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Physico-Chemical Characterization of the Interaction of Red Fluorescent Protein—DsRed With Thin Silica Layers

Marvine Soumbo, Alessandro Pugliara, Marie-Carmen Monje, Christine Roques, Bernard Despax, Caroline Bonafos, Robert Carles, Adnen Mlayah, and Kremena Makasheva,* *Member, IEEE*

Abstract—The *Discosoma* recombinant red fluorescent (DsRed) protein is the latest member of the family of fluorescent proteins. It holds great promise for applications in biotechnology and cell biology. However, before being used for rational engineering, knowledge on the behavior of DsRed and the underlying mechanisms relating its structural stability and adsorption properties on solid surfaces is highly demanded. The physico-chemical analysis performed in this study reveals that the interaction of DsRed with SiO₂ surfaces does not lead to complete protein denaturation after adsorption and dehydration. Nevertheless, the photoluminescence emission of dehydrated DsRed small droplets was found to be slightly red-shifted, peaking at 590 nm. The measured contact angles of droplets containing different concentration of DsRed proteins determine the interaction as hydrophilic one, however with larger contact angles for larger DsRed concentrations. The DsRed protein behavior is not pH-dependent with respect of the contact angle measurements, in agreement with previously reported studies.

Index Terms—DsRed, proteins, thin SiO₂ layers.

I. INTRODUCTION

RECENT advances in biotechnology offer the possibility to explore chemical, physical, and functional properties of proteins, in particular fluorescent proteins, in various applications such as biosensors, bioelectronics, photosensitizers,

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M. Soumbo, A. Pugliara, and B. Despax are with the LAPLACE laboratory, Université de Toulouse; CNRS, UPS, INPT; F-31062 Toulouse, France (e-mail: marvin.soumbo@gmail.com; alessandro.pugliara@laplace.univ-tlse.fr; bernard.despax@laplace.univ-tlse.fr).

M. Soumbo, M-C. Monje, and C. Roques are with the LGC laboratory, Université de Toulouse; CNRS, UPS, INPT; F-31062 Toulouse, France (e-mail: marvin.soumbo@gmail.com; marie-carmen.monje@ensat.fr; Ch.Roques@wanadoo.fr).

A. Pugliara, C. Bonafos, R. Carles, and A. Mlayah are with the “Nano-Optics and Nanomaterials for Optics” group, CEMES-CNRS, Université de Toulouse, F-31055 Toulouse, France (e-mail: alessandro.pugliara@cemes.fr; caroline.bonafos@cemes.fr; robert.carles@cemes.fr; adnen.mlayah@cemes.fr).

*K. Makasheva is with the LAPLACE laboratory, Université de Toulouse; CNRS, UPS, INPT; F-31062 Toulouse, France (e-mail: kremena.makasheva@laplace.univ-tlse.fr).

artificial implants, drug delivery systems, etc. [1]–[3]. These current achievements, however, lead to exposure and interaction of proteins with non-biological organic and inorganic solid surfaces, and determine the need of gathering knowledge on the underlying mechanisms. Proper description and control of protein interaction with solid surfaces add value to the report on the relation between structural stability of proteins and their adsorption behavior. The above given approach is entirely in line with the aim of this work. Considering further development our choice was to go with *Discosoma* recombinant red fluorescent (DsRed) protein and study its interaction with thin SiO₂ layers. Silica, an inorganic solid substrate, which is well known for its optically transparent properties in the visible range of the spectrum, is often used for surface adsorption studies of proteins [4]. When properly dimensioned, the thin SiO₂ layers can be used as anti-reflective substrates allowing detection of chemical substances on their surfaces. Studying thin SiO₂ layers is also based on their large use in plasmonics as a host matrix, in microelectronics as a diffusion barrier or as a thin electrical insulator, and especially for the properties of SiO₂/Si interface, etc. [5]–[8].

The fluorescent proteins are a family of proteins of 25–30 kDa, mainly applied to study the organization and function of living systems [1], [9]–[12]. The most extensively characterized member of this family is the green fluorescent protein (GFP). The recently cloned from reef coral *Discosoma* sp. DsRed protein [9] has the longest yet reported, for a wild-type spontaneously fluorescent protein, excitation and emission maxima at 558 nm and 583 nm, respectively. DsRed holds great promise for biotechnology and cell biology as a spectrally distinct companion or substitute for the GFP.

