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Citation: Appl. Phys. Lett. **111**, 142902 (2017); doi: 10.1063/1.4997446
View online: http://dx.doi.org/10.1063/1.4997446
View Table of Contents: http://aip.scitation.org/toc/apl/111/14
Published by the American Institute of Physics

The direct piezoelectric effect in the globular protein lysozyme

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(Received 24 July 2017; accepted 18 August 2017; published online 2 October 2017)

Here, we present experimental evidence of the direct piezoelectric effect in the globular protein, lysozyme. Piezoelectric materials are employed in many actuating and sensing applications because they can convert mechanical energy into electrical energy and vice versa. Although originally studied in inorganic materials, several biological materials including amino acids and bone, also exhibit piezoelectricity. The exact mechanisms supporting biological piezoelectricity are not known, nor is it known whether biological piezoelectricity conforms strictly to the criteria of classical piezoelectricity. The observation of piezoelectricity in protein crystals presented here links biological piezoelectricity with the classical theory of piezoelectricity. We quantify the direct piezoelectric effect in monoclinic and tetragonal aggregate films of lysozyme using conventional techniques based on the Berlincourt Method. The largest piezoelectric effect measured in a crystalline aggregate film of lysozyme was approximately 6.5 $pC\,N^{-1}$. These findings raise fundamental questions as to the possible physiological significance of piezoelectricity in lysozyme and the potential for technical applications. *Published by AIP Publishing.* [http://dx.doi.org/10.1063/1.4997446]

Many materials of biological origin such as wood,[1,2] bone[3] and tendon[4] as well as bioinspired peptide nanotubes[5] exhibit piezoelectricity. Piezoelectricity is a property of crystals and chiral molecules that originates from the absence of a center of symmetry in their structures. When such materials are stressed, their electrical neutrality is perturbed and a net polarization results at the surface of the material. The Curie brothers first demonstrated this property, known as the direct piezoelectric effect, in 1880.[6] There also exists an opposite effect known as the converse piezoelectric effect; in this case, an electrical field applied across a piezoelectric material causes a mechanical strain to develop. Of the 32 crystallographic point groups, 21 do not possess a center of symmetry and 20 of these classed as piezoelectric.[7]

Stemming from the work on bone, piezoelectricity has been studied extensively in collagen[4,8–10] and other fibrous proteins including keratin,[11] elastin,[12] and myosin and actin.[13] Unlike fibrous proteins, globular proteins can be easily crystallised. All natural protein crystals are non-centrosymmetric and thus fulfill the basic prerequisite of piezoelectricity. Based on reports that the hydration water density of lysozyme changes with pressure, previous reports have suggested that lysozyme might demonstrate electrostriction or piezoelectricity.[14] Kalinin *et al.* probed for piezoelectricity in amyloid lysozyme fibrils adsorbed onto mica using Piezoresponse Force Microscopy (PFM).[15] Recently, we reported evidence of the *converse* piezoelectric effect in individual monoclinic crystals of lysozyme using PFM.[16] Here, we show that crystalline aggregate films of lysozyme in monoclinic and tetragonal form exhibit the *direct* piezoelectric effect.

Lysozyme is an antibacterial enzyme found in the egg whites of birds and in mammalian tears, saliva, and milk. It catalyzes the hydrolysis of specific kinds of polysaccharides thereby weakening the cell wall, making the bacteria susceptible to osmotic lysis. Hen egg-white lysozyme (HEWL) is one of the most studied model proteins and can crystallize as tetragonal, monoclinic, orthorhombic, triclinic, or hexagonal crystals.[17] Here, we focus on the tetragonal and monoclinic forms of lysozyme which are typically described by point group 422 and point group 2, respectively. According to the classical theory of piezoelectricity, tetragonal crystals in point group 422 should demonstrate only shear piezoelectric coefficients (d_{14} and $-d_{14}$). Monoclinic crystals in point group 2 should demonstrate longitudinal (d_{22}), transverse (d_{21}, d_{23}), and shear (d_{14}, d_{16}, d_{25}, d_{34}, d_{36}) piezoelectric coefficients.

