Plasmonics-Nanofluidics Metamaterial: An Ultrasensitive Platform for Terahertz biosensing and Quantitative Measurement of Molecules

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Abstract:

The terahertz spectroscopy has attracted enormous attention for label free chemical and biological sensing, due to the presence of molecular fingerprints and remote sensing capabilities. However, because of the strong absorption of THz waves in aqueous solutions, detecting liquid-phase biological samples by THz spectroscopy has encountered strong constraints. Here, we demonstrate for the first time a rapid and label-free sensing for liquid-phase biological samples using THz spectroscopy based on microfluidic metasensors. The metasensors were integrated with microfluidic chip to precisely control the liquid sample thickness within 220 nanometer (MMS-N) and 50 micrometer (MMS-U). The metasensors include a periodic split ring resonator (SRR) with two resonant peaks in the THz range. The sensitivity(S) and corresponding figure of merit (FOM) of the two resonance peaks of MMS-N and MMS-U are analyzed with respect to different refractive index matching solution. Both the two resonance peaks of the metasensors are sensitive to the varies refractive index environment and high-frequency resonant of MMS-N shows the best sensitivity(~136GHz/RIU). In addition, the label-free biological sensing capability was conducted through qualitative and quantitative LDL and ox-LDL, where a shift of resonance frequency was observed as the LDL and ox-LDL concentration increased. The results indicated nanofluidic metasensor presented excellent sensing capability for liquid-phase molecular analysis by THz spectroscopy, and the proposed sensing system has the potentials to be developed as a rapid, label-free and highly sensitive detection tool for molecular analysis by THz sensing.

Key words: THz spectroscopy, microfluidic chip, metamaterial sensor, liquid-phase sensing
Introduction

Recently, spectroscopy detection has been considered as one of the most important methods in the field of bimolecular sensing. Terahertz (THz) time-domain spectroscopy technology has attracted the attention of many researchers because of its unique advantages, as a promising label-free, non-contact and non-destructive detection method. Nevertheless, the detection of the trace amount of analyte in aqueous environment using THz spectroscopy has encountered strong constraints because of lacking specific fingerprint and strong absorption of water in the THz domain. Thus, methods such as THz attenuated total reflection spectroscopy (THz-ATR), microfluidic chips and metamaterials have been developed, which increases the interaction strength between the analyte and the incident THz waves significantly and hence allows for a much higher sensitivity and try to achieve sensing in a liquid environment. The THz-ATR is limited by its relatively poor accessibility, integration limitations and high cost. Therefore, exploring a reasonable and effective liquid sensing method in THz region remains to worth studying.

The metamaterial has an excellent electromagnetic enhancement effect, which express as showing a strong resonance peak, and making the metamaterial sensitive to the dielectric change of the surface of the fabricated structure. However, the application of metamaterials for biomolecule sensing is mostly limited to solid or dry environments, and microfluidic chips can be used to overcome water absorption due to low cost, limited volume of detection samples and rapid detection. The integration of the microfluidics and metamaterials provides the possibility of reducing absorption by ultra-thin liquid to reduce analyte and increasing sensitivity by reasonable design. This strategy has clearly improved the THz measurement sensitivity and accuracy, as observed in studies on chemical mixtures, protein structure and ion concentration in liquid solutions, but the structural characterization and quantity of homologous samples has not been fully studied.

Natural low-density lipoprotein (LDL) contains a large amount of polyunsaturated fatty acids (PUFA), which can be easily oxidized to form oxidized low density lipoprotein (ox-LDL). ox-LDL is considered to be an independent risk factor for atherosclerosis (As) and is widely present in AVSD, non-alcoholic fatty liver disease (NAFLD), diabetes complications and systemic lupus erythematosus (SLE) patient’s blood. Therefore, the identification and content of LDL and ox-LDL are of great significance for the prediction and early diagnosis of cardiovascular diseases and lipid metabolism disorders.

