Optical activity of lysozyme in solution at 532 nm via signal-reversing cavity ring-down polarimetry

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An improved optical cavity-based polarimetry method is employed to measure the optical activity of lysozyme in water solution, in the concentration range of 0-2 mg/ml. We employ a signal reversing technique, which gives the absolute optical rotation, without needing to remove the sample for a null measurement. We report an absolute sensitivity limit on the order of 0.1 mdeg, corresponding to a detection limit of <50 μg/ml for a sample volume lower than 50 μL, thus surpassing the sensitivity of existing commercial polarimeters. We discuss how these sensitivity levels can be further improved using existing methods and technologies.

The existence of chiral asymmetry in biomolecules has profound implications for biology and is linked to the question of the origin of life [1]. On a more practical level, the fact that specific biomolecules are found exclusively in one of the two possible enantiomeric forms in living organisms makes chiral analysis an indispensable tool for pharmacology, medicinal chemistry, for agricultural chemistry and more [2].

Among the available experimental tools for probing chiral asymmetry in the solid and liquid phase, one can find X-ray crystallography [3], chiral chromatography [4], Nuclear Magnetic Resonance [5] and more, while in the gas phase, chirality can be probed by using optical methods such as microwave detection [6], femtosecond excitation [7, 8] and ionization imaging [9]. However, by far the most common method of chiral detection is the measurement of optical rotation of polarization of light that is transmitted through a chiral medium, i.e. polarimetric detection. Among the main advantages of polarimetry is the fact that it is a nondestructive and a nonresonant method. These advantages allow spectral characterization of the interrogated species, thus offering insights on the resonant transitions that produce chiral rotation and ultimately enabling the extraction of stereodynamical information in complex biomolecules.

Today, modern commercial polarimeters typically operate with a sensitivity of a millidegree, with the most sophisticated among them demonstrating sensitivities of 0.3 millidegrees. The main limitation of the sensitivity of commercial polarimeters is the need to frequently subtract the spurious backgrounds, which is caused by a combination of birefringence and misalignments of the probing beam. This requirement, along with the overall weakness of chiral signals, considerably limits the detection capability of chiral signals by conventional, single-pass polarimeters. An expansion of the standard polarimetric methods came with the introduction of Cavity Ring Down Polarimetry (CRDP) by Vaccaro and co-workers in the early 2000s [10], which used a linear optical cavity to enhance polarimetric signals, and has produced a variety of optical rotation measurements in the gas and liquid phase [11,12,13,14,15,16]. Linear cavities have also been used to measure the Faraday Effect in gases [17,18,19]. In 2014, our group improved CRDP

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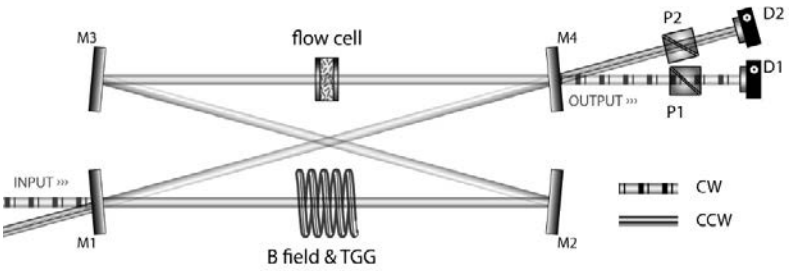

Figure 1 Drawing of the CRDP cavity set-up, showing the cavity mirrors (M1-M4), the flow cell and the coil used to produce the magnetic field, as well as the polarizers P1, 2 used at the output of the cavity. Adjusted on each side of the evacuated cell are 2 anti-reflection coated windows not shown here.

by using a ring cavity, allowing counter-propagating laser beams, and an intracavity Faraday Effect. Subtraction of the signals from the counter-propagating beams yields the chiral optical rotation, along with background contributions; however, changing the sign of the Faraday Effect reverses the sign of the chiral signals, while the backgrounds are unchanged. This use of signal reversals [20,21] allows the isolation of the chiral signals from sources of background, giving an absolute measure of the chiral optical rotation, without needing remove the sample for a null measurement. Here, we demonstrate these abilities by performing optical rotation measurements in lysozyme solutions in water, and extract specific optical rotation measurements at 532 nm. The sensitivity of our measurements is on the order of 10^{-4}-10^{-5} deg/pass, which translates to concentrations of 10-100 µg/ml.

