

# Terahertz spectroscopic techniques for the study of proteins in aqueous solutions

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## ABSTRACT

Liquid water is a very strong absorber in the THz frequency range. We have set-up a unique germanium laser spectrometer consisting of a Ge:Be laser, tunable from 1 to 4 THz, and a sensitive Ge photoconductor detector. The spectrometer uses a measurement scheme alternating sample and reference signal while placed in an environmentally controlled housing for high stability of temperature and humidity. The laser system leads to a very small statistical error in the absolute absorption coefficient ( $400\text{-}500\text{ cm}^{-1}$ ) of less than 0.1% corresponding to  $0.3\text{ cm}^{-1}$  while systematic errors due to filling of the sample cells become dominant. The high accuracy allows us to systematically investigate the effects of different solvates on water dynamics. Even a single point mutation in a protein can be measured in the THz absorption coefficient in the spectral range from 2 to 3 THz. The system has been recently used to study various solvates in liquid water like sugars and prototype proteins in aqueous buffer solutions in dependence of temperature, pH values, and denaturants. These studies are now augmented by time-resolved measurements using THz time-domain spectroscopy to analyze the kinetics of protein folding. We also discuss other THz sources and detection methods including the investigation of coherent synchrotron radiation at the synchrotron ANKA in Karlsruhe.

**Keywords:** Terahertz spectroscopy, terahertz lasers, semiconductors, biomolecules

## 1. INTRODUCTION

The development of powerful terahertz (THz) radiation sources and new detection schemes has opened up new research fields and applications<sup>1</sup> in the THz region. Semiconductor based table-top THz sources include time-domain THz systems,<sup>1</sup> quantum cascade lasers (QCLs),<sup>2-4</sup> germanium lasers and THz generation by photomixing.<sup>5</sup> Using THz radiation we can measure the dielectric function of the materials as a function of frequency. In absorption spectroscopy we can access the imaginary part of the dielectric function, e.g. by standard Fourier transform spectroscopy or tunable laser systems. The complete dielectric function can be obtained by dispersive Fourier transform spectroscopy or THz time-domain spectroscopy.

Figure 1 shows an illustration of the principle signatures of materials in different phases. Gases show characteristic absorption lines typically due to rotation and torsion (depending on temperature and pressure) with a small linewidth in the investigated spectral range. The gas is nearly transparent for frequencies which do not coincide with the absorption lines. The spectrometer has to provide sufficient intensity at the position of the absorption line to quantify the absorption correctly. Liquids have a typically broad THz spectrum with little structure if any (Fig 1). The low absorption of alcohol in comparison to very high absorption of water allows a very easy way to measure alcohol/water mixtures since the transparency with increasing alcohol content changes rapidly. Materials in the solid phase show a spectrum which is largely determined by the structure. In crystalline materials narrow lines can be observed corresponding to intermolecular crystalline networks which broaden in poly-crystalline materials due to the random orientation of micro-crystalline particles. In amorphous materials and materials with small particle structure the spectral features can be washed out.

For the latter and for liquids spectral measurements are only partly relevant to control possible global shifts in frequency or intensity. For those materials and liquids other physical, e.g. temperature or concentration, or chemical parameters, e.g. point mutations in proteins, are relevant. In our research we concentrate on strongly absorbing materials for which we need a high signal-to-noise ratio (SNR). Here we concentrate on the technical

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challenges and solutions which can provide improvements for investigations of samples with high absorption coefficients. The implications of the measurements and their impact in chemistry and biology is referenced.

We have developed a THz germanium laser based spectrometer<sup>6</sup> which is used to study strongly absorbing samples like water and solvates, including proteins, solved in water. The high absorption coefficient  $\alpha$  of (400-500  $\text{cm}^{-1}$ ) in the frequency range from 2 to 3 THz leads to a strong signal reduction and a sufficient signal-to-noise ratio is needed to obtain reproducible results.

