New Method to Study the Vibrational Modes of Biomolecules in the Terahertz Range Based on a Single-Stage Raman Spectrometer

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ABSTRACT: The low-frequency vibrational (LFV) modes of biomolecules reflect specific intramolecular and intermolecular thermally induced fluctuations that are driven by external perturbations, such as ligand binding, protein interaction, electron transfer, and enzymatic activity. Large efforts have been invested over the years to develop methods to access the LFV modes due to their importance in the studies of the mechanisms and biological functions of biomolecules. Here, we present a method to measure the LFV modes of biomolecules based on Raman spectroscopy that combines volume holographic filters with a single-stage spectrometer, to obtain high signal-to-noise-ratio spectra in short acquisition times. We show that this method enables LFV mode characterization of biomolecules even in a hydrated environment. The measured spectra exhibit distinct features originating from intra- and/or intermolecular collective motion and lattice modes. The observed modes are highly sensitive to the overall structure, size, long-range order, and configuration of the molecules, as well as to their environment. Thus, the LFV Raman spectrum acts as a fingerprint of the molecular structure and conformational state of a biomolecule. The comprehensive method we present here is widely applicable, thus enabling high-throughput study of LFV modes of biomolecules.

INTRODUCTION

The low-frequency vibrational (LFV) modes in the terahertz (THz) range (0.1–10 THz, 3–333 cm⁻¹) have been studied extensively due to their significance in providing information related to the dynamics and functional mechanisms of biomolecules’ activities, including collective modes of proteins, ligand binding, protein interaction, electron transfer, and enzymatic activity.

The importance of studying the LFV modes in biomolecules has led to the development of many methods to access the THz range. These include far-infrared Fourier transform infrared (FTIR), attenuated total reflectance (ATR), and Raman spectroscopies based on double- or triple-stage technology, inelastic neutron scattering, synchrotron irradiation, THz time-domain spectroscopy (THz-TDS), heterodyne-detected Raman-induced Kerr-effect spectroscopy (OHD-RIKES), and coherent anti-Stokes Raman scattering (CARS).

The correlation between the molecular mechanisms of biomolecule activity and LFV spectra can be more meaningful if the study is performed in a hydrated environment. Such studies are difficult to carry out because of the strong absorption of water in the THz range. Several solutions to overcome this problem have been suggested and subsequently implemented, depending on the spectroscopic method used to study the LFV modes. For far-infrared FTIR and THz-TDS, samples have been pressed with polyethylene (PE) powder into a pellet form or spin-cast as a thin film for ATR. When using synchrotron radiation, samples have been lyophilized in vacuum chambers and in some cases cryogenically cooled, and in the case of OHD-RIKES, highly concentrated protein solutions were used. A challenge in measuring LFV modes is to develop an affordable, nondestructive, noninvasive, and robust method that can allow high-throughput study of biomolecules in nearly any lab or field environment.

In this article, we demonstrate a new approach to studying the LFV modes of biomolecules based on Raman spectroscopy. Raman spectroscopy is a well-established technique to probe the vibrational modes of materials that can provide detailed information about the composition, stoichiometry, and crystaline phase of the materials under investigation. Even though...
Raman scattering has been used extensively in life-science research, studies of the LFV modes via Raman spectroscopy have been limited due to difficulty in performing the experiments. The traditional approaches for measuring the LFV Raman modes are accomplished by a triple spectrometer to reject the laser light or I₂ gas filters to absorb the narrow band laser light. Such optical setups are complicated and expensive and also suffer from low collection efficiency of the Raman signal. The recent development of notch filters based on volume holographic gratings (VHGs) has made it possible to measure LFV Raman modes down to 5 cm⁻¹ using a single-stage spectrometer. Using these filters, it is also possible to measure both Stokes and anti-Stokes LFV Raman modes simultaneously. Generally, Raman scattering arises from symmetric stretching and bending vibrations of molecules, whereas THz absorption deals mainly with asymmetric stretching vibrations. Thus, owing to the different selection rules governing Raman and IR transitions, both Raman and IR spectroscopies provide complementary spectral information about LFV modes of biomolecules.

Using a single-stage spectrometer and VHG notch filters, here, we study the previously unexplored LFV Raman modes of basic biomolecules such as amino acids, peptides, proteins, and DNA while taking considerations of earlier works by other techniques. We obtain Raman spectra for biomolecules in a hydrated environment with minimal sample preparation, thus providing a direct, robust, and relatively inexpensive method for high-throughput study of biomolecules.