Lysozyme is an enzyme found in secretions of animals and humans, such as tears, sweat and saliva. It is part of the innate immune system, having antibacterial effects; for example, it eliminates bacteria found in tears, and therefore its abundance in tears is a sign of a healthy eye. The normal level of Lysozyme in tears has been measured to be 1.5 mg/ml [22]. Low levels of lysozyme can indicate bacterial infections caused by dry eye syndrome [23]. The precise measurement of lysozyme in tears can thus provide a diagnostic tool about the eye's health.

The experimental setup, described in detail elsewhere [19,20] is shown in figure 1. The 532 nm output of a 10 µJ, 8 ns FWHM Nd:YAG DPSS laser (Roithner RLTMPL-532-100), operating at a repetition rate of 10 kHz, is split in two equal parts and is injected in a bowtie optical cavity. The cavity comprises two high reflectivity mirrors (Layertec, R>99.98%) and two high reflectivity, low loss output couplers (Layertec, R>99.5%). The total free-space cavity length is 4.8 m. A 3 cm-long intra-cavity, anti-reflection coated (<0.1% per side) Terbium Gallium Garnet (TGG) crystal is placed in the interior of a coil. The TGG can induce a rotation of a linear polarization plane of ~25 degrees per pass, when a 15 A current is applied. A 3mm thick flow cell is used to introduce the liquid solution, composed by 2 AR-coated windows, with one side coated for air-fused silica interface, and the other side coated for water-fused silica interface. The ring-down time of this configuration

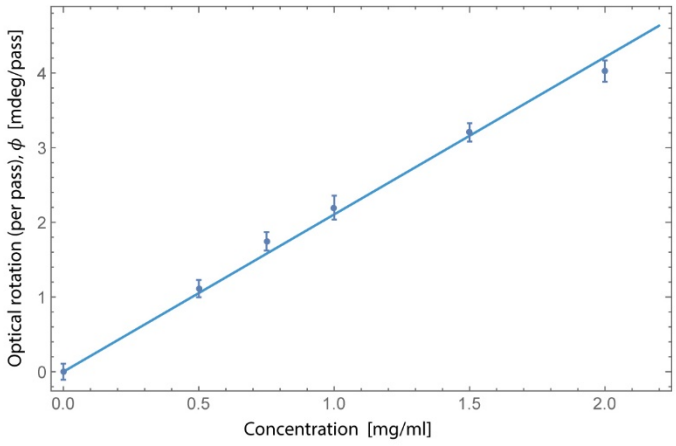

Figure 2 Optical rotation measurements of lysozyme solution in concentrations ranging from 0 to 2 mg/ml.

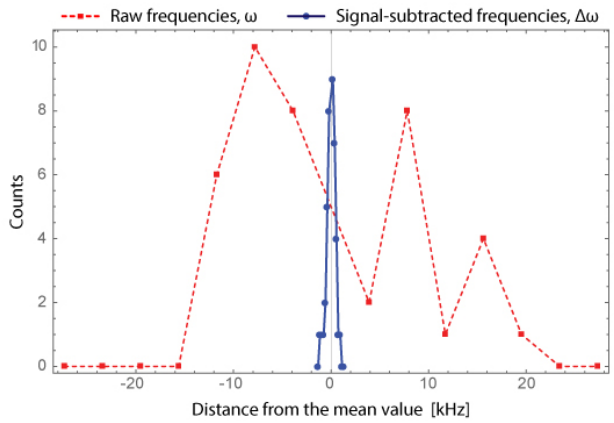

Figure 3 Plot of the distribution of our frequency measurements around their mean value for the raw measurements (dashed red) and the frequencies resulting from the signal subtraction (solid blue). Notice that the subtraction reduces frequency spread by over an order of magnitude, due to common mode noise.