In addition, due to the complex nature of biomolecules, small amounts available, and the possibility of long term biological degradation due to bacteria, it is necessary to obtain a good signal-to-noise ratio in a short measurement time. Each measurement should be performed as well in a reasonable time to be able to vary the sample in a large parameter space. The studies involve a range of temperatures, essentially from cryogenic via body temperature to high temperatures according to the various protein environmental conditions in which these can naturally occur.

Additional parameters are concentration of the solvate, buffer composition, pH value and concentration of denaturants. Since the samples consist of a multitude of components careful investigations of each component and their various combinations are needed as well. In the last years we have made considerable progress in the development of a germanium laser based spectrometer to obtain a very low statistical error in a reasonable measurement time.

For a total integration time of approximately 15 ms corresponding to 3000 pulses, each 5  $\mu\text{s}$  long, we obtain an accuracy of better than 0.1% corresponding to 0.3  $\text{cm}^{-1}$  in the absorption coefficient. With this low level of statistical error we were able to investigate systematic errors which become dominant. The stringent environmental control of temperature and humidity further improved the systems stability while still allowing a rapid change of sample cells and rapid return to equilibrium temperature conditions. Here we present the experimental setup and as well examples, illustrating that now even single point mutations of proteins can be measured by THz absorption spectroscopy.

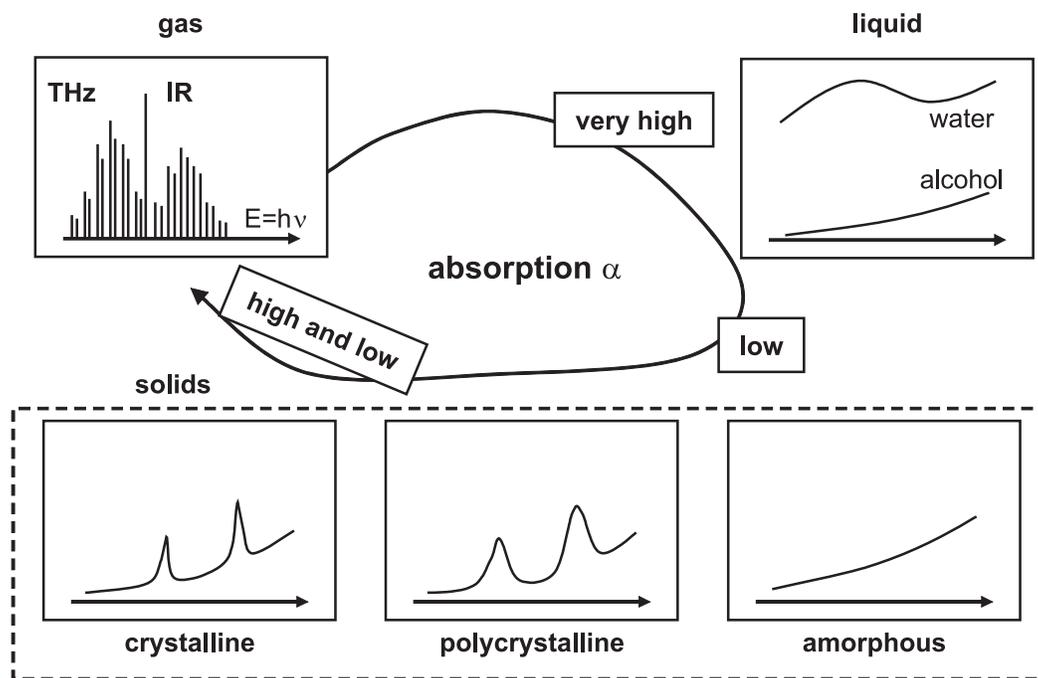


Figure 1. Illustration of absorption structures in the THz frequency range for different materials and phases.

## 2. TERAHERTZ GERMANIUM LASER SPECTROMETER

The germanium laser spectrometer system is based on germanium laser material<sup>7</sup> as shown in Fig. 2. Pure germanium, commercially available, is cleaned to ultra-pure conditions by float-zone purification. The crystal is then either grown by Czochralski growth with a doped charge like Ge:Be or doped by diffusion of copper. The ingot is further cut into a disk, oriented according to the crystal axes, polished and etched.

