

## Evidence of elastic to plastic transition in the zona pellucida of oocytes using atomic force spectroscopy

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We have investigated the mechanical properties of the zona pellucida (ZP), a multilayer glycoprotein coat that surrounds mammalian eggs, using atomic force spectroscopy. The response of the membrane to mechanical stress has been reconstructed using a modified Hertz model. The transition from elastic behavior, which occurs when low stress forces are applied (characterized by a Young's modulus  $E=(22 \pm 5)$  kPa), toward plastic behavior is observed. The critical indentation necessary to induce plastic deformations occurs at  $\delta_{\text{yield}}=(550 \pm 50)$  nm. This high critical value, corresponding to two ZP layers, well supports the noncovalent long lifetimes of interactions that take place between constituent glycoproteins. © 2009 American Institute of Physics.

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The zona pellucida (ZP) is a multifunctional porous glycoprotein coat that surrounds mammalian eggs. Penetration of this spherical glycoprotein shell by spermatozoa plays a crucial role in mammalian fertilization, and any incapacity of spermatozoa to penetrate the ZP inevitably leads to infertility. The ZP is a three-dimensional network of sulfated glycoproteins (ZP2, ZP3, and ZP4 in bovine) arranged to form fibrils.<sup>1-3</sup> Electron microscopy observation shows that several fibrils are arranged in cylindrical bundles distributed in concentric layers measuring about 250 nm in diameter oriented in strata parallel to the oocyte's surface. Bundles, randomly arranged in the inner and outermost areas, are organized in closely opposed parallel ranks in the core stratified layers<sup>4</sup> (see Fig. 1).

The physical properties of the ZP have not been studied extensively. Nevertheless, when oocytes with intact ZP are subjected to vigorous pipetting, the ZP can be made to rupture and consequently fragmentate. The shearing forces that cause rupture must involve considerable temporary distension of the ZP, but it is apparently elastic enough to recover its original shape. This suggests that the ZP behaves as an elastic solid over periods of time extending to minutes, and possibly much longer.<sup>5</sup> When the ZP is exposed to sodium dodecyl sulfate denaturation it separates into its constituent glycoproteins; however, this dissociation apparently leaves no residual scaffolding. Therefore it can be stated that the ZP is, by all appearances, a wholly noncovalent gel. Hence it follows that the noncovalent interactions that hold the ZP together probably have relatively long half-lives, and that the said interactions are of high affinity.<sup>6</sup>

Even in the light of these evidences, several questions remain unanswered in relation to the mechanisms, at the molecular level, responsible for the mechanical properties of the ZP: (i) how stable are the interactions (with half-lives sufficiently long to make the ZP behave elastically) between ZP proteins?, (ii) how much might they creep under load?, and (iii) what precisely is the function of the initial pattern of cross linking observed during ZP synthesis? The physical properties of a polypeptide network, such as ZP, have been found to largely depend on the chemical structure of constituent polymers that can be grouped into two limiting classes: covalently and noncovalently cross linked.

Noncovalently cross-linked polymers can be represented by viscous elements arranged alternately in series with the elastic elements (i.e., a Maxwell body).<sup>7</sup> Such a structure allows the network to stretch instantaneously but also per-

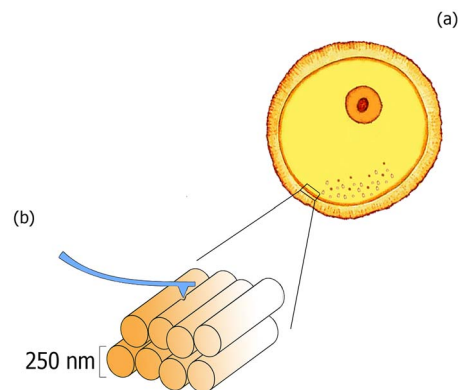


FIG. 1. (Color online) Schematic representation of ZP (a). The ZP membrane is composed of stratified layers of linear bundles, which, in the membrane's core, are also arranged in a parallel way (b).

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mits it to revert slowly to its original dimensions. In doing so, the system as a whole loses its “memory” of the position it formerly held. This phenomenon is known as stress relaxation, and the recovery dynamic is characterized by the lifetimes of the noncovalent interactions. When a network is gently stretched and immediately released, it returns to its original position; that is, it behaves like a Hookean spring. When, on the other hand, it is forcibly stretched, or held for a time before being released, it does not return to its original position but instead only to an intermediate position. At the molecular level, the noncovalent interactions that hold the gel together persist over the lifetime of a brief stretching. In contrast, when strain is applied for longer periods, the spontaneous dissociation of the noncovalent interactions allows the protein chains to recoil and establish new interactions closer to themselves, thereby eliminating the tension caused by elastic extension.