In this paper, we have developed two scales of label-free THz metamaterial sensor integrated with microfluidic channel. Meanwhile, we studied the resonance and transmission characteristics of both microfluidic metamaterials sensors (MMS), and
the nano-space fluidic metamaterials sensor (MMS-N) are applied to the identification of the oxidation state of LDL. The silicon-based biosensors include a periodic split ring resonator (SRR) with two resonant peaks in the THz range. The two resonance peaks of the both MMS-U and MMS-N are sensitive to the effective dielectric environment, and can effectively identify changes in the refractive index of the environment. At the same time, we compared the sensing capabilities of the two resonance peaks, and the THz transmission difference between LDL and ox-LDL is further discussed and analyzed.

**Experimental**

**THz time domain spectroscopy system (THz-TDs).**
A commercial THz-TDs system (TAS7500SP, Advantest Corporation) was used in the experiments. The system was used under normal circumstances with a stable frequency range of 0.5-2.3 THz, a frequency resolution of 7.6 GHz and a SNR of 60dB. During experiments, the humidity was kept less than 1.0% with dried nitrogen and temperature was kept around 25℃.

**Fabrication of Metamaterial**

The Metamaterials composed of metallic square arrays of single-ring resonant rings (SRRs) was fabricated by a conventional photo-lithography technique on a high-resistance silicon (Si) substrate with a thickness of 525 μm. The metallic SRRs consists of a 10 nm titanium layer and a 120 nm gold layer. The size of the SRRs is on the micron level, and detailed structural parameters are as follows: the length and width are 26μm, the line width is 6μm, the gap is 2μm, the period is 40μm, as depicted in Figure 1. (a). There is a different operation to make MMS-N. A region, with a length and width of 7.2 * 7.2mm and a depth of 220 nm, needs to be etched first, and then deposited metal. After lithography is completed, each chip is cut into 1.5 * 1.5cm for use.

**Preparation of Microfluidic metamaterials sensor**

Microfluidic sensor with two different liquid layer thicknesses have been developed, MMS-U with 50μm channel height and MMS-N with 220 nm channel height. Metamaterial devices are bonded to PDMS microfluidic chip with the structural parameters as follows: The outer length is 1.5cm, the inner length is 7.2mm, the outer width is 1.5cm, the inner width is 7.2mm, the thickness of PDMS is 1mm, the surface of the PDMS is perforated, the hole diameter is 1mm, and an external hose is inserted into the steel pipe (outer diameter 1mm, inner diameter 0.6mm). With the limitation of processing technology, we have to etched the flow channel with 220nm height on the si substrate for MMS-N, as mentioned above. Therefore, the channel height is 50μm and for MMS-U and it is just a cover with no height for MMS-N. The schematic and characterization of fabricated MMS-U and MMS-N is shown in Figure 2.

**Sample preparation**
LDL was purchase from Sigema; ox-LDL was from solabio; Analytical purity Methanol, Ethanol, Isopropanol were from Chuan-dong Chemical (Group) Co., Ltd. in Chongqing; CuCl$_2$, Sudan Black B, and PBS were purchased from Aladdin; DDW was derived from ultrapure water system. We also prepared oxidized low-density lipoprotein (ox-LDL) by Cu$^{2+}$ induction, the details is described as following: The concentration of the LDL protein in PBS was about 200 mg / mL; an equal volume of 400 µmol/L CuCl$_2$ solution was added, and the mixture was allowed to react at 37 °C for 17 h. The reaction was terminated by adding an equal volume of EDTA, and stored at 4 °C.

**Determination of relative mobility**

Adjust the concentration of LDL and ox-LDL to 2mg / ml, take each of them 20µl and mix with 2µl Sudan Black B staining solution, and incubate at 37 °C for 30 minutes; mix 1µl 10X loading buffer with 9µl pre-stained protein sample, 100V constant pressure electrophoresis 50min. The ratio of the electrophoresis distance of ox-LDL and LDL is the relative mobility of ox-LDL.