## RESULTS AND DISCUSSION

Here, we present the application of Raman spectroscopy to the study of the LFV modes of proteins and their building blocks, amino acids and peptides, and short synthetic DNA oligonucleotides. The LFV Raman system we developed allows measurement down to frequencies as low as 8 cm⁻¹ in both the Stokes and anti-Stokes regions. The air-dried spots of biomolecules hold enough water to maintain a hydrated environment.

### Amino Acids

Amino acids, which are the fundamental building units of proteins, are very important compounds as they take part in major metabolic processes, namely, growth, formation of new tissues, and biosynthesis of enzymes and hormones in the body. Phenylalanine is one of the 20 naturally occurring amino acids. It plays a key role in the biosynthesis of tyrosine and vital hormones like norepinephrine and epinephrine. Recently, phenylalanine was used as a building block for self-assembled metal nanowires. Previous studies of LFV modes carried out by THz-TDS or rapid-scan FTIR spectroscopy dealt with polycrystalline forms of phenylalanine mixed with PE powder pressed into round disks. However, to our knowledge, there is no theoretical as well as experimental published literature available that describes the LFV modes of phenylalanine using Raman spectroscopy. Figure 1 shows the LFV Raman spectrum of phenylalanine. The spectrum consists of several well-resolved

![Figure 1. LFV Raman spectrum of phenylalanine. (a) The Stokes and anti-Stokes spectra of phenylalanine and (b) Stokes spectrum only within the 0–200 cm⁻¹ range.](image-url)
peaks located at 9.4, 24.6, 39.6, 64.6, 98.7, 132.7, 142.8, and 178.8 cm$^{-1}$. Generally, the Raman modes between 130 and 200 cm$^{-1}$ arise from frustrated rotation and translation of one or more molecules (also known as intermolecular modes), whereas the modes below 130 cm$^{-1}$ are assigned to the lattice modes of organic crystals. As the rotational oscillations are stronger than the translational modes, the strongest peak that is observed at 98.7 cm$^{-1}$ can be attributed to rotation. The exact identification of the peaks needs to be carried out in a future study.

**Model Amphipathic Peptide.** LFV modes are important for the characterization of peptide structures as the observed spectra result from peptide backbone motions and hydrogen bond vibrations and therefore directly reflect the peptide secondary structure. Thus, the relationship between the delocalized backbone motions and the functional flexibility or rigidity of peptides can be probed with LFV Raman and THz spectroscopic approaches. Several peptides were previously studied in the LFV region, including lyophilized short-chain peptides by THz absorption and neutron vibrational spectroscopy, highly concentrated solution of di- and poly-L-alanine by OHD-RIKES, alanine-rich peptides by THz-TDS using custom-made copper cold finger, and by FTIR with synchrotron light source using liquid samples placed between the two polypropylene windows. In this study, we examined the LFV Raman modes of a model amphipathic peptide, $K_L L_R$. We found that the LFV Raman spectra of $K_L L_R$ did not show any vibrational peaks in the wavenumber range below 100 cm$^{-1}$ (Figure 2). However, a weak and broad shoulder centered at 140 cm$^{-1}$ was observed that can be assigned to the localized backbone torsions.

**Lysozyme and Bovine Serum Albumin (BSA).** Lysozyme, an enzyme which hydrolyzes the polysaccharides found in many bacterial cell walls, and BSA, the main protein in bovine plasma, are widely used models for studying the THz range of proteins. BSA studies include using THz spectroscopy, THz coherent synchrotron radiation, pulsed THz spectroscopy, and THz-TDS. Lysozyme studies include employing Raman spectroscopy with a double monochromator, OHD-EIKES, THz-TDS, FTIR-Raman spectroscopy with holographic grating, and femtosecond optical Kerr effect (OKE) Raman spectroscopy. Here, we measured the LFV Raman spectra of lysozyme and BSA for the first time in a hydrated environment, as described earlier in this work. Figure 3a shows the LFV Raman modes of lysozyme with Stokes (right) and anti-Stokes (left) spectra. The Raman spectra reveal a strong peak centered at 28 cm$^{-1}$, attributed to intermolecular vibration, and an additional broad shoulder centered around 70 cm$^{-1}$. The lysozyme contains both $\alpha$ and $\beta$ configurations of the peptide chains; therefore, the low-frequency line at 70 cm$^{-1}$ may arise from the torsional motion of peptide chains with $\alpha$ configuration and the weaker peak at 166 cm$^{-1}$ can be due to the bending motion of peptide chains with $\beta$ configuration, as explained by Genzel et al. Our observations are in agreement with the work that Genzel et al. carried out using a double-monochromator-based Raman spectrometer on crystalline lysozyme.