Films of lysozyme crystals were prepared using a modified crystallization protocol outlined by Hampton Research.[18] To prepare tetragonal films, 100 mg/ml of lysozyme powder (Sigma-Aldrich, Catalogue Number 62971-50G-F; used without further purification) was reconstituted in sodium acetate (50 mM, pH 4.6). For monoclinic films, the initial protein concentration was 50 mg/ml of lysozyme powder reconstituted in sodium acetate (50 mM, pH 4.6). For monoclinic films only, the protein solution was mixed with 4% sodium nitrate (w/v) in a 1:1 ratio. For all films, glycerol was added to the protein solution to prevent the films from cracking during drying. The glycerol was first diluted to 50% in deionized (DI) water. Then, 1 μl or 6 μl of 50% glycerol was added to the monoclinic and tetragonal solution, respectively. Typically, 100 μl of the final protein solution was dropcast on to the conductive side of an ITO-coated glass slide and left to dry overnight at

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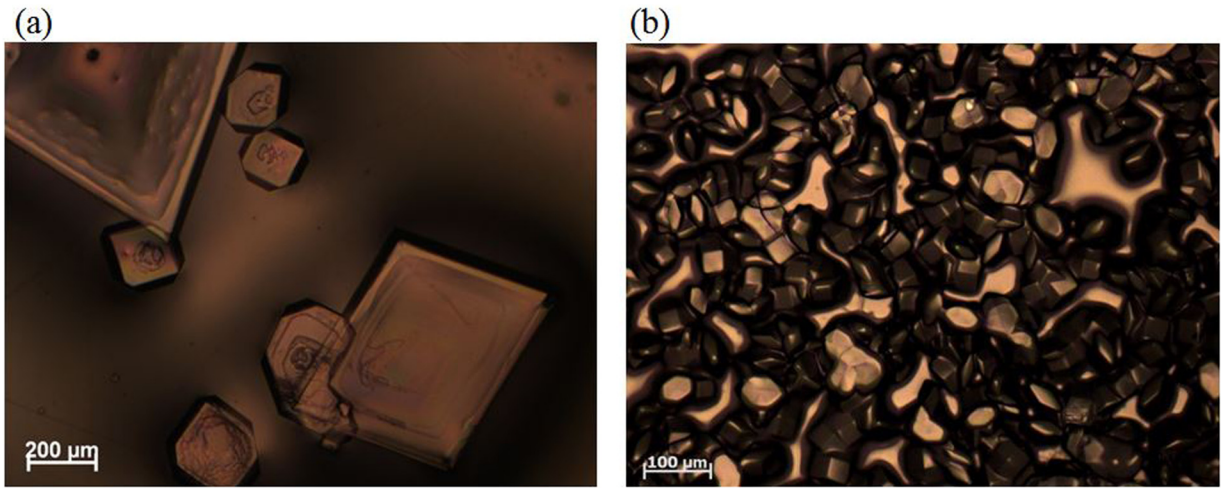

FIG. 1. Optical micrscopy images of (a) monoclinic and (b) tetragonal aggregate films of lysozyme.

20 °C in a temperature-controlled room. Once the film was dry, a second piece of ITO-coated glass, with the ITO coated side facing the film, was placed on top of the film acting as the second electrode.

Optical microscopy images of the films were taken using a Zeiss microscope (Axio Imager.A1m) in brightfield mode. Figures 1(a) and 1(b) show optical microscopy images of a monoclinic and a tetragonal aggregate film of lysozyme, respectively. Typically, monoclinic crystals of lysozyme were larger but less densely packed than tetragonal crystals within the film. For both types of films, the crystals were randomly orientated.

We measured the direct piezoelectric effect in both monoclinic and tetragonal aggregate films of lysozyme using a piezometer (Model PM300, Piezotest, UK). The piezometer gives a reading of the piezoelectric coefficient of the sample. If the sample is piezoelectric, the polarity of the coefficient will change when the sample is inverted. Rather than manually inverting the sample, a simple switch was used to effectively invert the electrode configuration. In this way, the same precise location of the lysozyme film was tested in both inverted and non-inverted states without disturbing the measurement.

