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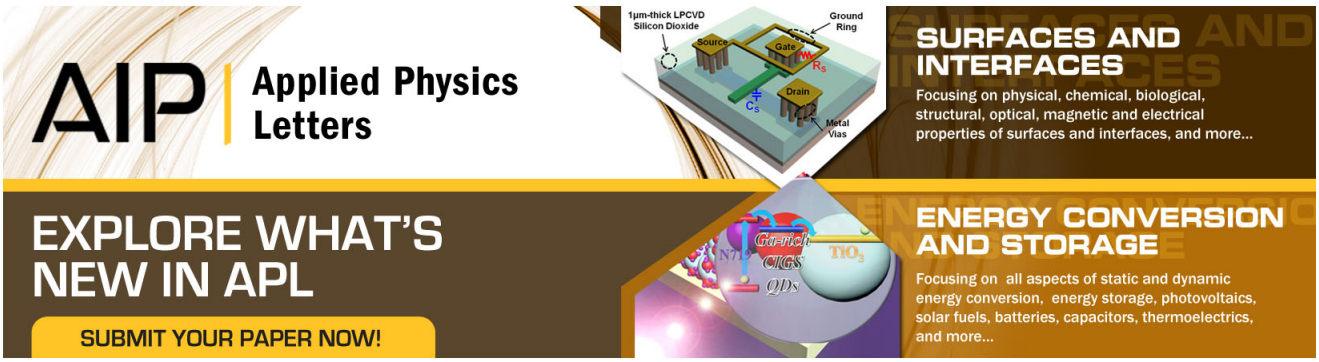

Probing viscosity of nanoliter droplets of butterfly saliva by magnetic rotational spectroscopy

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Magnetic rotational spectroscopy was employed for rheological analysis of nanoliter droplets of butterfly saliva. Saliva viscosity of butterflies is 4–5 times greater than that of water and similar to that of 30%–40% sucrose solutions at 25 °C. Hence, viscosity stratification would not be expected when butterflies feed on nectar with 30%–40% sugar concentrations. We did not observe any viscoelastic effects or non-Newtonian behavior of saliva droplets. Thus, butterfly saliva is significantly different rheologically from that of humans, which demonstrates a viscoelastic behavior. © *2013 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4788927]

Biofluids of insects possess specific combinations of physico-chemical properties that have neither human nor synthetic analogs.[1,2] Insect saliva, for example, significantly varies in physico-chemical properties, depending on the physiological conditions and food constituents.[3,4] While insect biofluids have attracted the attention of biochemists and bioengineers, the analysis of these fluids is challenging: the amount of available fluid often is so small that its physico-chemical characterization is not possible using conventional instruments. In this letter, we discuss the estimation of the viscosity of butterfly saliva when the droplet size is measured in nanoliters, a size which cannot be addressed by the best available rheological methods dealing with microliter droplets.[5–7]

The idea originated from the pioneering work of Crick and Hughes,[8,9] who used magnetic particles to probe the viscosity and elastic reaction of cytoplasm. An applied rotating magnetic field exerts a torque on a magnetic particle, which is balanced by the viscous and elastic torques acting from the medium. Crick and Hughes, therefore, suggested that the reaction of the medium on a step-like pulse of the external magnetic field could be studied by analyzing the particle relaxation to its equilibrium position. Instead of dealing with the analysis of the relaxation process, Frenkel[10] pointed out a phenomenon associated with a rotating magnetic field: the particle synchronously follows the rotating field only within a specific window of the field frequency. Above a certain frequency, the particle cannot keep up with the field. This critical behavior was first visualized with carbon nanotubes staffed with magnetic nanoparticles and used to estimate the magnetic properties of material.[11] The idea of using the transition from synchronous to asynchronous spinning was further developed by Kopelman's group employing magnetic beads with different chemical functionality.[12] This group demonstrated different sensoric applications of magnetic beads.[13–15]

