A hybrid characterization framework to determine the visco-hyperelastic properties of a porcine zona pellucida

A. Boccaccio1, L. Lamberti1, M. Papi2, M. De Spirito2, C. Douet3, G. Goudet3 and C. Pappalettere1

1Dipartimento di Meccanica, Matematica e Management, Politecnico di Bari, Bari 70126, Italy
2Istituto di Fisica, Università Cattolica del Sacro Cuore, Roma 00168, Italy
3UMR 85, Physiologie de la Reproduction et des Comportements, INRA-CNRS-Université de Tours, IFCE, 37380 Nouzilly, France

The zona pellucida (ZP) is a specialized extracellular matrix surrounding the developing oocyte. This thick matrix consists of various types of glycoprotein that play different roles in the fertilization process. Nowadays, several techniques are available for assessing ZP’s mechanical response. The basic assumption behind these methods is that the ZP behaves like an elastic body: hence, dissipative forces are neglected and Young’s modulus remains unaffected by probe dynamics. However, dissipative forces are strongly regulated by the slippage of ZP chains past one another while reaction forces related to elastic deformations (driven by the ability of each chain to stretch) depend on the ZP structure (i.e. number of cross-links and distances between knots). Although viscous reaction forces generated by the ZP are one of the main factors regulating sperm transit, their peculiar behaviour along the ZP structure remains poorly understood and rarely investigated. In order to overcome this limitation, a novel visco-hyperelastic model describing the porcine ZP reaction forces generated by nanoindentations at different probe rates is developed and verified in this study. Visco-hyperelastic parameters of porcine ZP membranes are determined by means of a hybrid characterization framework combining atomic force microscopy nano-indentation measurements, nonlinear finite-element analysis and nonlinear optimization. Remarkably, it is possible to separate the contributions of hyperelastic and viscous terms to ZP mechanical response and evaluate the error made in the determination of ZP mechanical properties if viscous effects were not considered.

1. Introduction

The zona pellucida (ZP) is the spherical extracellular membrane surrounding the mammalian oocyte at fertilization. The ZP is mainly composed of sulfated glycoproteins, each accomplishing specific functions; glycoproteins are assembled into a three-dimensional network organized according to a specific nanoarchitecture [1–3]. The principal tasks of the ZP are: (i) to regulate binding of sperm to ovulated eggs during fertilization, (ii) to prevent polyspermy after fertilization, and (iii) to protect early embryos while they traverse the female reproductive tract. Penetration of ZP membrane by spermatozoa is the crucial step in oocyte fertilization and any inability of spermatozoa to penetrate ZP inevitably leads to infertility [4–6]. A deep knowledge of ZP mechanical properties is hence at the basis of any study on the sperm–ZP interaction.

Techniques recently used to measure ZP mechanical properties include microtactile sensors made of piezoelectric material [7,8], micropipette aspiration [9,10], micropipette indentation [11], vision-based nanoforce estimation [12] and atomic force microscopy (AFM) [13–17]. While other techniques can characterize the mechanical behaviour of the entire oocyte as they induce...
macroscopic deformations of the whole cell, AFM allows nanoscale investigations to be carried out in specific regions of the cell specimen, thus detecting at very high resolution any source of inhomogeneity and/or anisotropy in mechanical response. Furthermore, AFM can operate in liquid environment that is in physiological conditions reproduced in vitro.

AFM was used to evaluate mechanical properties of immature, mature and fertilized bovine oocytes [13,14], also trying to assess the effect of ZP structure inhomogeneity across membrane thickness [15,16], and the role played by residual stresses that develop in the tested ZP membrane (which is originally spherical shaped) when specimens are spread out on a rigid substrate for nanoindentation measurements [17].

In spite of the fact that the structural response of polymeric networks like those contained in the ZP membrane is strongly affected by viscous/dissipative effects owing to the relative slippage of polymeric chains [18,19], the typical assumption made in the determination of ZP mechanical properties via AFM is that the cell membrane behaves as a linearly elastic or hyperelastic material and viscous forces are negligible. However, viscous reaction forces generated by ZP should be considered one of the main factors regulating the sperm transit.

