Eng. Sci., 2023, **22**, 820

Engineered Science
DOI: https://dx.doi.org/10.30919/es8d820

# Overview of Mechanical Characterization of Bone using Nanoindentation Technique and Its Applications

Vishwanath Managuli,[1,†] Yashwant Sing Bothra,[2,†] Sujith Kumar S,[3] Piyush Gaur[4] and Prasanna Lokadolalu Chandracharya[5,*]

## Abstract

The nanoindentation has proved to be a most promising technique in the investigation of the bone at the level of individual osteons and lamellae. It provides the nanomechanical properties of bone at submicron levels without being affected by its size, shape, and porosity. Its application in the study of bone requires the understanding of its functionality, modes, and influencing factors that have an impact on the outcome of the investigation. This necessitates a comprehensive a review. In the recent past, considerable attention is also paid to the elastic, time-dependent or viscoelastic response of the bone by conducting static, creep and nano-dynamic mechanical analysis (Nano-DMA) studies. Studies have also shown that at submicron levels, the mechanical properties of bone differ depending on the location and direction of testing within the bone, and among the individuals. Also, the impact of diseases progression and treatment procedures can be understood from variation in the biomechanical properties. This review focuses on the nanoindentation technique, its relevant modes used in the bone study, popular contact models used in the data analysis, various influencing parameters, recent applications such as the effect of aging, cancer, various diseases, and potential role in clinical studies such as to understand the effect of the treatment processes.

*Keywords:* Bone Mechanics; Elasticity; Hardness; Viscoelasticity; Nanoindentation Models.
Received: 17 December 2022; Revised: 13 February 2022; Accepted: 17 February 2023.
Article type: Review article.

## 1. Introduction

Bone is a naturally occurring complex composite material of primary importance in the structural integrity of a body. It supports the diverse set of functional demands during the life cycle, such as providing structure and support to the body and organs, anchoring muscles, and maintaining calcium homeostasis. The complexity in the bone structure arises due to the presence of various organic and inorganic components. From a biomaterial perspective, bone is viewed as a hydroxyapatite solid crystal stiffened by a collagen fiber network, hierarchically organized at multiple length scales, which are accountable for the material properties of the bone.[1-4] Fig. 1 shows the microstructure of compact bone consisting of an osteon, haversian canal, and collagen fiber at the microstructural level and the arrangement of collagen molecules, and hydroxyapatite crystals at the nanoscale level. This complex structure of bone and its relation with various mechanical and other functionalities has been the subject of investigation for many decades and is of utmost clinical importance.[5]

The mechanical behavior of bone is essential from a load-bearing viewpoint and its influence on other bio-functionalities. Its biomechanical properties are explored from the elastic nature to the impact or fatigue damage accumulation responsible for fragility or traumatic fractures.[1,6,7] These mechanical responses are very much dependent on their physiological state. The main objective of the biomechanical investigation is to determine the mechanical responses to the type of applied load, extract mechanical properties and develop efficient models to

---

[1] *Department of Mechanical and Industrial Engineering, Manipal Institute of Technology, Manipal Academy of Higher Education, Manipal, Karnataka, India -576104.*
[2] *Department of Mechanical Engineering, Faculty of Science & Engineering, University of Groningen, 9747 AG Groningen, The Netherlands.*
[3] *Department of Metallurgical Engineering and Material Science, Indian Institute of Technology Bombay, Mumbai, India.*
[4] *Mechanical Engineering Cluster, School of Engineering, University of Petroleum and Energy Studies, Bidholi Campus, Dehradun, Uttarakhand, India-248007.*
[5] *Department of Anatomy, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, Karnataka, India -576104.*
**Email:* prasanna.lc@manipal.edu (Prasanna L.C.)
*†These authors contributed equally to this work.*