**THz Measurement of liquids Sample by MMS**

MMS-U and MMS-N are applied to for liquid sensing, analyte (approximately 3µl for MMS-U and 2µl for MMS-N) was added through injected hole and ensure that the liquid fills the entire inspection area. For each sample, three consecutive measurements were conducted and each measurement was calculated 1024 times. By cutting off reflection peak of THz pulse in time-domain, Savitzkey-Golay smoothing and Normalized [0, 1], the frequencies corresponding to the minimum value of transmission were regarded as the resonant frequency. With dry nitrogen as a reference, the actual transmission of the sample is obtained through dividing the measured transmittance of the sample by the reference value.

**Results and discussion**

**Characterization of the MMS**

The fabricated metamaterial as shown in Figure 1. (a)-(c), consists of many square metal rings with a capacitive gap. When the THz waves were illuminated parallel to the MMS, we got two resonance peaks, one at sub-THz frequency (peak 1) and the other at THz frequency (peak 2), with frequency at 0.771 THz, 2.129 THz respectively, as shown in Figure 2., The appearance of the resonance peaks are due to LC (inductive-capacitive) resonance, which was determined by the inductance of the SRR (L) and the capacitance of the gap (C)$^{23}$. As for LC resonance, that is because the dielectric constant $\varepsilon_m$ of the metal is several orders of magnitude higher than the dielectric constant of the dielectric $\varepsilon_d$ in the THz state, so the resonance frequency of SRRs can be described by the dispersion relationship under normal incidence as follows $^{24}$:
\[
   f_{\text{SRR}} = |k_m + G| \frac{c}{2\pi} \sqrt{\frac{\varepsilon_m + \varepsilon_d}{\varepsilon_m \varepsilon_d}} \approx |k_m + G| \frac{1}{2\pi \sqrt{\varepsilon_d}} \quad (1)
\]

Where \( k_m \) is the in-plane wave vector of the incident THz field, and \( G \) is the reciprocal lattice vector of the periodic structure. Therefore, the refractive index \( n_d \) and the dielectric number \( \varepsilon_d \) of the dielectric medium satisfy the following relationship:

\[
   n_d = \sqrt{\varepsilon_d} \quad (2)
\]

Combining equations (1) and (2) shows that the index \( n_d \) is inversely proportional to the resonance frequency \( f_{\text{SRR}} \). Hence, the change of the refractive index around the SRR, especially capacitance gap, can be detected by the corresponding frequency shift of the resonance peak.

**Figure 1.** The schematic and characterization of fabricated MMS. (a) The Schematic of SRR. (b) Scanning electron microscope characterization of SRRs. (c) 3D topography of white light interferometer of SRRs. (d) Schematics of the ideal THz MMS and its cross-sectional device structure. (e) MMS made by micro-electro-mechanical processing.
**Figure 2.** Measured transmission spectra of MMS. (a) Transmission spectra of MMS-U. Inset graph shows schematics of the MMS-U. (b) Transmission spectra of MMS-N. Inset graph shows schematics of the MMS-N.

**The quality of the MMS**

Since the sensor works based on the frequency shift of the THz spectral resonant peak, the sensitivity and linewidth of peak play an important role in the precision of the refractive index sensing. In the study of sensing using THz metamaterials\(^{15}\), the refractive index sensitivity \( S \) of the sensor is defined as the resonance frequency shift corresponding to per-refractive index unit, and its unit is GHz/RIU.

Linewidth of peak is measured by full width at half maximum (FWHM), which was the full width when the semi-maximum value is obtained. The period and shape of SRRs affect the peak line width and the peak line width affects the frequency shift. The merit diagram (FOM) obtained by dividing the sensitivity by the resonant frequency linewidth is widely used to characterize the sensing capability of the SRRs. For spectral biosensor chips, the higher the FOM value, the better the performance of the biosensor.