An implantation with boron, under 7° to the crystal axis to avoid channeling, with subsequent metallization and annealing follows. Further cutting and polishing to the final laser sizes complete the laser devices. Figure 2 shows in comparison a larger crystal doped with Ga which however has a limited spectral coverage with a gap around 2 THz otherwise covering the band approximately from 1 THz to above 4 THz. The emission gap of Ge:Ga lasers is due to self-absorption within the Ga acceptor and subsequent lower duty cycles are measured.

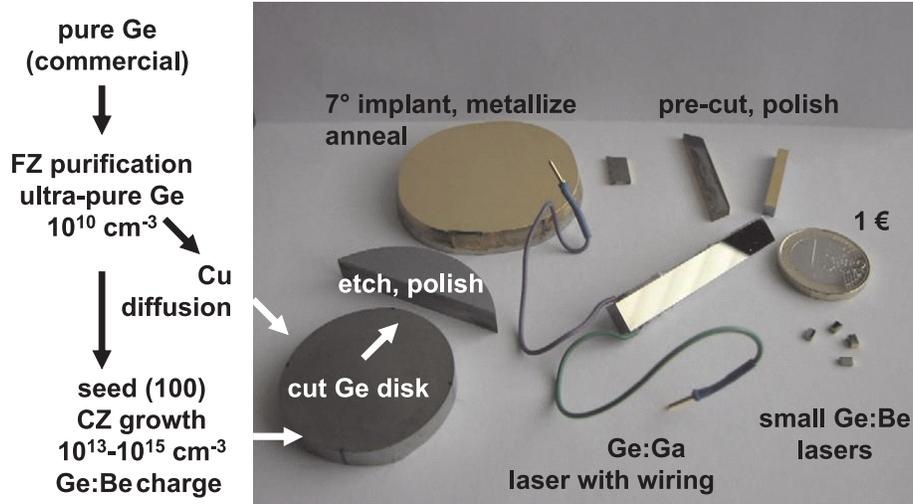


Figure 2. Processing steps for germanium terahertz lasers.

Ge:Be lasers can cover the THz range continuously as illustrated in Fig. 3(a). The broad gain profile is set by an external magnetic field indicated by the magnetic induction  $B$ . It is therefore smoothly tunable<sup>8</sup> and the induction can be provided by commercial small permanent magnets.<sup>9</sup> We currently use two  $50 \times 8 \times 8$  mm<sup>3</sup> NdFeB magnets with a remanence magnetic field of 1.4 T delivering a broad gain spectrum between 2 and 3 THz.<sup>6</sup>

The underlying longitudinal mode structure is determined by the Fourier limit of the used pulse length and the cavity optical length. A tunable resonator can select individual modes leading to narrow band emission.<sup>10</sup> In contrast to gas spectroscopy, the requirements for liquids in respect to the linewidth are not so stringent because on a small frequency band the absorption is nearly constant or linear.

The laser crystal is mounted in-between two permanent magnets within a closed cycle 3 Kelvin cooler (Sumitomo, Japan) and excited by an electric pulse generator. The emission can be split into different frequencies by a rotating blazed grating with a grating constant of 0.1 mm. The THz emission then passes through the sample cell which consists of quartz or diamond windows. Both types of windows are transparent in the visible which allows visual inspection of the liquid while it is mounted.

Special sample preparation is needed so that the liquid is free of air bubbles and particles. These can scatter the THz light leading to artifacts and systematic errors. The problem is more severe for protein solutions<sup>11,12</sup> which tend to aggregate at higher concentrations or tend to foaming in contrast to simpler mixtures like sugar solutions.<sup>13,14</sup>

The THz light is incident on a liquid helium (LHe) cooled photoconductor with a noise equivalent power (NEP) of approximately  $10^{-14}$  W Hz<sup>-1/2</sup> at 4.2 K. The Ge detector crystal with a cross-section of  $4 \times 4$  mm<sup>2</sup>