Figure 4 describes the Stokes (right) and anti-Stokes (left) LFV Raman spectra of BSA. The Stokes spectrum contains a peak at 28 cm$^{-1}$, which we assign to intermolecular vibration as described for lysozyme. It also possesses broad shoulders at 70 cm$^{-1}$. Lysozyme and BSA are typically used as models for studying the THz range of proteins. BSA studies include using THz spectroscopy, THz coherent synchrotron radiation, pulsed THz spectroscopy, and THz-TDS. Lysozyme studies include employing Raman spectroscopy with a double monochromator, OHD-EIKES, THz-TDS, FTIR-Raman spectroscopy with holographic grating, and femtosecond optical Kerr effect (OKE) Raman spectroscopy.

![Figure 3. LFV Raman spectrum of lysozyme. (a) The Stokes and anti-Stokes spectra of lysozyme and (b) Stokes spectrum only within the 0–200 cm$^{-1}$ range.](image1)

![Figure 4. LFV Raman spectrum of BSA. (a) The Stokes and anti-Stokes spectra of BSA and (b) Stokes spectrum only, within the 0–200 cm$^{-1}$ range.](image2)
and 164 cm\(^{-1}\), corresponding to H-bond bending and stretching modes, respectively, as reported by Mazur et al.\(^4\) by ultrafast OKE.

**Immunoglobulin G (IgG) and Fragment Antigen Binding (Fab) Immunoglobulins.** IgG is a large, Y-shaped molecule that consists of two identical light (L) and two identical heavy (H) polypeptide chains folded into 12 independent domains called β-barrels. Furthermore, each polypeptide chain is divided into variable (V) and constant (C) regions representing unique and evolutionarily conserved sequences, respectively. Therefore, with regard to both the sequence (V, C) and the polypeptide chain length (L, H), the β-barrels can be grouped into three categories as VL (two β-barrels), VH (two β-barrels), and CH/L (eight β-barrels).\(^4\) The two arms of the Y are each

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**Figure 5.** LFV Raman spectrum of whole IgG with (a) both Stokes and anti-Stokes spectra and (b) the region of the Stokes spectrum within 5–200 cm\(^{-1}\).

**Figure 6.** LFV Raman spectrum of the IgG-Fab fragment with (a) Stokes and anti-Stokes spectra and (b) the region of the Stokes spectrum within 5–200 cm\(^{-1}\).

**Figure 7.** Deconvolution of the LFV Raman spectrum of (a) whole horse IgG and (b) its Fab fragment in the region of the Stokes spectrum within 17–45 cm\(^{-1}\). The component peaks are the result of curve fitting using a Gaussian line shape. The sums of the fitted components superimpose on the experimental LFV Raman spectrum. The solid line represents the experimental LFV Raman spectrum after 2% Savitzky–Golay smoothing and the dashed lines represent the fitted components.
composed of $V_L$, $V_H$, and two $C_{H/L}$ $\beta$-barrels, whereas the base of the $Y$ (Fab fragment) comprises four $C_{H}$ $\beta$-barrels and contains the complementary binding site. The Fab fragment of IgG is composed of the $L$ chain and only the N-terminal part of the $H$ chain.

Chou et al. mathematically calculated the low-frequency Raman modes of $V_H$, $V_L$, and $C_{H/L}$ as 28.3, 35.8, and 28.6 cm$^{-1}$, respectively.$^5$ Their calculations were in agreement with earlier experimental LFV Raman measurements by a double-monochromator spectrometer of lyophilized and hydrated bovine IgG that showed three clear peaks at 28, 36, and 60 cm$^{-1}$, which were considerably broadened upon hydration.$^{50}$ Later, the same group attempted to reproduce these results with rabbit and human IgG and found that both spectra displayed a strong peak at 28 cm$^{-1}$. However, the second mode near 36 cm$^{-1}$ only appeared as a shoulder in the spectrum of rabbit IgG and was practically indiscernible in the spectrum of human IgG.$^{14}$ In a recent study, the Raman spectrum of murine IgG2a monoclonal antibody was examined in the THz region and a structured band with a maximum at 80 cm$^{-1}$ was observed. This band also had a shoulder on its lower frequency side near 40 cm$^{-1}$.