If viscous behaviour is neglected, the elastic modulus should actually be considered as an 'apparent elastic modulus' because it does not account for the nanoindentation rate. A recent study carried out by some of the present authors [20] has demonstrated that viscous forces play a role of crucial importance in the mechanical response of porcine and equine zona pellucidae submitted to AFM nanoindentation. From the physiological point of view, such a result indicates that for spermatozoa with excessive motility the reaction force of the ZP can be so large that the membrane may turn into an impenetrable barrier.

However, the peculiar behaviour of viscous forces along the ZP structure remains poorly understood. In order to overcome this limitation, this study presents for the first time in the literature a visco-hyperelastic model, which is able to reproduce the ZP reaction forces stressed by nanoindentation at different probe rates. This approach can provide some insight on the sperm–ZP interactions occurring in the fertilization process.

In a previous study conducted by some of the present authors [16], a hybrid mechanical characterization procedure combining AFM nanoindentation measurements, nonlinear finite-element (FE) analysis and nonlinear optimization was developed to determine the ZP hyperelastic parameters of immature, mature and fertilized bovine oocytes. This approach was successfully used to characterize both inner and outer sides of ZP specimens extracted from the fertilized oocyte. In this study, the characterization framework is further developed in order to include viscous effects. AFM measurements are conducted on the ZP of immature porcine oocytes at different indentation rates. By combining experimental data with a transient FE model reproducing the nanoindentation measurement and carrying out nonlinear optimization, it is possible to determine the visco-hyperelastic parameters of the porcine ZP. Remarkably, the proposed approach allows hyperelastic behaviour and viscous effects to be clearly separated. Furthermore, it is possible to evaluate the error made in the determination of ZP mechanical properties if viscous effects were not considered.

2. Characterization of zona pellucida’s visco-hyperelastic behaviour

2.1. Preparation of zona pellucida samples

Porcine immature cumulus–oocyte complexes (COCs) were collected from slaughtered gilts. Ovaries were obtained in local slaughterhouses, immediately after females had been killed, and transported to the laboratory within 2 h in 0.9% NaCl at 37°C. COCs were collected by aspiration of the follicles with a 16-gauge needle mounted on an aspiration pump and recovered under a stereomicroscope. COCs were washed in Dulbecco’s phosphate-buffered saline solution (DPBS, Dulbecco A, Paris, France). Before each experiment, porcine and equine COCs were flushed in and out of a glass pipette in DPBS to remove cumulus cells. The ZP was then mechanically removed from the oocytes, washed in DPBS and water and deposited onto a polylysine-coated slide.

2.2. Atomic force spectroscopy measurements

In order to evaluate changes in the ZP apparent Young’s modulus caused by different indentation rates, the mechanical response of ZP specimens was analysed by means of an AFM (NanowizardII, JPK, Berlin, Germany) combined with an optical microscope (Axio Observer, Zeiss, Jena, Germany). The AFM probe consisted of an ultrasharp silicon nitride cantilever of calibrated force constant, with a tip radius of less than 10 nm (CSC16, MikroMasch, San Jose, CA, USA).

All the samples were kept in the buffer at a constant temperature of 37°C throughout data acquisition. In order to carry out mechanical measurements, the exposed AFM tip was lowered onto the ZP surface at different rates: 0.5, 2, 4, 6, 8 and 10 μm s⁻¹; this range of indentation rate is comparable with the typical speed of spermatozoa observed during ‘in vitro’ fertilization [21]. Following contact, the AFM tip exerts a force against the ZP proportionate to cantilever deflection. The cantilever deformation Δ was recorded as a function of the piezoelectric transducer position z. The reaction force of the membrane F was determined from Hooke’s law \( F = K_c \Delta \) and the indentation \( \delta = z - \Delta \). The cantilever spring constant \( K_c \) nominally 0.01 N m⁻¹, was computed for each measurement by thermal calibration and the half opening angle of tip apex \( \alpha = 20° \) was accurately determined by means of scanning electron microscopy.

In order to obtain statistically significant results, for each indentation rate, force–indentation curves were recorded at 250 different points of each analysed sample and were repeated on 10 oocytes. Force–indentation curves were hence averaged to obtain the target data of the identification process. The standard deviation of experimental data with respect to average force–indentation curves was about 10%.

