Eur Biophys J (2010) 39:987–992
DOI 10.1007/s00249-009-0468-3

ORIGINAL PAPER

# Mechanical properties of zona pellucida hardening

**Massimiliano Papi · Roberto Brunelli · Lakamy Sylla · Tiziana Parasassi · Maurizio Monaci · Giuseppe Maulucci · Mauro Missori · Giuseppe Arcovito · Fulvio Ursini · Marco De Spirito**

Received: 14 February 2009 / Revised: 20 April 2009 / Accepted: 29 April 2009 / Published online: 27 May 2009


**Abstract** We have investigated the changes in the mechanical properties of the zona pellucida (ZP), a multilayer glycoprotein coat that surrounds mammalian eggs, that occur after the maturation and fertilization process of the bovine oocyte by using atomic force spectroscopy. The response of the ZP to mechanical stress has been recovered according to a modified Hertz model. ZP of immature oocytes shows a pure elastic behavior. However, for ZPs of matured and fertilized oocyte, a transition from a purely elastic behavior, which occurs when low stress forces are applied, towards a plastic behavior has been observed. The high critical force necessary to induce deformations, which supports the noncovalent long interaction lifetimes of polymers, increases after the cortical reaction. Atomic force microscopy (AFM) images show that oocyte ZP surface appears to be composed mainly of a dense, random meshwork of nonuniformly arranged fibril bundles. More wrinkled surface characterizes matured oocytes compared with immature and fertilized oocytes. From a mechanical point of view, the transition of the matured ZP membrane toward fertilized ZP, through the hardening process, consists of the recovery of the elasticity of the immature ZP while maintaining a plastic transition that, however, occurs with a much higher force compared with that required in matured ZP.

**Keywords** Zona pellucida · Atomic force spectroscopy · Cortical reaction

Proceedings of the XIX Congress of the Italian Society of Pure and Applied Biophysics (SIBPA), Rome, September 2008.

M. Papi · G. Maulucci · M. Missori · G. Arcovito · M. De Spirito (✉)
Istituto di Fisica, Università Cattolica del S. Cuore, L.go F. Vito 1, 00168 Rome, Italy
e-mail: m.despirito@rm.unicatt.it

R. Brunelli
Dipartimento di Scienze Ginecologiche, Perinatologia e Puericultura, Università di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy

L. Sylla · M. Monaci
Department of Patologia, Diagnostica e Clinica Veterinaria, Università di Perugia, Via San Costanzo 4, 06126 Perugia, Italy

T. Parasassi
Istituto di Neurobiologia e Medicina Molecolare, Consiglio Nazionale delle Ricerche, Via del Fosso del Cavaliere 100, 00133 Rome, Italy

F. Ursini
Dipartimento di Chimica Biologica, Università di Padova, V.le Colombo 3, 35131 Padova, Italy

## Introduction

The zona pellucida (ZP) is a 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 (Hoodbhoy and Dean 2004; Jovine et al. 2005; Noguchi et al. 1994). 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 surface. Bundles, randomly arranged in the inner- and outermost areas, are organized in closely apposed parallel ranks in the core stratified layers (Fléchon et al. 2004).

Physiological polyspermy, or penetration of the egg cytoplasm by more than a single spermatozoon, occurs in numerous species including insects, reptiles, and birds, while polyspermy is considered an abnormal phenomenon in mammals, resulting in developmental failure of the zygote. Following sperm penetration, cortical granules (CG), a special organelle in eggs, release their contents into the perivitelline space (PVS) in an event that is termed the cortical reaction. CG exudates alter the properties of ZP, which is known as zona reaction, and thus block polyspermic penetration.

There is evidence that the cortical reaction modifies the body of the ZP to prevent sperm penetration through a process of "zona hardening" (De Felici et al. 1985).

