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ARTICLES

Ion Mobility Spectrometry Coupled to Laser-Induced Fluorescence for Probing the Electronic Structure and Conformation of Gas-Phase Ions

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Abstract—We report on an improved design of a differential ion mobility analyzer (**DMA**) coupled to laser-induced fluorescence (**LIF**) for the simultaneous retrieval of two-dimensional information on the electric mobility and fluorescence spectroscopy of gas-phase ions. This enhanced design includes an ion funnel interface at the input orifice of the DMA and a nozzle beam stage at the output of the DMA. These improvements allow the detection of fluorescence not only from pure dyes and their clusters, as was demonstrated recently, but also from fluorophore-tagged biomolecules. Complex mixtures of fluorescent compounds can be separated by the DMA and studied by LIF. This unique combination of instruments also provides a powerful platform for probing fluorescent proteins in the gas phase. The green fluorescent protein (**GFP**) was tested on a new setup. In contrast to high vacuum, where no GFP fluorescence was detected, the presence of a LIF signal at the output of the DMA could explain some specific fluorescent properties of GFP in the gas phase. Given that both conformation and fluorescence are key properties of biological molecules in the gas phase, we expect that our enhanced design will answer the question whether gas-phase proteins retain their liquid-phase native structure or not.

Keywords: differential ion mobility analyzer, laser-induced fluorescence, green fluorescent protein, electrospray, gas-phase ions, soft ionization, native proteins

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INTRODUCTION

Laser-induced fluorescence (**LIF**) analyzers are based on measurements of fluorescence spectra induced in a test sample irradiated with a laser. The measured spectrum represents a set fluorescence signals from individual components present in the studied sample. Because the structure of the fluorescence signal is connected with the molecular structure of the studied objects, the analysis of fluorescence spectra in many cases allows the identification of a substance. Because of the high sensitivity of laser-induced fluorescence to the environment a molecule, the method is widely used to determine structures of molecules, biomolecules among them [1]. In studies of non-fluorescent proteins chemists use various fluorescent labels chemically attached to the protein molecule. Laser-induced fluorescence is used mainly to study properties of molecules in solution; however, for the fundamental understanding of processes occurring in molecules it would be desirable to record fluorescence spectra with no additional environment, i.e., to obtain them in the gas phase. Recently a new method combining mass spectrometry as a method for determining ion masses exclusively in the gas phase (vacuum) and laser-induced fluorescence has been developed [2]. This combination has allowed researchers to record fluorescence spectra of dyes and proteins in the gas phase [3–5].

Fluorescence spectra in the gas phase bear additional information on the structure of molecules and provide a necessary supplement to fluorescence spectra in liquids. An example of a study of the structures of proteins by a combination of mass spectrometry with laser-induced fluorescence was an experiment with the green fluorescent protein (**GFP**) reported in [6]. The polypeptide fold of this protein resembles a β-barrel, which contains a covalently bound fluorophore, spontaneously formed by the chemical modification of three amino acid residues in the center. Because of the presence of this fluorophore, the green fluorescent protein, which occurs in a natural state in aqueous solution, can emit strong fluorescence, highly sensitive to minor changes of its structure. A characteristic feature of this protein is the complete drop of fluorescence at a slightest change in its structure. Therefore, the detection of fluorescence of the green fluorescent protein is indicative of disturbances in its structure.

The experiments performed on an ion cyclotron resonance (**ICR**) mass spectrometer equipped with a