2.3. Finite-element modelling of the nanoindentation measurements on porcine zona pellucida samples

The FE model of the ZP membrane and the AFM indenter was built by using the general-purpose FE software ABAQUS v. 6.12 (Dassault Systèmes, France). Given the symmetry of the problem, an axisymmetric FE model was developed. As the ZP membrane is much softer than the AFM probe, the indenter was modelled as a rigid blunt cone with a tip radius of 10 nm and an angle of aperture of 40°. The ZP membrane was instead modelled as an incompressible visco-hyperelastic slab, 30 μm long.
and 6 μm thick (the value of thickness is consistent with data reported in [22] for porcine oocytes). The FE model simulating the AFM nanoindentation process is shown in figure 1.

The four-node hybrid axisymmetric CAX4H element available in the ABAQUS library was used to model the ZP slab. The mesh of the ZP membrane was properly refined in the contact region (see details in figure 1). The very small dimensions of the AFM indenter with respect to those of the ZP membrane along with the necessity of having small enough and regularly shaped elements in the contact region (this must be done to facilitate the convergence of FE analysis) have led to dividing the ZP slab domain in regions of different mesh density. The average element size is 0.06 nm in the contact region and about 180 nm in regions far from the AFM indenter. By using these element sizes and the above-described partition strategy, a good compromise between convergence of nonlinear analysis and computation time could be reached.

The nodes of the ZP bottom edge were clamped while symmetry constraints preventing only horizontal displacements were applied to the nodes of the left edge of the FE model. A vertical displacement of δ = 100 nm was imposed to the AFM indenter. The contact between the indenter and the membrane was assumed to be frictionless and the 'hard contact' option (i.e. surfaces come into contact when their gap reduces to zero; any contact pressure can be transmitted through surfaces in contact) available in ABAQUS was selected. FE analysis accounted for geometric nonlinearity (i.e. large deformations) and time-dependent behaviour (i.e. viscous effects).

2.4. Visco-hyperelastic constitutive model of the porcine zona pellucida

The porcine ZP membrane investigated in the study was modelled as a visco-hyperelastic material following the Arruda–Boyce (AB) constitutive law [23]. The Arruda–Boyce hyperelastic model, originally developed to reproduce the mechanical behaviour of polymer chain networks, was previously used in the literature to analyse biospecimens, including filamentous collagen networks [24,25], and has recently been found to describe very well the mechanical response of bovine ZPs at different stages of the fertilization process [16]. The Arruda–Boyce constitutive model requires that two constants be given as input to ABAQUS: the shear modulus \( \mu_{\text{chain}} \) and the distensibility \( \lambda_L \). The strain energy function can be expressed as (2.1)

\[
W = \mu_{\text{chain}} \left[ \frac{1}{2} (I_1 - 3) + \frac{2}{20A_1} (I_1^2 - 9) + \frac{33}{1050A_1} (I_1^3 - 27) \right. \\
+ \frac{76}{7000A_1} (I_1^4 - 81) + \left. \frac{519}{673,750A_1} (I_1^5 - 243) \right] \\
+ \frac{76}{7000A_1} (I_1^4 - 81) + \frac{519}{673,750A_1} (I_1^5 - 243) \\
+ \frac{76}{7000A_1} (I_1^4 - 81) + \frac{519}{673,750A_1} (I_1^5 - 243)
\]

(2.1)

where \( I_1 = \text{tr}[C] \) is the first strain invariant and \( [C] \) is the Cauchy–Green strain tensor. Young’s modulus of the material can be determined as \( E = 2(1 + \nu)\mu_{\text{chain}} \), where \( \nu \) is the Poisson ratio. By setting \( \nu = 0.5 \) to account for material incompressibility, it follows \( E = 3\mu_{\text{chain}} \).

The viscous behaviour of the ZP membrane (i.e. the rate-dependent part of mechanical response) is defined by an N-terms Prony series expansion of the dimensionless relaxation modulus. The effective relaxation modulus \( \mu_{\text{chain}}(t) \) is obtained as the product of the instantaneous elastic modulus (in the case of the Arruda–Boyce hyperelastic constitutive model, the shear modulus \( \mu_{\text{chain}} \)) by the dimensionless relaxation function. That is,

\[
\mu_{\text{chain}}(t) = \mu_{\text{chain}} \cdot \left( 1 - \sum_{k=1}^{N} \alpha_k \cdot (1 - e^{-t/\tau_k}) \right)
\]

(2.2)
where $g_k$ is the $k$th Prony constant ($k = 1, 2, \ldots, N$) and $\tau_k$ is the corresponding relaxation time constant.

