Hertz model or Oliver & Pharr analysis? Tutorial regarding AFM nanoindentation experiments on biological samples

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Abstract

The data processing regarding AFM nanoindentation experiments on biological samples relies on the basic contact mechanics models like the Hertz model and the Oliver & Pharr analysis. Despite the fact that the two aforementioned techniques are assumed to provide equivalent results since they are based on the same underlying theory of contact mechanics, significant differences regarding the Young’s modulus calculation even on the same tested sample have been presented in the literature. The differences can be even greater than 30% depending on the used model. In addition, when the Oliver & Pharr analysis is used, a systematic greater Young’s modulus value is always calculated compared to the Hertzian analysis. In this paper, the two techniques are briefly described and two possible reasons that accurately explain the observed differences in the calculated value of the Young’s modulus are presented.

1. Introduction

Atomic Force Microscopy nanoindentation is a powerful technique for the characterization of biological samples at the nanoscale [1–9]. The data processing is usually performed using basic models arising from the contact mechanics theory [7, 9]. If the sample that is being tested can be characterized as an elastic half space, the Young’s modulus of the sample can be calculated using the Hertz model or the Oliver & Pharr analysis [7, 9, 10]. Despite the fact that these techniques are based on the same theoretical tools, there are significant differences regarding the data processing [7, 9]. However, if the biological sample can be considered as homogeneous and isotropic the two techniques should theoretically provide the same results [7, 10]. Nevertheless, as it has been previously reported, in case of biological samples (e.g. collagen), the used model affects the results [11].

In this paper these methods and respective data processing techniques are briefly described. In addition, the related theoretical analysis is presented which proves that if the sample can be considered as homogeneous and isotropic, then the results provided by the two methods should be the same. Since this is not the case in research experiments [11], two possible explanations are provided with respect to the differences of the Young’s modulus calculations that have been reported when the same sample is tested using both methods.

2. Theory

2.1. AFM nanoindentation experiments on biological samples

2.1.1. Loading and unloading curves

In a typical AFM nanoindentation experiment the AFM tip indents the sample of interest and a load-displacement curve is recorded [9, 12]. The same procedure is also performed on a hard reference sample [9, 12]. As a result, the indentation values are calculated by the difference in the piezo-displacement between the hard and the soft sample for the same applied load [9]. Thus, a load—indentation curve is created. This curve consists
of two parts, the loading part in which the tip is moving towards the sample and the unloading part in which the motion of the tip is the opposite (figure 1) [9, 10]. These curves can be processed using contact mechanics models for the determination of the Young’s modulus value of the sample [7].

2.1.2. The Hertz model
The most widely used model for data processing in AFM nanoindentation experiments is the Hertz model. In particular, if the AFM tip has a spherical shape and if the indentation depth \( h \) is significantly smaller than the tip radius \( R \), then the loading data are fitted into the equation [7, 13, 14]:

\[
P = \frac{4}{3} \frac{E}{1 - \nu^2} R^{3/2} h^{1/2}
\]  

(1)

In equation (1), \( E \), \( \nu \) are the Young’s modulus and the Poisson’s ratio of the sample respectively and \( P \) is the applied load. As a result, the Young’s modulus can be easily calculated as a fitting parameter, under the condition that the dimensions of the indenter and the Poisson’s ratio of the sample are known.

The Hertz model can be applied only under specific conditions. The sample should be homogeneous (i.e. the material has uniform composition and uniform properties throughout), isotropic (i.e. the properties of the material are the same in all directions) and present a linear elastic response (i.e. stress is proportional to strain) [10]. However, biological samples at the nanoscale do not meet these requirements [15]. On the other hand, multiple experiments from different research groups have shown that for small indentation depths and for small indenters (compared to the sample’s dimensions) the load-indentation data follow the Hertzian mechanics and the sample can be approximately considered as an elastic half space [3, 16–21]. An elastic half space is an isotropic and homogeneous material that is assumed to extend infinitely in all directions and in depth, with the top surface as a boundary [22]. As it has been previously reported for spherical indenters, if the indenter’s radius is at least ten times smaller from each horizontal dimension of the sample, then the sample can be considered as an elastic half space [13]. In addition, the maximum indentation depth should not exceed the 5%–10% of the sample’s thickness in order to avoid measuring the sample’s substrate properties (Buckle’s rule) [12].