Table I reports the piezoelectric coefficients of five monoclinic aggregate films (labelled Monoclinic Lysozyme A-E) and five tetragonal aggregate films of lysozyme (labelled Tetragonal Lysozyme A-E). All films were prepared in an identical manner to that described earlier. The piezoelectric coefficient of each sample was measured five times in the upright configuration and five times in the inverted configuration.

The piezoelectric coefficient measured in aggregate films of monoclinic lysozyme ranged from 0.67 $pC\,N^{-1}$ to 1.34 $pC\,N^{-1}$ with an average piezoelectric coefficient of 0.94 $pC\,N^{-1}$. The standard deviation for each film was low, ranging from 0.75% to 4.17%. The standard deviation *between* films is high, 25.2% in the upright configuration and 28.4% in the inverted configuration. Although the films were prepared in the same manner, the large standard deviation between films reflects the fact that the piezoelectric coefficient across films is not constant. Such variations are not surprising given that protein crystallization is sensitive to small changes in temperature, humidity and pH. Care was taken to ensure that each drop of protein solution covered the same sized area of the ITO-coated slides. However, small variations in the spread of the protein solution on the ITO-coated slide would have resulted in slightly thicker or thinner protein drops. Thicker protein drops would take longer to dry and so have a longer crystallization time than thinner protein drops. For each film, the sign of the piezoelectric coefficient changed when the sample was inverted—this is confirmation of piezoelectricity.

The piezoelectric coefficient of tetragonal aggregate films of lysozyme ranged from approximately 1 $pC\,N^{-1}$ to

TABLE I. Piezoelectric coefficients of aggregate films of monoclinic and tetragonal lysozyme crystals measured with a commercial piezometer (Piezotest).

	Aggregate films of monoclinic lysozyme					
	Monoclinic lysozyme A	Monoclinic lysozyme B	Monoclinic lysozyme C	Monoclinic lysozyme D	Monoclinic lysozyme E	Average monoclinic lysozyme film
Upright *d*-coefficient ($pC\,N^{-1}$)	0.93 ± 0.01	1.34 ± 0.01	0.67 ± 0.02	1.25 ± 0.04	1.17 ± 0.05	1.07 ± 0.27
Inverted *d*-coefficient ($pC\,N^{-1}$)	−0.72 ± 0.03	−1.13 ± 0.02	−0.51 ± 0.01	−0.90 ± 0.03	−0.78 ± 0.01	−0.81 ± 0.23
	Aggregate films of tetragonal lysozyme					
	Tetragonal lysozyme A	Tetragonal lysozyme B	Tetragonal lysozyme C	Tetragonal lysozyme D	Tetragonal lysozyme E	Average tetragonal lysozyme film
Upright *d*-coefficient ($pC\,N^{-1}$)	1.04 ± 0.01	4.11 ± 0.03	2.93 ± 0.04	2.14 ± 0.03	6.44 ± 0.41	3.13 ± 2.07
Inverted *d*-coefficient ($pC\,N^{-1}$)	−0.89 ± 0.01	−3.97 ± 0.05	−2.76 ± 0.05	−1.78 ± 0.05	−6.50 ± 0.51	−3.18 ± 2.18

6.4 pC N^{-1} with an average piezoelectric coefficient of 3.16 pC N^{-1}. The standard deviation between measurements was low, ranging from 0.73% to 6.37%. Again, the large standard deviation between films reflects the fact that the piezoelectric coefficient between samples is not constant despite the fact that samples were prepared in the same manner. Slight differences in the number, size, and orientation of crystals within each film may have affected the measured piezoelectric coefficient.

The magnitude of the piezoelectric coefficient of lysozyme films is significant and is of the order of that in glycine.[19] It is possible that the magnitude of the piezoelectric coefficient may be increased further by poling, a process that is commonly used to enhance the piezoelectric coefficient of polycrystalline ferroelectrics that contain a spontaneous polarisation.[20] Poling of ferroelectrics typically involves heating the sample to a temperature near its Curie point and applying a high electric field. The electric field remains in place as the sample cools. If poling is successful, the majority of the dipoles will be orientated in the same direction, and the piezoelectric effect will be larger than before. In lysozyme, we have no proof yet that it is ferroelectric although the presence of longitudinal piezoelectricity in both monoclinic and tetragonal lysozyme suggests that it may be polar in nature and therefore possess a spontaneous polarisation. As of yet, there is no known method of poling proteins as they denature at relatively low temperatures.