Here, we measured the LFV Raman spectra of whole horse IgG as well as its Fab fragment. We found that the Raman spectrum of the whole horse IgG showed a peak at 14 cm$^{-1}$, a broad peak centered around 30 cm$^{-1}$, and a broad shoulder centered around 70 cm$^{-1}$ (Figure 5). In general, the broad appearance of the spectrum can be attributed to an increase in the scattering associated with the hydrated environment, in agreement with the observations of Painter et al.$^{50}$ by a double-monochromator spectrometer. Despite the major differences between the sequences and structures of the whole IgG and its Fab fragment, the spectrum of the Fab fragment displayed a very similar spectrum, differing primarily in the broader appearance of the 30 cm$^{-1}$ peak (Figure 6). At close observation, this broad peak at 30 cm$^{-1}$ presents shoulders that appear to originate from two closely spaced peaks near 28 and 35 cm$^{-1}$ (Figure 7b). The shoulders were not observable in the whole IgG spectrum (Figure 7a) because of the presence of 10 $\beta$-barrels (two $V_H$ and eight $C_{H/L}$ domains) that all generate low-frequency breathing modes near 28 cm$^{-1}$, which dominate the signal, as opposed to the two $V_L$ $\beta$-barrels (that generate the low-frequency breathing mode at 35 cm$^{-1}$). In contrast, the Fab fragment is composed of only six $V_H$ and $C_{H/L}$ $\beta$-barrel domains; thus, in its Raman spectrum, the low-frequency breathing modes of the $V_L$ $\beta$-barrels become more dominant, allowing for the partial appearance of the 35 cm$^{-1}$ peak. Together, these results show an observable change in the spectra arising from the structural differences between the Fab fragment and the whole IgG.

**Methylated Oligonucleotide.** As was shown for the above biomolecules, the LFV Raman spectroscopy is applicable to both natural amino acids and amino acid complexes of either small peptides or large protein molecules. In the case of DNA, it is particularly interesting to see if the LFV Raman spectrum shows measurable changes in frequency or intensity following DNA epigenetic modifications. DNA methylation is an important epigenetic modification that primarily occurs on cytosine (C) bases in the context of symmetric 5′ cytosine—guanine (CpG) dinucleotides. It has important implications in gene regulation, for example, X-chromosome inactivation and long-term gene silencing,$^{52−55}$ and in recent years it has become apparent that DNA methylation is potentially responsible for cancer initiation and various genetic diseases.$^{56−60}$
Several techniques have been developed to provide information on DNA methylation at different levels, but so far most of them are laborious and time-consuming. In this relation, vibrational spectroscopy techniques facilitate the acquisition of biochemical signatures based on chemical bonds presented within cellular material with minimum preparations and no requirement of radioactive or fluorescent labeling. A previous study with ATR-FTIR spectroscopy demonstrated specificity and sensitivity in determining single DNA base changes. More recently, it was shown that methylation-induced changes in DNA were specifically and sensitively detected in the 800−1700 cm$^{-1}$ region by Raman spectroscopy. Here, we measured the effects of DNA CpG methylation using LFV Raman spectroscopy. Figure 8 shows the LFV Raman spectrum of 5mC − DNA (unmethylated), with well-defined peaks at 20, 31, 65, 80, 103, 130, and 175 cm$^{-1}$. After methylation, the LFV Raman spectrum of 5mC + DNA shows peaks located at 27, 71, 103, 130, and 175 cm$^{-1}$ in comparison with 5mC − DNA (Figure 9). This change in the spectral features can be attributed to a decrease of the interhelical distance and/or to increases in the intrahelical spring constant due to methylation. We also examined the sample under microscope and observed a change in the morphology from the large crystalline domains of 5mC − DNA to needlelike crystallites of 5mC + DNA (Figure 10).