The physical properties of ZP have been investigated by performing atomic force microscopy (AFM) measurements using an SPMagic SX Atomic Force Microscope (Elbitech, Italy) in the contact operation mode. The samples—which were applied to glass coverslips—were kept in an aqueous environment (Dulbecco’s phosphate buffered saline, Sigma, USA) and at a constant 37 °C throughout the entire measurement acquisition phase. The microscope probe consisted in an ultrasharp silicon nitride cantilever of calibrated force constant, with a tip radius of less than 10 nm (MikroMash). In order to carry out the mechanical measurements, the exposed AFM tip was lowered on to the ZP surface at a preset rate, generally 3  $\mu\text{m/s}$ , a value comparable to that observed for the speed of a typical spermatozoon during “in vitro” fertilization.<sup>8</sup> Following contact, the AFM tip exerted a force against the ZP that was proportionate to the deflection of the cantilever. The deflection of the cantilever  $\Delta$  was recorded as a function of the piezoelectric translator position  $Z$ , and image analysis was performed using WSxM software (Nanotec Electronica S.L., Spain). Ovaries were obtained from cows and heifers at a local abattoir and were transported in saline solution at 37 °C to the laboratory within 2 h of slaughter. Cumulus-egg complexes (COCs) were isolated from sliced ovaries and were placed in Petri dishes and washed several times in PBS. Only the COCs with an intact, unexpanded cumulus oophorus and evenly granulated cytoplasm were chosen for the experiment. The selected COCs were washed three times in oocyte collection medium, a Tissue Culture Medium 199 (TCM-199) supplemented with 10% (w/v) heat-treated fetal bovine serum. The oocytes were matured to metaphase II in maturation medium, a TCM-199 buffered with bicarbonate and supplemented with 10% (w/v) heat-treated fetal bovine serum and 0.1 UI/ml follicle-stimulating hormone (FSH) and 10 UI/ml luteinizing hormone (LH), at 39 °C for 22–24 h at 5%  $\text{CO}_2$  in air.<sup>9</sup> Cumulus expansion was considered a normal feature of oocyte maturation.

In Fig. 2 a representative topographic image in an aqueous environment of a ZP extracted from a mature oocyte is shown. The ZP surface texture appears to be composed of a dense random meshwork of nonuniformly arranged fibril bundles with diameters of about 200–300 nm, as estimated directly from the image by measuring the size of a number ( $\sim 100$ ) of manually selected fibers. Such results are fully in agreement with previous observations made on mature pig oocyte where 200 nm bundles have been seen.<sup>3</sup> To quantify

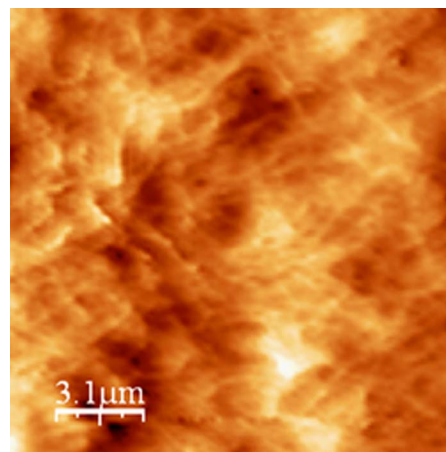


FIG. 2. (Color online) Characteristic atomic force image ( $15 \times 15 \mu\text{m}$ ) of a bovine ZP isolated from a mature ovarian egg. The surface topography shows a dense, random meshwork of nonuniformly arranged fibril bundles.

the morphology we report in Fig. 3 the distribution of heights. The bell-shaped distribution peaks at  $z \sim 450 \text{ nm}$ . The average roughness, calculated from the rms of  $z$  values, is  $\rho \sim 130 \text{ nm}$ . Assuming that the AFM tip is a rigid cone, the response of the membrane to a mechanically induced stress can also be recovered. Indeed, according to the modified Hertz model,<sup>10</sup> the force ( $F$ ) is related to the indentation ( $\delta$ ) as

$$F(\delta) = \frac{2E \tan(\alpha)}{\pi(1 - \nu^2)} \delta^2, \quad (1)$$

where  $E$  is the Young’s modulus and  $F$ , the reaction force of the membrane, are calculated by applying the Hooke relation ( $F = k_c \Delta$ ) and indentation  $\delta = Z - \Delta$ . Here we use  $k_c = 0.038 \text{ N/m}$  for the cantilever spring constant, as obtained by calibration,<sup>11</sup> and a Poisson’s ratio of  $\nu = 0.33$ .<sup>10</sup> From the electron microscopy image (see inset of Fig. 4), the half-opening angle of tip apex  $\alpha = 15^\circ$  has been accurately determined.

In Fig. 4 a characteristic  $\delta^2$  versus  $F(\delta)$  loading curve from a mature ZP is shown. Here we analyze only the load-

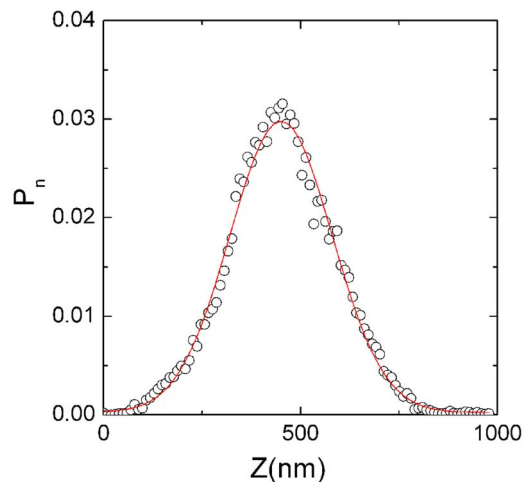


FIG. 3. (Color online) Normalized heights’ distribution of the ZP surface obtained from several atomic force micrographs ( $15 \times 15 \mu\text{m}$ ). The continuous line represents the fit of a Gaussian distribution to experimental data peaking at  $z \sim 450 \text{ nm}$ , with an average roughness  $\rho \sim 130 \text{ nm}$ .