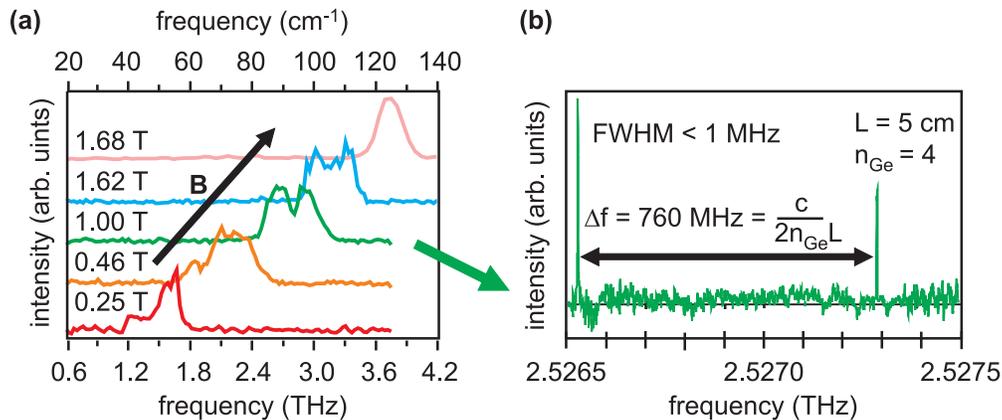


Figure 3. (a) Magnetically tunable emission of a single Ge:Be laser device measured with a germanium bolometer and Fourier transform spectrometer<sup>8</sup>. (b) Longitudinal laser mode structure for a 50 mm long laser crystals measured by laser heterodyne spectroscopy revealing a Fourier limited full width have maximum line width smaller and close to 1 MHz depending on the used laser pulse length<sup>10</sup>.

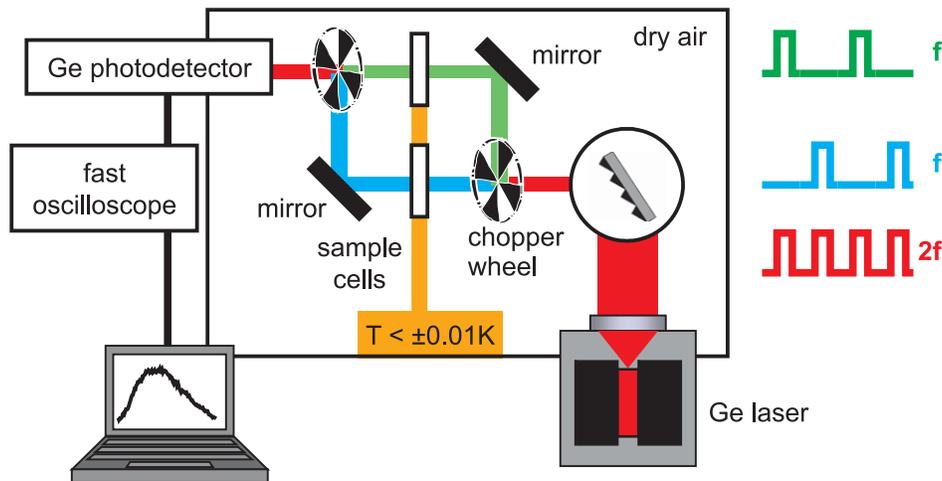


Figure 4. Germanium laser in-between two permanent magnets emits laser pulses typically set for  $5\ \mu\text{s}$  length. The pulses are synchronized to the rotation of the metallized chopper wheels which either reflect the pulse through a sample cell or reference cell. A germanium photodetector (response time less than 300 ns) detects the pulses and a fast oscilloscope card records a set of 3000 to 30000 pulses for each cell. The whole system is enclosed in a dry air environment on a laser table and the sample and reference cells are controlled in temperature to within 0.01 K. The repetition rate of the laser is limited to twice the mechanical speed (70 Hz to 140 Hz) of the chopper wheels for minimizing jitter of the control loop of the two choppers.

was mounted in a LHe cryostat. The THz emission incident on the Ge detector is filtered by a white 3 mm thick HD-PE vacuum window and two  $100\ \mu\text{m}$  thick cold filters of black low density PE at 77 K and 4.2 K. The detector is biased in the linear part of its I-V-curve ( $U_{BIAS} = 0.39\text{ V}$ ,  $I_{BIAS} = 0.32\text{ mA}$ ). Each laser pulse is read-out on a fast oscilloscope (GaGe Applied Technologies) and analyzed in the computer. The data acquisition and spectrometer operation is fully automated.