We report an application of this method to probe the shear viscosity of saliva droplets from three different species of butterflies. Magnetic rotational spectroscopy (MRS) with magnetic nanorods allows measurement of the viscosity of picoliter droplets.[11,16–18] It takes advantage of the instability of synchronous rotation of a magnetic nanorod having magnetic moment m in a uniform rotating magnetic field B: when the frequency of the driving field is increased beyond the critical frequency $f_c = mB/(2\pi\gamma)$, where γ is the drag coefficient of the nanorod, the nanorod stops rotating synchronously with the magnetic field.[10] For a long nanorod, the drag coefficient γ depends on the nanorod length l, its diameter d, and liquid viscosity η as $\gamma = \pi\eta l^3/(3ln\ (l/d) - 2.4)$.[19] This critical frequency is detectable by observing the nanorod spinning under a microscope. By gradually increasing the spinning frequency of the applied rotating field, one can detect a moment when the nanorod swings in the direction opposite to the direction of field rotation for the first time (supplementary video s1 (Ref. 28)). After that moment, the nanorod spinning does not follow the rotation field, i.e., the nanorod rotates asynchronously with the field.[11,13,16] Knowing the critical frequency and magnetic properties of the nanorod, one can extract the fluid viscosity as $\eta = mB(3ln\ (l/d) - 2.4)/(2\pi^2 l^3 f_c)$.

We used nickel nanorods 200 nm in diameter to measure the viscosity of butterfly saliva by MRS. Nickel nanorods were produced by electrochemical template synthesis.[18,20] To generate a rotating magnetic field, we used two magnetic coils placed perpendicular to each other and fixed under a BX-51 Olympus microscope equipped with a SPOT videocamera (SPOT Imaging Solutions, Inc.) The details of the generation of a rotating magnetic field can be found elsewhere.[21] The magnetic field was measured by a digital teslameter (133-DG GMW, Inc.), and in the center of the optical cell it was equal to 0.8 mT. Critical frequency was measured by analyzing the videos with VirtualDub software (http://www.virtualdub.org). Rotation of a single nanorod at $f = 1$ Hz frequency is shown in Fig. 2(a).

The nanorods were dispersed in ethanol by sonication. When the sonication time was increased above 10 min, the

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20-μm long nanorods broke, resulting in a nanorod polydispersity in the obtained dispersion. A droplet of the nanorod dispersion was placed on a glass slide and dried at room temperature. The dried spot on the glass slide contained multiple nanorods weakly attached to the slide by Van der Waals forces. Two rectangular pieces of double-sided tape (OfficeMax, 21009267, thickness $h = 90\,\mu$m) were placed on the glass slide, forming a well. The dried spot was positioned between the pieces of double-sided tape. The butterfly was held by its wings and its proboscis was extended to the glass slide, using a needle (Fig. 1).

The butterfly will release saliva upon contact with a sugary substrate. However, this method of salivation was avoided, as it could result in a saliva sample contaminated with sugar remnants. At least 5 h had passed since the butterfly's last meal. The proboscis of the butterfly was brought close to the spot with nanorods. To encourage salivation, the distal region of the proboscis was stroked using a toothpick. Once a droplet of saliva was produced, a cover slip was placed over the droplet and the tape to seal the chamber and prevent evaporation. Experiments were conducted at 24 °C. Temperature, measured with a thermometer, did not deviate during the experiments by more than 0.2 °C.

To seal the well and prompt formation of a liquid bridge, light finger pressure was applied to the cover slip. This method provided minute quantities of saliva ranging from 2.8 nl to 6.4 nl. The average volume was calculated assuming that the "liquid bridge" of saliva was a perfect cylinder with a volume $V = \pi r^2 h$, where h is the thickness of the double-sided tape and r is the radius of each droplet, which was measured using a microscope. The sample was placed in the magnetic system, producing a rotating magnetic field, and critical frequencies were measured for nanorods of different length. After nanorods were dispersed in the saliva, some nanorods attached to the slide and did not keep up with the rotating field even at low frequencies. To select a nanorod completely suspended in the saliva, we calibrated the microscope as follows. First, the focus was set on the lower glass slide and the position of the microscope knob was marked. We then rotated the knob to focus on the upper glass slide, and the position of the knob was marked again. Since the interslide gap was fixed by the 90-μm thick double-sided tape, we assumed a linear relation between the knob revolution angle and focal distance and calibrated the microscope accordingly. For the MRS analysis, only nanorods at least 30 μm from the surface were considered. These nanorods remained in the focal plane during the experiments.

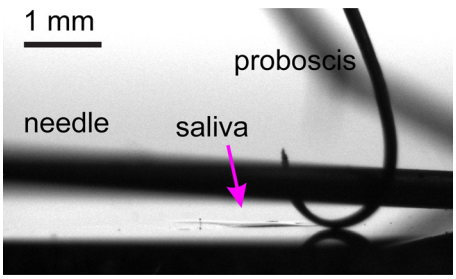

FIG. 1. Obtaining butterfly saliva. Black bar crossing the image is the needle used to extend the proboscis.

