for the SERS experiments.

6.3.2 SERS spectra

SERS spectra of cells treated with TCA molecules and nanoparticles simultaneously were collected. Figure 6.19. shows average and single spectra of cells treated with gold nanoparticles and desipramine simultaneously for 24 h and SERS spectra of the control samples. Exposure to antidepressants gives rise to different SERS spectra with significantly higher background than for controls cells. The higher background can be a consequence of relatively large nanoparticles aggregates (see also Figure 6.18.). Due to the significant background in the averages of the SERS spectra of the treated cells, the bands appear less

pronounced (Figure 6.19. A). In Figure 6.20., averages and examples of SERS spectra of cells treated with imipramine and amitriptyline are shown. The average SERS spectra of cells treated with amitriptyline show higher background, similar to the desipramine treated cells, which is not the case in imipramine treated cells (Figure 6.20. A and B).

Figure 6.19. Average of SERS spectra of cells treated with desipramine and gold nanoparticles simultaneously for 24 h (A) and control cells exposed to gold nanoparticles for 24 h (B). Examples SERS spectra from treated (C) and control cells (D). Excitation wavelength: 785 nm, acquisition time per spectrum: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar in: (A) and (B) 100 cps; (C) and (D) 20 cps.

Figure 6.20. Average of SERS spectra of cells treated with imipramine (**A**) and amitriptyline (**C**) gold nanoparticles simultaneously for 24 h. Example SERS spectra from cells treated with imipramine (**B**) and amitriptyline (**D**). Excitation wavelength: 785 nm, acquisition time per spectrum: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar in: (A) and (B) 100 cps; (C) and (D) 20 cps.

In the control cells, typical protein bands are pronounced, e.g., bands around 500 cm^{-1} , 1000 cm^{-1} , and 1230 cm^{-1} . In the averages of the SERS spectra of the treated cells, dominant bands are located around 1130 cm^{-1} , 1315 cm^{-1} , and 1350 cm^{-1} , as well as in the single spectra. The bands around 750 cm^{-1} , 960 cm^{-1} , and 1560 cm^{-1} have a higher relative intensity in the SERS spectra of the treated cells. These bands are assigned to different vibration of antidepressant molecules.

Randomly chosen examples of single spectra of the treated cells show a high similarity implying that the chemical surrounding of the nanoparticles is highly similar in the treated cells, for each of the antidepressants (Figure 6.19. C and Figure 6.20. C and D). In contrast, the individual SERS spectra of the control cells show more diversity (Figure 6.19. D). The similarities in single spectra may point to the preservation of the nanoaggregates formed before endocytosis. Assuming that the pre-formed aggregates carrying molecules produce the SERS signal inside the cells one would expect the TCA spectrum that is very similar the one of the model system (see Figure 4.4.). However, a clear SERS spectrum that shows the whole TCA fingerprint is not found in the SERS spectra of treated cells.

Figure 6.21. The frequency of occurrence of most prominent Raman bands in the SERS spectra of the cells treated 24 h with TCA molecules followed by 6 g incubation with gold nanoparticles. (A) Bands assigned to proteins, and (B) bands assigned to the antidepressants and lipids.

To evaluate the representation of certain bands in the SERS spectra of treated and control samples, the frequency of occurrence of the bands was calculated and shown in Figure 6.21. The frequency of occurrence is sorted according to band assigned to proteins (Figure 6.21. A) and to lipids and antidepressants (Figure 6.21. B) for easier comparison. From the frequency of occurrence, it can be seen that bands in the spectra of the desipramine treated cells show a significantly higher occurrence compared to the other two antidepressants and the control samples. The treated cells show similarities in the occurrence of bands at 755, 1005, and 1560 cm^{-1} assigned to proteins (Figure 6.21. A) and bands at 855, 1100, 1180, 1445, 1460, and 1420 cm^{-1} assigned to lipids (Figure 6.21. B) (for assignment see Table 6.1.). Some of the bands that show differences amongst treated and control cells are assigned to antidepressant molecules (see Tables 4.1., 4.2. and 4.3).

An interesting feature of the SERS spectra of the treated cells is the decrease in intensity of the strong mode of proteins at around 500 cm^{-1} assigned to S–S stretching from the disulfide bridges. It suggests two possibilities. First, the protein tertiary structure is strongly influenced by the presence of antidepressants and nanoparticles, and a dissociation of the disulfide bridges is observed. Such an effect of the antidepressants on the proteins was not observed in the other samples exposed to antidepressants. Another possible explanation for a decrease of the bands at around 500 cm^{-1} is the interaction of the gold nanoparticles and the antidepressants prior to cell treatment. Gold nanoparticles and antidepressants are mixed before the addition to the cells. The SERS signature of the antidepressants is visible even after mixing with the culture medium proving that the presence of the proteins in culture medium does not diminish the interactions (Chapter 4, Figure 4.6.). In contrast, in the interaction of antidepressant and gold nanoparticles with the liposome (Chapter 5), the close contact with the antidepressants are diminished, and only slightly changed SERS spectra of the lipid components are observed (see Figure 5.11.). These data suggest that antidepressants on the surface of nanoparticles can prevent close contacts between the proteins and the nanoparticles. Thereby the proteins may not experience SERS enhancement and the protein bands are not visible in spectra. It is very obvious in the case of the band at 500 cm^{-1} , but is also found for the bands around 650 and 830 cm^{-1} are influenced.

6.3.3 Analysis of SERS spectra by random forest algorithm and chemical imaging

Important bands selected using the MD method in the classification of treated and control cells are shown in Table 6.9. Important bands are assigned to different modes of antidepressants, lipids, and proteins.

Table 6.10. Important bands selected based on MD analysis of the SERS spectra of the cells incubated with TCA molecules and gold nanoparticles for 24 h. With red color are marked variables (bands) that are assigned to antidepressants, with yellow to lipids and with green to proteins. (Bands without color are unassigned modes).

Ami											
1037	868	1205	383								
1060	1084	1151	532	1167	532						
1561	1511	1213	948	933	1101	1576					
Des											
446	1037	1205	380								
1061	1082	1084	1090	1160	1044						
1007	1591										
Imi											
591	1037	1205	1207	1544	384	1111					
410	1160	1084	1095	1167	1178	1463	1488	1044	1488	1178	919 532
1560	1591	1248	948	1516							

Common bands assigned to vibrations of TCA molecules for all three antidepressants are around 380 cm^{-1} , 1037 cm^{-1} , and 1205 cm^{-1} . The band at 380 cm^{-1} is selected as important and assigned to the N–C modes from rings and aliphatic parts of antidepressants. The band at around 1037 cm^{-1} is also assigned to the aliphatic part of the antidepressants, while all others are assigned to the vibrational modes of ring moiety.

Most of important bands assigned lipids represent typical vibrations of phosphor- and sphingolipids (see Table 5.1.). However, bands at around 401 cm^{-1} and 532 cm^{-1} , assigned to cholesterol are selected as important variables [163]. This suggests that in the surrounding of nanoparticles cholesterol can be found and detected. Due to the presence of antidepressants on the surface of nanoparticles and/or in their close proximity, the interaction of gold nanoparticles with cholesterol is more likely to occur. Appearance of cholesterol assigned bands in spectra is in accordance with previous study that show a possibility of accumulation of cholesterol due to inhibition of ASM [52]. Thus the amount

of cholesterol could be important for discrimination between treated and control cells. The contribution of bands assigned to protein to important variables is significant in these samples. These protein modes are mainly assigned to the vibration of the protein backbone.

Based on the selection of important bands, as well as on the morphology of nanoparticles in the cells and SERS spectra, simultaneous delivery of nanoparticles and antidepressants to cells seems to be rather different compared to other two samples with different incubation sequences. In particular, most of the important bands are specific for this experiment and show very little correlation with previous experiments. Possible reason could be different aggregation state and surface properties of nanoparticle in the presence of antidepressants.

Selection of variables related to the important bands was performed using the SMD method. Results of this analysis are shown in Tables 6.11., 6.12., and 6.13. Related variables are divided according to the assignment of important variables. In Table 6.11. are shown important variables assigned to antidepressants and their related variables.

Table 6.11. Important bands assigned to antidepressants and their related variables selected based on SMD analysis of SERS spectra of cells incubated with TCA molecules and gold nanoparticles for 24 h. With red color are marked variables (bands) assigned to antidepressants, with yellow to lipids and with green to proteins. (Bands without color are unassigned modes).