These results were further verified using a custom-built rig, again constructed according to the Berlincourt method, but this time in a manner that allowed dynamic force pulses to be applied to the protein film while measuring the stress-generated voltage at the film surface. The apparatus was a modified version of an existing rig described earlier.[21] The apparatus consists of a 10 lbs. load cell (LPM530, Cooper Instruments) and a piezoelectric actuator (Pst 150/7/20, Piezomechanik) fixed to a stainless steel frame. The electroded film of lysozyme was placed on a platform above the load cell. A flat rectangular loading disc (8 mm^2) was placed on top of the sample to allow even loading of the sample. Initially, the actuator was brought into contact with the loading disc using a micrometer screw gauge attached to the actuator. The micrometer screw was then further adjusted in order to produce a preload force of 5 N on the sample.

A position controller (Posicon 150/1, Piezomechanik) provided fine control over the actuator. The actuator was then used to apply a series of dynamic force pulses to the sample. A load cell located beneath the sample determined the magnitude of the force applied. A digital multimeter (Keithley, model 195A) measured the voltage signal from the load cell. An electrometer (Keithley, model 6514) measured the voltage induced across the lysozyme film due to the direct piezoelectric effect. The entire apparatus was controlled using LabVIEW software (National Instruments®).

The monoclinic film of lysozyme produced a voltage peak in response to each force pulse, as shown in Fig. 2(a). The rig applied six force pulses to the sample, each with the same magnitude. At the instant that the force is applied, a voltage develops across the sample, which diminishes immediately after the impact. The insets in Fig. 2(a) indicate if the orientation of the sample is upright (0°) or inverted (180°). The voltage peaks are positive in the upright orientation and negative in the inverted configuration. The output voltage generated by the crystalline aggregate film increased linearly ($R^2 = 0.8968$) with increasing applied force [Fig. 2(b)] again demonstrating that piezoelectricity is the origin of the voltage generated.

The error bars in Fig. 2(b) are significant. The source of this large variation is most like experimental. The surfaces of monoclinic aggregate films of lysozyme were not perfectly smooth. Therefore, poor electrode contact may be the cause of the large variation of voltage peaks. The amount of voltage deviation from the average tends to increase with increasing force, suggesting that the adverse effects of poor electrode contact were exacerbated when larger forces were applied.

The piezoelectric charge coefficient (d) can be calculated as $d = g\varepsilon\varepsilon_o$, where g is the piezoelectric voltage constant, ε_o is the permittivity of free space, and ε is the permittivity of the sample. The g-coefficient is a measure of the electric field generated due to the piezoelectric conversion of stress. This can be determined from the slope of the graph of voltage (V) versus force (F) for lysozyme, the area of the film electroded (A) and film's thickness (t) as