A variety of experimental techniques have been employed to access and to assess the structural properties of adsorbed proteins on solid surfaces leading to formulation of a number of common trends [2], [4], [9]–[16]. It is generally acknowledged that proteins having strong internal coherence adsorb on hydrophobic surfaces. However, the electrostatic attraction is necessary for their adsorption on hydrophilic surfaces. In contrary, proteins with much lower structural stability adsorb freely on both hydrophobic and hydrophilic surfaces, even under unfavorable conditions of hydrophilic, electrostatically

repelling surfaces. Most of the applied diagnostic methods are adapted for studying the structural stability of proteins in solution. Nevertheless, some advanced diagnostic methods are based on probing proteins adsorbed on solid surfaces after dehydration. It is the case, for example, with matrix assisted laser desorption/ionization (MALDI) [13], [14] or the Fourier transform infrared (FTIR) spectroscopy [16], [17].

In this article we report results on the interaction of DsRed protein with solid silica surfaces and on the pH-dependent protein behavior with respect to the contact angle variation for a series of diluted protein solutions after adsorption and dehydration on solid silica surfaces. It also demonstrates a way to explore very small quantities of proteins, which allows investigation of rare proteins and/or of proteins demanding costly procedures for their extraction.

II. EXPERIMENTAL PART

A. Thin SiO₂ Sample Preparation

Silica layers, 100 nm-thick, were thermally grown on intrinsic Si-substrates at 1100 °C under slightly oxidizing atmosphere using a N₂-O₂ gas mixture containing 1.0% of O₂. Before being exposed to protein deposition, the SiO₂/Si substrates were consecutively cleaned in ethanol (95% vol.) and acetone (95% vol.) and then rinsed in deionized water. The deionized water was filtered through 200 nm pore size filter. The rinsing procedure continued until attaining zero surface conductivity on the SiO₂ surfaces. Cleaning the SiO₂ surface is an essential step in this study because of avoiding electrostatic interactions and offering a better control of the experimental conditions. It was performed in a clean room with well controlled environmental conditions, temperature and relative humidity.

B. Proteins and Preparation Procedure

DsRed was purchased from Biovision. According to the SDS-PAGE, the recombinant DsRed was at least 97% pure and in a freeze dried form. A stock solution of DsRed was made to a concentration of 1 mg/mL in water for injectable preparations (European Pharmacopoeia, COOPER) [18]. The pH-value of water for injection was measured to 7.0 with conductivity of 1.2 μS/cm. The pH-value of DsRed stock solution was also determined to 7.0 and its stability was repetitively controlled during all the measurements. The assays were performed at room temperature (23 °C). Aliquots of DsRed stock solution were diluted 4-20 times into water for injectable preparations for characterization of DsRed sessile droplets with different protein concentrations. The pH-dependent behavior of DsRed was studied with the 0.05 g/L concentration. The pH was varied in the range from 3.0 to 11.0. Stock solution at 1 mg/mL was diluted to 0.05 g/L with water for injection. pH-values of 3.0 and 5.0 were obtained with citric-phosphate buffer (0.5 M). The 9.0 and 11.0 pH-values were obtained with a buffer NaHCO₃/NaOH solution of 0.05 M and 0.1 M, respectively. When required, the pH of DsRed solution was further readjusted to pH = 3.0 and 5.0 by addition of predetermined volumes of 0.5 M citric acid solution. Similarly, the 9.0 and 11.0 pH-values were realigned by addition, if necessary, of predetermined volumes of 0.1 M of NaOH.

C. Deposition of DsRed Proteins on Thin SiO₂ Layers

Sessile droplets of different concentrations of DsRed and with very small volume ($3.8 \pm 0.1 \mu\text{L}$) were deposited on the surface of SiO₂ layers by using Digidrop goniometer, a contact angle meter from GBX Scientific Instruments to measure the adhesion process of DsRed proteins on silica surfaces. The droplets were deposited with microsyringe Gastight 1700 series fixed on the goniometer giving the possibility of fine control of the droplet volume. To record and analyze the sessile droplets a Visiodrop software was coupled to the contact angle meter. The measurement precision of the applied method is of $\pm 0.1^\circ$ degree. The results given in this work are averaged over three independent measurements per sample.