It is important to be clear about what is meant by zona hardening because superficially the term appears to indicate an alteration of the physical properties of the ZP, that is, a stiffening of the ZP matrix. However, what is normally meant by zona hardening is not increased stiffness but rather an increased resistance of the ZP to proteolytic digestion, normally tested with α-chymotrypsin. These two factors need not be coupled since there is some evidence that increased resistance to chymotrypsin digestion is not always accompanied by an increase in mechanical stiffness (Drobnis and Katz 1991).

Recently, it has been observed, by means of atomic force spectroscopy, that local mechanical stress can induce plastic deformation of matured bovine oocyte ZP and that both the Young's elastic modulus and the critical stress value (yield point) required for such deformation are related to polymer molecular interactions (Papi et al. 2009).

To investigate the occurrence of an increase in the ZP stiffness, we extend our previous investigations by measuring the mechanical response to a local stress of immature, matured, and fertilized bovine oocyte's ZP.

## Materials and methods

### Atomic force microscopy

The physical properties of ZP have been investigated by performing AFM measurements using an SPMagic SX atomic force microscope (Elbatech, Italy) in contact operation mode, maintaining the samples, which were laid on glass coverslips, in an aqueous environment (Dulbecco's phosphate-buffered saline, PBS; Sigma, USA), and at a constant temperature of 37°C, throughout the measurement acquisition phase. The microscope probe consisted of 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 pre-established rate, typically 3 μm $s^{-1}$. 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 Δ was recorded as a function of the piezoelectric translator position $Z$, and image analysis was performed using WSxM software (Nanotec Electronica S.L., Spain). Surface roughness has been quantified by measuring the root mean square ($RMS_Z$) of the cantilever deflections

$$\mathrm{RMS}_z = \sqrt{\frac{1}{(n-1)}\sum_{i=1}^{n}(Z-\langle Z\rangle)^2},$$

where $n$ is the total number of pixels and $\langle Z\rangle$ is the average $Z$ position.

Assuming that the AFM tip is a rigid cone, the response of the membrane to a mechanically induced stress can also be recovered. Within the limits of small deformation, the Hertz contact theory can be applied to describe the elastic contact behavior (Cappella and Dietler 1999). Indeed, according to the Hertz model, before plastic deformation 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, is calculated by applying the Hooke relation ($F = k_c\Delta$) and indentation $\delta = Z - \Delta$. Here we use $k_c = 0.038$ N $m^{-1}$ for the cantilever spring constant, as obtained by calibration (Sader et al. 1999), and a Poisson's ratio of $\nu = 0.33$ (Cappella and Dietler 1999). From the electron microscopy image, the half-opening angle of tip apex $\alpha = 15°$ has been accurately determined. The derivative of the approach contact line is proportional to the stiffness of the sample. Hence, by observing the changes in the derivative, it is possible to infer the variations in sample stiffness due to changes in one or more experimental parameters. Once the maximum equivalent stress inside the material reaches the yield stress, yielding will start and the indentation force–depth curve will start to deviate from the purely elastic indentation curve described by Eq. 1. Because this yield point signals the start of plastic deformation in the sample, it is an important characteristic point on the elastic–plastic indentation curve. It is important to remember that the elastic continuum theories may be applied only to the elastic part of the indentation curve and not to the plastic part, and that this model treats the yielding region as a transition from the first elastic deformation to a second deformation with a lower stiffness. Thus, a plastically deformed polymer can be treated, only from a

mathematical point of view, as an elastically deformed polymer with a smaller stiffness.

## Sample preparation

### *Oocyte collection and maturation in vitro*

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 $ml^{-1}$ follicle-stimulating hormone (FSH) and 10 UI $ml^{-1}$ luteinizing hormone (LH), at 39°C for 22–24 h at 5% $CO_2$ in air. After in vitro maturation (IVM) an aliquot of the completely denuded oocytes were placed on glass slides and routinely stained with a working solution of lacmoid. Cumulus expansion and the first polar body expulsion were considered as occurrence of oocyte maturation.