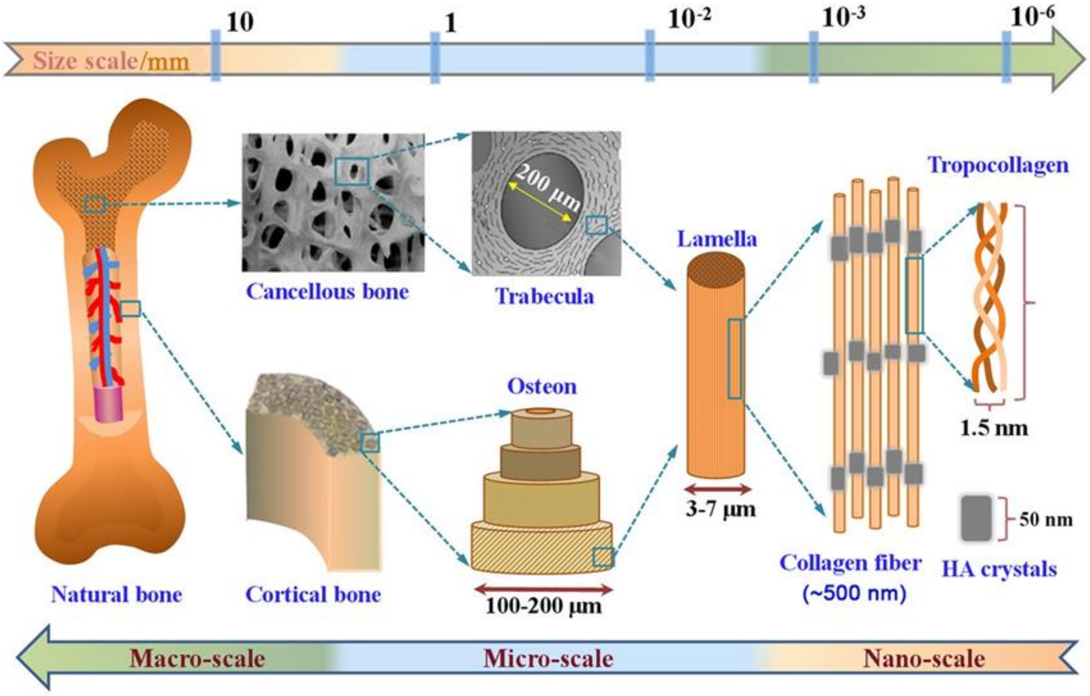

**Fig. 1** The hierarchical structure of typical bone at varying length scales. Reproduced with the permission from [5], Copyright 2017, The Author(s).

evaluate such responses, identify the recoverable and irrecoverable strains in bones, develop optimum treatment methods, and finally use the extracted information in bionics and biomaterials development.[3,8-10] The bone mechanical properties at the macro-level have been fairly investigated.[11-14] In past studies, it has been reported that the biomechanical properties of bones helped select implants of appropriate mechanical properties. So, in this regard, tensile, compression, bending studies, buckling analysis, and ultrasonic measurements were mainly carried out.[8,13] However, these traditional characterization methods are done with different objectives in mind. Many of these studies need a considerable amount of bone, which may not be feasible all the time due to ethical and availability issues. Also, unlike passive engineering materials, biological tissues like bone are highly heterogeneous, and their global mechanical behavior depends on each hierarchical level of organization. The macro-mechanical studies have disadvantages as it lacks to detect and characterize the peculiarities of bone microstructures at the cellular level, *i.e.*, at the trabecula or osteon level.[15,16] Thus, the experimental studies on bone biomechanics are now extended to the submicron level. The studies of geometry, composition, heterogeneity, structural organization, and mechanical properties with nano-level accuracy provide profound information on the mechanical function and dysfunction of bone tissue. The instrumented nanoindentation tool stands out as indispensable in the mechanical study of bone, implants, and other biomaterials.[17-19]

## 2. Nanoindentation

Nanoindentation is a depth-sensing technique in which the depth of penetration is measured with nanometer resolution and provides a mechanical fingerprint of material *i.e.*, load vs. displacement curve. This technique involves a probe of selected tip geometry brought in contact with a sample surface, whose surface mechanical properties need to be investigated. The probe is indented onto the surface of the material and retracted. In this entire process, the variables like displacement ($h$), load ($P$), and time ($t$) are the variables that are recorded. The data are typically used to assess the elastic modulus and hardness. In addition, fracture toughness, viscoelasticity, stress-strain behavior, and creep can be thoroughly investigated.[20-23] Nanoindentation standards are defined by EN ISO 14577-1: 2015, and details listed in Table 1 discern the indentation test at different scales.[5] Fig. 2 shows the schematic of a typical nanoindenter consisting of an upper