D. Structural and Optical Characterization

Optical images of sessile droplets after their dehydration were recorded with a digital microscope Keyence VHX-1000. The droplet diameter (d) was measured on the images using the associated to the microscope VHX 1.3.0.7 software. The other parameters of the dehydrated droplet: thickness (e) measured inside the droplet just before the droplet ring, droplet ring width (l), and droplet ring height (h), were measured with a 2D surface profilometer Alpha-Step IQ from KLA-Tencor.

The morphological state of DsRed after dehydration and adsorption on solid silica surface was observed with a field emission scanning electron microscopy (SEM), ZEISS CrossBeam 1540 XB.

The photoluminescence emission of the DsRed was excited with an argon ion laser operating at 514 nm. The emitted light was dispersed using a Jobin Yvon spectrometer with a 150 grooves/mm grating.

FTIR spectra were acquired with a Bruker Vertex 70 spectrometer in transmission mode in the range 400-4000 cm⁻¹ with a resolution of 2 cm⁻¹ to obtain information about the composition of dehydrated protein layers. The transmission mode was attainable due to the transparency of the used intrinsic Si-substrates to infrared light.

III. RESULTS AND DISCUSSION

The interaction of DsRed proteins with SiO₂ surfaces was analyzed on the basis of the adhesion process of diluted protein solutions when approaching the SiO₂ surface, their subsequent behavior during the droplet dehydration, and the related DsRed morphological modifications.

A. Adsorption and Optical Properties of DsRed Sessile Droplets

The adsorption of DsRed proteins on silica surfaces is an interdependent process involving the protein concentration, the solution in which the proteins are diluted, the silica surface state, and the environmental air conditions. When a droplet containing given concentration of proteins is brought to a contact with a solid surface, the organization and protein behavior rely primarily on the contact angle hysteresis (CAH) at the solid-vapor and liquid-vapor interfaces [19]–[22]. The dehydration process, however, involves mechanisms related to irreversible thermodynamic functions giving rise to mechanical

TABLE I
DEHYDRATION DYNAMICS OF SESSILE DROPLETS

θ_A^a	θ_R^b	θ_R	θ_R	θ_R
$t = 1 \text{ s}^c$	$t = 1 \text{ min}$	$t = 5 \text{ min}$	$t = 10 \text{ min}$	$t = 15 \text{ min}$
Control solution sessile droplet, pH = 7.0 and 23°C				
55.3°	51.2°	50.4°	47.3°	26.8°
DsRed sessile droplet with 0.05g/L, pH = 7.0 and 23°C				
65.4°	60.1°	40.3°	5.0°	n/a

^aAdvancing contact angle - θ_A

^bReceding contact angle - θ_R

^cStandard deviation of the contact angle measurements for all the presented times does not exceed $\pm 1.7^\circ$.

hysteresis, due to topographical surface defects, and to chemical hysteresis associated with rearrangements of chemical groups due to positional and orientational changes of the surface molecules.

To reveal the DsRed interaction with the SiO₂ surface, we compare the measured advancing contact angles when droplets with very small volume ($3.8 \pm 0.1 \mu\text{L}$) of the control solution (water for injection, pH = 7.0) and of the DsRed (concentration of 0.05 g/L diluted in the control solution) are brought to contact (Table I).

The obtained value for the control solution ($\theta_A = 55.3^\circ$) is consistent with the values measured on SiO₂ surfaces with deionized water ($\theta_A = 54.7^\circ$, verification not shown here) confirming the hydrophilic nature of the used SiO₂ surfaces. The contact angle measured with DsRed is higher ($\theta_A = 65.4^\circ$) suggesting chemical hysteresis induced by the presence of proteins and their organization on the silica surface. Most likely the DsRed proteins adsorb on the SiO₂ surface with their polar side chains towards the available α -polar bonds of the clean SiO₂ surface. The droplet diameter is determined at the moment of contact and remains constant till full dehydration of the droplet.

The very small droplet volume leads to a rapid dehydration. The dehydration process of DsRed ($c = 0.05 \text{ g/L}$) solution is completed for 10 min at 23 °C. In the same experimental conditions the water droplet is not evaporated for the same duration; it lasts for almost 20 min. It is also worth to notice that whereas the contact angle of the control solution is preserved for the whole process of dehydration except at the very end due to very small residues present in the water for injection, i.e., the contact angle remains almost constant, the contact angle for different DsRed solutions varies with time (Fig. 1) expressing a CAH. The latter observation is related to the adsorption of proteins on the SiO₂ surface. The physical description of this effect is the following. The DsRed proteins deposit at the contact line due to surface tension interactions. They pin the contact line and prevent from receding during the evaporation process. Consequently, they impose a diffusion-limited evaporation process with a larger evaporation rate at the edge as for the water droplet the evaporation is rather uniform over the droplet surface.