At this point it must be noted that the Buckle’s rule is not the only restriction regarding the maximum allowed indentation depth. Another significant restriction is the depth dependence of the biological samples’ mechanical properties [23]. Usually, the mechanical properties of biological samples are depth-dependent and if a certain limit is surpassed, equation (1) cannot be applied. In addition, despite the fact that equation (1) is used when the indentation experiments are performed using spherical indenters (when \( h \ll R \)), it can be accurately used for indenters with the shape of a paraboloid of revolution as well. Thus, the indenter’s radius provides an additional significant limitation regarding the maximum indentation depth [24]. Last but not least, the contact geometry must be axisymmetric, smooth and continuous [7]. Under the aforementioned requirements, the data related to nanoindentation experiments on bio-samples (such as cells, proteins, articular cartilage etc) can be processed using the equation provided by the Hertz contact mechanics (equation 1).

2.1.3. Sneddon’s equations and an accurate solution for spherical indenters
As it was presented in section 2.1.2, the Hertz model can be applied for parabolic indenters and approximately for spherical indenters under the condition \( h \ll R \). In this section, the general case, that can be applied for any axisymmetric indenter will be presented. In particular, assume an indenter that is described by an arbitrary
function \( Z = f(r) \) which is rotated about z-axis to produce a solid of revolution. The function is infinitely differentiable and is chosen so as \( f(0) = 0 \). The indenter elastically deforms an elastic half-space at a depth \( h \) and produces a circle of contact at the surface with radius \( r_\text{c} \). If the non-dimensional parameter \( x = r/r_\text{c} \) is used \((0 \leq x \leq 1)\) the indenter’s shape can be described by the function \( Z = f(x) \) [25]. Ian N Sneddon in 1965 succeeded to derive simple expressions for the indentation depth \( h \) and the applied load \( P \) in terms of integrals of the shape function [26]:

\[
h = \int_0^1 \frac{f'(x)}{\sqrt{1 - x^2}} \, dx
\]

(2)

\[
P = \frac{2E r_\text{c}}{(1 - \nu^2)} \int_0^1 \frac{x^2 f'(x)}{\sqrt{1 - x^2}} \, dx
\]

(3)

Where \( E, \nu \) are the Young’s modulus and the Poisson’s ratio of the elastic half-space respectively. For example, assume a spherical indenter with radius \( R \). In this case,

\[
f(x) = R - \sqrt{R^2 - r_\text{c}^2 x^2}
\]

(4)

Using equations (3) and (4), the applied load on the elastic-half space results in [27]:

\[
P = \frac{E}{2(1 - \nu^2)} \left[ (r_\text{c}^2 + R^2) \ln \left( \frac{R + r_\text{c}}{R - r_\text{c}} \right) - 2r_\text{c}R \right]
\]

(5)

The indentation depth is related to the contact radius with the following equation [27]:

\[
\ln \left( \frac{R + r_\text{c}}{R - r_\text{c}} \right) = \frac{2h}{r_\text{c}}
\]

(6)

Equation (5) is the equation to accurately fit the data when a spherical indenter is used in an experiment. In addition, it must be noted that the contact radius is related to the contact depth with the equation [9, 28]:

\[
r_\text{c} = \sqrt{2Rh_\text{c} - h^2}
\]

(7)

In equation (7), \( h_\text{c} \) is the contact depth [9]. Other basic solutions of Sneddon’s equations are targeted for indenters in the form of a paraboloid of revolution and for conical indenters [26]. For parabolic indenters, Sneddon’s equations result in equation (1). In this case [7],

\[
r_\text{c} = \sqrt{R h_{\text{max}}}
\]

(8)

In addition, for conical indenters [9],

\[
P = \frac{2E}{\pi (1 - \nu^2)} (\tan \theta) h^2
\]

(9)

In equation (9), \( \theta \) is the semi-angle of the cone.