is 640 ns, corresponding to 40 round-trips inside the cavity and an effective sample length of 12 cm. The main limiting factor of the photon lifetime is the flow cell, specifically at interior interfaces between the sample and the window. Since the absorption of the sample at 532 nm should be negligible for this sample thickness, we believe that losses arise from coating imperfections or degradation at the window-sample interface, which produce visible scattering. Two thin-film polarizers are employed after the cavity, to filter the projection of the rotating polarization plane. The signal is detected by two Biased Si Photodiodes (Thorlabs DET36A), and recorded by an oscilloscope with a sampling rate up to 10 Gs/s, and a maximum bandwidth of 3 GHz. The oscilloscope monitors four signals; the two photodiode outputs; the laser trigger; and the magnetic field signal. In the experiments reported here, the coil used to produce the magnetic field applied to the TGG crystal is driven using custom electronics and its direction can be inverted at a repetition rate that can be varied between 0.1 and 100 Hz. A home-made software monitors the status signal of the magnetic field reversal circuit. When change in the status signal is detected (i.e., the magnetic field is reversed), the average of a predetermined number of pulses is recorded.

The cavity supports two distinct counter-propagating modes, designated as clockwise(CW) and counterclockwise(CCW). Upon each consecutive round trip of the light inside the cavity, the polarization plane is rotated by a finite angle, and part of the light leaks out of the cavity mirrors. Thus, by placing polarizers in the output of the two modes we observe a time-dependent signal which has the form of a damped oscillation. The frequency of this oscillation is proportional to the sum and difference of the magneto-optic and the chiral optical rotation for the CW and the CCW modes, respectively. By inverting the direction of the magnetic field produced in the coil, the sign of the magneto-optic rotation is reversed. Thus, by subtracting the CW and CCW polarization oscillation frequencies for each direction of the magnetic field, and subsequently subtracting these differences for the two directions of the magnetic field [20,21], any signals that don't have chiral symmetry are cancelled out. Therefore, absolute optical rotation measurements are obtained. The subtraction yields a frequency that is directly equivalent to the intracavity chiral rotation as $\omega = \frac{\varphi_c}{4\,FSR}$, where ϕ_c the chiral optical rotation and FSR the free spectral range of the cavity.

In Figure 2 we show the dependence of the optical rotation of Lysozyme solution as a function of the sample concentration. The samples of various Lysozyme concentrations were obtained by diluting an 10 mg/ml aqueous (ph 7) solution of Lysozyme from chicken egg white (Sigma Aldrich, 98% protein), stored at -20oC. The errors shown for each concentration are the standard errors (confidence level 95%) of a batch of 40 consecutive measurements, obtained in less than 1 minute, and vary from 0.1 to 0.15 mdeg.

The standard error can be used in this context since the signal variation appears to follow normal distribution, as shown in Figure 3. In this figure, we examine the distribution of 40 consecutive measurements, and compare raw measurements (i.e., frequencies obtained by analyzing one channel only) versus measurements after the two signal reversals. We plot the number of measurements which lie within a specific distance from their average value. We see that the raw frequencies are scattered across a wide frequency range while the subtracted frequencies follow a narrow bell-like distribution reminiscent of the normal distribution.

An additional benefit of the signal reversals is the cancellation of drifts, which can arise from temperature variations or mechanical instabilities and which do not change chiral rotation. An example of this is demonstrated in Figure 4 where, again, we plot 40 consecutive measurements: a) CW mode frequencies for one direction of the magnetic field and b) the values resulting after the subtraction of the CW and CCW frequency beatings, and the subsequent subtraction of frequencies corresponding to the two orientations of the magnetic field (+B and -B). As we see, the drifts that dominate any of the single channel measurements (only one channel shown here) are largely cancelled by the two signal subtractions, and are reduced by over a factor of 20. These drifts are a result of the large change in the coil temperature, as it warms up after initialization, which induces a change in the TGG refractive index. This temperature variation does not contribute to the optical rotation of the sample, as a change in the temperature of the sample itself would do, and therefore it is canceled out by the signal reversals. The variations in frequency after the signal reversals, on the other hand, are a combination of real optical rotation fluctuations, mainly due to temperature fluctuations, and other, spurious noise sources (air fluctuations, vibrations) that are not canceled out because of experimental imperfections cited below.