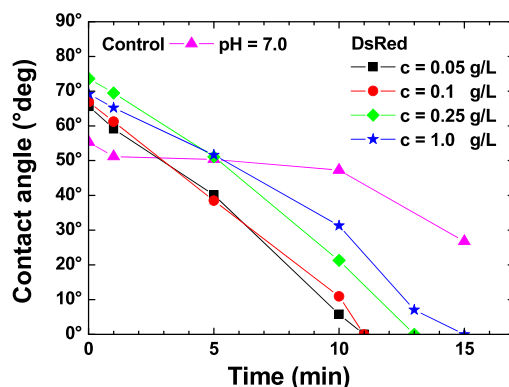




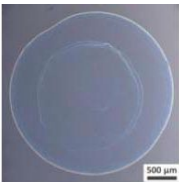

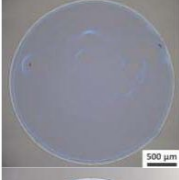

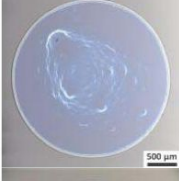

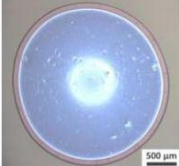
Fig. 1. Measured contact angle at 23 °C as a function of time during sessile droplet dehydration.

Fig. 1 shows the DsRed behavior during dehydration for different protein concentrations. The droplet dehydration is more rapid for small concentrations of DsRed (up to 0.1 g/L). The linear decrease over time of the contact angle is common for all the studied DsRed concentrations. This effect can be related to a large surface energy variation on the droplet profile close to the triple line (solid-liquid-vapor) and to protein adsorption mechanisms induced by the liquid convective drive inside the droplet (Marangoni effect) [21]. According to theory, a region with high surface tension exerts a pulling force on neighboring regions with lower surface tension inducing a flow across the gradient. For an evaporating droplet [22], the contact line is the place where the lowest surface tension occurs. It will induce an outward flow resulting in accumulation of proteins at the contact line. Given the dependence of Marangoni stress on the contact angle, it is stronger for larger contact angles [23], the dehydration process takes longer times for larger DsRed concentrations as observed here. Additional reason, to the surface energy variation, is the tetrameric nature of the DsRed protein that can be at the origin of imprisonment of extremely small volumes of water, thus slightly delaying the dehydration process. However, to go further in this explanation by quantification of the dehydration process a thorough theoretical analysis must be performed considering also in details the proteins as surfactant in the sessile droplets.

Table II summarizes the characteristics of sessile droplets with different concentrations of DsRed diluted in water for injection (pH = 7.0) studied at room temperature (23 °C). Due to the hydrophilic properties of SiO₂ surface the measured contact angles for droplets containing DsRed proteins remain in the range $\theta_A = 65^\circ - 75^\circ$ determining the interaction as hydrophilic one, however with larger contact angles for larger DsRed concentrations. Because of the adsorption of the proteins on the silica surface, the wettability of the DsRed droplet is reduced in comparison with water for injection (control solution droplet). The increased standard deviation for larger DsRed concentrations reflects the dynamic character of the interaction between DsRed proteins and silica surface in relation with the large DsRed-interfaces imposed by its tetramerization [10].

The optical images of dehydrated DsRed droplets indicate that the visual aspect of the sessile droplet becomes more

TABLE II
CHARACTERISTICS OF DsRED SESSILE DROPLETS AT pH = 7.0 AND 23 °C

DsRed (g/L)	Droplet image at 1s	Contact angle $-\theta_A$	Optical image after dehydration	Dehydrated sessile droplet ^a
control pH=7.0		54.3° ± 0.1°	n/a	n/a
0.05		65.4° ± 0.2°		$d = 2.8 \text{ mm};$ $e = 20 \text{ nm};$ $l = 46 \text{ }\mu\text{m};$ $h = 0.6 \text{ }\mu\text{m}$
0.10		65.5° ± 1.6°		$d = 2.8 \text{ mm};$ $e = 30 \text{ nm};$ $l = 67 \text{ }\mu\text{m};$ $h = 1.4 \text{ }\mu\text{m}$
0.25		73.7° ± 1.5°		$d = 2.7 \text{ mm};$ $e = 30 \text{ nm};$ $l = 84 \text{ }\mu\text{m};$ $h = 3.0 \text{ }\mu\text{m}$
1.00		72.0° ± 2.7°		$d = 2.6 \text{ mm};$ $e = 60 \text{ nm};$ $l = 200 \text{ }\mu\text{m};$ $h = 5.2 \text{ }\mu\text{m}$