2.1.4. The Oliver & Pharr analysis

A significant step regarding data processing in nanoindentation experiments was the formulation of a simple formula that can be applied between a rigid, axisymmetric punch and an elastic half space [25]. The aforementioned formula was proved by Pharr, Oliver and Brotzen using the general equations (2) and (3) [25]. In particular, it was proved that the Young’s modulus can be calculated using the equations [25, 28, 29]:

\[
E = \frac{\sqrt{\pi} S}{2 (1 - \nu^2)} \sqrt{A}
\]

(10)

In equation (10), \( S \) is the contact stiffness of the sample and is calculated as the derivative of the curve \( (S = dP/dh) \) at the inception \( h = h_{\text{max}} \) [25]. In addition, \( A \) is the projected area of the indenter at contact depth [28, 29]. Equations (1) and (5) satisfy the general equation (10), since equation (10) can be used for any axisymmetric indenter. More specifically, for spherical indenters, the relation between the applied load and the indentation depth is provided by equations (5)–(7) and the projected area of the indenter at contact depth is given by the following equation [28]:

\[
A_{\text{sphere}} = \pi (2Rh_\text{c} - h^2)
\]

(11)

For indenters than can be considered as a paraboloid of revolution, the relation between the applied load and the indentation depth is provided by equation (1) (Hertz model). In this case, the projected area of the indenter at contact depth is given by the equation:

\[
A_{\text{paraboloid}} = \pi Rh_{\text{max}}
\]

(12a)
It should be emphasized that the two aforementioned cases provide similar results for small $h/R$ ratios. In particular, for small indentation depths comparing to the tip radius (i.e. $h \ll R$), equation (11) can be modified as follows:

$$A = 2\pi Rh_c$$

In addition, it must be noted that under the condition $h \ll R$, $h_c = h_{\text{max}}/2$, thus [29, 30]:

$$A = \pi R h_{\text{max}}$$ \hspace{1cm} (12b)

Furthermore, for conical indenters [9]:

$$A = \pi h_c^2 \tan^2 \theta$$ \hspace{1cm} (13)

For conical indentations, $h_c = 2h_{\text{max}}/\pi$ [30].

2.1.5. The dependence of the Young’s modulus to the used model

As it has been previously reported when a spherical indenter is used (on a purely elastic sample) for small $h/R$ ratios it is irrelevant which equation will be used for the data processing [25]. Hence, equation (1), (i.e. Hertz model) and equations (10) and (12a) (Oliver & Pharr analysis) can be equally used for the case of purely elastic samples, since in this case the paraboloid of revolution is approximated to a sphere and the projected area of the indenter at contact depth is provided by equation (12b) in both cases. It is easy to show that these two equations are identical. More specifically, equation (10) can be written as follows:

$$\frac{dP}{dh} = \frac{2E}{1-v^2} \sqrt{Rh}$$

The solution of this differential equation under the condition that $P = 0$ of $h = 0$ is:

$$P = \frac{4ER^{3/2}}{3(1-v^2)} h^{3/2} \quad \text{(equation (1), Hertz model)}$$

However, experimental results have shown that the model for data processing affects the results regarding the Young’s modulus calculation [11]. For example, it has been reported by Andriotis et al., that the Young’s modulus calculation using Hertzian analysis resulted in a 34% smaller Young’s modulus compared to the value that was calculated using the Oliver & Pharr analysis [11]. It must be noted that the influence of the data analysis model on the Young’s modulus results has been proved in many other studies. For example, the results provided by Heim et al [31], Strasser et al [32], Jolandan and Yu [33] and Grant et al [34] who calculated the Young’s modulus of collagen fibrils type I using Hertzian analysis, resulted in significantly smaller Young’s modulus values in comparison with the values provided by Wenger et al [28] who used the Oliver & Pharr analysis. In particular, there is a systematic increased value of Young’s modulus if the Oliver & Pharr model is used for data processing. It is undeniable from a mathematical perspective that these differences are a consequence of a misuse of the related equations and ‘bad’ approximations during data processing.

2.2. Two possible explanations for the differences observed in Young’s modulus calculated values using different models for data processing

2.2.1. The misuse of the equation provided by Hertz

2.2.1.1. Spherical indenters

In this section, the errors which result when using equation (1) for spherical indentations and for big values of $h/R$ will be explored. In particular, the values of the dimensionless factor $P (1 - v^2) / ER^2$ will be examined for a wide range of indentation values. The aforementioned factor will be calculated using the approximate (for spherical indenters) equation (1) and the accurate equation (5). It should be noted that equation (5) does not directly relates the applied load to the indentation depth, since it is expressed in terms of contact radius $r_c$.