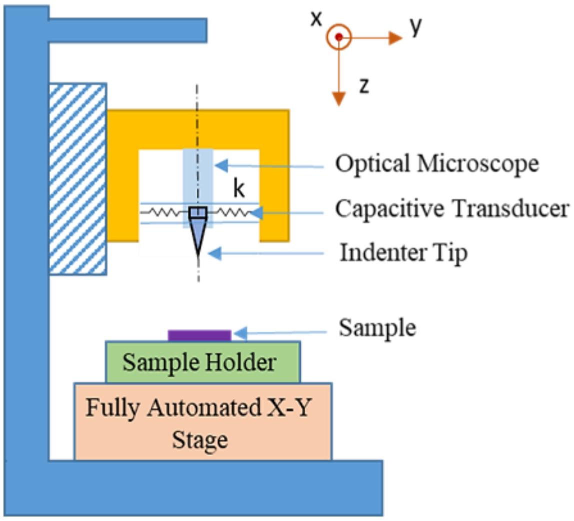

**Fig. 2** The schematic of the nanoindenter setup. Reproduced with permission from [24].

frame consisting of a force and displacement sensing mechanism, an indenter tip holder, an XY sample positioning table, and an optical microscope for precise positioning.

It is seen that in the nanoindentation study of bone and biomaterials, there is no clear boundary between micro and nanoindentation test parameters. The research objective and requirement of capturing accurate material response dictate these parameters' final values. But as a tool, the nanoindenter stands out clearly from the micro indenter by offering a significant advantage in control, resolution, data quality, variety of tests leading to different properties other than hardness.

**Table 1.** Classification of static-indentation tests corresponding to depth and force range.[1,23]

| Scale | Depth of Indentation (δ) | Force (F) |
|---|---|---|
| Macro | δ ≥ 12 μm | 30 kN ≥ F ≥ 2 N |
| Micro | 12 μm ≥ δ ≥ 0.2 μm | 2 N ≥ F ≥ 0.6 mN |
| Nano | 0.2 μm ≥ δ | 0.6 mN ≥ F |

### 2.1 Importance of nanoindentation technique

Nanoindentation has emerged as a leading technique for the nano-mechanical investigation of biological materials.[25-28] One of the reasons for its wide acceptance in bone research is the precise measurements at the nano-scale that helps in understanding the inter-individual biological variations at a microstructural level without being affected by their shape and porosity.[29,30] The contact methodology of this technique allows for region-specific property mapping of bone and biomaterials. It has high spatial resolution and does not require a powerful microscope to measure the impression of the indentation. Fig. 3 elastic property map obtained from nanoindentation test showing spatial and temporal changes in the woven bone of sheep metatarsus generated during the bone transport process at different points of time after surgery. The results helped in identifying the presence of different trends in the evolution of spatial and temporal elastic modulus ($E_r$) in the distraction and docking site calluses. The ability to extract a wide variety of mechanical properties with no restriction on the sample has propelled its application in various research domains, especially in bone mechanics. Now it has become an indispensable mechanical instrument in the interdisciplinary research domain. But its use in any interdisciplinary field dictates the necessity of better understanding its modes, contact models used in data analysis, and influencing parameters.

### 2.2 Modes and contact models

### 2.2.1 Static mode

The nanoindenter has two modes: static and dynamic. Each has different data output. The static test is the most popular mode employed in the interdisciplinary research domain due to its easily understandable indentation physics and direct interpretable properties. The static indentation test aims to extract the biological specimen’s elastic modulus and hardness from loading/unloading data. Fig. 4 shows a typical load-displacement curve (non-DMA mode). In this test, the depth of penetration and the force are recorded as the load is applied from zero to maximum and then retracted back to zero with a pre-defined rate. During retraction, the material attempts to regain its original shape. This is due to the relaxation of elastic strains within the material. Also, there will be a residual permanent deformation remaining on the material's surface due to plastic deformation. The extent of penetration and indenter geometry provides an indirect measurement of the contact area, from which the mean contact pressure can be obtained. This mean pressure ($P$) is used in evaluating the hardness ($H_C$), $H_C = {}^{P}/_{A_C}$, here $A_C$ is the projected contact area. The actual hardness can be evaluated by following the description provided by Oliver-Pharr (2004).