Ami															
Imp.	Related bands														
1037	970	883	980	874	862	1531	1516	1541	1466	1492	1503	1483	1453	761	730
868	886	925	768	792	719	724	1274	1267	1286	1362	1367	1376	1333	962	
1205	1240	1251	1701	1711	1689	1288	1166	1155	1300	1277	1309				
383	1642	1651	1666	1729	453	438	483	478							
Des															
Imp.	Related bands														
446	461	400	472	379	356	363	393	484	520	538	645	663	634		
1037	874	900	911	970	748	921	584	742	720	784	773	565	1262	1408	704
1205	1242	1228	1251	1175	1155	1284	1277	1711	1701	1112	1121				
Imi															
Imp.	Related bands														
591	568	720	727	770	774	874	893	1253	1241	1086	1275	1548	1438		
1037	747	757	687	683	968	977	990	675							
1205	1193	1769	1228	1703	1239	1253	1751	1717	1735	1693	1264				
1207	1228	1239	1706	1765	1251	1688	1718	1264	1172						
1544	1508	1495	1489	1474	1450	1463	1439								
384	364	400	450	411	461	421	431	1650	1636	481	1668	651	671	664	
1111	1264	1722	1715	1704	1257	1289	1303	1219	1666	1313					

Important bands assigned to antidepressants are correlated to other bands of antidepressant molecules, as expected. However, most of them are also correlated with different vibrational modes of proteins and lipids. It seems that the correlation between important bands chosen in this case is higher compared to the other experiments. Specifically, the band at around 1030 cm^{-1} is almost exclusively related with antidepressants and lipids in all three cases. The band at 1037 cm^{-1} is assigned to C–C stretching in the aliphatic chain of antidepressants, is correlated with vibrational modes assigned to alkyl chains. In addition, other important bands assigned to antidepressants are also related to the vibration of the lipid alkyl chains. These data suggest a close spatial distribution of antidepressants and lipid molecules in the nanoparticles surrounding. Considering the fact that antidepressants and gold nanoparticles are delivered together to cells, correlation of antidepressants and lipids suggest an interaction between these components. Furthermore, these bands are also in relation with bands assigned to proteins.

Table 6.12. Important bands assigned to lipids and their related variables selected based on SMD analysis of SERS spectra of the cells incubated with TCA molecules and gold nanoparticles for 24 h. With red color are marked variables (bands) that are assigned to antidepressants, with yellow to lipids and with green to proteins. (Bands without color are unassigned modes).

Ami																	
Imp.	Related bands																
1084	1064	1191	1184	1166	1200	1175	1548	1577									
1151	1704	1697	1681	1690	1675	1237	1229	1104									
532	1112	1226	1192	1124	1106	1268	1235	1245	1253	1316	1306	1278	1089	1184			
1167	1704	1692	1679	1372	1239	1228	1219	1247	1367	1211	1384						

Des																	
Imp.	Related bands																
1061	1046	1087	502	654	1618	1597	1128	972	827	900	956	949	933	941	1272	415	
1082	1058	503	827	832	1193	1184	1179	1591	1584	1171	1200	654	1163	1573	1607	1617	415
1084	1057	1191	1184	1180	502	1591	1585	1200	1173	1162	827	834	654	416	1156	1572	1565
1090	1071	1057	1191	1173	1200	502	1162	655	827	1590	1481	1154	415				
1160	1170	1820	1770	1705	1728	1241	1231	1688	1677	1251							
1044	900	948	932	964	911	874	756	709	719	727	585	773	1130	502	1330	1309	1408

Imi																	
Imp.	Related bands																
410	357	1634	375	384	460	488	493	1620	502	1679	1737						
1060	498	759	705	975	965	744	753	956									
1084	1061	1192	1184	1200	1279	1383	1173										
1095	1191	1174	1201	1166	1211	1422											
1167	1708	1691	1241	1211	1679	1255	1224										
1178	1196	1703	1693	1237	1211	1715	1782	1746	1734	1250	1262	1272					
1463	1440	1549	1538	1559	1522	1576	1388	1376	768	726	1190	1240	1190				
1488	1526	1548	1452	1576	1438												
1044	748	758	972	962	901	741	728	913	895	924	949	700	720				

In Tables 6.12. are shown variables related to the important bands assigned to lipids. Analysis of the related bands shows that lipid bands are correlated with other lipids, but also in a larger extent they are correlated to bands assigned to proteins. Several bands are also related to antidepressant bands, specifically, bands at 1060, 1084, 1456 cm^{-1} . All of these bands are assigned to different modes of the alkyl chain and are related to the bands from ring moiety of antidepressants, showing similar correlation like in the case of important bands assigned to antidepressants.

In the case of the related variables to protein assigned important bands, shown in Table 6.13. correlation to antidepressants, lipids and other protein bands is visible.

Table 6.13. Important bands assigned to proteins and their related variables selected based on SMD analysis of SERS spectra of the cells incubated with TCA molecules and gold nanoparticles for 24 h. With red color are marked variables (bands) that are assigned to antidepressants, with yellow bands that are assigned to lipids and with green bands that are assigned to proteins. (Bands without color are unassigned modes).

Ami														
Imp.	Related bands													
1561	1528	1518	1467	1474	1485	1498	1454	1437	1434	1198	1191	1575	1587	
1511	1548	1478	1573	1458	1442	1440	1191	1181	1187	1198				
1213	1237	1250	1285	1702	1711	1259	1277	1307	1184	1174	1166	1155		
948	895	872	906	1336	1345	1327	1315	779	1280	730	721	1135		
933	894	906	880	1331	1338	1358	1344	1297	1311	1279	1288	779	794	1135
1101	1205	1192	1218	1182	1175	1427	1434	1252	1244	1264	527			
356	405	454	1648	1654	1659	1666	1747	1735	1727					

Des														
Imp.	Related bands													
1591	1559	1533	1524	1515	1493	1485	1476	1470	1458	1437				
1007	1088	1454	1120	829	657									

Imi														
Imp.	Related bands													
1560	1535	1529	1517	1495	1482	1475	1468	1451	1445	1431				
1591	1556	1529	1517	1506	1488	1475	1456	845	515					
1248	1217	1699	1292	1280	1369	1192	1177	1116	1154					
948	927	901	872	781	1280	1338	1355	1361	720	730	767	1272	793	1309
1700	1693	1707	1774	1728	1669	1674	1680							
901	920	878	781	774	789	1281	1296	1336	1327	1259	1361	1351	730	724
919	895	1283	1329	1340	1315	780	1361	1290	1270	870	796	768	1133	721
933	911	899	1280	1313	1335	1327	1296	1275	1365	877	773	1261	1254	1135
356	383	460	1650	1659	1666	1636	432	1735	1744	1725	1776			
375	364	400	440	432	451	425	1652	1637	672	660	479			
532	1277	1114	1245	1123	588	614	564	579	755	712	766	1305	1315	1252
1516	1548	1565	1576	1459	1450									
1192	1231	1238	1245	1156	1702	1696	1264	1378	1369	1323	1331	1710	1688	

In the case of co-delivery of antidepressants and nanoparticles important bands show correlation between all molecules. This might be primarily due to delivery conditions suggesting that nanoparticles and antidepressants remain in cells together, even though clear SERS signature of antidepressants could not be observed. However, correlation between lipid molecules and antidepressants suggests a potential interaction of these molecules.

6.4 Summary

In cells incubated with antidepressants for 24 h prior to exposure to nanoparticles for 6 h, nanoparticles are located in the close proximity of accumulated lipids in lysosomes as evidenced by TEM and cryo-XRT data. The nanoparticles are also associated with other vesicles of the endo-lysosomal system. The SERS spectra reflect the chemical composition of all vesicles with nanoparticles and data analysis shows that bands assigned to antidepressants and lipids are important for the classification between treated cells and controls. An analysis of the SERS spectra showed that the antidepressants colocalize with lipids, while lipids do not colocalize always with antidepressants. When the incubation sequence is reversed, and cells are incubated with the nanoparticles prior to the treatment with antidepressants, most of the nanoparticles are located in the lysosomes together with antidepressants and lipids. In the classification of these spectra predominantly lipid and antidepressant bands are selected as important. Random forest analysis shows a strong correlation between all components, antidepressants, lipids, and proteins. Chemical maps showing distribution of these molecules also suggests colocalization of lipids, antidepressants, and proteins. When cells are treated with the antidepressants and gold nanoparticles simultaneously, the gold nanoparticles are found in the aggregates. Analysis of SERS spectra showed a high influence of lipids to the classification of spectra. Correlation between all molecules is observed, and the distribution maps show high colocalization. However, the interactions of antidepressants with nanoparticles when delivered simultaneously differ from those when the antidepressants and gold nanoparticles are delivered separately.

Regardless of the incubation sequence, all data show that in living cells the interactions between the lysosomal membrane and antidepressants is very likely to occur. The most informative results are obtained from the cells incubated first with nanoparticles and then with antidepressants, due to predominant localization of nanoparticles in the lysosomes.

Chapter 7

SERS characterization of Leishmania infected cells

Use of SERS as a sensitive tool to characterize the interaction of nanomaterials with molecules inside animal cells [27, 112], and to gain better insight into endosomal transport, [29] was established. Its potentials are used in this thesis to study of antidepressant interaction with cells in this thesis as well as for characterization of the interaction between liposomes and gold nanoparticles. Possibilities of SERS for studying lipid-rich environments are exploited and applied for studying biologically relevant Leishmania infection in macrophages. Leishmania-infected cells harbor large amounts of lipids in the special compartments called parasitic vacuoles. Due to their topological characteristics and possibility to clearly distinguish lipid-rich vacuoles, SERS mapping experiments are possible. SERS will be used to assess the distribution of lipids and other molecules in infected cells. Results presented here are done in collaboration with Dr. Geo Semini and Dr. Tony Aebischer from Robert Koch-Institut, Berlin. This chapter was published and reprinted with permission from *Živanovic et al., Anal. Chem. 2018, 90, 13, 8154-8161* [236]. Copyright 2018 American Chemical Society.