For the case of spherical indenters, the contact radius is accurately provided by equation (7). A significant question that should be clearly answered in this case is the relationship between the contact and the maximum indentation depth for spherical indentations. Under the approximation $h \ll R$ it is known that $h_c = h_{\text{max}}/2$ [26]. It will be tested if this equation produces accurate results for big $h/R$ ratios as well. According to Oliver & Pharr analysis the contact depth is related to the maximum indentation depth by the following equation [28, 29]:

$$h_c = h_{\text{max}} - \frac{\varepsilon}{\text{p}_{\text{max}}}$$ \hspace{1cm} (14)

where $\varepsilon$ is a constant parameter that depends on the geometry of the indenter. Furthermore, it has been experimentally proven that for any axisymmetric indenter the applied load during indentation is related to the contact depth by the following equation [20, 35]:

$$P \approx \frac{h_{\text{max}}}{\text{h}_{c}}$$

$$\frac{\text{h}_{c}}{\text{h}_{\text{max}}}$$
\[ P = ah^m \]  

In equation (15) the coefficients \( a, m \) are determined as fitting constants. In addition, the coefficients \( \varepsilon, m \) are related to the equation [28, 29, 36]:

\[ \varepsilon = m \left[ 1 - \frac{2\Gamma \left( \frac{m-1}{2m} \right)}{\sqrt{\pi} \Gamma \left( \frac{1}{2m-1} \right)} \right] \]  

Thus, if the exponential coefficient \( m \) is known, then the coefficient \( \varepsilon \) will be easily calculated using equation (16) and as a result the relation of the contact and the maximum indentation depth will be revealed. For this purpose, equation (5) is written in the form:

\[ P = \frac{ER^2}{2(1 - v^2)} \left( \frac{r^2}{R^2} + 1 \right) \ln \left( \frac{1 + \frac{z}{R}}{1 - \frac{z}{R}} \right) - \frac{2R^2}{R} \]

or

\[ \frac{P}{Q} = \frac{1}{2} \left( z^2 + 1 \right) \ln \left( \frac{1 + z}{1 - z} \right) - 2z \]  

In equation (17), \( Q = \frac{ER^2}{1 - v^2} \) and \( z = \frac{h}{R} \). In addition, equation (6) can be written in the form:

\[ \frac{h}{R} = \frac{1}{2} z \ln \left( \frac{1 + z}{1 - z} \right) \]  

For \( 0 \leq z \leq 0.775 \), it is derived that \( 0 \leq \frac{h}{R} \leq 0.80 \). Using equations (17) and (18) the graph \( \frac{P}{Q} = f \left( \frac{h}{R} \right) \) can be plotted (figure 2(a)). The \( \frac{P}{Q} = f \left( \frac{h}{R} \right) \) data can then be fitted to equation (15). The fitted function was \( \frac{P}{Q} = c \left( \frac{h}{R} \right)^m \), where \( c = 1.189 \) and \( m = 1.5 \). The fitting was accurate since \( R^2 = 0.9997 \). Subsequently, using equation (16) it is easy to calculate \( \varepsilon = 0.75 \). In conclusion, using equation (14):

\[ h_c = h_{\text{max}} - \varepsilon \frac{ah_{\text{max}}^m}{amh_{\text{max}}^{m-1}} \Rightarrow h_c = \frac{h_{\text{max}}}{2} \]  

As a result, equation (19) can be also used for deep indentations using spherical indenters. Hence, using equation (19), the contact radius for spherical indenters can be written in the form:

\[ r_c = \sqrt{Rh - \frac{h}{4}} \]  

By combining equations (5), (20):

\[ P = \frac{E}{2(1 - v^2)} R^2 \left( \frac{h}{R} - \frac{h^2}{4R^2} + 1 \right) \left[ \frac{2h/R}{\sqrt{R^2 - h^2/4R^2}} - 2 \sqrt{R^2 - h^2/4R^2} \right] \]

or else

\[ \frac{P}{Q} = \frac{1}{2} \left( \frac{h}{R} - \frac{h^2}{4R^2} + 1 \right) \left[ \frac{2h/R}{\sqrt{R^2 - h^2/4R^2}} - 2 \sqrt{R^2 - h^2/4R^2} \right] \]  