The selection of a contact model for static indentation data analysis depends on probe geometry. For example, the Hertz model is used in the case of a spherical probe. The equations developed by Oliver and Pharr (OP) are employed when a Berkovich indenter tip is selected. The elastic modulus is determined through a linear fitting of an initial portion of the unloading curve, presuming the pure flexible response from the material. The extracted modulus is measured as plane strain modulus indicating resistance to elastic deformation. The OP model can provide accurate estimates of the properties of matured bones where the influence of viscoelastic nature is minimal.[23] While in biomaterials or newly formed bones, this model fails to provide an accurate estimate of properties due to the presence of viscoelastic nature in the bone material. In this case, mathematical models that account for the viscoelastic nature are better suited.

Bones are known to exhibit both elastic and viscous qualities. For a deeper understanding of its mechanical nature, capturing time and frequency-dependent response is vital in several contexts, *i.e.*, understanding irradiation effects and energy dissipation properties of bones,[10] bone response during growth, to measure bone quality associated with initial damage formation that affects fatigue behavior,[12] to get a better insight of bone regeneration process[21] and healing.[32] Nanoindenter offers a promising technique to determine these viscoelastic properties of the bone sample. There are two techniques to capture the viscoelastic response: Creep Test and Nano-Dynamic Mechanical Analysis (Nano-DMA).

The creep test is a traditional method of significant practical interest to study the time-dependent behavior of bone. There is a considerable presence of literature showing attempts to understand the viscoelastic response of bone by creep tests.[12,20,32-34] The creep test is conducted under constant load. The analysis accounts for the changes in contact area based on tip shape while the stress decreases with time. The main difference between static and creep studies lies in data analysis. Here the values of elasticity and viscosity are

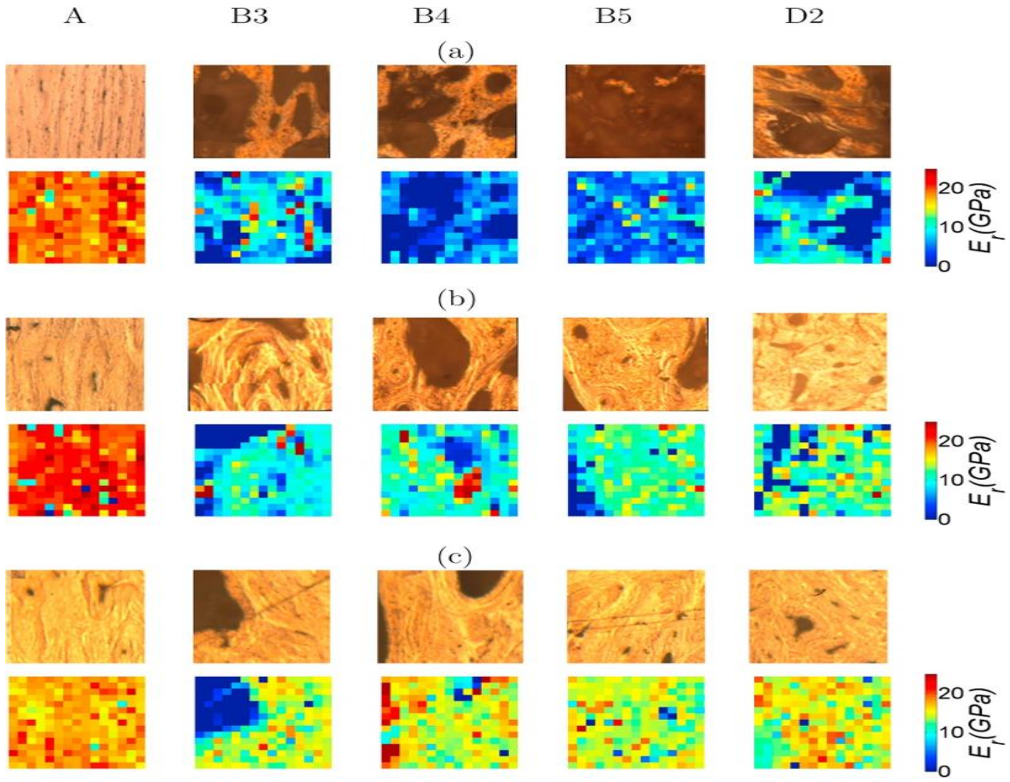

**Fig. 3** Detail of the micrographs in the matrix regions indented and the corresponding elastic modulus maps (below) for the sheep sacrificed (a) 35, (b) 161, and (c) 525 days after surgery (A, in the cortical bone, B3, B4, B5, in the distraction callus, and D2, in the docking site). Reproduced with the permission from [31], Copyright 2014 Elsevier Ltd.