In summary, Table 1 compares the LFV Raman modes for the biomolecules studied here with those obtained using LFV studies carried out by other spectroscopic techniques. In half of the cases, no previous data was available. As noted in Table 1, where previous literature was found, similar peak positions were observed but with different relative intensities. The changes in the relative intensity can be attributed to the different selection rules applicable to Raman scattering versus other techniques, as well as the experimental conditions of each method.

Table 1. Summary and Comparison of the LFV Modes Obtained Using Present and Other LFV Techniques$^a$

| biomolecule                  | LFV modes of present study (cm$^{-1}$) | previous studies (cm$^{-1}$) |
|------------------------------|----------------------------------------|------------------------------|
| phenylalanine                | 9(90%), 25(80%), 40(70%), 65(70%), 99(100%), 133(60%), 143(50%), 179(10%) | THz-TDS$^{-1}$ − 41(20%), 66(30%), 84(100%), 90(50%) |
| Ku4 peptide                  | 140(100%)                              | FIR$^2$ − 92(35%), 139(45%), 195(100%) |
| lysozyme                     | 28(100%), 70(80%), 166(20%)             | no literature available      |
| BSA                          | 28(100%), 164(20%)                     | Raman using double monochromator$^3$ − 25(100%), 75(80%), 160(20%) |
| horse IgG                    | 14(80%), 30(100%), 70(80%)             | OHD−OKE$^4$ − 25(80%), 75(100%), 160(50%) |
| IgG-Fab fragment             | 14(80%), 28(100%), 35(100%), 70(80%)   | OHD−OKE$^4$ − 30(70%), 75(100%), 160(60%) |
| DNA − 5mC (ATACGCGTAT)       | 20(40%), 31(20%), 65(30%), 80(60%), 103(100%), 130(30%), 175(10%) | Raman using double monochromator$^3$ bovine IgG − 28(100%), 36(75%), 60(70%) |
| DNA + 5mC (ATACCH$_3$GCCH$_3$GTAT) | 27(100%), 71(80%), 103(100%), 130(50%), 175(10%) | no literature available |

$^a$Relative peak intensities are given in the parentheses.

CONCLUSIONS

LFV Raman spectroscopy is a promising approach to elucidate the structural and functional information hidden in the LFV modes of biomolecules, which in many aspects have thus far not been studied in detail. The approach we present in this article introduces a step forward in LFV Raman spectroscopy. Using an experimental setup based on a single-stage monochromator and volume holographic filters, we were able to obtain the LFV Raman spectrum of a single amino acid phenylalanine in a hydrated environment, in contrast to the previous studies on the polycrystalline form or pressed pellets mixed with PE powder. We presented LFV Raman measurements for a...
model amphipathic peptide made of a combination of two amino acids, lysine and leucine. Furthermore, using LFV Raman spectroscopy, we were able to distinguish spectroscopically between truncated IgG (Fab) and its whole molecule counterpart (IgG) and between methylated and nonmethylated DNA. The range of biomolecules we examined in this study illustrates the wide applicability of LFV Raman spectroscopy and the ability of experimental setup to provide a robust and affordable solution to study biomolecules in the LFV/THz range. From the viewpoint of biological functions, the current study can open up exciting frontiers in molecular biology. By providing a nondestructive, label-free characterization technique with submicron spatial resolution and three-dimensional scanning capability, LFV Raman spectroscopy offers a unique analytical tool for the study of biomolecules.

■ MATERIALS AND METHODS

Materials. L-Phenylalanine (>98%) was purchased from Sigma-Aldrich and dissolved in phosphate-buffered saline (PBS, 0.1 M, pH = 7.5, Sigma-Aldrich) to a final concentration of 50 mg/mL. BSA fraction V (Roche Life Science) and hen egg white lysozyme (>90%) were purchased from Sigma-Aldrich and dissolved in PBS to a final concentration of 50 mg/mL.

The K4L8 peptide (12-mer; >95%) with the KLLLKLLLKLLK sequence (K and L for lysine and leucine, respectively) was synthesized by Cellmano Biotech, Ltd. (Hefei, China). For stock solution, the peptide was dissolved in high-performance liquid chromatography (HPLC)-grade water to a final concentration of 25 mg/mL, incubated for 1 h at room temperature (RT), and stored at −20°C until used. For measurements, the peptide was diluted to a final concentration of 12.5 mg/mL (in 50% acetonitrile and 0.05 M HCl), briefly sonicated, and allowed to equilibrate by 60–90 min incubation at RT.