^aThe DsRed sessile droplet characteristics after dehydration are as follows: d is the droplet diameter; e is the droplet thickness measured inside the droplet, just before the droplet ring; l is the droplet ring width; and h is the droplet ring height.

homogeneous with increasing DsRed concentrations. The droplet diameter (d) remains almost unchanged over the wide DsRed concentration range from 0.05 to 1.0 g/L. A slight reduction of the diameter of dehydrated droplets is observed with increasing concentration of DsRed. As it is shown in Table II, all the other droplet characteristics (droplet thickness, e , measured inside the droplet, just before the droplet ring, droplet ring width, l , and droplet ring height, h) are increased when the concentration of DsRed in solution is larger. It means that the DsRed adsorbs on the entire area covered by the droplet with preferential deposition close to the triple line as discussed earlier. The obtained results imply that the thickness of the adsorbed DsRed layer on solid surfaces (SiO_2) can finely be controlled by the protein concentration. Moreover, the DsRed layer thickness can be varied in the nanometer range. DsRed layers as thin as 20 nm ($c = 0.05 \text{ g/L}$) can be deposited. The characteristics of dehydrated DsRed droplets are important to be determined for future Raman or fluorescence spectroscopy studies of DsRed adsorbed on silica surfaces requiring very thin protein layers.

The photoluminescence (PL) of the DsRed was excited using the 514 nm line of an Argon laser focused to $1 \text{ }\mu\text{m}^2$ spot size. The incident laser power was limited to 5 mW in order

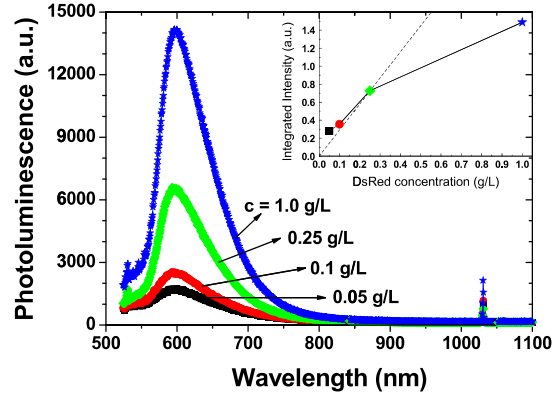


Fig. 2. Photoluminescence spectra of DsRed dehydrated droplets adsorbed on SiO_2 surface for different concentrations at pH = 7.0 and 23 °C. The inset represents the integrated intensity of the photoluminescence peak as a function of the DsRed concentration.

to avoid laser induced heating and subsequent degradation of the samples. Uniform regions of the dehydrated droplets containing DsRed (pH = 7.0) with different concentrations were selected for the optical measurements. During measurements, the photoluminescence signal of the DsRed exhibited photo-bleaching on the time scale of 2 minutes [24], [25]. After 3 minutes the PL intensity was quite stable and the spectra were acquired with 10 s accumulation time. The so-obtained photoluminescence spectra are presented in Fig. 2. As can be noticed the photoluminescence emission increases with increasing DsRed concentration. However, it does not scale up linearly most likely due to quenching induced by protein aggregation at high concentration (Fig. 2, inset). The photoluminescence emission band is peaking at 590 nm. The slight red-shift with respect to the DsRed emission in solution (583 nm) [9] can be attributed to conformation effects and to interaction of the DsRed with the silica substrate.