7.1 Leishmania parasite

Leishmania are protozoan parasites that alternate between extracellular flagellated forms transmitted by insects and intracellular forms called amastigotes that replicate in vertebrate cells. They are the cause of cutaneous and deadly visceral leishmaniasis with ~1 billion people worldwide living at risk [237, 238]. Amastigotes replicate in an endo-lysosomal compartment, the parasitophorous vacuole (PV) [239, 240]. The biology and composition of this compartment is thought to hold the key to understand what supports parasite replication or mediates containment [241] with likely impact on the course of disease. To date, PV biology has either been studied by (i) conventional immunofluorescence microscopy where analytes have to be known to allow detection by, e.g. fluorescently labelled antibodies [241] or in situ produced fluorescent proteins [242] or tracers [243], or (ii) the PV and parasites within them have been characterized by destructive approaches such as proteomics [239] or mass spectrometry of purified lipids [244].

The PV is thought to contain some particularly abundant molecular species of sterols and their esters [245], and proteophosphoglycans (PPG) [246, 247] that are secreted by the parasites. Studies showed that parasite- and host-derived lipids, phospho- and sphingolipids, and sterols are particularly important for the virulence of *Leishmania* [248-251]. For example, staining by filipin [245] suggested that cholesterol and cholesterol ester sequestration and local concentration around and within the parasites is relevant for the cholesterol-associated effects that govern disease outcome [245, 252]. However, this approach requires fixation, which is notoriously difficult for lipids and, in the case of filipin, suffers from a relatively low specificity of available reagents for the target molecular species (ref. [245] and refs therein).

Simultaneous probing of different molecular species and their localization in live macrophage cells infected with *Leishmania mexicana* in vitro, without purification or labelling, by SERS is achieved. Gold nanoparticles are employed, making use of the connection and interaction of endosomal compartments with the PV [253] in the macrophage infection model. The strong enhancement in SERS, in principle enables the detection of individual molecules [25]. Therefore, SERS microscopy could report on the biochemical environment of gold nanoparticle probes that are introduced into the infected cells and gives information about the molecular composition of different subcellular areas, e.g., the amastigote, the PV, and non-parasitized endocytic structures.

In most reported applications of SERS nanoprobe to cultured cells, the cellular interior is reached by the process of endocytosis, where gold nanoparticles are taken up from the culture medium into endosomal vesicles. Macrophages and the *Leishmania* parasites represent a uniquely suitable, real biological model because of the size of distinct cellular compartments formed by the infected cells [239, 254], and because of the easy microscopic identification of the PV and the amastigote parasites. The mobility and interaction of endosomal structures that contain SERS probes with the vacuoles [253] is exploited and spectra are acquired from different positions in the infected cells, in particular the PV and the immediate surroundings of the parasites themselves. As data show, the nanoscopic SERS approach allows monitoring of the molecular milieu in the vacuole at very high sensitivity, identifying high abundance locations of cholesterol and ergosterol, as well as of PPG, all known to be characteristic components of *L. mexicana* harbouring PV. Further hallmarks of *Leishmania*'s molecular fingerprint, including lipids, proteins, and polysaccharides and compare the spectra to those obtained from typical, non-parasite infested phagolysosomal structures in the same macrophage cells are discussed. The characterization of the biochemistry of both the parasite and the host cells, off-resonance with electronic transitions in the biomolecules will be discussed, and the distribution of several different molecular species is analyzed.

7.2 SERS spectra from *Leishmania* infected cells

In the cells, SERS spectra are only generated from spots where the gold nanoprobe is located. Therefore, gold nanoparticles had to be taken up by primary macrophage cells which were infected by *L. mexicana* and had to reach the PV. To investigate precise localization of the nanoprobe, parallel to the samples for SERS micromapping, samples were prepared for transmission electron microscopy (TEM), and ultrathin sections of the infected cells were imaged (Figure 7.1.). As is visible in the TEM micrographs, in the amastigote stage inside the macrophage cells, *L. mexicana* is part of the parasitophorous vacuole. Exemplary micrographs in Figure 1 illustrate that, apart from being contained in endolysosomal structures scattered throughout the cytoplasm (Figure 7.1. A, Figure 7.1. D), many nanoparticles are localized in the proximity of the amastigotes (Figure 7.1. C, Figure 7.1. D) and in the PV (Figure 7.1. B). Thus, in spite of the *Leishmania* infection, the macrophages take up individual nanoparticles by endocytosis, and consistent with previous reports the endosomal pathway communicates with phagolysosomal compartments that

includes the PV. [253] Figure 7.1. C shows that the nanoparticles are also internalized by the parasites inside the PV.

Figure 7.1. TEM micrographs of *L. mexicana* infected macrophages showing localization of the gold nanoparticles in the parasitophorous vacuoles, parasites, and phagolysosomes. Arrowheads indicate gold nanoparticles (not all nanoparticles are labeled). P: parasites (amastigotes); V: parasitophorous vacuole. Scale bar: 1 μ m. Figure reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154-8161. Copyright 2018 American Chemical Society.

The parasites can easily be identified in the bright field images of the cells, and Raman maps, yielding approximately 500 SERS spectra each, were obtained. Representative SERS spectra of the parasite region are shown in Figure 7.2 A, for an average spectrum with standard deviation see Figure 7.3 A.

Figure 7.2. SERS spectra, extracted from the mapping datasets of several *L. mexicana* infected primary macrophage cells from the areas of (A) parasite, (B) the parasitophorous vacuole, and (C) endolysosomes located in the cytoplasmic regions. Excitation wavelength: 785 nm, acquisition time: 1s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar: 50 cps. Figure reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154-8161. Copyright 2018 American Chemical Society.

They exhibit a high level of similarity, unusual for the SERS spectra of typical cell samples [31, 112, 127]. Several very strong bands dominate the spectra, they are listed with their tentative assignments in Table 7.1. In the region of the parasite, most of the bands can be assigned to vibrations of proteins and lipids. They include modes around 500 cm^{-1} , 650 cm^{-1} and 830 cm^{-1} originating from different vibrations of proteins [112, 164, 222]. The bands around 1080 cm^{-1} and 1140 cm^{-1} are both assigned to C-C stretching modes of lipid chains [210, 223]. In accord with the presence of long aliphatic chains, the band at 1440 cm^{-1} due to the deformation vibrations of the CH_2 groups from fatty acids [74] is also found. The band at 420 cm^{-1} is typical of cholesterol [210], but has in its very close proximity at only slightly lower frequency also contributions from saccharides [234]. Moreover, vibrations of phosphate groups are evident at 990 cm^{-1} and 1280 cm^{-1} in the SERS spectra of areas containing the parasite [222]. The pronounced contributions by proteins, phosphate, and carbohydrates to the spectral fingerprints is in accord with the fact that the surface of the parasite is covered with unusual PPG and an abundance of PPG that is secreted by the amastigote [246, 247]. These molecules consist mainly of several phospho-disaccharide repeats which are connected via serine to a core protein [247].

Figure 7.3. Average of normalized SERS spectra, extracted from the mapping datasets of several *L. mexicana* infected primary macrophage cells from the areas of (A) parasite, (B) the parasitophorous vacuole, and (C) endolysosomes located in the cytoplasmic regions, shown with their standard deviation. Figure reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154-8161. Copyright 2018 American Chemical Society.

Table 7.1. Raman Shifts and Tentative Assignments of Important Bands in the SERS spectra from Leishmania Infected Macrophage Cells Assignments Based on Refs: [74, 112, 164, 210, 222, 223, 234, 235]. Table reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154-8161.