### *Sperm capacitation and in vitro fertilization*

Commercial frozen semen was used for fertilization purposes. Motile sperm separation was carried out by using the Percoll gradient technique. Sperm concentration was determined with a hemocytometer. After 22–24 h of maturation, the COCs were washed three times in H-SOF medium and placed in four-well culture dishes containing pre-equilibrated fertilization medium (TALP-IVF) supplemented with heparin (1.2 g $ml^{-1}$). Spermatozoa were then added at a final concentration of $1 \times 10^6$ cells $ml^{-1}$ in 300 µl medium per well containing a maximum of 20 COCs. In vitro fertilization was accomplished by coincubating oocytes and sperm cells for 20 h at 39°C at 5% $CO_2$ in air. The male and female pronuclei and the second polar body expulsion were considered to be signs of fertilization.

### *Examination of zonae pellucidae*

In order to evaluate zonae pellucidae (ZP) of immature, matured, and in vitro fertilized oocytes, the COCs were denuded by vortexing for 3 min and washed three times in H-SOF medium. Thereafter, ZP of denuded oocytes were isolated by aspirating the cells in a narrow-bore pipette. The isolated ZP were mounted with outer surface upside on polylysine-treated slides and heated on warming plate for 5 min. Slides were stained with methylene blue and stocked at room temperature until observation (Sylla et al. 2005).

## Results

Three representative topographic images of ZP obtained from immature (a), matured (b), and fertilized (c) oocytes immersed in a PBS buffer environment, are reported in Fig. 1. All the ZP surface textures appeared to be composed of a dense, random meshwork of nonuniformly arranged fibril bundles. Whereas ZP of immature and fertilized oocytes showed a thicker and more compact bundles structure with diameters of about 500–1,000 nm, the ZP of matured oocytes, more porous and wrinkled, appeared to be composed by thinner bundles with diameters of about 200–300 nm. From the $Z$ distribution (not shown) it was possible to determine an $RMS_Z$ of 80, 130 and 62 nm for ZP extracted from matured, immature, and fertilized oocytes, respectively.

In Fig. 2 three characteristic $\delta^2$ versus $F(\delta)$ curves from an immature, matured, and fertilized ZP are shown. Initially, when increasing the applied force, $\delta^2$ increases linearly. This is a pure elastic response of ZP to the applied force, as predicted by Eq. 1 (dashed lines). When increasing the force a clear deviation from pure elastic behavior is observed in the matured and fertilized ZP

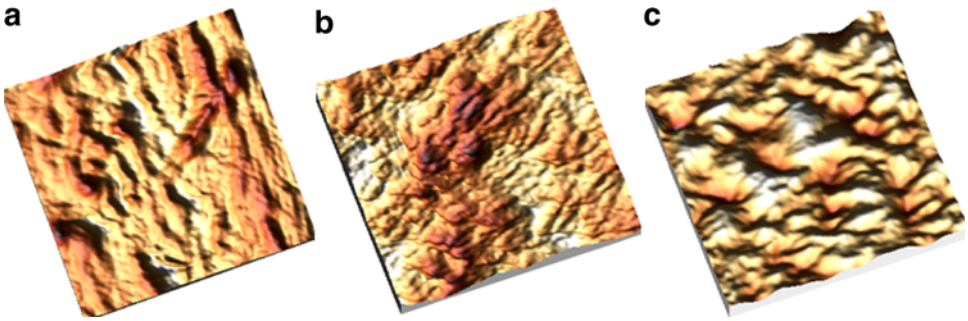


**Fig. 1** Characteristic atomic force images (15 × 15 µm) of bovine zona pellucida isolated from immature (**a**), matured (**b**), and fertilized (**c**) bovine oocytes. The surface topography shows always a dense, random meshwork of nonuniformly arranged fibril bundles but with different diameters