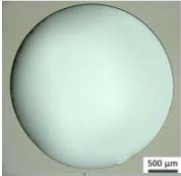

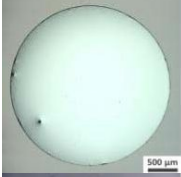

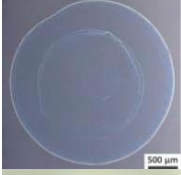

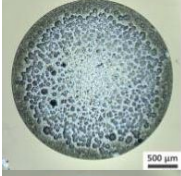

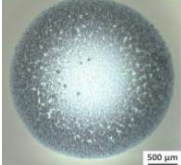
B. PH-Dependent Behavior of DsRed Proteins

The DsRed proteins seem rather stable under pH-variations with respect to the contact angle variation. This is in agreement with previous studies reporting on the pH-stability of DsRed [11], [12]. The pH-dependent behavior of DsRed ($c = 0.05 \text{ g/L}$) is summarized in Table III. It does not appear important on the measured diameter of the droplet after dehydration. However, the contact angle value and the dehydration time increase under mildly acidic conditions. The visual aspect of the DsRed droplets after dehydration is imposed by the corresponding pH-solution. The low DsRed concentration was selected in relation with the finest thickness of the adsorbed DsRed layer after dehydration. Further works on the pH-behavior of larger DsRed concentrations should be performed considering the biochemical properties of DsRed.

C. Morphology of Dehydrated DsRed Layer

To get insight the morphology in the organization of DsRed on the silica surface after dehydration and adsorption on solid hydrophilic surfaces with zero conductivity, the samples were observed with SEM. The image corresponding to $c = 0.05 \text{ g/L}$, pH = 7.0 is shown in Fig. 3. Given the

TABLE III
PH-DEPENDENT BEHAVIOR OF DsRED SESSILE DROPLETS
WITH 0.05 g/L AT 23 °C.

pH	Droplet image at 1s	Contact angle $-\theta_A$	Optical image after dehydration	Dehydrated sessile droplet
3.0		$73.9^\circ \pm 2.1^\circ$		$d = 2.7 \text{ mm}$
5.0		$67.6^\circ \pm 1.3^\circ$		$d = 2.7 \text{ mm}$
7.0		$65.4^\circ \pm 0.2^\circ$		$d = 2.8 \text{ mm}$
9.0		$69.2^\circ \pm 0.8^\circ$		$d = 2.7 \text{ mm}$
11.0		$67.7^\circ \pm 0.3^\circ$		$d = 2.7 \text{ mm}$

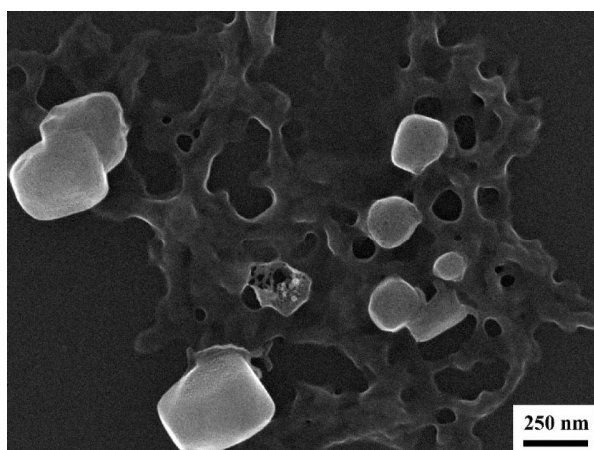


Fig. 3. SEM image of DsRed dehydrated droplet adsorbed on SiO_2 surface for $c = 0.05 \text{ g/L}$ at $\text{pH} = 7.0$ and $23 \text{ }^\circ\text{C}$.

limited resolution of SEM technique one cannot go further but to say that the DsRed forms a very thin continuous layer on the SiO_2 surface. However, the DsRed structural rigidity is at the origin of the obtained porous aspect of the protein

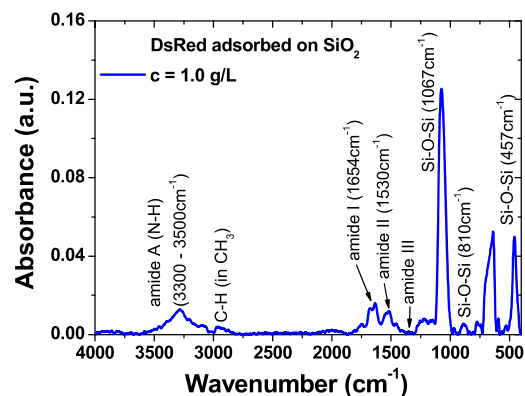


Fig. 4. FTIR spectrum of DsRed dehydrated droplet adsorbed on SiO_2 surface for $c = 1.0 \text{ g/L}$ at $\text{pH} = 7.0$ and $23 \text{ }^\circ\text{C}$.

overlayer with pores of different shape and size. The whitish rhomboidal structures observable on the image are due to some unidentified residues from the solution. The observed protein morphology after dehydration suggests that the interaction of DsRed with a solid silica surface does not completely break the protein native state. The recorded photoluminescence spectra (Fig. 2) confirm this key aspect, as the fluorescence of DsRed is critically dependent on the fidelity of protein folding. Potential modification of the excitation and emission spectra due to the DsRed – SiO_2 interaction in solution should be addressed to reveal eventual changes in the DsRed protein optical properties.