A significant point that should be clarified is that equation (21) can be written in the simple form:

\[ \frac{P}{Q} = c \left( \frac{h}{R} \right)^{3/2} \]

Where, the fitting factor \( c \) varies depending on the \( h/R \) ratio. In particular, for

\[ 0 \leq h/R \leq 0.8 \]

It results to,

\[ 1.333 \leq c \leq 1.189 \]

If \( c \to 1.333 = 4/3 \) then the paraboloid of revolution approximation can be used. On the other hand, equation (1) can be always written in the form:

\[ \frac{P'}{Q} = \frac{4}{3} \left( \frac{h}{R} \right)^{3/2} \]  

(22)
Figure 2. (a) The $\frac{P}{Q} = f(h/R)$ function and a power-law fitted curve. The applied load is related to the indentation depth with the following law $P \sim h^{1.5}$. (b) The comparison of the $\frac{P}{Q} = f(h/R)$ values when a parabolic and a spherical indenter is used. (c) The $\frac{P}{Q} = f(h/R)$ curve for small $h/R$ ratios. In this case the approximation of a spherical indenter to a paraboloid of revolution is valid. (d) The ratio $E^*/E$ with respect to the ratio $h/R$. (e) A load indentation curve on a H4 human glioma cell. In this case $\lambda = 0.19$ and the ratio $\frac{E^*}{E} = 1.025$. (f) A load indentation curve on a H4 human glioma cell using a bigger $h/R$ ratio. In particular, $\lambda = 0.24$. In this case $\frac{E^*}{E} = 1.031$. 
In figure 2(b), the functions \( P/Q = f\left(\frac{h}{R}\right) \) and \( P'/Q = f\left(\frac{h^*}{R}\right) \) are presented for comparison. Both equations are following the law \( P \sim h^{3/2} \) as it has already been mentioned. In figure 2(c) the \( P'/Q = f\left(\frac{h^*}{R}\right) \) function is presented at the domain \( 0 \leq h/R \leq 0.05 \). In this case, the Hertz equation (equation 22) fits perfectly to the data \((R^2 = 1.0000)\). As a result, the errors become significant when the Hertzian analysis is used for big \( h/R \) ratios due to the approximation in the contact radius. Nevertheless, equation (1) can be easily modified for big \( h/R \) ratios. The general equation (10) can easily provide the differences regarding the Young’s modulus calculation when a parabolic approximation is used (since it was proved that the load-indentation data can be fitted to equation \( P = ah^{3/2} \) in both cases). In particular,

\[
\frac{E}{E^*} = \sqrt{\frac{A^*}{A}} = \sqrt{\frac{\pi \left(Rh_{\text{max}} - \frac{h_{\text{max}}^2}{4}\right)}{\pi Rh_{\text{max}}} = \left(1 - \frac{h_{\text{max}}}{4R}\right)^{1/2}}
\]

In equation (23), \( E \) is the Young’s modulus value as calculated if it is assumed that the contact area is given by the equation \( A = \pi Rh_{\text{max}} \) (Hertzian analysis) and \( E^* \) is the real value (i.e. when the real projected area \( A^* = \pi \left(Rh_{\text{max}} - \frac{h_{\text{max}}^2}{4}\right) \) is used). Thus, function (21) was fitted to equation \( \frac{P}{Q} = \frac{4}{3}\frac{E}{1 - v^2}R^{1/2}h^{3/2} \left(1 - \frac{h_{\text{max}}}{4R}\right) \)

\((R^2 = 0.9997)\) (figure 2(b)). As a result, the Hertzian modification for big \( h/R \) ratios is the following:

\[
P = \frac{4}{3}\frac{E}{1 - v^2}R^{1/2}h^{3/2} \left(1 - \frac{h_{\text{max}}}{4R}\right)(24)
\]

In figure 2(d), the ratio, \( E^*/E \) is presented. For example, in case that \( h/R = 0.8 \) the approximate Young’s modulus (using the Hertz model) value equals to 89.3% of the real value which is a significant error in the analysis.