Raman shift /cm ⁻¹	Tentative assignments
410	Phosphoinositol, CH ₂ bending
420	Sterols, CH ₂ bending in the ring
505	Proteins, S–S stretching
641 / 651	Amino acids, C–S stretching
713 / 728	Sterols, ring deformation
806	RNA, O–P–O stretching
835	Tyr, ring breathing
990	Phosphorylated proteins , P–O–P stretching
1030	Tyr, in plane ring deformation
1086	Phospholipids, C–C stretching
1130	Proteins, Tyr, C–C stretching
1140	Lipids, C–C stretching
1211	Tyr, Phe, C–C ₆ H ₅ stretching
1278	Lipids and proteins, PO ₄ ³⁻ stretching and amide III
1305	Proteins and lipids, CH ₂ /CH ₃ deformation
1322	Proteins, CH ₂ , CH ₃ deformation
1337 / 1344	Carbohydrates, C–O–H
1381	Lipids, CH ₃ deformation
1418	Amino acids, CO ₂ ⁻ stretching
1440	Lipids, CH ₂ deformation
1452	Lipids, proteins, carbohydrates, CH ₂ deformation
1514	Proteins, N–H deformation
1550	Trp, C=C stretching
1572	Proteins, Amide II
1602	Ergosterol, C=C stretching
1630	Lipids, C=C stretching

Examples of SERS spectra of the large PV that is formed after the infection with *L. mexicana* parasites and that surrounds the amastigote are shown in Figure 7.2. B. At first glance, they show greater variation compared to the spectra of the parasite (Figure 7.2. A, Figure 7.3. B), which points towards a distinct molecular composition of this compartment. The different signals of proteins, e.g., around 500 cm^{-1} or 830 cm^{-1} are present. The signatures of lipids are very pronounced in the vacuole, for example, the band at 1440 cm^{-1} that is assigned to the CH_2 deformation of the long lipid chains, or the band at 1140 cm^{-1} . Many of the bands in the spectral region around 1350 cm^{-1} , which is obvious in many of the vacuole spectra, are assigned to different protein and sugar components, as well as to inositol [235], likely reflecting the presence of modified lipids and proteins. In *Leishmania*, glycoinositol-phospholipids have been shown to be an abundant molecular class [255, 256], in addition to lipids that have an additional ether double bond at the sn-1 position [257]. In addition to the high abundance of lipids and glycosylated lipids, distinct vibrational modes of cholesterol as discussed above, and also of ergosterol are observed. Specifically, the band at 1602 cm^{-1} is assigned to the stretching of $\text{C}=\text{C}$ bonds of the latter [258]. In the normal Raman spectra of the pure sterols ergosterol and cholesterol (data not shown), the band at 1602 cm^{-1} is found in the ergosterol spectrum and not in the cholesterol spectrum. As will be discussed below, the localization of cholesterol and ergosterol differs at different positions in the infected cells. The combined appearance of signals from both proteins and carbohydrates also evidences the presence of PPG released by the parasite.

The SERS spectra that were collected from regions outside the vacuole must originate from the non-parasitized endolysosomal compartment of the infected cells. Representative spectra are shown in Figure 7.2. C (for an average see Figure 7.3. C). In accord with previous SERS spectra obtained from macrophage cells [112], they show a very high variability, indicating variation in composition of different endo-/lysosomes, and the many degradation products that are found in them. Some of the bands are more frequent, but can vary greatly in intensity. Several protein signals are quite abundant, e.g., the bands at 505 cm^{-1} and around 650 cm^{-1} that are assigned to cysteine or methionine. Similar to the SERS spectra of the parasite and vacuole, many of the spectra from the endolysosomal structures show contributions from lipid signals as well, in particular the bands at 990 cm^{-1} , 1140 cm^{-1} , and 1440 cm^{-1} can be assigned to the different vibrations of the lipids and sterols (see also Table 7.1.). The similarity between the spectra of the vacuole and the phagolysosome, specifically with respect to the lipid signature, are in good agreement with

the similarity of the membranes and cargo of both types of the vesicles that has been described previously [259].

7.3 SERS mapping of the biochemistry in live cells

By analyzing the distribution of the intensities of the vibrations discussed so far, the location of the respective molecular species is obtained from chemical images of the infected macrophage cells. Figure 7.4. shows mapped regions from three exemplary cells, together with the corresponding bright field images that clearly indicate the positions of the PV and the amastigotes inside of them. It should be noted that in this study, the Raman probed volume in the z -dimension included the whole thickness of the cell at a given position. Therefore, although the extension of the vacuoles is large in the z -direction, some nanoprobe in endosomes outside (that is, above or beneath) could also contribute to the signal in a region denoted as ‘vacuole’ or ‘parasite’ region. The bands at 420 cm^{-1} , 505 cm^{-1} , 990 cm^{-1} , 1140 cm^{-1} , 1278 cm^{-1} , and 1602 cm^{-1} are discussed here, as they represent the distribution of different molecular species, based on lipids (Figure 7.4., green maps), proteins (Figure 7.4., blue and red maps), phosphate (Figure 7.4., red maps) and sugar components that are characteristic of *Leishmania* infection (cf. assignments in Table 7.1.).

Figure 7.4. (A)–(C) Distribution of signals that are related to molecular species characteristic of the infection by *L. mexicana*, representing lipids (green), proteins (blue), and phosphate (red) species together with bright field images of the three exemplary cells. Chemical images are generated by mapping the intensity of the bands at 420 cm^{-1} assigned to cholesterol, 505 cm^{-1} of disulfide in proteins, 990 cm^{-1} mainly assigned to phosphate in phosphoproteoglycans, 1140 cm^{-1} of phospholipid alkyl chains, 1278 cm^{-1} of phosphate in proteins and phospholipids, and 1602 cm^{-1} of ergosterol. Arrowheads in the brightfield images indicate individual parasites (amastigotes); V: parasitophorous vacuole. Scale bar: 10 μm . Figure reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154–8161. Copyright 2018 American Chemical Society.

The maps are particularly conclusive regarding the distribution of the lipid components in the infected cells: The bands at 420 cm^{-1} , 1140 cm^{-1} and 1602 cm^{-1} can serve as representatives of different types of lipids (Figure 7.4. A to C, all green maps). The band at 1140 cm^{-1} can be considered as a marker band for the phospho- and sphingolipids. In Figure 7.4. A, B and C, high intensities for this band are mainly found in the phagolysosomes and less frequently in the vacuole part of the cells. The distribution pattern of the band at 420 cm^{-1} of cholesterol is similar to that of the band at 1140 cm^{-1} , indicating that often, although

not always, cholesterol co-localizes with phospholipids. In Figure 7.5. A, another mapping data set showing the distribution of the intensities at 420 cm^{-1} and the 1140 cm^{-1} bands is displayed together with some example spectra (Figure 7.5. B) from the labeled positions, confirming the assignment to very lipid rich locations by the presence of several different characteristic lipid bands.

Figure 7.5. (A) Chemical images displaying the distribution of lipids (the intensity of the bands at 420 cm^{-1} and 1140 cm^{-1}) and (B) example spectra of a *L. mexicana* infected cell that are labeled in both panels of (A). The SERS spectra represent highest intensity pixels in the maps. Excitation wavelength: 785 nm, acquisition time per spectrum: 1 s, excitation intensity: $2 \times 10^5\text{ W cm}^{-2}$. Scale bar in (A): $10\text{ }\mu\text{m}$. Scale bars in (B): 50 cps. Figure reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154-8161. Copyright 2018 American Chemical Society.

In the spectra 1 and 2 of Figure 7.5. B, both cholesterol and the phospholipid signals are present, while spectra 3 and 4, collected from outside the vacuole, exhibit the band at 1140 cm^{-1} , yet no cholesterol signal at 420 cm^{-1} . As can also be seen in all the cells displayed in Figure 7.4., in the region of the vacuole, the cholesterol signal is mostly found around the vacuole membrane. From filipin staining it has been proposed recently that the amastigotes, which in the investigated cells sit in the proximity to the vacuole membrane (Figure 7.1. and Figure 7.4., arrowheads) are surrounded by a cholesterol halo, and that cholesterol is sequestered to the PV and parasites in infected cells [245]. This is confirmed here in live cells and is strongly arguing against the criticism that the cholesterol halo as detected by filipin may be the result of a fixation artefact. The intensity of the band at 1602 cm^{-1} , attributed to the C=C double bond of ergosterol and/or ergosterol derivatives [258] is high in the region of the vacuoles (Figure 7.4.). While the sterol specific filipin staining cannot discriminate between cholesterol and ergosterol, here it can be seen that they do not necessarily co-localize in the same focal volume. Furthermore, different from the signal of cholesterol, the ergosterol spectrum is not observed in the regions of the endolysosomes away from the vacuole regions. This confirms its very specific abundance in the parasite [260] and indicates that it is also abundant in the vacuole, possibly as part of membrane delimited extracellular vesicles thought to be released by the parasite [261, 262].

The Raman cross sections of the many different vibrations of proteins can vary significantly, with aromatic amino acid side chains providing the highest signals [263, 264]. To include contributions from as many protein species as possible, so the band at 505 cm^{-1} that can be assigned to an S-S stretching vibration of disulfide bonds that are found in many proteins is chosen. In agreement with the observation of pronounced protein signals in the individual spectra discussed above (Figure 7.2. C), the highest contribution of the protein is found in the cytoplasmic regions containing endo-/phago-/lysosomes (Figure 7.4., blue maps). Also in the vacuoles, proteins are present, but to a lesser extent.

The signals from phosphate groups (Figure 7.4., red maps) enable further interpretation and assignments of characteristic intensities to molecular species. As known from previous work, PPG is identified as a very abundant and important species in the infected cell [246, 247]. As the protein core is glycosylated via a particular serine-O-glycosylation [246, 247], with phospho-di-ester disaccharides containing mannose and other sugars, bands that belong to the phosphate group can show the distribution of the compound. The bands at

990 cm^{-1} of the phosphate P-O-P stretching mode and at 1280 cm^{-1} show a similar distribution, although the band at 1280 cm^{-1} has significant presence in the endolysosomes compared to the band at 990 cm^{-1} (compare the two red maps in each of the of Figure 7.4.). Both of these maps indicate contributions from the phosphate group of phospholipids (compare the red maps with 1140 cm^{-1} maps), however, there is also overlap with the protein distribution (e.g., Figure 7.4. B compare the red 990 cm^{-1} maps with the blue maps), showing that the phosphorylation represented by the red map co-localizes with the signals of proteins (blue). In the region of the vacuole, this points directly to the presence of PPG. As another indication of PPG species, the band at 1278 cm^{-1} can be assigned to the symmetric stretch of PO_4^{3-} group, but also to the amide III bands of proteins. The differences between the phosphate based on the bands at 1278 cm^{-1} and 990 cm^{-1} could be caused by the phosphate contained in the phospholipids, and indeed this difference matches the distribution of the signal at 1140 cm^{-1} .