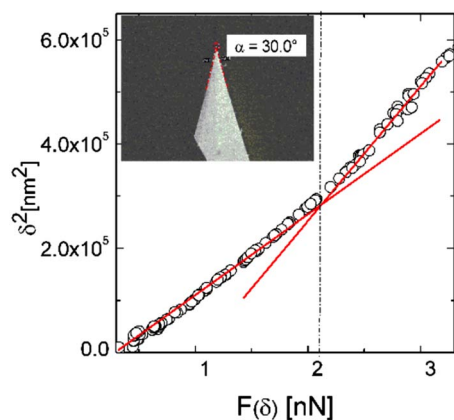


FIG. 4. (Color online) The square of indentation is reported vs the reaction force of the ZP membrane (open circles). Two limiting regimes can be distinguished: elastic and plastic. Continuous lines are fits of Eq. (1) to each of these regimes. The intersection of the two lines serves to estimate the yield point that defines the transition between the two regimes. In the inset, the electron microscopy image of the tip used is shown.

ing curve since the unloading one is much less sensitive to the dissipative contribution.<sup>12</sup> Initially, when increasing the applied force,  $\delta^2$  increases linearly. This is a pure elastic response of ZP to the applied force. By increasing the force, i.e., when  $F(\delta) > 2.5$  nN, a clear deviation from pure elastic behavior is observed. This behavior, called “plastic,” is still characterized by a linear increase of  $F(\delta)$ , but with a larger slope as compared to that observed in the first region. Since the first derivative of  $F(\delta)$  is proportional to the stiffness of the sample, the two behaviors have to be attributed to variations in the stiffness of the samples, caused by changes in the noncovalent interactions that stabilize the local polymeric structure.<sup>13</sup> From a molecular point of view, this phenomenon can be seen as the critical local stretch—or deformation—that permits the dissociation of the noncovalent interactions, thereby allowing protein chains to recoil and establish new interactions that reduce the tension caused by elastic extension.<sup>14</sup> Thus, from the intersection between the two asymptotic linear behaviors shown in Fig. 4, it is possible to estimate the critical force ( $F_{\text{yield}}$ ) and the critical indentation ( $\delta_{\text{yield}}$ ), at which membranes undergo plastic deformations.

By investigating several membrane areas and 15 membranes, we obtained an average Young’s modulus  $E = (22 \pm 5)$  kPa, a yielding force  $F_{\text{yield}} = (2.1 \pm 0.4)$  nN, and an indentation  $\delta_{\text{yield}} = (550 \pm 50)$  nm.

Interestingly, the very low dispersion of all the parameters determined, together with the absolute value of the Young’s modulus, which is not very different from those obtained by exerting macroscopic mechanical stress on mature bovine oocytes ( $E = 25 \pm 8$  kPa),<sup>15</sup> suggests that local properties of fibrous bundles possess a considerable degree of homogeneity. Hence, the ZPs mechanical properties are not affected by their nonuniform planar spatial distribution; instead, they probably depend on the fibrils’ structure. Finally protein chains, when stretched, recoil, and establish new noncovalent interactions, which result in a diminution of elastic extension tension, thereby provoking a breakdown in

Hookean-type spring behavior. In this letter, on local scales, we observed that this phenomenon, never before observed on macroscopic scales (i.e., by pipetting), occurs at a critical indentation value of  $\sim 500$  nm. This high critical value, which corresponds to two ZP bundles ( $\sim 250$  nm each), satisfactorily corroborates the long lifetimes of interactions observed at the macroscopic level.<sup>5,6</sup>

In conclusion, we demonstrate the possibility of combining high-resolution images with quantitative local mechanical parameters, obtained by using atomic force spectroscopy, so as to characterize the elastic behavior of the ZP extracted from a mature bovine oocyte. A distinctive feature of this approach consists in the possibility of discriminating between the elastic and plastic responses of a polypeptide network. Indeed, when employing classical experimental techniques that involve macroscopical straining of the ZP, the elastic and plastic responses cannot be distinguished, therefore an inevitable underestimation of  $E$  occurs.<sup>5,16</sup> Moreover, since the Young’s modulus and the yielding force, necessary to induce a plastic deformation, are strictly dependent on noncovalent interaction lifetimes, our approach would be an extremely useful tool when dynamically investigating polymer modifications, which occur during the course fertilization,<sup>17</sup> or to identify structural pathologies responsible for infertility related to the spermatozoon’s head’s success (or failure) in fracturing the ZP surface during the initial mechanical penetration stage of fertilization.<sup>18</sup>

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