**Sensing the refractive index matching solution**

In order to verify the sensing capabilities of MMS-U and MMS-N and compare the performance of the sub-THz frequency and THz frequency resonance peaks for liquid sensing, analytically pure methanol, ethanol, isopropanol, and DDW were used as the refractive index matching solution to compute the \( S \) and FOM of the MMS through their transmission spectra. Their refractive indices are as follows: Water (refractive index \( n=2.20 \)), methanol (\( n=1.69 \)), ethanol (\( n=1.54 \)), and isopropyl alcohol; IPA (\( n=1.51 \))\(^{31}\).

When IPA, ethanol, methanol, and water were detected, the peak 1 of MMS-U was red shifted from 0.771 to 0.717 and Peak 2 red shifted from 2.129 to 2.022, with maximum \( \Delta f \) were 53.4 and 102.8 GHz, respectively, as shown in Figure 3. (a)-(b). When those solutions were detected by MMS-N, their resonance frequencies still showed the tendency of redshift, but their maximum \( \Delta f \) were 58.5 and 135.9 GHz for peak 1 and peak 2, respectively [Figure 3. (c)-(d)].

For peaks 1 and peak 2, whether MMS-U or MMS-N, the refractive index and frequency shift change show a proportional trend, as shown in Figure 4. (a). Corresponding to the relationship between refractive index and \( \Delta f \) with blank as reference, the slope of the fitted line in the Figure 4. (b) are the sensitivity of the sensor. the R square of fitting line for MMS-U was 0.80293 and 0.97978 for the peak 1 and peak 2, respectively. Meanwhile, the number of Peak 1 is 0.95879, and Peak 2 is 0.94711 for MMS-N. FWHM also can be calculated from black line of Figure 3., Since the sensitivity of double peaks of MMS-N are higher than MMS-U, the FOM also have the same trend.

In summary, for MMS-U and MMS-N, the sensitivity peak 2 are significantly higher...
than those of peak 1, indicating that the sensitivity of high-frequency resonance may be better than low-frequency. These results are attributed to the strong electric field limitation and is basically consistent with the reported solid-phase. In addition, it is worth noting that the sensitivity and FOM of MMS-N are higher than MMS-U, indicating that limiting the detection volume to the nanometer level may not only reduce water absorption but also improve the detection efficiency of THz metamaterials.

Figure 3. Measured transmission spectra of MMS under different liquid sample environments. Normalized Transmission spectra of peak 1 (a) and peak 2 (b) used MMS-U. Normalized Transmission spectra of peak 1 (c) and peak 2 (d) used MMS-N.

Figure 4. The fixed quantity between the resonant frequency of MMS and different liquid sample. (a) The measured resonance frequency shift with different liquid sample. (b) Fitting of resonance
Sensing different concentrations of ethanol
In order to further explore the ability of MMS-U and MMS-N to sense in complex liquid environments, we further measured the transmission response of the vary concentrations of ethanol in water solution. Figure 5. (a) and (b) present the measured transmission spectra of the double peaks of MMS-U with varied ethanol concentrations. The resonance frequencies of the peak 1 and peak 2 were at 0.732 THz and 2.090 THz, respectively, when pure ethanol was injected. They gradually redshifted to 0.710 THz and 2.045 THz as the ethanol concentration decrease. Figure 5. (c) and (d) are transmission spectra of ethanol by using MMS-N. The result trend is similar to the MMS-U measurement results. In general, with the ethanol concentration increasing, resonance frequencies gradually blueshift with water as reference, because ethanol has a lower refractive index compared to water.

**Figure 5.** Measured transmission spectra of MMS with ethanol/water solution of different ethanol concentrations. Normalized transmission spectra of peak 1 (a) and peak 2 (b) using MMS-U with different ethanol concentrations; Measured transmission spectra of peak 1 (c) and peak 2 (d) using MMS-N with of different ethanol concentrations.
Figure 6. Sensing different concentrations of ethanol. (a) The Δf (vs. water) of peak 1 is plotted against different ethanol concentrations. (b) The Δf (vs. water) of peak 2 is plotted against different ethanol concentrations.