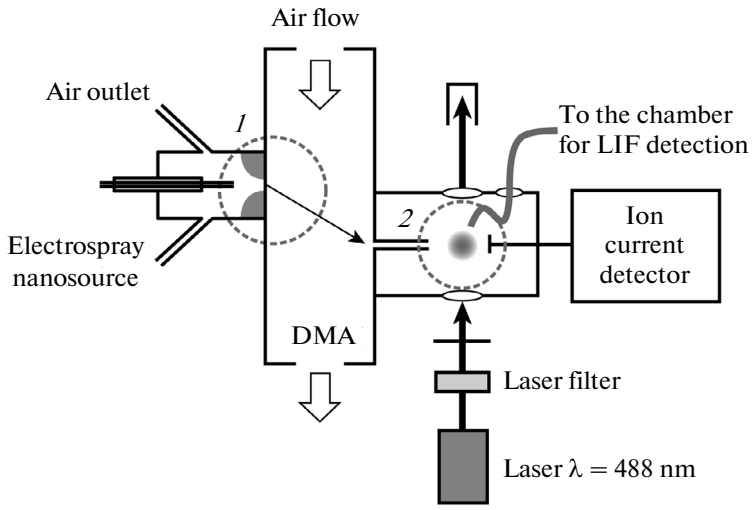

Fig. 1. Differential ion mobility detector with an analyzer of laser-induced fluorescence.

special optical cell for measuring laser-induced fluorescence in the gas phase showed the absence of fluorescence of the green fluorescent protein [6], which is indicative of irreversible changes in its structure in the high vacuum of the mass spectrometer.

For the ionization of biomolecules analysts use mild methods, such as atmospheric pressure electrospray ionization and matrix-assisted laser desorption/ionization [7, 8]. These methods ensure the ionization of even large biomolecules without their destruction and fragmentation. It was shown that electrospray under usual conditions, i.e., atmospheric pressure, gives gas-phase ions [9]. This was proved by comparing spectra of laser-induced fluorescence of the Rhodamine 6G laser dye, obtained in an electrospray plume under atmospheric pressure and in high vacuum. The identity of the spectra indicated that gas-phase processes can be investigated even under atmospheric pressure. It is a quite simple method, allowing the investigation of gas-phase processes by laser-induced fluorescence without using additional devices for ion capture and transport. Among the drawbacks of this method is the impossibility of selective analysis: in experiments one can record only the total fluorescence signal. Selective analysis can be performed only by mass spectrometry; however, the experiments are very laborious and expensive.

Recently we have developed an instrument on which ions in the gas phase can be selectively investigated by laser-induced fluorescence [10]. This simple and reliable instrument is based on a combination of a differential mobility analyzer (**DMA**) [11] with LIF. Separately DMA and LIF are widely used to study various samples. The tandem instrument offers a unique possibility of simultaneously detecting ion mobilities and recording fluorescence spectra. However, the sensitivity of this instrument was sufficient only for the study of dye molecules with quantum yields close to unity.

In this work we propose an advanced instrument combining DMA with LIF. The sensitivity of the proposed instrument is sufficient not only for studying dyes, but also for the investigation of proteins with fluorescent labels. The most vivid demonstration of the possibilities of the proposed instrument is the LIF spectrum of the green fluorescent protein, recorded in the gas phase for the first time.

EXPERIMENTAL

The scheme of experimental setup is shown in Fig. 1. The electrospray source was a capillary (inner diameter 30 μm), to which a potential of 3000 V was applied; the distance between the capillary and the input orifice of the DMA is 5 mm. Sample solutions in methanol were fed through a capillary under a pressure in 7000 Pa. A vacuum chamber with two optical windows and an electrometer (Lazcano Inc., Spain) were installed at the exit from the commercial DMA (P4, SEADM, Spain). A plastic optical fiber (NA = 0.51, PGRFB 3000, SEDI, France) for collecting the fluorescence signal was installed perpendicularly to the direction of the ion flux and the exciting light beam. The fluorescence signal was excited with a laser (OBIS LS, Coherent, United States) with a wavelength of 488 nm, 20 mW. The opposite end of the optical fiber was attached to a holographic spectrograph (HoloSpec F/1.8i, Kaiser Optical Instruments Inc., AnnArbor, United States) with a CCD array (LN/CCD-2500-PB/VISAR, Princeton Instruments, Trenton, United States). A laser filter (Thorlabs, United States) was used to remove scattered laser radiation.