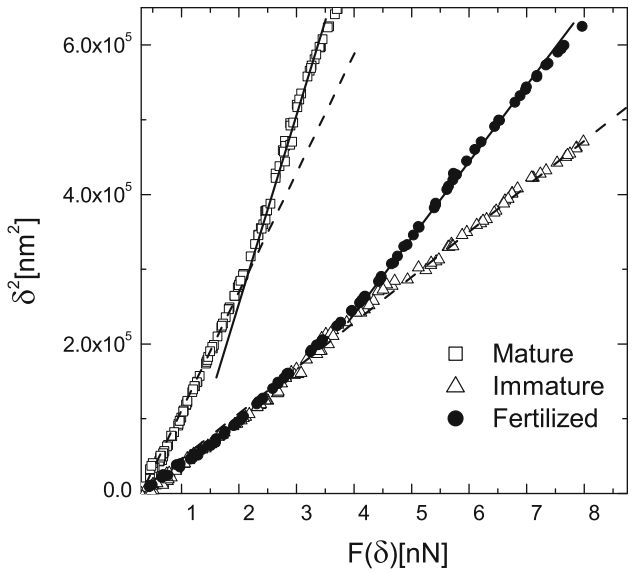


**Fig. 2** The square of indentation is reported versus the reaction force of the ZP membrane isolated from immature (*open triangle*), matured (*open square*), and fertilized (*circles*) bovine egg. Two limiting regimes can be distinguished: elastic and plastic. *Dashed* and *solid lines* are fits of Eq. 1 to each of these regimes. The intersection of the two lines allow to estimate the yield point that defines the transition between the two regimes

while, in the same force-indentation range, the immature ZP shows a pure elastic response. This behavior, observed in matured and fertilized ZP, called "plastic," is still characterized by a linear increase of $F(\delta)$, but with a larger slope as compared with that observed in the first region (continuous lines). 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 sample stiffness, caused by changes in the noncovalent interactions that stabilize the local polymeric structure. 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. Thus, from the intersection between the two asymptotic linear behaviors shown in Fig. 2, 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 of 15 different membranes, we obtained the average value of the Young's modulus, and yielding force $F_{\text{yield}}$ and indentation $\delta_{\text{yield}}$ reported in Table 1. By quantitatively comparing these obtained data we can point out that: (1) ZP of immature and fertilized oocytes have a very similar Young's modulus value, about four times higher than that of the matured oocyte's ZP, (2) fertilized ZP have a breakdown of the Hookean-type spring behavior for a very similar critical indentation, but in the fertilized ZP almost double the force is necessary, (3) immature ZP always shows an elastic behavior in the limit of our experiments, and (4) the very low dispersion of all the parameters determined suggests that local properties of fibrous bundles possess a considerable degree of homogeneity.

**Table 1** Young's modulus, yield force and indentation, and surface roughness of ZP isolated from immature, matured, and fertilized bovine oocytes

| ZP | $E$ (kPa) | $F_{\text{yield}}$ (nN) | $\delta_{\text{yield}}$ (nm) | RMS (nm) |
|---|---|---|---|---|
| Immature | 89 ± 7 | – | – | 80 |
| Matured | 22 ± 5 | 2.1 ± 0.4 | 550 ± 50 | 130 |
| Fertilized | 84 ± 10 | 3.9 ± 0.8 | 490 ± 40 | 60 |

## Conclusions

### Morphology of the zona pellucida surface

The surface structure of the ZP from different mammals was described by several authors. In mice, hamsters, pigs, and cattle a net-like structure formed out of different layers of a string-like material perforated by numerous pores was observed. Several papers assumed a correlation between the type of surface morphology and the stage of maturity. Some authors (Calafell et al. 1992; Familiari et al. 1992; Motta et al. 1991) described a net-like porous surface mainly in matured oocytes while immature and degenerated oocytes had a compact type with no pores. Some other authors (Sathananthan 1994; Suzuki et al. 1994; Magerkurth et al. 1999), instead, did not found any correlation between the appearance of zona surface and the maturity of the oocytes. Fixation artefacts due to variable fixation/dehydration conditions may account for these last discrepant observations. Here, by using a minimally perturbative technique (AFM), we investigated, in a hydrated environment, the morphology of ZP membranes extracted from bovine oocytes at different stages of maturation (Fig. 1a–c). ZP surface always appears to be composed mainly of a dense, random meshwork of nonuniformly arranged fibril bundles; however, we observed an increase of the roughness on the surface of matured oocytes ZP.