7.4 PCA analysis of spectra from different cellular compartments

Principal component analysis (PCA) was carried out with the spectra from different regions in the cell, in order to use the full SERS spectral fingerprint for the discrimination. Variation in a set of SERS data can have many different origins, apart from different molecular structure or composition. For example, since different nanoaggregates are residing in the focal volume at the time each spectrum is acquired, the difference in signal-to-noise ratio within the spectra from the different intrinsic biomolecules of one particular cell can be very high. However, due to the high abundance of particular molecules, and the dominating effect of the PV in the model used here, which provides a suitably coarse granularity of the cellular landscape encountered by the nanoprobe, signal-to-noise ratios were found to be very similar, and PCA using vector-normalized first derivatives of the spectra could be applied. This finding is in accordance with other recent works on the application of multivariate analyses of SERS data in bioanalytics, e.g., in diagnostics [92] or microorganisms classification [87, 88]. PCA was applied to the SERS spectra from the three different regions in a few data sets, with 108 spectra in total, in the range from 300 cm^{-1} to 1900 cm^{-1} (Figure 7.6.).

The scores plots of the 1st and 2nd (Figure 7.6. A), and the 2nd and 3rd PC (Figure 7.6. B), respectively, indicate that the second PC represents variation in the data set that separates spectra from the endolysosomal compartment from those of the PV (Figure 7.6. A and B,

black points) and the parasite, (Figure 7.6. A and B, green and red data points). Overlap in the spectra can be due to the impossibility to clearly distinguish the border between parasite / vacuole and vacuole/ cytoplasm in all cases. PCA scores of PC1 (Figure 7.6. A, green points) reveal higher inner variation within the group of the PV spectra, compared to the spectra of the parasite and to the endo-/phago-/lysosome spectra, in agreement with the high variation discussed for the example spectra from this compartment (Figure 7.2. B). The *p*-values obtained in a Kruskal-Wallis test suggest that the separation in the PCA scores plot is significant. Figure 7.6. C shows the corresponding loadings. The variance revealed in the loadings agrees very well with the bands selected as marker bands for the specific compounds and the maps discussed above: In the loading of PC2, explaining the differences between endo-/phago-/lysosome spectra on the one hand and PV and parasite spectra on the other, differences in the contributions of phospholipids (signals $\sim 1140\text{ cm}^{-1}$) and of proteins and ergosterol / cholesterol ($\sim 850\text{ cm}^{-1}$).

Figure 7.6. Result of a principal component analysis of vector-normalized, first derivatives of 108 SERS spectra extracted from three data sets using the spectral range from 300 to 1900 cm^{-1} . Scores plots using (A) the first and second principal component (PC) and (B) the second and third PC. Spectra collected in the area of the parasitophorous vacuole are represented in green, of the parasite in red, and phagolysosomes in the non-parasitized areas in black. (C) Loadings of the first three PCs. Figure reprinted with permission from Živanovic *et al.*, *Anal. Chem.* 2018, 90, 13, 8154-8161. Copyright 2018 American Chemical Society.

So far, only little is known about the dynamics and homogeneity of the vacuole's molecular composition, since fluorescence imaging approaches reveal the position only of known and of a few markers or molecular species [245, 253]. It should be noted that the gold nanoparticle SERS probes probably display a different behavior in the different compartments of the infected cells, as they obey the changes in dynamics of the cell caused by transport, or different viscosity / diffusion, or electrostatic interactions. While it is known that the movement of nanoparticles, including SERS nanoprobe [29], inside the endosomal system occurs in a very controlled fashion, and nanotomography gives evidence that the nanoparticles interact with the distinct ultrastructure of the endosomes [27], nanoparticles may experience much less guidance by supra/-molecular structures inside the PV and may rather work similar to dipsticks that are loosely immersed in the vacuole and detected in the Raman microscope. The observation of very different spectra in different positions within the region of the PV with different SERS probes here indicates that its composition is very heterogeneous. Considering the low affinity of lipid molecules for the surface of the gold nanoparticles in an aqueous environment [217] and the relatively small Raman cross sections of many vibrations of the lipid molecules [265], high lipid signals obtained specifically in the vacuole and the proximity to the parasite are the result of a very local high abundance of the lipids there. It is possible that, in the phagolysosomal compartment containing the parasite, local sequestration of the hydrophobic lipid molecules is a consequence of repulsion by the very hydrophilic PPG molecules that are synthesized and released by the amastigote. The combination of SERS with the characteristics of this infection model, i.e. the high abundance of distinct analytes such as lipids and PPG is particularly well suited for chemical mapping in live infected cells. Future refined studies using *z*-slicing, and correlation of the SERS data with cryo-X-ray tomography as shown for other systems recently [112], or serial thin section electron microscopy will improve our understanding of the distribution of the different molecular components over time and how this may be correlated with infection outcome.

Informative SERS experiments are possible using gold nanoparticles that reach the *L. mexicana* phagolysosomal habitat in infected primary macrophage cells. Respective spectra obtained off-resonance, without selectivity for specific molecular species could be obtained from gold nanoprobe contained in the PV, located in close proximity to the parasite and the parasite itself, and within non-infected endocytotic compartments distributed throughout the cytoplasm. The spectra are very characteristic of the respective

compartments, so that fingerprints from inside and outside the vacuole can be discriminated by multivariate analysis. Spectra obtained from PVs show the strong contributions from lipids and sterols. This suggests particularly high concentration of these molecules in PV. The simultaneous mapping of many molecular constituents with micron spatial resolution in individual live cells allowed the identification of spectral contributions by proteophosphoglycans, by ergosterol, and by phospholipid inositol, all specific for the parasite. The localization agrees with previous results of sterol staining procedures regarding the distinct distribution of cholesterol and ergosterol, but, importantly, extends and confirms this now to live cells.

The characteristics of the particular cellular infection model and of plasmon-enhanced Raman scattering with gold nanoprobe offer a unique combination to investigate the molecular aspects of *Leishmania* infection, and yield insight into a pathogen-host interaction that could be of benefit to further studies of this and related diseases.

Chapter 8

Summary and outlook

Lysosomes are important for the degradation of membranes and proteins, playing a crucial role in different cellular processes. This thesis focuses on monitoring lysosomes in living healthy and diseased cells with sufficient temporal and spatial resolution. Surface-enhanced Raman scattering (SERS) was used as an approach to study intact lysosomes, in particular, lysosomes that have a disturbed lipid metabolism. In order to achieve probing of lysosomes by SERS, cells were treated with tricyclic antidepressants, which are known to accumulate in lysosomes and to inhibit acid sphingomyelinase, an important enzyme in the metabolism of sphingomyelin. Exploiting both, localization of gold nanoparticle in lysosomes and their beneficial optical properties, SERS spectra from intact lysosomes were obtained. A novel approach using random forest algorithm to analyze complex SERS data obtained from the cells was carried out. The results of the random forest analysis were compared with spectra of two model systems, the combination of gold nanoparticles with antidepressants and of liposome with gold nanoparticles. Based on SERS and SEHRS data, the interactions of antidepressant molecules with the surface of the nanoparticles was determined. The interactions between liposomes and gold nanoparticles were assessed by SERS and cryo-EM. The exact binding sites of the lipids and the gold nanoparticles were proposed.

Moreover, it was shown that, depending on the concentration of the stabilizing agent of the nanoparticles, the lipids interact differently with the particles. These results improve our understanding of SERS spectra from the lipid-rich environment in cells. Leishmania infection was used for the SERS analysis as a model for lipid-rich environment in cells. As the results show, SERS can be used to assess the distribution of molecular species important for the infection in the cells and to distinguish between chemically similar lipids such as ergosterol and cholesterol. This progress in the analysis of lipids in the living cells is also evidenced by lipid signals in the antidepressants treated cells.

To study the interactions of three TCA molecules with gold nanoparticles, SERS spectra were obtained at two different excitation wavelengths (532 and 785 nm), and SEHRS spectra were excited at 1064 nm. The data show in all three molecules, the methylaminopropyl side chain plays a very important role in the interaction with gold nanoparticles, along with parts of the ring system. In contrast, the molecules interact with silver nanoparticles mainly via their ring moiety and less intensely with the alkyl chain. The NIR excited SERS spectra of the TCA and gold nanoparticles are greatly invariant concerning changes in TCA concentration and size of the biocompatible gold nanostructures. They show a remarkable stability in the presence of the cell culture media, and upon decrease of pH in the typical ranges of pH values in endo-lysosomal vesicles. These findings suggest the antidepressants–nanoparticles composites can be used in drug delivery. Furthermore, combined one- and two-photon vibrational spectroscopic approach is demonstrated to be a useful tool for monitoring and characterization of such nanoparticle-based drug delivery platforms and their interactions in real biological environments.