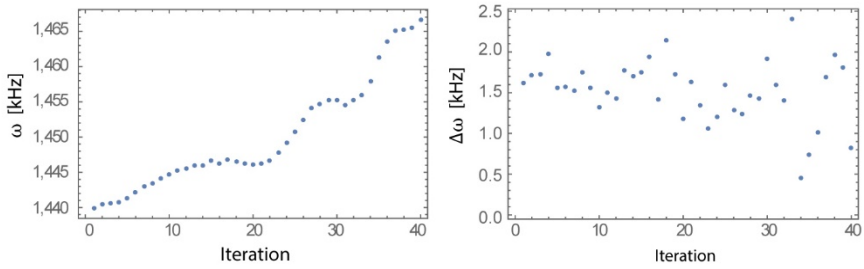

Figure 4: Consecutive measurements of a) the polarization beating frequency observed in one channel (CW propagation, positive magnetic field) b) the beating frequency corresponding to optical rotation, obtained after two signal reversals.

The main limitation of our data acquisition scheme at this time is the inability to capture all light pulses emitted from the laser, and to allow measurements of rapid signal reversals. Oscilloscopes typically introduce a dead time between acquisitions of a few tens of milliseconds, which in our instrument results in the loss of roughly 99% of the emitted pulses, since the laser repetition rate is 10 kHz. A remedy for this effect, which would improve our measurement time by a factor of 100, would be to deploy a dedicated data acquisition card, described in Ref. [24], where our group demonstrated running ellipsometric measurements with a μs temporal resolution. This way, more ring-down traces would be averaged in a much shorter times, and the sensitivity levels of 0.1 mdeg would be achieved in acquisition times of less than 1 sec. Together, these improvements should allow real-time monitoring of liquid flows, with applications in HPLC and chiral dynamics. In addition, much faster signal reversals at 10-100 Hz instead of 0.1 Hz, will likely improve the sensitivity further, by canceling slow drifts.

Measurements of the optical activity of lysozyme have been reported in [25], in [26] and in [27] at 589 nm, 365 nm and at 578 nm respectively. The extracted value for the specific rotation at 532 nm reported here is $68.1 \pm 2.3 \frac{deg}{dm}\left(\frac{g}{ml}\right)^{-1}$, and it is in good agreement with the expected value at 532 nm. In reference [23] the dependence of optical rotation to wavelength was calculated with two methods, the Moffitt-Young and the Shechter-Blout expression. In Figure 5, we plot the predictions of both these expressions, with the confidence intervals calculated using the values for the standard deviation of the experimentally determined fit parameters reported in [27]. Together, we plot the values reported in reference [25,26,27] and the measurement reported here. We see that our measurement lies in the high side of the experimental curves; however, lysozyme is a macro-molecule whose spectral features have been reported to vary significantly with pH [27] or depending on the molecule's origin, for example if the molecule is of human origin or of white-egg origin [28], so we attribute the small discrepancies of the values to reasons such as the above.

In conclusion, we present measurements of optical rotation in lysozyme-water solution using a compact set-up, which employs a four-mirror optical cavity, a magneto-optical crystal and a low-power microchip laser and a custom, programmable electronics board. We demonstrate absolute determination of the specific rotation of lysozyme at the wavelength of 532 nm. We were able to measure various concentrations of lysozyme in aqueous solutions, with a sensitivity of 100 μg/ml. This corresponds to a precision down to ~7% of the normal levels, which is enough to precisely determine whether lysozyme levels in tears are normal.

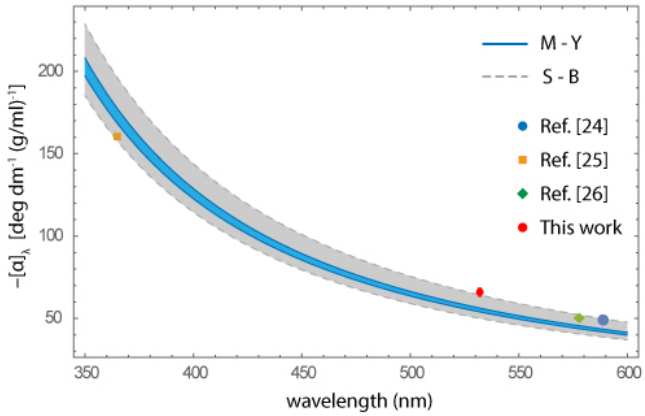

Figure 5: Specific Optical Rotation of lysozyme as a function of wavelength, (blue line: Moffitt-Young model, gray line: Shechter-Blout)

Acknowledgments: This research was partially supported by ERA-NETs co-fund action (EPOCHSE, grant no. T3EPA-00043). This research is co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Reinforcement of Postdoctoral Researchers" (MIS-5001552), implemented by the State Scholarships Foundation (IKY). We also acknowledge the European Commission Horizon 2020, ULTRACHIRAL Project (grant no. FETOPEN-737071) for the financial support. GK acknowledges funding from the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT), under grant agreement No [1789].