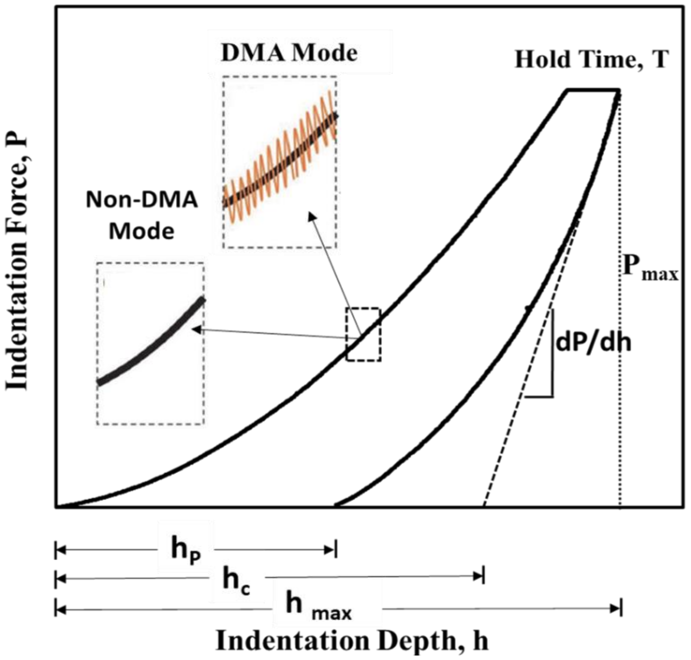

**Fig. 4** Schematic of a *P-h* curve. The maximum ($h_{max}$), contact ($h_c$), and permanent ($h_p$) depths and as well as maximum indentation force ($P_{max}$) are in the schematic. The dotted line signifies the slope during elastic unloading. Insets in the figure highlight the difference between Dynamic Mechanical Analysis (DMA) and static indentation modes (non-DMA). Reproduced with the permission from [36 & 37], Copyright 2008 Elsevier Ltd and 2007, Springer Science Business Media, LLC.

evaluated from the displacement, load, and time data from the hold period or dwell region (as depicted in Fig. 4) using a curve-fitting approach.

The use of an appropriate mathematical model can yield values for the elastic and viscous properties. Most of the reported mathematical models are based on an arrangement of three elements: (1) spring (representing elastic nature); (2) dashpot (representing viscous nature), and (3) slider (representing plastic nature) in a different combination,[23,32] shown in Fig. 5. These spring-dashpot-based models focus on observed phenomena rather than their causes. The creep behavior of murine cortical bone was studied using an equation for conical tip indenter combined with the Maxwell-Voigt model,[15] Details are provided in Table 2. The model is an appropriate choice when there is a linear relation between displacement and time. This model has been used to describe the viscoelastic nature of cortical bones.[34] Oyen M L *et al.* developed a model to capture viscoelastic-plastic (VEP) behavior, and it has three quadratic elements: a spring, a dashpot, and a slider arranged in the series form, shown in Fig. 5. Usually, a sharp Berkovich indenter deforms the bone sample in a viscoelastic-plastic manner. The contribution of viscous, elastic, and plastic components can be linked to the underlying composition and hierarchical microstructural organization of bone tissue.[20] For example, $E_R$ and $H_C$ values