Morphological correlates, evidenced by electron microscopy, of the so-called zona reaction, which occurs after penetration of the spermatozoon into the ZP, are also contradictory. Some authors (Familiari et al. 1992) could not find any changes correlated to fertilization, whereas some others (Suzuki et al. 1994; Nikas et al. 1994;

Vanroose et al. 2000) reported a more compact surface for fertilized oocytes compared with a porous structure of matured ones. Our results using AFM match both these latter findings and those reported specifically in bovine ZP, as we observed that ZP of fertilized oocytes (Fig. 1c) displayed a more compact surface ($RMS_Z = 60$ nm) in contrast to matured ones (Fig. 1b, $RMS_Z = 130$ nm).

## Physical properties of the zona pellucida

The physical properties of a membrane, 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.

When ZP is exposed to sodium dodecyl sulfate it separates into its constituent proteins without any residual scaffolding. Furthermore, ZP behaves as an elastic solid over periods of time extending to minutes, and possibly much longer (Green 1987). Therefore, ZP is a wholly noncovalent gel whose interactions have relatively long half-lives of high affinity.

Noncovalently cross-linked polymers can be represented by viscous elements arranged alternatively in series with elastic elements (a Maxwell body) (Green 1997). Such a structure allows the membrane to stretch instantaneously, but also permits it to slowly revert 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 dynamic of recovery is characterized by the lifetimes of the noncovalent interactions. When a membrane is gently stretched and immediately released, it returns to its original position; that is, it behaves like a Hookean spring.

Accordingly we found that local mechanical properties of immature ZP, within the range of the applied forces, evidenced a pure elastic behavior characterized by a Young's modulus $E = 89 \pm 7$ kPa. Matured oocyte's ZP exhibited significantly changed mechanical properties such as dramatic softening ($E = 22 \pm 5$ kPa). Contextually a transition towards a plastic behavior, occurring at $F_{yield} = 2.1 \pm 0.4$ nN, can be clearly observed. This behavior cannot be associated to definitive biochemical changes, but clearly facilitates sperm's binding and penetration through ZP.

Following sperm penetration, CG are released from the oocytes into the PVSs in an event called cortical reaction. Cortical reaction modifies ZP, preventing polyspermic penetration through a process of zona hardening (De Felici et al. 1985).

Zona hardening is dependent upon enzymes-induced changes, as evidenced by its prevention with addition of protease inhibitors such as fetuin (Schroeder et al. 1990; Kalab et al. 1991). Other factors include a cortical granule protease which converts ZP2 to ZP2f (Moller and Wassarman 1989), and an ovoperoxidase which may cross-link tyrosine residues (Gulyas and Schmell 1980).

Changes in the zona architecture, which cause hardening of the zona during fertilization, is accompanied by changes in the secondary structure of the zona protein with a significant increase in the beta structure content. The dissociation of noncovalent bindings along with the increase of new cross beta mediated bindings induce a polymerization into higher-order structures while hampering the pure elastic behavior. Here, we observed that fertilized ZP possesses exactly the same Young's modulus as immature ZP (Table 1), but exhibits a breakdown of the Hookean-type spring behavior.

Our results qualitatively match those obtained by means of a microtactile sensor (MTS) on mouse ZP where the Young's modulus of immature and fertilized ZPs was three times higher than that found on matured ZP (Murayama et al. 2006).

In conclusion, from a mechanical point of view, the transition of the matured ZP membrane towards fertilized ZP, through the hardening process, consists of the recovery of the elasticity of the immature ZP, while maintaining a plastic transition that, however, occurs with a much higher force compared with that required in matured ZP.

Therefore, the block of polyspermy through zona hardening is achieved not only by increasing the resistance of the ZP to proteolytic digestion but also by an increase of the stiffness of the ZP along with the occurrence of the elastic to plastic transition only at large forces.

In this context we demonstrated that the term "zona hardening" indicates not only an increased resistance of the ZP to proteolytic digestion but also a real stiffening of the membrane.

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