D. On the Possibility to Perform FTIR Analysis of Dehydrated DsRed Adsorbed on Solid Surfaces

The recorded FTIR spectrum of dehydrated DsRed droplet ($c = 1.0 \text{ g/L}$, $\text{pH} = 7.0$) adsorbed on SiO_2 surface is presented in Fig. 4. Due to the fine protein layer thickness after dehydration, the concentration of 1.0 g/L is the only one authorizing acquisition of FTIR spectra in transmission mode. For lower concentrations the dehydrated protein layer is very thin to allow detectable absorbance. The FTIR spectrum contains all characteristic bond vibrations of the SiO_2 layer and those typically associated with the secondary structure of proteins. The three typical TO modes of amorphous silicon dioxide, the Si-O-Si rocking vibration at 457 cm^{-1} , the symmetric stretching mode at 810 cm^{-1} , and the asymmetric stretching mode at 1067 cm^{-1} , are clearly observable on the spectrum [26]. One can also notice the SiO_2 characteristic shoulder centered around 1250 cm^{-1} .

In general, the protein structures are relatively unstable when exposed to solid surfaces, allowing internal protein regions to interact with the surface. Proteins tend to unfold in this case. Protein denaturation is often associated with complete loss of their secondary structure. The recorded FTIR spectrum (Fig. 4) suggests that the DsRed protein is not fully denatured after dehydration and adsorption on the silica surface in accordance with the observed photoluminescence. The Amide I band with peaks centered at 1654 cm^{-1} , assigned the protein α -helix, and the one at 1684 cm^{-1} , belonging to the protein β -sheet are well present on the spectrum [16]. Amide II band centered at 1530 cm^{-1} , and most likely

the Amide III band around 1340 cm^{-1} , are also detectable. Other bands on the spectrum are the Amide A (N-H) one ($3300\text{--}3500\text{ cm}^{-1}$) and the C-H bonds in CH_3 environment (symmetric at 2900 cm^{-1} and asymmetric at 2960 cm^{-1} stretching bonds). The large band around 670 cm^{-1} can be attributed to possible N-H out-of-plane bending vibrations ($640\text{--}750\text{ cm}^{-1}$) [27]. Further studies will be directed to deconvolution of the FTIR spectrum consistent with identification of positions of the protein secondary structure and possible protein-protein interactions. However the applied here procedure to study dehydrated proteins adsorbed on solid surfaces by FTIR analysis in transmission mode offers the possibility to work on reduced quantities of proteins, especially for quite costly proteins, like DsRed and/or for rare proteins. This attempt will be further developed to extract information about the structural stability of proteins.

IV. CONCLUSION

Physico-chemical analysis of the interactions of red fluorescent protein, DsRed with thermal SiO_2 surfaces was performed aiming at identification of the relation between structural stability of DsRed proteins and their adsorption behavior. The obtained characteristics of dehydrated DsRed droplets imply that the thickness of the adsorbed DsRed protein layer on solid SiO_2 surfaces can finely be tuned by the protein concentration. The measured contact angles of very small droplets containing different concentration of DsRed proteins determine the interaction as hydrophilic one, however with larger contact angles for larger DsRed concentrations. With respect to the contact angle measurements, the DsRed proteins appear stable under pH-variations in line with previously reported studies. The adsorption of DsRed on SiO_2 surfaces and the following dehydration processes do not lead to complete protein denaturation. The photoluminescence emission of dehydrated DsRed proteins adsorbed on SiO_2 layers is preserved and found to peak at 590 nm , which is slightly red-shifted compared to the reported value for a solution (583 nm). The procedure for recording FTIR spectra in transmission mode confirms the possibility to explore the protein secondary structure after dehydration and adsorption on SiO_2 surfaces. It also largely supports future spectroscopic studies about the behavior of the proteins with modified surfaces (less hydrophilic), taking advantage of the silica layer and the fact that its surface can be easily functionalized. Potential modification of the DsRed excitation and emission spectra due to the DsRed – SiO_2 interactions in solution will also be addressed further.

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