**Table 2.** List of most common Mathematical models used in the indentation data analysis.

| Mode | Name and Equation | Tip / Data / Application |
|---|---|---|
| Static | Oliver-Pharr (OP)<br>$E_R = \frac{S\sqrt{\pi}}{2\sqrt{A_c}}$<br>$\frac{1}{E_R} = \frac{(1-\upsilon_{sp}^2)}{E_{sp}} + \frac{(1-\upsilon_{ind}^2)}{E_{ind}}$<br>$S$= Slope of unloading curve at peak load<br>$E_R$= Reduced modulus<br>$E_{ind}$= Indenter modulus<br>$\upsilon_{ind}$= Indenter Poisson's ratio<br>$E_{sp}$ & $\upsilon_{sp}$= Sample elastic modulus and Poisson's ratio | *Tip:* Berkovich indenter, *Data:* Unloading curve, *Application:* Engineering and hard biomaterials, matured Bones[15,21] |
| | Hertz<br>$P = \frac{4}{3}\sqrt{R}E_R\delta^{3/2}$<br>$P$= Applied external load<br>$R$= Radius of the sphere<br>$\delta$= Elastically recovered depth after unloading | *Tip:* Spherical Probe, *Data:* Loading curve *Application:* Biomaterials, Soft materials[23] |
| Creep | Maxwell-Voigt (Burger)<br>$h^2(t) = \frac{\pi}{2}P_0 \cot\alpha \left[\frac{1}{E_1} + \frac{1}{E_2}\left(1 - e^{-tE_2/\eta_2}\right) + \frac{1}{\eta_1}t\right]$<br>$P_0$= Peak force<br>$\alpha$= Equivalent cone semi angle<br>$E_1, E_2$=Elastic parameters (GPa)<br>$\eta_2$= Long-term creep viscosity (GPa-s)<br>$\eta_2/E_2$=Creep time constant (s) | *Tip:* Conical probe, *Data:* Load-displacement data in hold period,<br>*Application:* Materials showing creepy nature like young bones[12,15,20,34] |
| | Viscoelastic plastic (VEP)<br>$H_C = \frac{1}{\alpha_1\left(2t_R/3\left(\alpha_3\eta_Q\right)^{-1/2} + (\alpha_2E_R)^{-1/2} + (\alpha_1H)^{-1/2}\right)}$<br>$\alpha_1$, $\alpha_2$ & $\alpha_3$= Constants<br>$t_R$= Time rises<br>$\eta_Q$= Indentation viscosity<br>H= Hardness<br>Hc= Resistance to the total deformation | *Tip:* Berkovich Probe, *Data:* Loading, holding period and unloading,<br>*Application:* Young bones, Biomaterials[21,29,32] |
| Nano-DMA | Viscoelastic<br>$E^* = E' + E''$, $E' = \frac{K_s\sqrt{\pi}}{2\sqrt{A_c}}$, $E'' = \frac{\omega C_s\sqrt{\pi}}{2\sqrt{A_c}}$,<br>$\tan\theta = \frac{E''}{E'}$ $H = \frac{P}{A_c}$<br>$E^*$= Complex modulus, $E'$ = Storage modulus, $E''$=Loss modulus, $\theta$=Phase angle ($0^0$ to $90^0$), $A_c$= Contact area, $\omega$ = Oscillating frequency, $K_s$= Stiffness, $C_s$= Damping coefficient | *Tip:* Berkovich tip, *Data:* Holding period data collected during oscillating load<br>*Application:* Young bones and biomaterials. with viscoelastic nature[38] |

are compelled by the amount of mineral phase. If the same properties were evaluated using the OP model, they would be coupled to the level of maturity of collagen fibers.[35] The material properties like $\eta_Q$, $E_R$, and $H$ is extracted by curve fitting the creep data, unloading curve, and loading curve data using respective equations in the model.[21,32] The details of popular models like the viscoelastic-plastic (VEP) model and Maxwell-Voigt model (Burger Model) are provided in Table 2.

The VEP model assumes the material is a viscoelastic plastic with a linear creep rate. Limitations of this model are, (a) it is a single-time constant model, and (b) its prediction is limited when studying hierarchical material. The Burger and Generalized Maxwell models have two-time constants in stress relaxation, while the standard linear solid model has only a single time constant.[12,13] Overall, these models are helpful for the comparison of groups of samples in a study. These models express mechanical properties in a broader spectrum of deformation components. In a study conducted on osteonal cortical bone bovine femora, it is noted that values of creep time constants increase with an increase in indentation load hold time, and for accurate property measurements, a hold time greater than 15 secs is recommended and beyond 30 sec, properties get affected by the thermal drift of the instrument.[13] During the study, the sample was thawed in PBS and tested after removing the excess water without drying otherwise it will influence extracted time constant. Also, it should be noted here that macroscopic and microscopic viscous behavior is distinctly different, and extracted properties from these tests vary by several magnitudes.[12] So nanoindentation-based creep tests cannot substitute macroscopic behavior study and vice versa. This is because the macroscale viscous behavior of bone is likely a result of fluid flow in pores and interaction with microstructure. These effects cannot be captured in small-scale nanoindentation.