A model system composed of liposomes and gold nanoparticles was developed and studied. Liposomes were composed of phosphatidylcholine (PC), sphingomyelin (SM), a combination of both, and mixtures of the respective lipids with cholesterol (CH) were obtained. As a model system of the intralysosomal membrane, liposomes containing 10 and 20 mol % phosphatidic acid were prepared, and their interactions with gold nanoparticles were studied by SERS. Evidenced by the structural information from SERS and cryo-EM, gold nanoparticles change the structure of the liposomes upon the interaction with the lipids in the presence of a high amount of citrate in the solution. At lower citrate concentration, the nanoparticles are adsorbed on the liposomal surface, and they were found to stabilize smaller vesicles. The spectra of PC and PC/SM liposomes provide direct evidence that, at

the site of interaction, the lipid chains are highly ordered and in the gel phase, induced by the contact of the nanoparticles with the liposomal surface. The SERS spectra are characteristic of the vesicles' lipid. In intact vesicles, the data identify the polar head region as the main contact site with the nanoparticles. Due to different interaction of the head groups of PC and SM, both with the gold nanoparticles and with CH, the presence of CH has different effects on the contacts of the vesicles with the nanoparticles. The interaction between the gold nanoparticles and the liposomes that carry negative charge due to the presence of phosphatidic acid are similar to the interaction of lipids in the presence of a high citrate concentration. From this, it can be concluded that the negative charge on the surface of liposomes induces different interaction with the surface of nanoparticles. SERS spectra obtained here are used for the interpretation of SERS spectra from lipid-rich environments in cells. Moreover, the study of the interactions between the lipids and the nanoparticles, has implications in the development of new, well-defined, optically monitored drug delivery systems. By combining cryo-EM and SERS, it is possible to fine-tune and structurally characterize a lipid–nanoparticle model yielding wrapped, adsorbed, or encapsulated nanoparticles.

To study the influence of TCAs in the lysosomes of live cells, the SERS analysis of cells treated with antidepressants and gold nanoparticles was carried out. The morphology and localization of the gold nanoparticles, together with the accumulation of the lipids due to the effects of antidepressants are assessed by TEM and cryo-XRT data. These data show that after 24 h exposure to antidepressants, lipid accumulation occurs in cells and gold nanoparticles are associated with the accumulated lipids, thus allowing probing of these vesicles by SERS. The sequence of incubation of nanoparticles and antidepressants has a significant influence on the obtained data. When cells are incubated first with nanoparticles, and then with antidepressants, the nanoparticles, antidepressants, and accumulated lipids are localized together in lysosomes, revealing the interactions between these components. Random forest analysis of the SERS spectra shows a high correlation between signals of the antidepressants and the lipids, suggesting the mutual influence of these molecules. Chemical maps based on variables (i.e. bands) selected by random forest approach show high colocalization of lipids and antidepressants. SERS data obtained from the cells were compared with the data obtained from the model systems and, based on the analysis, it can be proposed that the interactions between the positively charged antidepressants and the negatively charged inner lysosomal membrane is very likely to occur in cells.

The application of SERS for the analysis of lipids in biologically relevant systems has been demonstrated. Informative SERS data are obtained using gold nanoparticles that reach the *Leishmania mexicana* phagolysosomal vacuoles in infected primary macrophage cells. Respective spectra could be obtained from gold nanoprobe contained in the parasitic vacuoles (PV), located near the parasite, and in the parasite itself and within noninfected endocytotic compartments distributed throughout the cytoplasm. The spectra were found to be very characteristic of the respective compartments, so that fingerprints from the inside and outside the vacuole can be discriminated by PCA. Strong spectral contributions from lipids and sterols from the PVs suggest particularly high abundance of these molecules, possibly due to sequestration and/or synthesis by the parasite. The simultaneous mapping of many molecular constituents with micron spatial resolution in individual live cells allowed the identification of spectral contributions by proteophosphoglycans, by ergosterol, and by phospholipid inositol, all specific to the parasite. The distinctive spatial distribution of the assigned pronounced signals indicates that the PV is structured with respect to the local abundance of these molecules, possibly as a consequence of segregation into hydrophobic and hydrophilic regions. The localization agrees with previous results of sterol staining procedures, particularly regarding the distinct distribution of cholesterol and ergosterol and confirms the same principle in the live cells. The characteristics of the particular cellular infection model and surface-enhanced Raman scattering with gold nanoparticles offers a unique approach to investigate the molecular aspects of *Leishmania* infection and yields insights into the lipid distribution.

In conclusion, with the right analysis, TCA molecules can be detected in live cells. However, it is desirable to develop a model system that improves studying the interactions between TCA molecules and ASM in living cells. For example, one could envision a system where nanoparticles are coated with molecules such as 1-decanethiol, on top of which lipid molecules could be assembled, forming the so-called hybrid liposomes. This construct can serve as a docking site for ASM, and SERS would provide detailed information about the interactions with the membrane and the inhibitors. In principle, this model can be used, not only to study interactions of ASM, but also for other membrane proteins and their interactions either with membranes itself, and/or with active molecules. Moreover, the detection of antidepressants in cells can be improved by chemical binding of these molecules to the nanoparticles. In that way, molecules can lead nanoparticles inside the

cells to the lysosomes and at the same time investigate changes in their environment by SERS.

In this thesis, the possibilities of SERS for the analysis of lipid-related processes in living cells are demonstrated. The SERS data point very clearly to the presence of lipids stored in the lysosomes. In future work, SERS can be used to further address the challenges of different lysosomal storage disease, such as Niemann-Pick disease (NPD), Gaucher, or Fabry disease. In NPD, the main stored component is cholesterol [43], while in Gaucher and Fabry diseases it is sphingomyelin [266, 267]. The investigation of the mechanism of lipid accumulation or the evaluation of therapy treatments by SERS in living cells would be beneficial in understanding these diseases and improving their treatment.

The work with model systems in this thesis showed that combinations of TCA molecules with gold nanoparticles and liposomes with gold nanoparticles are very promising systems to study drug delivery by SERS. In particular, the observation that the interaction of gold nanoparticles modifies lipid membranes can be helpful in improving and optimizing drug release or interactions of liposomal carriers with the cell membrane. At the same time, SERS can be used to provide information about the structure and interaction of the lysosomal cargo.

The selection of variables by a random forest approach prior to chemical mapping showed co-localization of important components characteristic for inhibition of ASM by TCA molecules. This combination showed to be very useful in understanding complex SERS data and for extracting information relevant for studying induced lysosomal disease in live cells. In the future, it must be shown whether such an approach is feasible to study other processes in cells, for example, endosomal maturation, apoptosis, and autophagy.

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List of Figures

Figure 2.1. The substrate for the acid sphingomyelinase is sphingomyelin, a lipid found in cellular membranes. Upon enzymatic reaction, sphingomyelin is split into two products: ceramide and phosphorylcholine.	7
Figure 2.2. Schematic representation of the interaction between antidepressants and acid sphingomyelinase (ASM) in lysosomes. In figure uptake of desipramine by cell and its enrichment in lysosomes is depicted.	10
Figure 2.3. Schematic representation of Stokes Raman scattering (A), anti-Stokes Raman scattering (B), and hyper-Raman scattering (C). Only non-resonant processes are displayed. ν_0 represents the initial state for Stokes and hyper-Raman scattering, while ν_1 is initial state for anti-Stokes scattering.	13
Figure 2.4. Schematic representation of the surface-enhanced Raman scattering process. Molecules are positioned in the close proximity of gold nanoparticles. Laser light interacts with plasmonic nanoparticles and enhances incident light (ν_L) and Raman light (ν_{RS}).	15
Figure 2.5. Endocytic uptake of metal nanoparticles by a cell.	17
Figure 4.1. Structure and labeling of antidepressant molecules, imipramine (Imi), desipramine (Des) and amitriptyline (Ami). Figure reprinted with permission from ref. <i>Živanovic et al., J. Phys. Chem. C</i> 2017, 121, 41, 22958-22968. Copyright 2017 American Chemical Society.	34
Figure 4.2. Transmission electron micrographs of (A) the gold nanoparticles with average size 30 nm (Au30) (B) gold nanoparticles with average size 60 nm (Au60), and (C) silver nanoparticles (Ag). (D) UV-Vis absorbance spectra of the AgNP (black line), Au30 (red line), and Au60 (blue line) and (E) with addition of Des.	35
Figure 4.3. (A) SEHRS and (B) SERS spectra of the antidepressants imipramine, desipramine and amitriptyline obtained with silver and gold nanostructures. Concentration of the TCA molecules: 9×10^{-5} M for Au and 9×10^{-4} M for Ag nanostructures. (A) Excitation wavelength: 1064 nm, intensity: 2.1×10^{11} W cm ⁻² , acquisition time: 5 min. (B) Excitation wavelength: 532 nm, laser	