Four different monarch butterflies were used for salivation, and viscosity was measured for eight droplets. Two droplets were taken from each butterfly at 10:00 and 14:30 h. Salivation by tiger swallowtails and painted ladies was less frequent, and we were able to obtain only a single drop from each butterfly out of 4 of each tested. The nanorod diameter, measured with scanning electron microscopy, was equal to 200.6 ± 2.6 nm. For the viscosity of 4 mPa s, this error results in 2% deviation (± 0.08 mPa s). The nanorod length was estimated from the dark-field pictures with ± 200 nm accuracy. For the viscosity of 4 mPa s, this error produces 5.3% deviation (± 0.21mPa s). Three nanorods of different lengths suspended in these droplets were used to average the viscosity data.

To interpret the results, one needs to know the magnetic properties of nanorods. They were measured using an alternating gradient magnetometer (AGM MicroMag 2900 by Princeton Measurements, Inc.) prior to the experiments with saliva samples. Magnetic hysteresis of an alumina membrane with the electrodeposited nickel nanorods is shown in Fig. 2(b). The solid line shows the sample behavior before magnetization and the dashed line corresponds to the magnetized sample. In the inset, we plot the magnetization curve in the milliTesla range of the magnetic field, which is below the coercive filed. In this field range, the average magnetization is linearly dependent on the field and can be approximated as $M = 0.00023 \cdot B + 1.8 \times 10^{-7}$. Using the obtained magnetization curves, we calculated the magnetic moment of a single nanorod as $m = M/N = M/(S_m/S_n) = M/(S_m/\pi r^2)$, where S_n is the cross-sectional area of a single nanorod, S_m is the membrane area, M is a magnetic moment of the membrane at

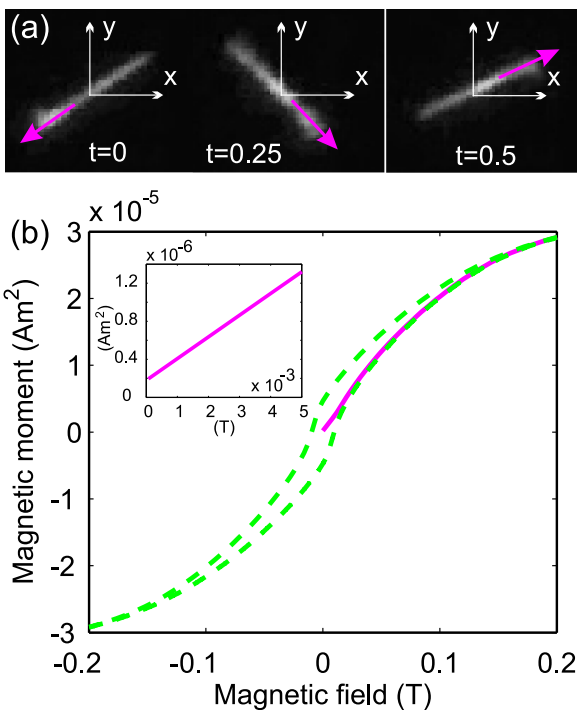

FIG. 2. (a) Counter-clockwise rotation of nickel nanorod ($d = 200$ nm) at 1 Hz frequency. (b) Hysteresis of the membrane with electrodeposited nanorods. The solid line shows the magnetization curve of "as-prepared" sample and the dashed line shows the hysteresis loop. Insert shows the magnetization of the sample in the mT range of magnetic fields (enhanced online) [URL: http://dx.doi.org/10.1063/1.4788927.1].

$B = 0.8\,\text{mT}$, $r = 100\,\text{nm}$ is the radius of a single nanorod, and N is the number of nanorods in the tested membrane. The radius and length $l = 20\,\mu\text{m}$ of the nanorods were measured with scanning electron microscopy (Hitachi S4800). The magnetic moment of the 20-μm long nanorod was $m = 2.6 \times 10^{-14}\,\text{Am}^2$.