Considerable effort has been put into the stabilization of the measurement routine. The choppers direct the laser pulses alternating to the sample and reference cell, respectively. One chopper serves as the trigger for the first laser pulse and as a master chopper, while the other chopper is synchronized to the first. The best performance of the mechanical chopper wheels can be obtained between 70 and 140 Hz.

Nevertheless there is a frequency jitter between both chopper wheels. Since the laser pulse is very short ( $5\ \mu\text{s}$ )

in comparison to the chopper speed, we can trigger the second laser pulse directly from the second chopper which guarantees that the pulse is reflected always on the same metallized area of the chopper blade. The automated data acquisition gives online control and evaluation of all pulses in short and long-term segments so that the system is routinely operating at an statistical error of less than  $0.3 \text{ cm}^{-1}$  for absorption coefficient of 400 to  $500 \text{ cm}^{-1}$ . The measurement time is secondary to the time spent of filling the sample cells and examining the sample preparation.

### 3. TERAHERTZ ABSORPTION OF BIOMOLECULES

Figure 5 shows an example with a simple solvate in which the small "systematic" error of the Ge laser spectrometer is apparent. We have compared the measurements to Fourier transform spectroscopy using our vacuum spectrometer with the FIR light source and a liquid helium cooled Si-bolometer as detector (FTIR Bruker Vertex80v). The statistical error bars due to the Ge laser spectrometer are not visible, the systematic errors by exchanging the sample three times, exchanging the position of sample and reference, and repeating the measurement are within in the plotted data points.

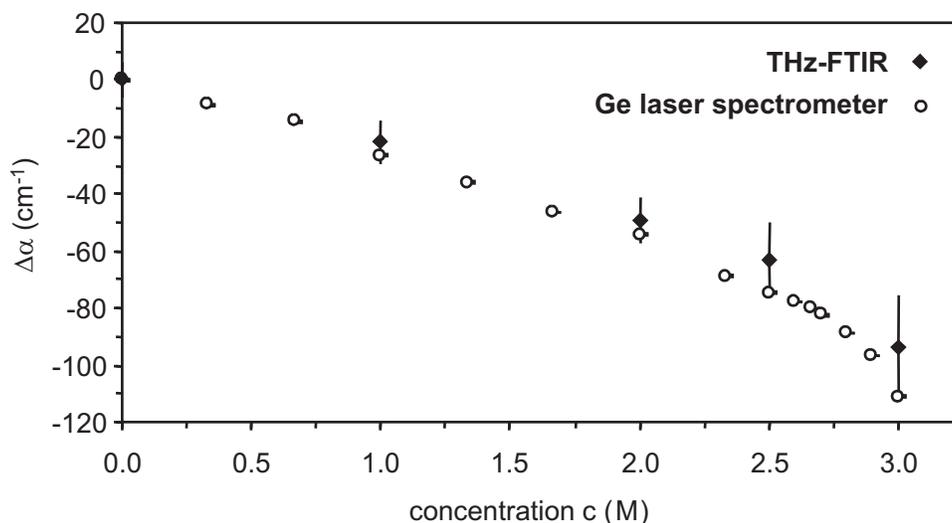


Figure 5. Comparison of systematic errors in the absorption coefficient as a function of concentration  $c$  during a THz measurement of solvates in water using the Ge laser spectrometer system and a THz-FTIR spectrometer while measuring for comparable time.

The measurement time for 30000 pulses for each channel (sample and reference) and the set chopper speed was approximately 7.5 minutes. With exchange, cleaning, new filling and thermal equilibrium of the solution a measurement time of 20 to 30 minutes is needed for each measurement which corresponds to the total scanning time used in each FTIR measurement. The systematic errors are especially evident for high concentrations. Nevertheless, both measurement systems show the same trend although this conclusion is a result of knowing the data obtained with the Ge laser system. The error bars, increasingly larger for higher concentrations, would not have allowed to detect the strong non-linearity above 2.5 M. At 3.0 M we obtain an error in the absorption coefficient of  $\pm 0.3 \text{ cm}^{-1}$  in comparison to  $\pm 20 \text{ cm}^{-1}$ .