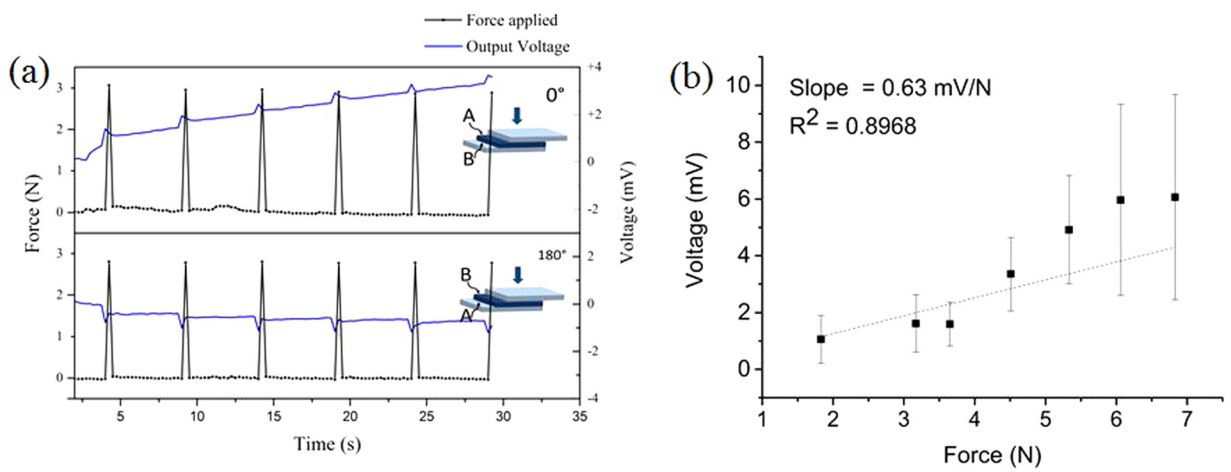

FIG. 2. (a) Piezoelectric voltage generated from a monoclinic film of lysozyme in response to dynamic force pulses when the sample is upright (0°) and inverted (180°). (b) The magnitude of the voltage generated increases with increasing force applied.

$$g = \frac{Electric\,field\,developed}{Applied\,stress} = \frac{\Delta V/t}{\Delta F/A} = \frac{Slope\,x\,A}{t}. \quad (1)$$

To quantify the piezoelectric effect in monoclinic lysozyme films, we estimate its g-coefficients using the slope of the best-fit line in Fig. 2(b). The thickness of the sample was approximately 30 μm, and the area exposed to force pulses was 64 μm^2. The capacitance of the sample was approximately 1000 pF (measured with piezometer). From this, the permittivity of the film was determined to be 43.2. The calculated permittivity value lay in the range of values reported in the literature for lysozyme ($2 < \varepsilon < 80$).[22–25] From Eq. (1), the piezoelectric voltage co-efficient g is $\sim$1340 μV m N^{-1}. Hence, the piezoelectric coefficient of this monoclinic lysozyme film is approximately 0.5 pC N^{-1}. This is in good agreement with the piezoelectric coefficient of this film determined with the commercial piezometer of 0.7 pC N^{-1}.

The tetragonal aggregate films of lysozyme also generated voltage peaks in response to force pulses. The voltage peaks were positive when the film was in the upright configuration and negative in the inverted configuration as shown in Fig. 3(a). The output voltage generated by the tetragonal aggregate film increased linearly with increasing applied force [Fig. 3(b)].

As before, we calculated the g-coefficient and d-coefficient as $\sim$1300 μV m N^{-1} and $\sim$0.5 pC N^{-1}, respectively. This same film showed a piezoelectric coefficient of 2 pC N^{-1} in the piezometer.

The observation of direct piezoelectricity in films of monoclinic lysozyme crystals corresponds with the classical theory of piezoelectricity. Monoclinic crystals belonging to point group 2 support longitudinal piezoelectricity (d_{22}). That is to say, the application of force along its 2-axis, generates a charge perpendicular to the 2-axis. However, the piezometer measures the so-called d_{33}-coefficient. The term longitudinal coefficient describes this measurand more completely. The longitudinal coefficient measured by a piezometer may be d_{11}, d_{22} or d_{33}, depending on the choice of coordinate axis assigned to the laboratory space. Rotating the laboratory coordinate system such that the b-axis lies along the vertical laboratory direction, the piezometer becomes a "d_{22}-meter." The same logic applies to the custom-built rig.

The point group most commonly associated with tetragonal lysozyme crystals (422) is limited to just two shear piezoelectric coefficients, d_{14} and $-d_{14}$. Point group 422 does not permit longitudinal piezoelectric coefficients. However, we measured longitudinal piezoelectricity with a commercial piezometer and confirmed the observation with the custom-built rig. Noting that the tetragonal crystals within the film are randomly oriented, an averaging effect may have caused the shear piezoelectric components to appear as an apparent longitudinal piezoelectric response.

An alternative argument may be that the point group of the tetragonal aggregate films of lysozyme is lower than that typically assigned to tetragonal crystals of lysozyme. Lowering the symmetry to tetragonal point group 4 would permit longitudinal piezoelectricity. This type of symmetry lowering is not unprecedented. Both bone[3,4,7,26] and wood[2,27] were reassigned to lower point group symmetries after experimental evidence contradicted the description of their original point group. Recently, Yamada *et al.* have reported that increasing the pressure to 950 MPa causes lysozyme to undergo a phase transition from point group 422 to the lower symmetry of point group 4.[28] While the pressure used in this study is not in this range, the study by Yamada *et al.* does show that symmetry lowering of tetragonal lysozyme crystals is possible. In our case, the substrate may strongly influence the lysozyme crystals during growth.[29,30] The substrate may restrict 3D growth and alter the overall symmetry of the crystals.