As the ethanol concentration increased, Δf showing an increasing trend with water as reference, and indicated the resonance frequency shift from pure ethanol and water was 15.3 GHz for MMS-U and 30.5 GHz for MMS-N [Figure 6.]. The sensing result of peak 2, with larger Δf, were similar to the peak 1, which were 68.7 GHz and 91.6 GHz for MMS-U and MMS-N, respectively. In general, the frequency shift and R² of peak 2 is better than peak 1, and the performance of MMS-N is a little better than MMS-U, which is basically the same as the previous results.

Qualitative and quantitative of LDL and ox-LDL with MMS-N

The above experimental results prove that the THz element sensor constructed can detect changes of refractive index in a highly absorbing liquid environment, and MMS-N has better expressive power, so it is applied to distinguish the low-density lipoprotein oxidation state.

Natural low-density lipoprotein (LDL) contains a large amount of polyunsaturated fatty acids (PUFA), which can be easily oxidized to form oxidized low density lipoprotein (ox-LDL). As it’s shown in Figure 7. (a), the electrophoresis distance of ox-LDL is greater than LDL, because the positive charge of apo B was neutralized during the formation of ox-LDL. 

Figure 7. Identification of LDL and ox-LDL. (a)Agarose gel electrophoresis of LDL and ox-LDL; Measured transmission spectra of peak 1 (b) and peak 2 (c) with same concentration of LDL and...
ox-LDL.

Each liquid has its different refractive index and absorption coefficient, which can be distinguished by the frequency shift of the resonance peaks. The transmission of both LDL and ox-LDL solutions were further measured by using that MMS-N. The average curves of 3 measurements for each sample are plotted in Figure 7. (b)-(c). We observed the difference in resonance frequency of 7.8GHz for peak 1 between same concentration of LDL and ox-LDL with the resonance frequency 0.724, 0.732 respectively. However, peak 2 showed no changes in resonance frequency for sensing LDL and ox-LDL. Using water as a reference, the addition of salt to water causes red shift of the resonance peak due to a decrease of the refractive index, as described by the institute of Masson\textsuperscript{35} and Asaki\textsuperscript{36}. When adding LDL and ox-LDL, the result is reversed because the addition of protein will reduce the absorption coefficient of the solution as described by the institute of the protein in cell\textsuperscript{37} and BSA and lysozyme\textsuperscript{38}. During the identification of oxidation states, peak 1 performed better than peak 2. This is different from our previous results in sensing ethanol solution.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Sensing different concentrations of LDL. Measured transmission spectra of peak 1 (a) and peak 2 (b) with different LDL concentrations. (c) The relation between resonant frequency and solution of concentration for LDL. Inset graph shows the relation between $\Delta f$ and solution logarithm of LDL with concentration from 0.1 to 2 mg/ml.}
\end{figure}

We subsequently performed the quantitative sensing studies of LDL and ox-LDL at different concentrations for further confirmation and research. When detecting
different concentrations of LDL, the blue shift of the resonance frequency of both peaks could be observed with water as a reference [Figure. 8. (a) and (b)]. With the increasing of lipoprotein concentration, the resonance frequency shift of double peaks increased then decreased and finally reached the plateau phase [Figure. 8. (c)]. The concentration range is between 0.1 ~ 2mg, and the frequency shift is linearly related to the log₂C [insert graph of Figure. 8. (c)]. The sensing result of ox-LDL([figure 9.]) is very similar to LDL, but it has a larger frequency shift for peak 1, and peak 2 are easier to the plateau phase. On the other hand, the slope and R² of the fitted line of peak 2 are both greater than peak 1, which further validates our previous conjecture.