In figures 2(e)–(f) two load indentation curves on a H4 human glioma cell are presented. The protocol regarding AFM nanoindentation experiment is presented in the appendix. A spherical indenter was used to perform the experiments with radius 2.5 \( \mu \)m. The Poisson’s ratio of the cells was assumed to be \( v = 0.5 \). The curve which is presented to figure 2(e) was fitted to the equation \( P = 6.942 h^{3/2} \) \((R^2 = 0.9994)\) and the curve which is presented to figure 2(f) was fitted to the equation \( P = 5.684 h^{3/2} \)

\((R^2 = 0.9993)\). If equation (1) is used for the Young’s modulus determination it results in 2.47 kPa and 2.02 kPa respectively. However, the \( h/R \) ratios for the two curves are small but not negligible \((0.19 \text{ and } 0.24)\), thus equation (1) cannot be applied. As a result, using the modified equation (24) it is easy to find that the real Young’s modulus values are 2.53 kPa and 2.09 kPa respectively.

2.2.1.2. Conical indenters

For the case of conical indenters, the equation (9) (Sneddon’s extension of the Hertz model for conical indenters) and (10) (Oliver & Pharr analysis) will provide the same results since there is no approximation in contact area. More specifically, for conical indentations, \( A = \frac{2\pi}{\tan \theta} \). Thus, equation (10) can be written as follows:

\[
\frac{dP}{dh} = \frac{2E}{\pi (1 - v^2)}2h(\tan \theta)
\]

As a result, under the condition that \( P = 0 \) of \( h = 0 \), it is concluded:

\[
P = \frac{2}{\pi} E (\tan \theta)h^2,
\]

which is equation (9) for conical indentations.

2.2.1.3. Pyramidal tips

Pyramidal tips can be also used in AFM nanoindentation experiments to combine high resolution imaging with the determination of mechanical properties. In this case the AFM tip can be analyzed in terms of a sphere (using the analysis presented by this paper) if the indentation depth is smaller than the tip apex radius \((h < R)\) or in terms of a cone if \( h \gg R \). The significance of the presented analysis can be revealed using the following example. Assume a tip with a tip apex radius equal to \( R = 10 \) nm. In this case the Hertzian analysis as provided by equation (1) can be used only if \( h \ll R \) (for example \( \frac{h}{R} = 0.1 \)) as it is presented in figure 2(b). However, a nanoindentation experiment with so small maximum indentation depth will be inaccurate, thus the modified equation (24) can be used for data processing. As a result, the presented analysis can be used in cases that both high resolution imaging and mechanical properties measurements are needed (e.g. experiments on collagen fibrils).
2.2.1.4. Carbon nanotube tips (CNTs)

CNTs have been proven as an exceptional tool for high resolution imaging of biological samples such as proteins, DNA, etc [37, 38]. In addition, a CNT probe due to its mechanical strength and nanoscopic dimensions (i.e., too sharp tips) can be used as a ‘nanoneedle’ in a nanoscale cell injection to deliver cargo into cells [37]. The aforementioned penetration is controlled by the AFM. However, the use of CNT probes is not appropriate for obtaining typical load-indentation curves [37]. Thus, the CNT’s cannot be used for measuring the mechanical properties of biological materials using the standard techniques as described in this paper.

2.2.2. The possibility of minor viscoelastic effects

The Hertz model takes into account the loading curve [39], while the Oliver & Pharr analysis the unloading curve (this fact will be analyzed in section (2.3)) [29]. However, if the sample is purely elastic these curves are identical.