The magnitude of the piezoelectric effect in lysozyme is appreciable and may motivate further research in the area of energy harvesting and flexible electronics for biomedical devices. Being naturally biocompatible and piezoelectric, lysozyme may present an alternative to conventional piezoelectric energy harvesters, many of which contain toxic elements such as lead. The finding of piezoelectricity in a globular protein such as lysozyme may have relevance in biology. Perhaps, for example, protein piezoelectricity plays a role in the hydration and folding of these soluble proteins. Future applications may include controlling the release of drugs *in vivo* by using lysozyme as a physiologically mediated pump that scavenging energy from its surroundings.[31] We also imagine that lysozyme may be employed as a

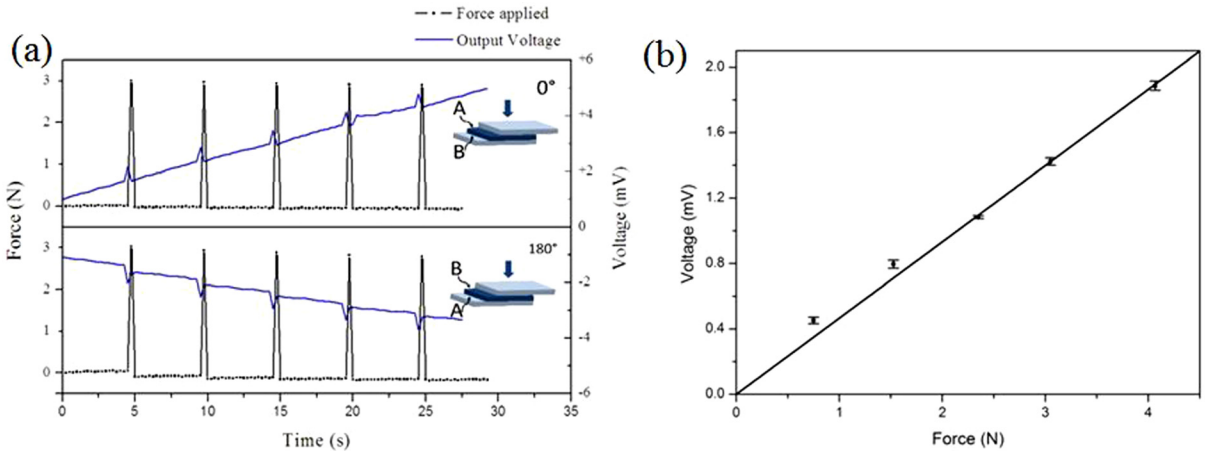

FIG. 3. Application of dynamic force pulses induces voltage peaks in tetragonal lysozyme films, which switch polarity when the sample is changed from its upright (0°) position to the inverted (180°) position (b). The voltage generated increases linearly with the magnitude of force applied.

biodegradable, piezoelectric, and antimicrobial additive/coating to conventional implants.

In summary, we have measured the direct piezoelectric effect in crystalline aggregate films of lysozyme. On average, the piezoelectric coefficients of films of monoclinic and tetragonal lysozyme were $0.94\,\mathrm{pC\,N^{-1}}$ and $3.16\,\mathrm{pC\,N^{-1}}$, respectively. The highest piezoelectric coefficient (6.5 ± 0.51 $\mathrm{pC\,N^{-1}}$) was measured in a tetragonal aggregate film of lysozyme. The observation of longitudinal piezoelectricity in monoclinic aggregate films of lysozyme is predicted by the classical theory of piezoelectricity. Our findings of longitudinal piezoelectricity in tetragonal aggregate films of lysozyme suggest an averaging effect or potentially a symmetry lowering effect.

Funding from the Irish Research Council EMBARK Postgraduate Scholarship (RS/2012/337) to A.S. is acknowledged. Part of this study was also facilitated by a HEA grant under the Programme for Research in Third-Level Institutions (PRTLI 5) to the University of Limerick.

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