The number of terms included in the Prony expansion was limited to 1 to minimize the computational cost of the FE analyses entailed by the ZP characterization process. This choice was supported by the fact that preliminary numerical tests carried out for the highest indentation rate considered in this study revealed that the Prony expansion is largely dominated by the first term of the series. Prony series expansion (2.2) was not implemented for the distensibility parameter $\lambda_l$ basically for two reasons. In the first place, the structural response of ZP membranes is mostly driven by the shear modulus (see the findings of Boccaccio et al. [16] for the bovine oocytes). Second, the viscous response of a polymeric network like that included in the ZP membrane depends on the relative slippage of filaments [18,19] rather than on material distensibility which instead determines the ability of filaments to stretch and hence affects the overall stiffness of the material. The latter assumption sounds very logical, as distensibility does not change much through the material.

Time-dependent FE analyses were carried out for each indentation rate $v_i$ (i.e. 0.5, 2, 4, 6, 8 and 10 $\mu$m s$^{-1}$, respectively) considered in the experimental tests. The total duration of the indentation process $t_{\text{step}}$, corresponding to the analysis time specified in ABAQUS, hence is $t_{\text{step}} = n_i$.

For each indentation rate, the displacement given to the AFM indenter is ramped over the time step $t_{\text{step}}$:

$$
2.5. \text{Optimization-based algorithm for extracting zona pellucida visco-hyperelastic properties}
$$

The visco-hyperelastic properties of the ZP membrane were determined with the optimization-based identification algorithm schematized in figure 2. A similar approach was used in

Figure 2. Flow chart of the optimization-based algorithm implemented in Matlab to identify ZP’s mechanical properties.
other mechanical characterization studies of nonlinear materials at the micro- and nanoscale [26–28,16].

The identification algorithm, coded in the Matlab (The Mathworks Inc., Austin, TX, USA) software environment, minimizes the difference between nanoindentation data and FE analyses via nonlinear optimization. The flow chart shown in figure 2 includes several steps. Initial values are assigned to the four unknown material parameters \( \mu_{\text{8chain}}^{0}, \lambda_{t}^{0}, g_{1}^{0} \) and \( \tau_{1}^{0} \) included as optimization variables (Step 1). An input file containing data on model geometry and material properties is prepared (Step 2) and given as input to the ABAQUS FE solver (Step 3). ABAQUS saves the results of FE analysis in another file (Step 4) and the computed force–indentation curve is compared with its counterpart measured experimentally (in the schematic of figure 2, AFM measurements carried out at a given indentation rate correspond to Step 0) in order to evaluate the difference between numerical results and experimental data (Step 5). This leads to formulate an optimization problem including the unknown material properties as design variables where the objective is to minimize the error functional \( \Omega \)

\[
\min \left\{ \Omega(\mu_{\text{8chain}}, \lambda_{t}, g_{1}, \tau_{1}) = \frac{1}{N_{\text{CNT}}} \sum_{j=1}^{N_{\text{CNT}}} \left( \frac{F_{\text{FEM}} - F_{j}}{F_{j}} \right)^{2} \right\}
\]

\[0.1 \text{kPa} \leq \mu_{\text{8chain}} \leq 100 \text{kPa}, 1 \leq \lambda_{t} \leq 10.
\]

\[0.001 \leq g_{1} \leq 1, 10^{-5} \leq \tau_{1} \leq 10^{-1}.
\]

(2.3)

As mentioned above, there are four unknown material properties: two hyperelastic constants \( \mu_{\text{8chain}} \) and \( \lambda_{t} \) and two viscous parameters \( g_{1} \) and \( \tau_{1} \). The bounds of the shear modulus \( \mu_{\text{8chain}} \) were chosen so as to cover the range of variation of the apparent Young’s modulus of porcine oocytes reported in Papi et al. [20] for the same indentation rate range (by scaling the Poisson ratio from the value of 0.33 used in Papi et al. [20] to the value of 0.5 used in this study, the apparent Young’s modulus would range from 25 to 135 kPa; because for the Arruda–Boyce model it holds \( E = 3\mu_{\text{8chain}} \), the shear modulus would be between 8.3 and 45 kPa), and to account for the fact that values of elastic moduli determined under the assumption of hyperelastic behaviour may be up to one or two orders of magnitude smaller than those determined with the classical Hertzian model, which assumes instead linear elasticity, ‘infinite space’, perfect spherical indenter and small contact area (e.g. [16,29–31]). In general, material property bounds were set large enough to increase design freedom and rapidly converge to global optimum.