In biological samples significant differences are recorded and the two curves are not identical. The reason is the viscoelastic behavior of biological samples. A viscoelastic behavior is observed when a material exhibits both elastic and viscous behavior [39]. The term ‘viscous’ implies that the material deforms slowly when exposed to an external load. The term ‘elastic’ implies that the material will return to its initial configuration when the applied load has been removed. If a material presents a purely elastic response then the loading and the unloading curves are superimposed. On the other hand, for the case of viscoelastic materials there is a ‘hysteresis’ loop [39]. The area within the loop is equal to the energy loss, dissipated as heat [39]. In addition, a characteristic behavior of viscoelastic materials is that their mechanical properties depend on the deformation rate [40]. In particular, the material’s stiffness increases with the loading rate. As a result, choosing different loading rates will result in a family of different load-indentation curves; each curve represents the mechanical properties of the sample at each loading rate [39]. The viscoelastic behavior of biological samples is due to the aqueous environment surrounding the biological material, since biological materials are usually tested in liquid environment (so as to be near physiological conditions). A common practice to minimize viscoelastic effects is to use an appropriate ramp frequency [40]. However, even small viscoelastic effects can play a significant role in the analysis. In figure 3, the loading and the unloading load indentation curves on a H4 human glioma cell are presented. As it can be seen in figure 3, small viscoelastic effects are present (the area under the loading curve is approximately 7.65% bigger compared to the area under the unloading curve).

Each of these curves can be fitted to a 4th degree polynomial curve as it has been previously reported [10, 36]:

\[ P(h) = c_4 h^4 + c_3 h^3 + c_2 h^2 + c_1 h + c_0 \]  

(26)

The fitting factors for the loading curve are provided below:

\[ c_4 = 8.6066 \cdot 10^{-12} \]

\[ c_3 = -1.2737 \cdot 10^{-8} \]
\[ c_2 = 1.072 \cdot 10^{-5} \]
\[ c_1 = 0.0010149 \]
\[ c_0 = -0.0069072 \]

In addition, the fitting factors for the unloading curve are presented:
\[ c_4 = 5.5848 \cdot 10^{-12} \]
\[ c_3 = -8.7743 \cdot 10^{-9} \]
\[ c_2 = 1.0669 \cdot 10^{-5} \]
\[ c_1 = 0.000433031 \]
\[ c_0 = -0.00364440 \]

The derivative of each curve at the inception \( h = h_{\text{max}} \) can be easily calculated:
\[
S = \left( \frac{dP}{dh} \right)_{h_{\text{max}}} = 4c_4 h_{\text{max}}^3 + 3c_3 h_{\text{max}}^2 + 2c_2 h_{\text{max}} + c_1 \tag{27}
\]
For the loading curve,
\[ S_l = 0.0065 \text{ N m}, \]
while for the unloading curve,
\[ S_u = 0.0073 \text{ N m} \]

As a result, the aforementioned small difference between the loading and the unloading curve resulted in a 12.3% bigger value regarding contact stiffness for the unloading curve.

### 2.2.3. A comparative example

Assume now a nanoindentation experiment using a pyramidal indenter with round tip apex on a purely elastic sample. The tip radius can be assumed equal to \( R = 20 \text{ nm} \) and the maximum indentation depth equal to \( h = 16 \text{ nm} \). As a result, \( h = 0.8R \). In this case, according to the analysis presented in section 2.2.1:
\[
\frac{E}{E^*} = \sqrt{\frac{A^*}{A}} = \sqrt{\frac{\pi (2Rh_{\text{c}} - h_{\text{c}}^2)}{2\pi Rh_{\text{c}}}} = 0.893 \tag{28}
\]

In equation (28), \( A^* \) is the accurate contact area (provided by equation (11)) as it is taken into consideration in Oliver & Pharr analysis and \( A \) is the approximate contact area as it is considered in Hertzian analysis (equations (1), (12a)).

In addition, using equation
\[
\frac{dP}{dh} = \frac{2E}{1 - v^2} \sqrt{Rh_{\text{c}}},
\]
which is equivalent to equation (1) (if it is assumed that \( P = 0 \) of \( h = 0 \)) it is easy to calculate the Young's modulus if the derivative at the inception \( h = h_{\text{max}} \) for the loading curve \( S_l \) is known. Using this approach, the Young's modulus is equal to the Young's modulus provided by equation (1) (Hertzian analysis). On the contrary, the Young's modulus using the Oliver & Pharr analysis can be calculated by using the derivative at the inception \( h = h_{\text{max}} \) for the unloading curve \( S_u \). In case of small viscoelastic effects (e.g. figure 3), it is possible that \( S_u = 1.12 \cdot S_l \). Thus,
\[
\frac{E^*}{E} = \frac{1 - v^2}{(\frac{\pi}{2})\sqrt{h_{\text{c}}}} = \frac{S_u \sqrt{A}}{S_l \sqrt{A^*}} = 1.254
\]

As a result, for the same nanoindentation experiment the Oliver & Pharr analysis will provide a Young’s modulus value 25.4% bigger comparing to the Hertzian analysis. The deviation using the two methods for data processing will be bigger for significant viscoelastic effects and for big \( h/R \) values. In addition, the deviation between the two models is bigger for spherical indenters comparing to the conical indenters due to the projected contact area approximation.