In comparison to the setup described in [10], in the new one we made some important changes shown by

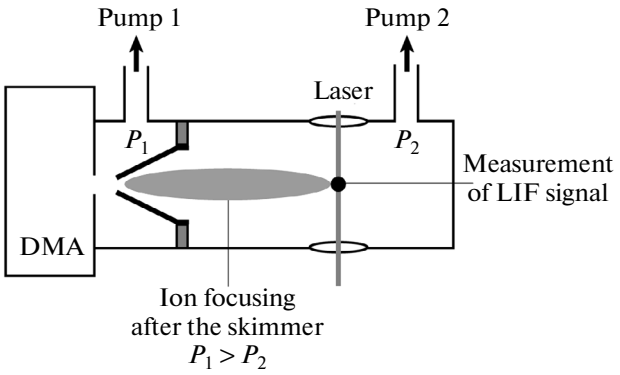

Fig. 2. Exit chamber of the differential ion mobility detector with a built-in skimmer and a differential pump.

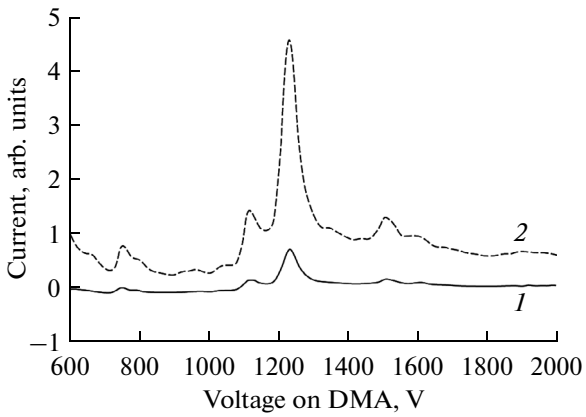

Fig. 3. Sensitivity of differential ion mobility detector: *1*, in traditional configuration; *2*, in the presence of an ion funnel after the electrospray source.

circles in Fig. 1. These zones are critical for the amount of test substance (ions) entering and leaving the differential mobility analyzer. To improve the efficiency of sample injection into the differential mobility analyzer, we used a cone of a type of ion funnel in zone *1*, made by analogy with [12]. The diameter of the input orifice was 1 mm; the diameter of the outer funnel parts was 10 mm. The actual modification of zone *2* is shown in Fig. 2. The vacuum chamber at the exit from the differential mobility analyzer was divided into two sections with a skimmer used to focus ions at the point of intersection with the laser beam. Pressures P_1 and P_2 were selected with regard to recording the maximum fluorescence signal and varied in the range from 1 to 10000 Pa using vacuum regulators (SL015, Kurt J. Lesker, United States) installed before two additional forevacuum pumps (Alcatel 2010, France). The inner diameter of the skimmer was 0.1 mm.

RESULTS AND DISCUSSION

As has been shown recently, gas-phase ions form in an electrospray ionization source just under atmospheric pressure [9]. The transfer from the liquid to the gaseous state begins just at a distance of several millimeters from the capillary end. The formation of gas-phase ions proceeds more efficiently in nanocapillaries. As was shown in [10], the ions present at a distance of 5 mm from the capillary to the differential mobility analyzer in an electrospray plume are mainly in the gas phase. To efficiently focus and improve the efficiency of transportation of all ions to the detector, it was proposed to install an additional mechanical funnel. This funnel, to which the potential of the detector was applied, allowed Rauschenbach et al. to increase the amount of ions entering the instrument [12]. The spectrum of Rhodamine 6G in Fig. 3 shows a 7.5-fold increase in sensitivity in the presence of an ion funnel. As was shown in [12, 13], the use of an ion funnel at the entrance into the mass spectrometer can enhance sensitivity by several orders of magnitude. However, this can be done under certain conditions, such as certain fluxes, pressure, etc. For our setup, we could not change the experimental parameters in the entrance chamber of the differential mobility analyzer within a wide range; this resulted in a decrease of the collecting ability of the funnel.