In equation (2.3), \( F_{\text{FEM}} \) and \( F_{j} \), respectively, are the indentation force values for the \( j \)-th load step computed by ABAQUS and those measured experimentally by AFM. The number of control points \( N_{\text{CNT}} \) corresponds to the number of load steps executed for completing nonlinear FE analysis.

The computed error functional \( \Omega \) is compared with a pre-defined convergence limit \( \varepsilon_{\text{CONV}} \) set as 0.0001 (i.e. 0.1%) (Step 6). If it occurs \( \Omega < \varepsilon_{\text{CONV}} \), the identification process terminates and material properties are listed in output (Step 8). Conversely, if it occurs \( \Omega > \varepsilon_{\text{CONV}} \) material parameters \( \mu_{\text{8chain}} \), \( \lambda_{t} \), \( g_{1} \) and \( \tau_{1} \) are perturbed in the subsequent design cycles until convergence (Step 7).

The above-described identification process was carried out to determine the corresponding material properties \( \mu_{\text{8chain}} \), \( \lambda_{t} \), \( g_{1} \) and \( \tau_{1} \) for each indentation rate. The inverse problem stated in (2.3) was solved with the Sequential Quadratic Programming method, a globally convergent gradient-based optimization algorithm, coded in the Matlab v. 7.0 commercial software. For each indentation rate, optimization runs were started from five different sets of material properties randomly generated. This multi-start optimization strategy together with the large range of variability chosen for material parameters allowed to cover the whole search space and increased the probability of finding the global optimum. Serial optimization runs were carried out for each test case to avoid premature convergence. This process ended as soon as relative variations of error functional \( |(\Omega_{k} - \Omega_{k-1})/\Omega_{k-1}| \) and design vector \( |X_{k} - X_{k-1}|/|X_{k-1}| \) between the last two serial runs became smaller than 0.0001 (i.e. 0.1%).

It should be noted that the common hypothesis behind most of the studies reported in the literature is that the viscous forces involved in the nanoindentation process are negligible. Further optimization runs were hence carried out in order to evaluate the error on hyperelastic material properties that would be made if viscous effects were neglected. For that purpose, only the hyperelastic parameters \( \mu_{\text{8chain}} \) and \( \lambda_{t} \) were identified, without including any Prony term, and time-independent hyperelastic FE analyses were executed. The ‘static’ values of \( \mu_{\text{8chain}} \) and \( \lambda_{t} \) minimizing the error functional (2.3) for each force–indentation curve relative to a given indentation rate were then compared to the corresponding ‘dynamic’ values determined by including viscous effects. This allowed assessing the limit of validity as well as the practical implications of the assumption of time-independent behaviour usually followed in the literature.

3. Results

Table 1 presents the values of visco-hyperelastic properties determined with the hybrid mechanical characterization procedure described in §2 for the \( (0.5 \div 10) \mu \text{m s} \) indentation rate range considered in this study. The table also reports the average values of identified properties and the corresponding standard deviations over the indentation rate range as well as the values of the correlation coefficient \( R^{2} \) between AFM data and force–indentation curves reconstructed numerically for each indentation rate.

It can be seen that values of visco-hyperelastic properties were rather insensitive to the indentation rate. In fact, the ratio of the standard deviation on identified parameters to the average material property values was 9.06%, 0.845%, 3.32% and 8.87%, respectively, for shear modulus, distensibility, Prony constant and relaxation time. Such a level of statistical dispersion on identified material properties is owing to the fact that the target force–indentation curve taken for each indentation rate was the average of the corresponding force–indentation curves recorded experimentally in 250 different points of 10 samples. In fact, the standard deviation of experimental data with respect to the target data used in the characterization process was on average about 10%, hence consistent with the values of standard deviation found on identified material properties.

Figure 3 compares the average force–indentation curves measured experimentally with those reconstructed numerically by giving in input to ABAQUS the visco-hyperelastic properties identified via nonlinear optimization. The overall
quality of data fitting was always good over the entire range of indentation rates: in fact, the correlation coefficient ranged between 0.9 and 0.975 with an average value of 0.939. Interestingly, no trend of variation of the data correlation coefficient with respect to the indentation rate could be found.