Figure 6 shows absorption measurements as a function of concentration using the Ge laser spectrometer. It is possible to distinguish a single point mutation in the protein ubiquitin. The error of the measurement of the buffer reference at concentration zero is very small. Both points coincide within 0.3% corresponding to the error bar while the difference for both proteins is clearly visible exceeding to 3%. The somewhat higher error in reference to the previous example is a result of the more difficult sample preparation and tendency of proteins to foaming. Further details and measurements can be found in the references.<sup>15</sup>

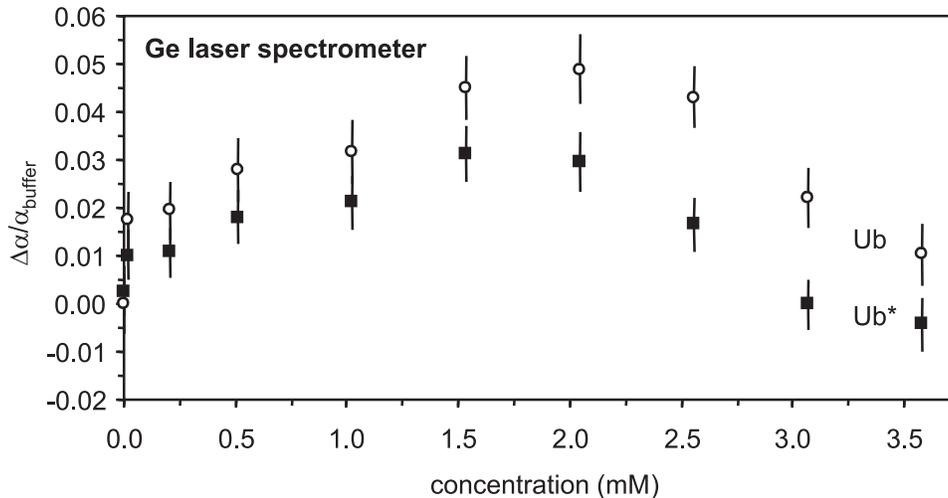


Figure 6. Relative absorption difference of the ubiquitin wild-type (Ub) and a pseudo-wild-type (Ub\*) with a single point mutation in reference to the used buffer solution (mainly water). The point mutation F45W replaces at position 45 the amino acid phenylalanine by a tryptophane. Ubiquitin contains 76 amino acids.

#### 4. TERAHERTZ TIME-DOMAIN SPECTROSCOPY

In the lower THz frequency range transmission experiments are possible with other sources. Terahertz time domain spectrometry (THz-TDS) is frequently used with a good signal-to-noise ratio (SNR) below 1.5 THz. The signal is generated in a THz emitter which is excited by a femtosecond laser pulse.<sup>16</sup> A 80:20 beam splitter splits the fs laser pulse. The high power fs pulse is focused on the emitter. The weaker pulse is directed via a translation stage introducing a time delay to the THz detector.

The emitter (Tera-SED, GIGAOPTICS GmbH, Germany) is a planar large-area GaAs-based photoconductive emitter using a interdigitated electrode structure. The Tera-SED array structure allows to omit the typically used silicon lens since the emitted pulse beam profile has already a small beam divergence. The emitter is biased with a modulated voltage of up to 30 V at a frequency of 40 to 50 kHz. This modulation frequency is also connected to the reference channel of the lock in amplifier. The fs laser pulse leads to the emission of a THz pulse of sub-picosecond length. This pulse is directed via an off-axis mirror to the sample cell containing the liquid or to the volume where two liquids are mixed. The whole THz set-up is also enclosed in a chamber flushed with dry air to reduce water vapor absorption.

Recently this setup has been used for kinetic THz absorption (KITA) spectroscopy showing that protein folding can be followed in real-time.<sup>15,17</sup> The initial steady-state absorption experiments with the Ge laser spectrometer have enabled to define the correct concentrations and the necessary mixing volumes for sufficient signal-to-noise in the experiments for KITA.