The low sensitivity of the instrument previously described in [10] was due to not only insufficient ion focusing before the input orifice of the DMA, but also the defocusing of the ion cloud at the exit from it, namely, at the point of intersection with the laser beam. The low ion density at the exit from the DMA hindered the detection of fluorescence signals from substances with low quantum yields. The exit beam can be focused with a device shown in Fig. 2. A skimmer installed in the exit chamber of the spectrograph efficiently focused the ion beam at certain pressures P_1 and P_2. The optimum ratio of pressures was determined experimentally by comparing the ion current signal with the fluorescence signal for Rhodamine 6G. The maximum ion current and a twentyfold increase in the fluorescence signal were attained at P_1 = 1000 Pa and P_2 = 300 Pa (Fig. 4). A considerable (to above the detection boundary) increase in current (peak at 1250 V) was observed when a skimmer was used in the exit chamber of the DMA. The high sensitivity of the combined instrument allowed us to study not only dyes but also other substances. Figure 5 presents an ion current signal for Rhodamine-tagged ubiquitin protein. An intense (exceeding the detection boundary) signal at 1300 V corresponds to ubiquitin in the charge state 7+.

The fluorescence signal shown in Fig. 6 was recorded by scanning voltage over the range 1200–1600 V for 5 min. The peak of laser-induced fluorescence at the wavelength 560 nm corresponds to the fluorescence of labeled ubiquitin in the liquid phase. For small molecules, such as Rhodamine, a big difference (several dozen nanometers) was observed between the fluorescence peaks in the liquid and gas

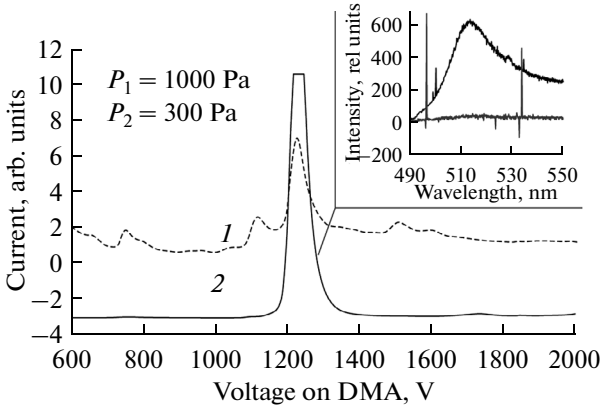

Fig. 4. Sensitivity of differential ion mobility detector: *1*, in traditional configuration; *2*, using a skimmer and differential pump at the exit from DMA chamber. The insert demonstrates a 20-fold increase of the signal laser-induced fluorescence of Rhodamine 6G with using a skimmer and the optimum pressure in the chambers.

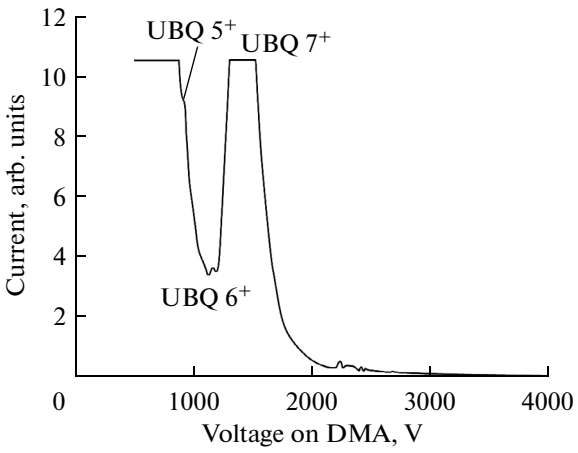

Fig. 5. An ion mobility spectrum of ubiquitin. Peaks corresponding to different charge states of ubiquitin are shown.

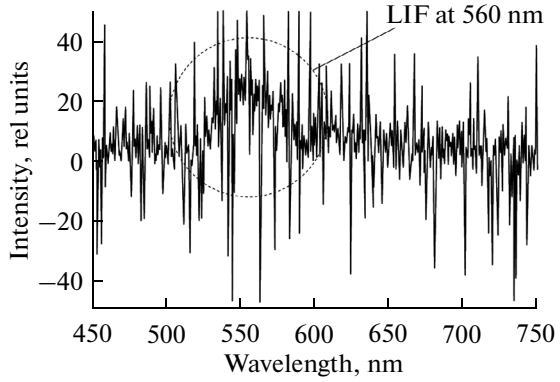

Fig. 6. A fluorescence spectrum of Rhodamine-tagged ubiquitin recorded using DMA with scanning voltage at the exit from DMA in the range 1200–1600 V.