The time-independent hyperelastic parameters determined for the very low indentation rate of 0.5 \( \mu \)m s\(^{-1}\) without considering viscous effects are \( \mu_{\text{chain}} = 1.415 \) kPa and \( \lambda_L = 2.988 \), practically the same as the corresponding values determined with the visco-hyperelastic model, \( \mu_{\text{chain}} = 1.406 \) kPa.

### Table 1. Visco-hyperelastic properties of the porcine ZP determined with the hybrid characterization procedure. The values listed in the table were obtained by taking the force–indentation curves averaged over AFM measurements done for each indentation rate as targets.

<table>
<thead>
<tr>
<th>indentation rate (( \mu )m s(^{-1}))</th>
<th>( \mu_{\text{chain}} ) (kPa)</th>
<th>( \lambda_L )</th>
<th>( g_1 )</th>
<th>( \tau_1 ) (s)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.406</td>
<td>3.001</td>
<td>0.9031</td>
<td>0.00109</td>
<td>0.975</td>
</tr>
<tr>
<td>2</td>
<td>1.586</td>
<td>2.962</td>
<td>0.9742</td>
<td>0.00134</td>
<td>0.900</td>
</tr>
<tr>
<td>4</td>
<td>1.692</td>
<td>2.960</td>
<td>0.9797</td>
<td>0.00136</td>
<td>0.925</td>
</tr>
<tr>
<td>6</td>
<td>1.835</td>
<td>2.927</td>
<td>0.9819</td>
<td>0.00140</td>
<td>0.920</td>
</tr>
<tr>
<td>8</td>
<td>1.816</td>
<td>2.941</td>
<td>0.9848</td>
<td>0.00140</td>
<td>0.950</td>
</tr>
<tr>
<td>10</td>
<td>1.694</td>
<td>2.959</td>
<td>0.9849</td>
<td>0.00137</td>
<td>0.963</td>
</tr>
<tr>
<td>average ± s.d.</td>
<td>1.688 ± 0.153</td>
<td>2.958 ± 0.0250</td>
<td>0.9681 ± 0.0321</td>
<td>0.00133 ± 0.000118</td>
<td>0.963</td>
</tr>
</tbody>
</table>

**Figure 3.** Comparison of force–indentation curves experimentally measured by AFM with their counterpart 'optimized' by ABAQUS for the different indentation rates. (Online version in colour.)
and \( \lambda_\text{L} = 3.001 \). Conversely, figure 4 shows that the ‘static’ value of shear modulus increased significantly with indentation rate (i.e. from 1.42 kPa for the 0.5 \( \mu \text{m s}^{-1} \) indentation rate to 31.3 kPa for the 10 \( \mu \text{m s}^{-1} \) indentation rate) and differed by more than one order of magnitude from the ‘dynamic’ value of shear modulus obtained by accounting for time-dependent behaviour. By recalling the relationship \( E = 3\mu_{\text{chain}} \) derived for the Arruda–Boyce model applied to incompressible materials, it follows that the range of variation of the apparent Young’s modulus found in the ‘static’ case is \([4 \div 94]\) kPa. This range is qualitatively consistent with the [25 \div 135] kPa range of variation found in Papi et al. [20] using the classical Hertzian model.

4. Discussion

This study presented the first visco-hyperelastic model ever developed in the literature to describe the viscous response of the porcine ZP. The purpose of the research was to analyse the variation of viscous reaction forces exerted by the porcine ZP under different conditions of sperm motility. As these forces are one of the main factors regulating sperm transit, their detailed knowledge must be the start point of any study on sperm–ZP interaction. In fact, even small variations of spermatozoa velocity may lead to significant changes in the ZP reaction force turning the ZP shell into an impenetrable barrier and leading to fertility impairments. Variations of sperm motility were simulated by carrying out AFM nanoindentation tests at different rates between 0.5 and 10 \( \mu \text{m s}^{-1} \), the typical range adopted in \( \text{in vitro} \) fertilization. The force exerted by AFM probe on ZP membrane simulates the interaction between the spermatozoa and the ZP itself.