intensity, $1.2 \times 10^{10} \text{ W cm}^{-2}$, acquisition time 5 s. Scale bars: (A) SEHRS, 0.1 cps, (B) SERS 50 cps.	37
Figure 4.4. SERS spectra of the antidepressants Imi, Des and Ami obtained using sgold (A) and silver (B) nanostructures. Excitation, 785 nm; laser intensity, $2.0 \times 10^5 \text{ W cm}^{-2}$; acquisition time, 1 s; concentration, $9 \times 10^{-7} \text{ M}$ (gold), $9 \times 10^{-5} \text{ M}$ (silver); scale bars: 40 cps (gold), 4 cps (silver)..	46
Figure 4.5. UV-Vis absorbance spectra of the Au30 (black line), Au30 with the addition of desipramine (red line), and Au30 with addition of desipramine and DMEM-FCS (blue line).....	49
Figure 4.6. SERS spectra of the antidepressants desipramine obtained using gold nanostructures without the DMEM-FCS and in the presence of DMEM-FCS. Excitation wavelength: 785 nm; excitation intensity: $2.0 \times 10^5 \text{ W cm}^{-2}$; acquisition time: 1 s, Concentration of desipramine: $9 \times 10^{-7} \text{ M}$. Scale bars: 50 cps.	50
Figure 4.7. SERS spectra of the antidepressant desipramine obtained using gold nanostructures at pH 3.4, pH 4.4, and pH 6.0.	51
Figure 5.1. Schematic representation of possible interactions of gold nanoparticles and lipid membranes	56
Figure 5.2. Schematic representation of gold nanoparticles-liposome composites preparation using thin layer hydration and reverse phase separation methods.....	57
Figure 5.3. TEM micrographs of liposomes and citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 8 \times 10^{-10} \text{ M}$) prepared using the thin layer hydration method.....	58
Figure 5.4. Cryo-EM micrographs of liposomes and citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 8 \times 10^{-10} \text{ M}$) prepared using the thin layer hydration method (A)-(C) and reverse phase evaporation method (D)-(F).....	59
Figure 5.5. Cryo-EM micrographs of a mixture of liposomes and citrate stabilized gold nanoparticles prepared in situ using thin layer hydration.	60
Figure 5.6. Cryo-EM micrographs of the samples composed of liposomes and citrate stabilized gold nanoparticles. Liposomes are composed of (A)-(B) PC, (C)-(D) PC/CH, (E)-(F) PC/SM....	61
Figure 5.7. Representative SERS spectra of liposome-gold nanoparticles composites prepared using reverse phase evaporation method (black trace) and thin layer hydration method (blue trace). Used excitation wavelengths are 633 nm (A) and 785 nm (B).....	63
Figure 5.8. Representative SERS spectra of (A) liposome and citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 8 \times 10^{-10} \text{ M}$) prepared using the thin layer hydration method, (B) mixture of liposomes with gold nanoparticles, (C) liposome and citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 1 \times 10^{-10} \text{ M}$), after removal of excess citrate ions.....	65
Figure 5.9. Representative SERS spectra of citrate stabilized gold nanoparticles ($c_{\text{AuNP}} = 1 \times 10^{-10} \text{ M}$) and liposomes composed of: (A) PC and PC/CH, (B) SM and SM/CH, (C) SM/PC and SM/PC/CH.	69

Figure 5.10. Representative SERS spectra of citrate-stabilized gold nanoparticles ($c_{\text{AuNP}} = 1 \times 10^{-10}$ M) and liposomes composed of (A) 10 mol % PA, 10 mol % SM 20 mol % CH, 60 mol % PC, and (B) 20 mol % PA, 10 mol % SM 20 mol % CH, 50 mol % PC..	71
Figure 5.11. Representative SERS spectra of liposome-gold nanoparticles composites (blue trace) and liposomes-desipramine-gold nanoparticles composite. Liposomes were composed of 10 mol % PA, 10 mol % SM 20 mol % CH, and 60 mol % PC. s.	73
Figure 6.1. Schematic representation of performed SERS experiments in the cells. (A) Cells were incubated with TCA molecules for 24 h prior to the incubation with gold nanoparticles for 6h. (B) Cells were incubated with gold nanoparticles for 6h prior to the incubation with TCA molecules for 24 h. (C) Cells were incubated with TCA molecules and gold nanoparticles for 24 h simultaneously.	77
Figure 6.2. TEM micrographs of 3T3 fibroblast cells incubated with desipramine for 24 h prior to the incubation with gold nanoparticles for 6 h showing vesicle with a significant amount of lipids accumulated and associated with single or aggregated gold nanoparticles.	78
Figure 6.3. TEM micrographs of 3T3 fibroblast cells incubated with desipramine for 24 h prior to the incubation with gold nanoparticles for 6 h showing vesicles with significant myelin accumulation and associated with gold nanoparticles (A, C, D) and endolysosomal vesicle with aggregated gold nanoparticles (B).	78
Figure 6.4. TEM micrographs of 3T3 fibroblast cells incubated with gold nanoparticles for 6 h showing localization of the gold nanoparticles. Scale bars in: A, C, D, E, F 500 nm, B 1 μm .	80
Figure 6.5. X-ray microscopic image (A) and a slice of tomographic reconstructions (B) of a vitrified 3T3 fibroblast cells after incubation with desipramine for 24 h and nanoparticles for 6 h in the culture medium.	81
Figure 6.6. Projection images (A, B, C) and a slice of tomographic reconstructions (D) of a vitrified 3T3 fibroblast cells after incubation with gold nanoparticles for 6 h in cell culture medium. Arrowheads are pointing to the particles that are enclosed in vesicular structures.	82
Figure 6.7. Average of SERS spectra of the cells treated with Des for 24 h, prior to the incubation of gold nanoparticles for 6h (A) and control cells exposed to gold nanoparticles for 6 h (B). Example SERS spectra from treated (C) and control cells (D).	83
Figure 6.8. Averages (A) and examples (B) of SERS spectra of the cells treated with Imi for 24 h, prior to the incubation of gold nanoparticles for 6h. Averages (C) and examples (D) of SERS spectra of cells treated with Ami for 24 h, prior to incubation of gold nanoparticles for 6h.	84
Figure 6.9. Frequency of occurrence of most prominent Raman bands in the SERS spectra of the cells treated 24 h with TCA molecules prior to 6 h incubation with gold nanoparticles. (A) Bands assigned to the antidepressants and proteins, and (B) bands assigned to lipids.	88
Figure 6.10. Distribution of signals related to molecular species characteristic of amitriptyline treated cells, representing lipids (green), proteins (blue), and antidepressants (red) species together	

with bright field images of two exemplary cells. Chemical images are generated by mapping the intensity of the bands at 505 cm^{-1} of disulfide bond in proteins, 830 cm^{-1} of tyrosine, 960 cm^{-1} and 1030 cm^{-1} assigned to antidepressants, 1130 cm^{-1} of phospholipid alkyl chains, 1458 cm^{-1} to CH_2 deformation in phospholipids, and the full spectral range $300 - 1800\text{ cm}^{-1}$ 95

Figure 6.11. X-ray microscopic image (A, B, C) and a slice of tomographic reconstructions (D) of a vitrified 3T3 fibroblast cells after incubation with nanoparticles for 6 h and desipramine for 24 h in the culture medium. Arrowheads are pointing to the particles that are enclosed in vesicular structures. 97

Figure 6.12. AverageS of SERS spectra of cells incubated 6 h with gold nanoparticles prior to exposure to desipramine for 24 h (A) and control cells exposed to the gold nanoparticles 6 h and 24 h to standard culture medium (B). ExampleS SERS spectra from treated (C) and control cells (D). 99

Figure 6.14. The frequency of the occurrence of most common bands in the SERS spectra of fibroblast cells that are treated 6 h with gold nanoparticles prior to 24 h incubation with TCA molecules. (A) Bands assigned to the antidepressants and proteins, and (B) bands assigned to lipids. 101

Figure 6.15. Distribution of signals related to molecular species characteristic of amitriptyline treated cells, representing lipids (green), proteins (blue), and antidepressants (red) species together with bright field images of two exemplary cells. Chemical images are generated by mapping the intensity of the bands at 620 cm^{-1} , 780 cm^{-1} , 960 cm^{-1} , and 1030 cm^{-1} assigned to antidepressants, 1140 cm^{-1} of phospholipid alkyl chains, 1280 cm^{-1} to CH_2 deformations of phospholipids alkyl chains, 1420 cm^{-1} to CH_2 deformation in phospholipids, and 830 cm^{-1} of tyrosine, 1340 cm^{-1} of CH_2 deformations in proteins, and the full spectral range $300 - 1800\text{ cm}^{-1}$ 108

Figure 6.16. TEM micrographs of 3T3 fibroblast cells incubated with desipramine and gold nanoparticles simultaneously for 6 h showing nanoparticles localized in different vesicles inside the cells (endosomes, lysosomes) and formation of the myelin figures (A, B). 110

Figure 6.17. Slices of tomographic reconstructions of a vitrified 3T3 fibroblast cells after incubation with nanoparticles and desipramine simultaneously for 6 h. Arrowheads are pointing to the particles that are enclosed in vesicular structures. 111