To confirm this average value of the magnetic moment, we used another series of experiments, rotating the nanorods from a different piece of the same alumina membrane in water droplets. According to Fig. 2(b), the magnetic moment should linearly depend on the field in the milliTesla range. The magnetic moment was represented in the form $m = VB\chi/\mu_0$, using the scaling arguments, where V is the nanorod volume, μ_0 is the magnetic permeability of the vacuum, and χ is a constant to be determined. Measuring the critical frequency of nanorods spinning in water with known viscosity $\eta = 1$ mPa·s, we obtained $\chi = 63$. Substituting this value into the equation for magnetic moment, we obtained $m = \pi r^2 lB\chi/\mu_0 = 3.3 \times 10^{-14}$ Am^2, which is close to the value obtained with AGM.

Saliva viscosity of butterflies, measured with the MRS method, is 4–5 times greater than that of water (Table I).

We did not observe any viscoelastic effects[22] or non-Newtonian behavior[16] of saliva droplets. To confirm this result and to demonstrate the robustness of the developed MRS method, we measured viscosities of 10%–40% sucrose solutions. Values of critical frequencies for nanorods of different length in 10%–40% sucrose solutions are shown in Table SII in supplementary information.[28] Results of the viscosity measurements are summarized in Table I and agree with published data for sucrose solutions.[23]

Thus, the saliva of butterflies is significantly different from that of humans in which mucin and other proteins provide a viscoelastic behavior to the saliva.[24] Butterfly saliva is incompletely characterized, owing largely to the challenge of analyzing the limited quantities produced, but it contains enzymes such as amylase and invertase[25] and, in rare diet-specific cases, proteases.[26] The saliva viscosity of the butterflies is close to that of 30%–40% sucrose solutions at 25 °C.[23,27] Figure 3 compares the viscosity of saliva with the viscosity of 10%–40% sucrose solutions.

In insects, saliva lubricates the mouthparts, aids digestion, and dissolves viscous and dried substances.[25] Our results suggest that saliva should not be needed for liquefying nectars with sugar concentrations up to 30%–40%; viscosity stratification would not be expected when butterflies feed on nectar with 30%–40% sugar concentrations. Rather,

TABLE I. Viscosity (mean ± SD) of saliva of 3 species of butterflies and of 10%–40% (weight/weight) sucrose solutions.

	Viscosity, mPa·s
Monarch (*Danaus plexippus*)	3.9 ± 0.7
Tiger swallowtail (*Papilio glaucus*)	5.0 ± 0.5
Painted lady (*Vanessa cardui*)	5.2 ± 1.0
10% sucrose	1.3 ± 0.2
20% sucrose	1.7 ± 0.2
30% sucrose	3.8 ± 0.4
40% sucrose	6.9 ± 0.7

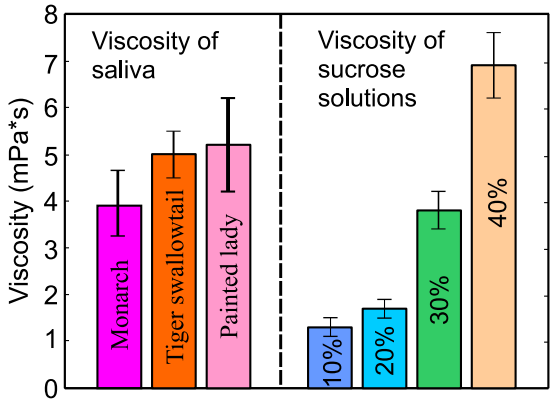

FIG. 3. Comparison of the viscosity of butterfly saliva with that of sucrose solutions of different concentrations.

our results support the observed role of butterfly saliva in solubilizing encrusted films and highly viscous nectar.[26] The results suggest that saliva viscosities differ among some species (Table I), possibly as a function of feeding habits. The intriguing possibility that saliva viscosities are adapted to particular feeding habits (e.g., nectar-feeding versus fruit-feeding) and that they might help optimize foraging efficiency invites further study using magnetic rotational spectroscopy.

In summary, we showed that magnetic rotational spectroscopy is a useful tool for rheological analysis of nanoliter droplets of biofluids, such as butterfly saliva. It also would allow measurements of even smaller droplets, for example, saliva from minute moths. We expect that this method will find broader applications in experimental biology.

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[28]See supplementary material at http://dx.doi.org/10.1063/1.4788927 for the video of nanorod rotating at different frequencies of applied rotating field in a droplet of butterfly saliva and for the critical frequencies for nanorods of different lengths suspended in 10%–40% sucrose solutions.