Although visco-elastic behaviour of human and animal cells, including oocytes, has been extensively analysed in the literature (e.g. [32–37,11,12,20]), no systematic study has ever been carried out on the visco-hyperelastic behaviour of ZP membranes. However, hyperelasticity is the best constitutive model to describe the mechanical response of the ZP in the different stages of the fertilization process [16]. Nonlinear elasticity and, more specifically, hyperelasticity should always be used to model soft materials made of polymeric chain networks, namely the ZP membrane. Very recent studies have correctly pointed out the viscous mechanical response of mouse [11,12], porcine and equine ZPs [20] but provided just qualitative information on material properties that were determined via standard fitting based on more or less sophisticated models of contact mechanics yet without assuming visco-hyperelastic behaviour.

This study attempted to overcome the above-mentioned limitation trying to separate the time-independent part of the ZP’s mechanical response, which is driven by the ZP polymeric network structure (i.e. number of cross-links and distances between knots), from the viscous part (i.e. time-dependent), which is driven instead by the sliding of molecules over one another. For that purpose, visco-hyperelastic properties of the porcine ZP membrane were determined by combining AFM measurements and optimization-based inverse FE analysis. The hyperelastic behaviour of the ZP membrane was described by the Arruda–Boyce constitutive model, whereas the viscous behaviour (i.e. the rate-dependent part of material behaviour) was described by means of a Prony series expansion of the dimensionless relaxation modulus.

A gradient-based optimization algorithm minimizing the difference between the force–indentation curve measured experimentally by AFM and its counterpart computed by ABAQUS from the visco-hyperelastic model allowed the hyperelastic material parameters, \( \mu_{\text{chain}} \) and \( \lambda_\text{L} \), and the viscous terms, \( g_i \) and \( \tau_i \), to be determined for each indentation rate. Remarkably, all material properties determined with the identification procedure changed by less than 10% (table 1) in spite of the fact that the indentation rate varied by 20 times (i.e. from 0.5 to 10 \( \mu \text{m s}^{-1} \)). This sounds logical because the visco-hyperelastic parameters describing the time-independent and time-dependent parts of the ZP’s mechanical response are inherent properties of the investigated material. The viscous reaction force increases with the loading rate but the relationship between these two quantities must always be described by the same material properties. Therefore, the standard deviation seen on material properties

![Figure 4. Comparison between the average value of shear modulus of the porcine ZP determined by including viscous effects and counterpart values determined not considering viscous effects. (Online version in colour.)](image-url)
was not caused by the large variation of loading rate, but it is the direct consequence of having averaged a huge amount of experimental data to obtain target force-indentation curves for the optimization-based identification process.

This model could actually separate the rate-independent and the viscous contributions to the ZP’s mechanical response. In fact, the lowest standard deviation on material properties (i.e. 0.845%) was found for the distensibility parameter \( \lambda_t \), which affects only the elastic response of the ZP, as it represents the ability of polymeric chains to stretch. The Prony series terms \( g_i \), which is directly related to relaxation, exhibited the second lowest dispersion. The most significant elastic parameter, the shear modulus, was also the most largely dispersed material property soon followed by the relaxation time, which is however three orders of magnitude smaller than the other material parameters and hence intrinsically more sensitive to dispersion of experimental data. It is worthy to note that correlation between experimental data and force-indentation curves computed by ABAQUS for the material parameter values determined via optimization was good for each indentation rate, ranging between 0.9 and 0.975 (table 1 and figure 3). As no clear trend of variation between the correlation coefficient \( R^2 \) and the indentation rate could be established, it appears that the proposed identification framework is robust and able to correctly describe the real mechanical behaviour of the porcine ZP.

Errors made in the determination of material properties if viscous terms were neglected can also be evaluated with the present approach. Hyperelastic properties obtained with the ‘static’ hyperelastic model without considering viscous effects (i.e. by including only the shear modulus and the distensibility as design variables) were very close to those obtained from the visco-hyperelastic model for the lowest indentation rate of 0.5 µm s\(^{-1}\). 1.415 versus 1.406 kPa and \( \lambda_t = 2.988 \) versus 3.001 for, respectively, shear modulus and distensibility. This was largely expected because at low indentation rates the structural response of ZP is mostly driven by the elastic deformations that depend on the structure of the ZP’s polymeric network (i.e. number of cross-links and distances between knots) and the ability of polymeric chains to stretch. As the indentation rate increases, viscous effects dominate and neglecting them leads to significant errors (more than one order of magnitude, as it can be seen from figure 4) on material stiffness: in the present case, the apparent Young’s modulus determined without considering viscous effects ranged from approximately 4 to approximately 94 kPa (those values are in qualitative agreement with the findings of Papi et al. [20]) versus 5.1 ± 0.46 kPa found with the visco-hyperelastic model. Viscous effects were already significant for the second lowest indentation rate of 2 µm s\(^{-1}\); the ‘static’ model predicted an apparent Young’s modulus of 27.6 kPa, which is more than five times larger than that found by the visco-hyperelastic model.