Figure 6.18. Slices of tomographic reconstructions of a vitrified 3T3 fibroblast cells after incubation with nanoparticles and desipramine simultaneously for 24 h. Arrowheads are pointing to the particles that are enclosed in vesicular structures. 112

Figure 6.19. Average of SERS spectra of cells treated with desipramine and gold nanoparticles simultaneously for 24 h (A) and control cells exposed to gold nanoparticles for 24 h (B). Examples SERS spectra from treated (C) and control cells (D). Excitation wavelength: 785 nm , acquisition time per spectrum: 1 s , excitation intensity: $2 \times 10^5\text{ W cm}^{-2}$. Scale bar in: (A) and (B) 100 cps ; (C) and (D) 20 cps 113

Figure 6.20. Average of SERS spectra of cells treated with imipramine (A) and amitriptyline (C) gold nanoparticles simultaneously for 24 h. Example SERS spectra from cells treated with imipramine (B) and amitriptyline (D).	114
Figure 6.21. The frequency of occurrence of most prominent Raman bands in the SERS spectra of the cells treated 24 h with TCA molecules followed by 6 g incubation with gold nanoparticles. (A) Bands assigned to proteins, and (B) bands assigned to the antidepressants and lipids.	115
Figure 7.1. TEM micrographs of <i>L. mexicana</i> infected macrophages showing localization of the gold nanoparticles in the parasitophorous vacuoles, parasites, and phagolysosomes. Arrowheads indicate gold nanoparticles (not all nanoparticles are labeled). P: parasites (amastigotes); V: parasitophorous vacuole. Scale bar: 1 μm	126
Figure 7.2. SERS spectra, extracted from the mapping datasets of several <i>L. mexicana</i> infected primary macrophage cells from the areas of (A) parasite, (B) the parasitophorous vacuole, and (C) endolysosomes located in the cytoplasmic regions. Excitation wavelength: 785 nm, acquisition time: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar: 50 cps..	127
Figure 7.3. Average of normalized SERS spectra, extracted from the mapping datasets of several <i>L. mexicana</i> infected primary macrophage cells from the areas of (A) parasite, (B) the parasitophorous vacuole, and (C) endolysosomes located in the cytoplasmic regions, shown with their standard deviation..	128
Figure 7.4. (A)-(C) Distribution of signals that are related to molecular species characteristic of the infection by <i>L. mexicana</i> , representing lipids (green), proteins (blue), and phosphate (red) species together with bright field images of the three exemplary cells. Chemical images are generated by mapping the intensity of the bands at 420 cm^{-1} assigned to cholesterol, 505 cm^{-1} of disulfide in proteins, 990 cm^{-1} mainly assigned to phosphate in phosphoproteoglycans, 1140 cm^{-1} of phospholipid alkyl chains, 1278 cm^{-1} of phosphate in proteins and phospholipids, and 1602 cm^{-1} of ergosterol.. Arrowheads in the brightfield images indicate individual parasites (amastigotes); V: parasitophorous vacuole. Scale bar: 10 μm	132
Figure 7.5. (A) Chemical images displaying the distribution of lipids (the intensity of the bands at 420 cm^{-1} and 1140 cm^{-1}) and (B) example spectra of a <i>L. mexicana</i> infected cell that are labeled in both panels of (A). The SERS spectra represent highest intensity pixels in the maps. Excitation wavelength: 785 nm, acquisition time per spectrum: 1 s, excitation intensity: $2 \times 10^5 \text{ W cm}^{-2}$. Scale bar in (A): 10 μm . Scale bars in (B): 50 cps.	133
Figure 7.6. Result of a principal component analysis of vector-normalized, first derivatives of 108 SERS spectra extracted from three data sets using the spectral range from 300 to 1900 cm^{-1} . Scores plots using (A) the first and second principal component (PC) and (B) the second and third PC. Spectra collected in the area of the parasitophorous vacuole are represented in green, of the parasite in red, and phagolysosomes in the non-parasitized areas in black. (C) Loadings of the first three PCs.	136

List of Tables

Table 4.1. Raman shift values in the SEHRS and SERS spectra of amitriptyline, and assignment to vibrations of the amitriptyline molecule, based on refs [127, 162-164].....	38
Table 4.2. Raman shift values in the SEHRS and SERS spectra of desipramine, and assignment to vibrations of the desipramine molecule, based on refs.[127, 162-164].....	39
Table 4.3. Raman shift values in the SEHRS and SERS spectra of imipramine, and assignment to vibrations of the imipramine molecule, based on refs. [127, 162-164].....	40
Table 5.1. Concentration, zeta potential, and pH of nanoparticles used in the experiments.	61
Table 5.2. Raman Shifts and Assignments of Important Bands in the SERS spectra of liposome-gold nanoparticles composites. Assignments Based on Refs: [163, 208, 209, 212, 218-220].	67
Table 6.1. Raman shifts and tentative assignments of important bands in the SERS spectra from antidepressants treated and control cells. Assignments based on Refs: [74, 112, 163, 208, 220, 221, 233, 234].....	87
Table 6.2. Important bands selected based on minimal depth analysis of the SERS spectra of cells treated with TCA for 24 h, prior to 6 h incubation with gold nanoparticles.	90
Table 6.3. Important bands assigned to antidepressants and their related variables selected based on SMD analysis of SERS spectra of cells treated 24 h with TCA prior to 6 h incubation with gold nanoparticles. With red color are marked variables (bands) are assigned to antidepressants, with yellow to lipids and with green bands to proteins. (Bands without color are unassigned modes).	91
Table 6.4. Important bands assigned to lipids and their related variables selected based on SMD in the cells treated with 24 h TCA prior to 6 h incubation with gold nanoparticles.).....	93
Table 6.5. Important bands assigned to proteins and their related variables selected based on SMD in the cells treated with 24 h TCA prior to 6 h incubation with AuNP.).....	94
Table 6.6. Important bands selected based on minimal depth analysis of the SERS spectra of the cells incubated 6 h with gold nanoparticles prior to treatment with TCAs for 24 h.....	102

Table 6.7. Important bands assigned to antidepressants and their related variables selected based on SMD in the cells incubated 6 h with gold nanoparticles prior to the treatment with TCA for 24 h.	104
Table 6.8. Important bands assigned to lipids and their related variables selected based on SMD in the cells incubated 6 h with gold nanoparticles prior to the treatment with TCAs for 24 h.	105
Table 6.9. Important bands assigned to proteins and their related variables selected based on SMD in the cells incubated 6 h with gold nanoparticles followed by treatment with TCA for 24 h. ...	106
Table 6.10. Important bands selected based on MD analysis of the SERS spectra of the cells incubated with TCA molecules and gold nanoparticles for 24 h.	117
Table 6.11. Important bands assigned to antidepressants and their related variables selected based on SMD analysis of SERS spectra of cells incubated with TCA molecules and gold nanoparticles for 24 h.	119
Table 6.12. Important bands assigned to lipids and their related variables selected based on SMD analysis of SERS spectra of the cells incubated with TCA molecules and gold nanoparticles for 24 h.	120
Table 6.13. Important bands assigned to proteins and their related variables selected based on SMD analysis of SERS spectra of the cells incubated with TCA molecules and gold nanoparticles for 24 h.	121
Table 7.1. Raman Shifts and Tentative Assignments of Important Bands in the SERS spectra from Leishmania Infected Macrophage Cells Assignments Based on Refs: [74, 112, 163, 208, 220, 221, 233, 234]. /.	129

List of Abbreviations

(NPD)	Niemann-Pick Disease
(ASM)	acid sphingomyelinase
(TCAs)	tricyclic antidepressants
(Ami)	amitriptyline
(Des)	desipramine
(Imi)	imipramine
(FIASMA)	“functional” inhibitors of ASM
(SERS)	surface-enhanced Raman scattering
(SERRS)	enhanced resonance Raman scattering
(LSPR)	localized surface plasmon resonance
(PCA)	Principal component analysis
(LDA)	linear discriminant analysis
(cryo-EM)	cryogenic electron microscopy
(SEHRS)	surface-enhanced hyper-Raman scattering
(BMDMs)	bone marrow derived macrophages
(cryo-EM)	cryogenic electron microscopy
(RF)	Random Forest
(BMP)	bis(monoacylglycero)phosphate
(TEM)	transmission electron microscopy
(cryo-XRT)	cryo X-ray tomography
(MD)	minimal depth
(SMD)	surrogate minimal depth
(SM)	sphingomyelin
(PC)	phosphatidylcholine
(CH)	cholesterol
(PA)	phosphatidic acid
(PV)	parasitophorous vacuole

Declaration

I declare that I have completed the thesis independently using only the aids and tools specified. I have not applied for a doctor's degree in the doctoral subject elsewhere and do not hold a corresponding doctor's degree. I have taken due note of the Faculty of Mathematics and Natural Sciences PhD Regulations, published in the Official Gazette of Humboldt-Universität zu Berlin no. 126/2014 on 18/11/2014.”

I declare that the written and electronic version of the dissertation written by me in the University Library agrees with the accepted dissertation. I have only remedied the deficiencies and fulfilled the conditions which had been communicated to me in the defense and which had been confirmed by the chairman of the doctoral committee.

Berlin, February, 2020

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