The major limitation of this protein folding experiment is the process of mixing two solutions which renders the protein unusable for further experiments. Considerable time and effort is necessary to obtain and purify a small amount of protein especially if mutants are investigated. After mixing, the protein has to be purified again. So only up to 100 or fewer mixing processes can be performed. All data has to be collected within these 100 shots each lasting a few tens of milliseconds. The knowledge of the optimum concentration for these experiments is therefore crucial.

With an asynchronous optical sampling (ASOPS) time-domain spectrometer (GIGAOPTICS GmbH, Germany) we can obtain a full 0.1 to 3 THz spectrum within 100  $\mu\text{s}$  with an spectral resolution of 1 GHz corresponding to the repetition frequency of the twin-femtosecond laser. Processes and kinetics on this time scale are therefore feasible or even faster if the sampled region is limited to a few picosecond at the expense of spectral resolution which is not required for liquids as has been discussed (cf. 1).

## 5. THZ SOURCES AND DETECTION COHERENT SYNCHROTRON RADIATION AT ANKA

Chemistry and biology suggest various spectroscopy problems for which we try to find optimal solutions. In the case of materials with lower absorption it is possible to use room temperature detectors and small liquid nitrogen cooled quantum cascade lasers.<sup>4</sup> Micro-bolometer cameras optimized for the thermal band from 7 to 14  $\mu\text{m}$  wavelength can still detect at THz frequencies.<sup>18</sup> We were able to detect laser radiation from liquid nitrogen cooled 3 to 4 THz quantum cascade lasers and of a far-infrared gas laser at a small frequency of 1.76 THz<sup>19</sup> using a micro-bolometer camera based on amorphous silicon. Recently, we have used the micro-bolometer camera at the free electron laser FELBE. With the emission power of 8 W lowered to 3.6 mW, it was possible to focus the 87  $\mu\text{m}$  laser beam onto a single 45  $\mu\text{m}$  pixel of the camera. The optimization of these types of cameras for the THz range by adapting the micro-bolometer chip to THz frequencies might enable an easy way for imaging active sources.

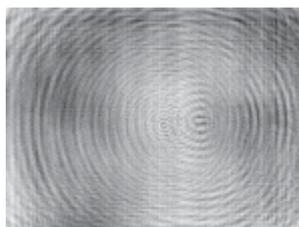


Figure 7. Diffraction pattern of two off-set circular apertures at 87  $\mu\text{m}$  (3.4 THz) using 200 mW power at FELBE (Dresden-Rossendorf) measured with a micro-bolometer camera.

Synchrotron radiation has the advantage of covering a large range of frequencies from microwaves to X-rays continuously. With decreasing frequency the power level diminishes. However, measurements at the synchrotron ANKA (Karlsruhe, Germany) have shown that THz coherent synchrotron radiation can provide sufficient power even for experiments with strongly absorbing samples. We have used a room temperature Golay detector<sup>4</sup> to estimate the average power emitted in the frequency range from 0.1 to 1.8 THz from the ANKA storage ring. The measured average power of up to 0.2 mW suggests a peak power of more than 1 W.<sup>20</sup> Currently we are setting up a new scanning near-field infrared microscope<sup>21</sup> for a planned commission in 2010 at the new ANKA infrared beamline. Demonstrated experiments with a THz laser power of 5 mW for near-field microscopy<sup>22</sup> suggest also the feasibility of a synchrotron based THz near-field microscope.

## 6. SUMMARY

We have compared systematic errors in different experimental setups referencing to well known Fourier transform spectrometers. The germanium laser based spectrometer provides a high accuracy to allow the systematic study of biomolecules, like proteins in water, even though liquid water has a very high absorption coefficient in the THz frequency range. A single point mutation in a protein can be distinguished in the absorption coefficient. Time-domain spectroscopy allows to measure kinetics like protein folding. Coherent synchrotron radiation can provide sufficient signal for THz applications especially for a THz near-field microscope.

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