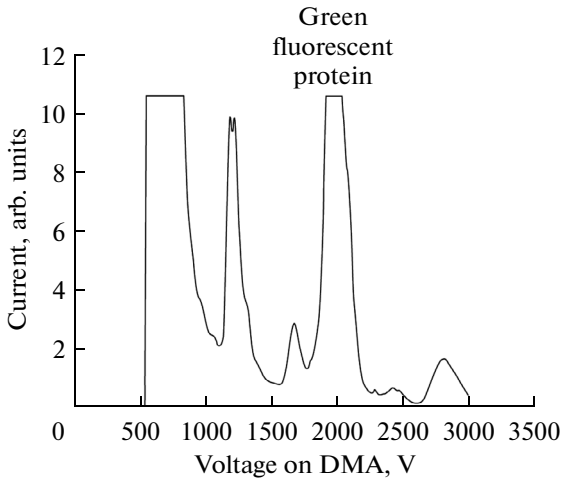

Fig. 7. An ion mobility spectrum of the green fluorescent protein.

phase [3]. This is basically due to the absence of additional channels of the relaxation of excited molecules through solvent molecules, leading to an increase of the Stokes shift. For Rhodamine-tagged ubiquitin, we observed the relaxation of the excited states of Rhodamine similar to that occurring in the liquid phase because of a great number of the degrees of freedom in the protein molecule. As a result, the spectrum of laser-induced fluorescence recorded for Rhodamine-tagged ubiquitin in the gas phase was quite similar or even identical to its spectrum in solution.

Figure 7 presents an ion mobility spectrum of the green fluorescent protein. The peak at 2000 V corresponds to the GFP charge state 10+. It corresponds to value of ion mobility calculated for a substance with the known mobility [10]. For the additional identification of GFP peaks, we used laser-induced fluorescence. The LIF signal was recorded at different scanning voltages. Figure 8 presents fluorescence spectra of GFP recorded at different voltages in the DMA. The fluorescence signal corresponding to protein fluorescence in the liquid phase can be seen only on scanning in the range 1800–2200 V. This means that the peak at 2000 V in Fig. 7 corresponds to a GFP peak. Similarly to the experiment with labeled ubiquitin, we demonstrated the identity of fluorescence spectra at the exit from DMA and in solution. This is due to the big weight of the protein (28 kDa) and the possibility of the relaxation of the excited states of the chromophore via numerous chemical bonds.

The use of laser-induced fluorescence in combination with DMA not only opens a possibility for gaining additional spectral information, but also provides an additional method for the identification of peaks of fluorescent components. The experiment with the

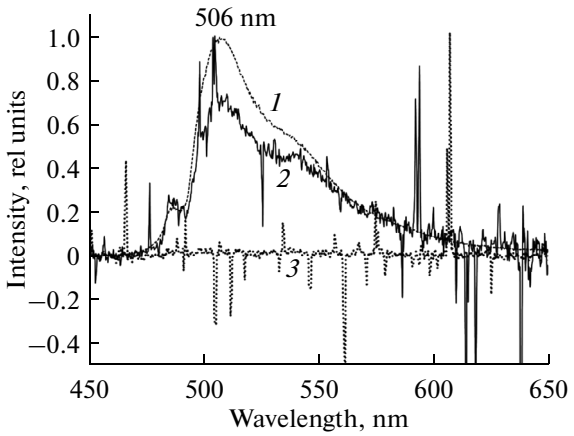

Fig. 8. Fluorescence spectra of the green fluorescent protein recorded *1*, in the liquid phase; *2*, *3*, in the gas phase using a DMA. Voltage at the exit from DMA is *2*, 1800–2200 V and *3*, 500–1500 V.

green fluorescent protein is also of other fundamental importance. This protein possesses unique properties: it fluoresces exclusively in the natural state. The fluorescence disappears at minor changes in its structure. The experiments performed on an ion cyclotron resonance spectrometer in high vacuum have shown the absence of protein fluorescence. This means that the protein lost its ability to fluoresce as a result of changes in its structure. At the same time, it was shown that the green fluorescent protein fluoresces in the electrospray plume. One of plausible explanations for of the drop of GFP fluorescence in deep vacuum was the assumption that the protein can lose water molecules from its structure. These molecules are essential constituents of the protein, necessary for the correct packing of the polypeptide chain and the formation of the so-called "native" structure and are, consequently, important for its fluorescence properties.