### 2.3. Non—biological samples

It must be noted that for the case of hard samples (non-biological) the Oliver & Pharr analysis is the best choice regarding data processing. The reason is that the big forces that are used to deform a hard sample (e.g. polymer of metal) usually result in permanent deformation of the sample [29]. As a result, the contact is elastic-plastic. For this reason, the Oliver & Pharr analysis uses the unloading curve, as they clearly mention [29]:
During unloading, it is assumed that only the elastic displacements are recovered; it is the elastic nature of the unloading curve that facilitates the analysis.

On the contrary for purely elastic contacts, equation (10) can be applied for both curves (loading and unloading). It is important to mention this fact since equation (10) has been related to the unloading—load indentation curve only; this fact is true only for elastic-plastic contacts.

3. Summary

The main key points of this tutorial paper are summarized below:

- Equation (1) (equation as provided by Hertz analysis) assumes the indenter as a paraboloid of revolution. It can be used for spherical indenters for small $h/R$ ratios (i.e. $h/R < 10$).
- Equations (1) and (10) (i.e. Hertz model and Oliver & Pharr analysis respectively) provide identical results for purely elastic samples and for parabolic indenters. Thus, there is no dependence of the Young’s modulus on the used model for data processing.
- For spherical indentations on a purely elastic sample, equation (10) provides accurate results since it takes into account the real projecting area of the indenter at contact depth (equation 11) and not the approximated equation (12b).
- The dependence of the applied load $P$ from the term $h^{3/2}$ holds for spherical indentation as well. Thus, for spherical indentations the equation $P = ah^{3/2}$ is valid; However, the fitting factor $a$ depends on the $h/R$ ratio.
- The relationship $h_c = h_{max}/2$ holds for spherical indentations for big $h/R$ ratios.
- Equation (24) can be used for spherical indentations and for big $h/R$ ratios.
- The Oliver & Pharr analysis takes into account the unloading load-indentation curve since it is usually applied for elastic-plastic contacts on hard non-biological samples.
- If a sample presents a purely elastic response it does not matter if the loading or the unloading curve will be used for data processing. In the literature, the Hertz model is usually used for fitting the loading curve and the unloading curve is usually processed using the Oliver & Pharr analysis. If the indenter can be well approximated to a paraboloid of revolution or a cone then the differences in the Young’s modulus calculations are not a consequence of the used model. The differences are usually a consequence of minor viscoelastic effects.

4. Conclusion

In this paper a comparative report regarding the data processing in AFM nanoindentation experiments using the Hertz model and the Oliver & Pharr analysis was presented. Despite the fact that the two aforementioned methods are equivalent for purely elastic samples, significant differences have been reported for approximated elastic samples depending on the model for the data processing used even when the same biological sample is tested. Thus, in this paper a logical explanation regarding these significant deviations was provided. The presented by this paper analysis clarifies significant issues regarding data processing and enlightens the fact that the Hertz model can be approximately used only for purely elastic response of the sample and for small $h/R$ ratios.

Appendix

Cell culturing: H4 human glioma cells (ATCC) were used for the evaluation of the proposed technique. Briefly, before each experiment, the cells were washed twice with PBS and new complete media was added. The petri dish (35 mm) was mounted directly on the AFM specimen disc and all the experiments were performed under liquid condition (cells’ complete media) at room temperature [10, 41, 42].

AFM experiments: A Molecular Imaging-Agilent PicoPlus AFM system (now known as AFM 5500 Keysight technologies) with a round (2.5 μm radius) ball-shape tip (CP-PNPL-BSG), and spring constant of 0.08 N m$^{-1}$. 

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was used for spherical indentation experiments. The spring’s constant of the cantilever was determined using the thermal noise method [10, 43, 44].

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