[1]B. A. McGuire et al., *Science* **352,** 1449 (2016)
[2] Prasad L. Polavarapu (Ed.): Chiral analysis: advances in spectroscopy, chromatography, and emerging methods, 2nd ed. (Elsevier 2018) ISBN: 978-0-444-64027-7
[3] H. D. Flack and G. Bernardinelli Chirality **20,** 681 (2008)

[4] S. C. Moldoveanu and V. David Selection of the HPLC Method in Chemical Analysis (Elsevier 2017) ISBN: 978-0-12-803684-6
[5] J. Labuta et al., Nat. Comm. **4,** 2188 (2013)
[6] Patterson, D., Schnell, M. & Doyle, J. M. Enantiomer-specific detection of chiral molecules via microwave spectroscopy. Nature **497**, 475–477 (2013)
[7] Rhee, H. J. et al. Femtosecond characterization of vibrational optical activity of chiral molecules. *Nature* **458**, 310–313 (2009)
[8] Beaulieu, S., et al. "Photoexcitation circular dichroism in chiral molecules." Nature Physics 14.5 (2018): 484-489
[9] Janssen, M. H. M. & Powis, I. Detecting chirality in molecules by imaging photoelectron circular dichroism. Phys. Chem. Chem. Phys. **16**, 856–871 (2014)
[10] Muller, T., Wiberg, K. B. & Vaccaro, P. H. Cavity ring-down polarimetry (CRDP): a new scheme for probing circular birefringence and circular dichroism in the gas phase. J. Phys. Chem. A **104**, 5959–5968 (2000)
[11] S. M.Wilson, K. B.Wiberg, J. Cheeseman, M. J. Frisch, and P. H. Vaccaro, J. Phys. Chem. A **109**, 11752-11764 (2005).
[12] T. Muller, K. B. Wiberg, P. H. Vaccaro, J. R. Cheeseman, and M. J. Frisch, J. Opt. Soc. Am. B **19**, 125 (2002).
[13] S. M.Wilson, K. B.Wiberg, M. J. Murphy, and P. H. Vaccaro, Chirality **20**, 357 (2008).
[14] P. Lahiri, K. B. Wiberg, and P. H. Vaccaro, J. Phys. Chem. A **116**, 9516 (2012).
[15] P. Lahiri, K. B. Wiberg, and P. H. Vaccaro, J. Phys. Chem. A **117**, 12382 (2013).
[16] M. Caricato, P. H. Vaccaro, T. D. Crawford, K. B. Wiberg, and P. Lahiri, J. Phys. Chem. A **118**, 4863 (2014).
[17] C.L. Patrick, J. Westberg, G. Wysocki, Anal. Chem. 91, 1696 (2019).
[18] M. Gianella, S.A. Press, K.M. Manfred, H.C. Norman, M. Islam, G.A.D. Ritchie, *J. Chem. Phys.* **151**, 124202 (2019).
[19] R. Engeln, G. Berden, R. Peeters, G. Meijer, *Rev. Sci. Instrum.* **69**, 3763 (1998).
[20] D. Sofikitis, L. Bougas, G. E. Katsoprinakis, A. K. Spiliotis, B. Loppinet, and T. P. Rakitzis, *Nature* **514**, 76 (2014).
[21] Lykourgos Bougas et al*., J. Chem. Phys.* **143**, 104202 (2015)
[22] Pertti Morsky and Esko Aine, Clinica Chimica Acta, **129**, 201 (1983)
[23] V. P. deLuise and K. F. Tabbara, Arch Ophthalmol. **101**, 634 (1983)
[24] D. Sofikitis et al., Applied Optics **54,** 18 (2015)
[25] B. Jirgensons Arch. Biochem. Biophys. **41,** 333 (1952)
[26] C. N. Pace and T. McGrath J. Biol. Chem. **255,** 3862 (1979)
[27] J. A. Gordon J. Biol. Chem. **243,** 4615 (1967)
[28] J. P. Halper, N. Latovitzki, H. Bernstein and S. Beychok PNAS **68,** 517 (1971)