Figure 9. Sensing different concentrations of ox-LDL. Measured transmission spectra of peak 1 (a) and peak 2 (b) with different ox-LDL concentrations. (c) The relation between resonant frequency and solution of concentration for ox-LDL. Inset graph shows the fit of Δf to log₂C.

Since the study of protein behavior in aqueous solutions in THz region is very limited, the emergence of this result has attracted our interest. To further clarify this result, we try to explain using a three-component dielectric model of solvated molecules. The solvated molecule is described as a sphere with a radius R, then a three-component model exists in the solution: a solute (in this paper, it’s protein), a solvated shell around the solute, and free water. Assuming the total solution volume is V and the volumes of solute, solvated shell and free water are Vp, Vs, Vw, the effective refractive index of the solution is given by:

$$n_{eff} = \frac{V_p}{V} n_p + \frac{V_s}{V} n_s + \frac{V_w}{V} n_w$$  \hspace{1cm} (3)
Where \( n_{\text{eff}} \) is the refractive index of the solution. Meanwhile, \( n_p, n_s, \) and \( n_w \) represent the refractive indices of the protein, solvated shell, and free water, respectively. For macromolecular proteins (\( M > 2 \text{kDa} \)), the contribution of \( n_s \ll n_p \) and solvated shell is negligible compared to the contribution of solute. At the same time, because \( n_p \approx 0.86 n_w \), the effective refractive index of the solution is transformed into:

\[
  n_{\text{eff}} = \frac{v_p}{v} 0.86n_w + \frac{v_w}{v} n_w = 0.86\left(\frac{v_p + v_w}{v}\right)n_w
\]

Therefore, for large protein solutions, the effective refractive index of the solution is mainly given by the refractive index of the protein, and the \( n_{\text{eff}} \) of the solution is smaller than the refractive index of water. Thence, adding protein to solution causes a blue shift of resonance peaks, and the amount of frequency shift for different proteins may diverse, such as LDL and ox-LDL. But the contribution of protein is limited compare with water and , so there will be prone to a plateau period.

The sensor can qualify and quantify LDL and ox-LDL without labels by reflecting the change in the refractive index of the solution. Although based on the design of our devices and THz-TDS equipment, we only get a few GHz differences, the sensitivity still needs to be improved in future clinical applications. However, this can be readily overcome through further exploration of selective functionalization methods in the gap region as well as optimization of the resonator design. The improved metamaterial sensor is expected to realize more complete and comprehensive study of molecular behavior in the THz region.

**Conclusion**

In summary, we designed and fabricated both Si-based SRR microfluidic and nanofluidic resonators and examined their sensing capabilities using the alcohol-water mixture, LDL and ox-LDL solution. Through confinement of aqueous solution in a micro/nano space, clear resonance was observed for the design of both SRR resonators. Moreover, the change of their surrounding environment led to the resonance frequency shift in the measured spectra. The nano space THz metamaterial sensor requires small sample volume of \(~2\mu l\) and has better sensitivity to diversification dielectric environment \((\sim 136 \text{GHz/RIU})\), which makes it a good option to inspect expensive biomaterials. This work verifies the feasibility that our sensor has the potential to be an effective approach for molecular analysis through advanced selective functionalization methods, optimization of metamaterial design, and integration with more efficient micro/nano-fluidic system and opens up a research area for highly sensitive, label-free and non-destructive molecular study in the near future.
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None.

Authors’ contributions
Weiling Fu, Yang Zhang and Jinchun He conceived the research and designed the experiments. Mingzhen Xie performed the experiment part and wrote the manuscript. Yu Liu and Huiyan Tian contributed to the manuscript preparation and data validation. Xinyu Zhan, Ke Xia and Mengya Li performed sample pretreatment and THz sensing. Marc Lamy de la Chapelle provided advice and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors have approved the manuscript and agree with submission to your esteemed journal.

Competing interests
The authors declare no competing financial interest.
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