ChromPure Horse IgG whole molecule and ChromPure Horse IgG (Fab fragment only) were purchased from Jackson ImmunoResearch Inc. and diluted in PBS to a final concentration of 50 and 25 mg/mL, respectively.

Methylated (5mC+) and unmethylated (5mC−) short DNA oligonucleotides with the ATACCGGTAT primary sequence were synthesized by Integrated DNA Technologies (IDT, Belgium). The methylated DNA oligonucleotide includes two cytosine modifications (ATACCH3GCCH3GTAT) within the CpG motif, representing the DNA methylation from naturally occurring eukaryotic cells. For measurements, both DNA oligonucleotides were dissolved in HPLC-grade water to a final concentration of 6 mg/mL.

Sample Preparation. For LFV Raman measurements, 3 μL of each sample was spotted onto a glass slide in two consecutive layers. Between the layers, the samples were allowed to dry completely at RT. The measurements were carried out at RT (293 K). Reproducibility of data was checked by repeating each experiment in triplicates using fresh samples. For every sample, spectra were collected from three randomly selected areas and averaged. No appreciable variation of band shapes was noticed in the course of the experiments, indicating preservation of the sample integrity during the measurements. To estimate the contribution of the buffer solutions, we measured the LFV Raman spectrum of HPLC-grade water, PBS, and 50% acetonitrile with 0.05 M HCl in HPLC-grade water. No measurable peaks were obtained for all of the liquids in the spectral region of interest; this confirms that the detected signal belongs to the samples alone.

Raman Measurement Apparatus. The Raman system used in the measurements is a lab-built apparatus that consists of several components. A single-mode continuous-wave laser with a wavelength of λ = 532 nm (Samba, Cobolt) was used to excite the samples via an upright microscope (BXFM, Olympus) and a long-working-distance 100× objective with a numerical aperture of NA = 0.9 (MPlanFL WD = 1 mm, Olympus). The Raman filters had a 5 cm−1 transition (BragGrate 532, OptiGrate). In the LFV Raman set-up, the excitation laser is first reflected off a “clean-up” bandpass filter (BPF) to remove residual spontaneous emission and also to direct the beam toward the sample. Three bandpass notch filters (BNF) then reject the Rayleigh scattering.
of the laser, with each BNF providing 3 orders of magnitude of light attenuation. The Raman signal from the sample is collected in back-scattered geometry. Leaked light is used to align all of the optics in the collection path. After alignment is complete, the three BNF filters are used to achieve a combined 9 orders of magnitude rejection of the laser excitation. Figure 11 shows the schematic of the system along with the positioning of the filters in the apparatus. The samples were kept on a computer-controlled motorized stage (SCANplus 100 × 100, Marzhauser Wetzlar), which can maintain the exact position below 1 μm resolution. This prevents the possibility of the drift in the sample position during the measurement. The Raman signal is collected with a confocal configuration using a 50 μm diameter fiber to define the confocal pinhole and is spectrally resolved in a 0.5 m long imaging spectrometer (SP-2500i, Princeton Instruments) and charge-coupled device (PIXIS 400, Princeton Instruments). The Raman measurements were carried out using a 1800 grooves/mm grating. Each spectrum was averaged over five accumulations, for an overall integration time of 300 s. The laser power at the sample was measured to be 60 mW. The Raman setup was calibrated using silicon and polystyrene samples, before carrying out the measurements. All experiments were conducted at RT (293 K).

Data Analysis. To resolve overlapping bands, the spectra were processed using PEAKFIT (Jandel Scientific, San Rafael, CA) software. First, second-derivative spectra accompanied by 2% Savitzky–Golay smoothing were calculated to identify the position of the component bands. Next, the resulting values (in wavenumbers) were used as initial parameters for curve fitting with Gaussian component peaks. Finally, positions, bandwidths, and amplitudes of the peaks were varied until good agreement between the calculated sum of all components and the experimental spectra was achieved ($r^2 > 0.99$).

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank the Israel Strategic Alternative Energy Foundation (ISAEF) and the “Tashtiyot Program” of the Israeli Ministry of Science & Technology for funding this research. We would also like to acknowledge the Israel National Nanotechnology Initiative for providing support through a Focal Technology Area project, FTA grant number 458004. Authors also like to thank the European Research Council, ERC-STG grant number 309600 (D.G.), for funding the research.

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