An interesting issue emerging from this study is the following. The correlation coefficient \( R^2 \) evaluated for the ‘static’ material properties was on average 0.97, hence higher than the 0.939 average value found by including visco-elastic effects. Looking uncritically at these results, it might seem that the ‘static’ model is more efficient than the viscous model. However, the elastic modulus determined in the ‘static’ case represents only an apparent stiffness. The actual stiffness results from the combination of an ‘elastic’ stiffness and a ‘viscous’ stiffness. In the case of a polymeric network, namely the ZP, the former term depends on the network structure and on the ability of polymeric chains to deform independently one from the other; the latter term represents the equivalent stiffness of the spring-dashpot system describing the viscous behaviour of the material. In summary, much care should be taken in interpreting results of the identification process: a better fitting of experimental data does not necessarily imply that the hypothesized constitutive model is the most accurate modelling assumption especially if the material shows time-dependent behaviour.

There is another important consideration regarding protocols and instrumentation used to characterize biotissues and cells. The fact that the mechanical response of the ZP membrane is rate dependent may lead to conclude that it is practically impossible to compare mechanical properties derived for ZP specimens tested with instruments operating at different loading rates. This statement is very misleading because, as it was demonstrated in this research, mechanical properties are inherent characteristics of materials and cannot depend on experimental protocols, testing equipments and conditions. However, neglecting viscous behaviour may lead to the erroneous conclusion as mentioned above.

A number of assumptions and simplifications were made in this study. First, residual stresses developing in the ZP membrane specimens spread out on the rigid substrate for indentation were not considered. However, as shown in a previous study [17], using a very sharp AFM tip like that employed in the present measurements makes the determination of mechanical properties marginally sensitive to residual stresses casually developed in the preparation of the indentation tests. Second, a three-dimensional model of the ZP membrane and the indenter would have better reproduced the real nanoindentation process. However, axisymmetric FE models are very common in the literature and allow a more detailed description of the contact problem between the AFM tip and the ZP membrane. Third, the ZP–indenter contact was assumed to be frictionless. However, as the influence of friction in the extraction of elastic properties becomes significant if the indentation depth reaches half of the sample thickness and considering that the 100 nm indentation depth represents only 1.6% of the total sample thickness of 6 µm, one can reasonably conclude that the error made in neglecting friction is very small. Fourth, the axis of symmetry of the AFM tip was assumed to be exactly orthogonal to the ZP slab. Indeed, for the indentation depth of 100 nm considered in this study, the very small rotation of the AFM tip yields a lateral displacement three–four orders of magnitude smaller than the contact arc length between the indenter and the ZP membrane. Last, the visco-hyperelastic behaviour of the ZP membrane was described by a Prony series expansion of the dimensionless relaxation modulus. However, the visco-elastic relaxation response of bio-membranes could also be described by the three-parameter standard linear or the five-parameter Maxwell–Weichert models [38]. Studying the implications of using different viscous models on the determination of material properties of cell membranes will be an interesting subject for future investigations.

5. Conclusion

This study presented the first visco-hyperelastic model ever developed in the literature to describe the viscous response


References

6. Green DPL. 1997 Three dimensional structure of the oocyte membrane. This ability represents a significant step further with respect to the current literature and may be a good start basis for future studies on the sperm–ZP interactions to assess the complex relationships between sperm motility, mechanical behaviour of ZP and fertility impairments. The next step of the research will be to analyse the visco-hyperelastic response of the ZP at different stages of the fertilization process to confirm the relationships between mechanical hardening and architecture of polymeric network [16]. Furthermore, the effect of the indenter shape on the visco-hyperelastic response will be studied in order to understand the relationship between sperm head shape and penetration [39].