In this work we observed the fluorescence of ions of the green fluorescent protein at the exit from the DMA. This means that, in contrast to mass spectrometry utilizing high-vacuum chambers, the proposed method is really "mild" for biomolecules.

CONCLUSIONS

A combination of mass spectrometry with a differential mobility analyzer detector and an analyzer of laser-induced fluorescence was used to study the electronic structure and conformations of ions in the gas phase. Changes in the detector design allowed us to use the proposed tandem instrument both for studies of complex mixtures of biomolecules in the gas phase, which could not be done previously, and for the determination of small dye molecules and their derivatives with high sensitivity. Using this instrument, the fluorescence of the green fluorescent protein in the gas phase could be recorded for the first time. Using this example we have demonstrated the retention of the liquid-phase structure of this protein in its transfer into the gas phase. This is an important advantage of the new method over conventional mass spectrometry, because the initial structure of proteins can be lost under the conditions of deep vacuum. The proposed instrument provides an efficient tool, expanding the possibilities of the analysis of proteins for gaining new information on their physical and chemical properties.

REFERENCES

1. Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, Berlin: Springer, 2006.
2. Frankevich, V., Guan, X., Dashtiev, M., and Zenobi, R., *Eur. J. Mass Spectrom.*, 2005, vol. 11, no. 5, p. 475.
3. Chingin, K., Chen, H.W., Gamez, G., and Zenobi, R., *J. Am. Soc. Mass Spectrom.,* 2009, vol. 20, no. 9, p. 1731.
4. Chingin, K., Balabin, R.M., Barylyuk, K.V., Chen, H.W., Frankevich, V., and Zenobi, R., *Phys. Chem. Chem. Phys.*, 2010, vol. 12, no. 37, p. 11710.
5. Frankevich, V., Barylyuk, K., Chingin, K., Nieckarz, R., and Zenobi, R., *Chem. Phys. Chem.,* 2013, vol. 14, no. 5, p. 929.
6. Zimmer, M., *Chem. Rev.*, 2002, vol. 102, no. 3, p. 759.
7. Fenn, J., Mann, M., Meng, C., Wong, S., and Whitehouse, C., *Science*, 1989, vol. 246, no. 4926, p. 64.
8. Karas, M. and Krüger, R., *Chem. Rev.*, 2003, vol. 103, no. 2, p. 427.
9. Chingin, K., Frankevich, V., Balabin, R.M., Barylyuk, K.V., Chen, H.W., Wang, R., and Zenobi, R., *Angew. Chem.*, 2010, vol. 49, no. 13, p. 2358.
10. Frankevich, V., Martinez-Lozano, SinuesP., Barylyuk, K., and Zenobi, R., *Anal. Chem.*, 2013, vol. 85, no. 1, p. 39.
11. Fernandez de la Mora, J., *The Differential Mobility Analyzer (DMA): Adding a True Mobility Dimension to a Preexisting API-MS,* in *Ion Mobility Spectrometry – Mass Spectrometry: Theory and Applications,* Wilkins, C. and Trimpin, S., Eds., CRC Press, 2010.
12. Rauschenbach, S., Pauly, M., Küster, A., Kern, K., Reiss, J., Sesterhenn, J., and Sroka, M., European Patent Application EP 2631930A1, 2013.
13. Kelly, R.T., Tolmachev, A.V., Page, J.S., Tang, K., and Smith, R.D., *Mass Spectrom. Rev.*, 2010, vol. 29, no. 2, p. 294.

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