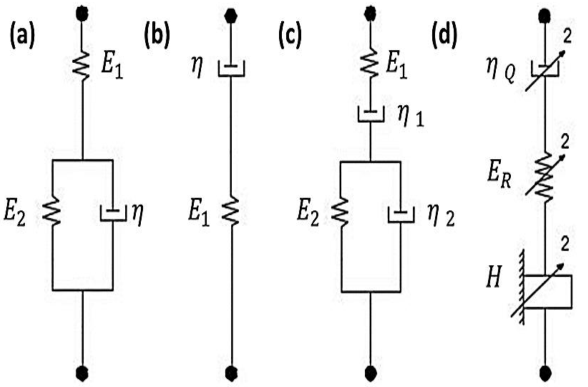

**Fig. 5** (a) Three-element Voigt model for a viscoelastic material (delayed elasticity), (b) Maxwell representation of a viscoelastic material (steady creep), (C) Four-element combined Maxwell-Voigt (Burger's) model, (d) Viscoelastic-plastic model (VEP) on loading. Reproduced with the permission from [23], Copyright 2020 Acta Materialia Inc.

### 2.2.2 Nano-DMA

Nano-Dynamic Mechanical Analysis (Nano-DMA) or continuous stiffness mode (CSM) is an alternate method to study the viscoelastic properties of bone tissue.[20,39,40,41] In this test, a small oscillating displacement (10-200 Hz) is imparted to the indenter in contact with the sample surface, as shown in Fig. 4. The displacement and the load signals will provide data whereby the specimen's elastic, viscous, and frequency-dependent responses can be captured. Here, the data analysis provides a complex modulus ($E^*$) consisting of two distinct components. The first one is storage modulus ($E'$) and is proportional to the energy stored during the oscillating cycle, representing the stiffness of viscoelastic material. The other is loss modulus ($E''$), which represents the energy dissipated during the oscillating cycle and is associated with friction and various internal molecular motions. Combined storage and loss modulus represent the viscoelastic property of the bone material ($E^* = E' + E''$). The loss tangent ($\tan\theta = E''/E'$) is evaluated by taking the ratio of two parameters and it provides a phase angle (θ), details provided in Table 2. The phase angle varies between $0^0$ (pure elastic) to $90^0$ (pure viscous) and its value signifies the extent of the viscoelastic nature present in the material. The nano-DMA technique can be employed to generate a force map that can be used to generate a map of bone viscoelastic properties.[41] In a study of mouse femora bones in a hydrated state, it was noted that the loss tangent might act as necessary information in studying the porosity and its distribution.[39] A study by Pathak S (2015), noted the existence of an inverse relationship between viscoelastic response and mineral-to-matrix ratio. Also, a decrease in tan δ values is an indication of a higher degree of maturity of the bone in the region. Compared to static nanoindentation, nano-DMA is less explored in the interdisciplinary domain of research. This technique helps elucidate the compositional and property relationship in bone. Also, it helps to know how such relationships might affect the whole bone performance.[40]

### 2.3 Influence of testing parameters

The continuous advances in instrumentation and software interface have led to automation in nanoindenter operation and data analysis. However, to assure the accuracy of extracted properties and take complete advantage of a nanoindenter in its various applications necessitates a clear understanding of fundamental principles involved in its working mechanism, selection of experimental parameters like loading/unloading rate, maximum force or penetration depth, and probe geometry.[17,20,42] Also, clarity in the steps to be followed while preparing the bone samples, understanding of the influence of specimen surface roughness, limitations of mechanical contact models used in the data analysis, and the effect of the sample testing environment are necessary. Here an attempt is made to shed light on some of the important influencing parameters.

The mechanical deformation strongly depends on the indenter tip geometry and it influences the outcome significantly. Spherical tips generate a larger initial elastic regime compared to shaper tips. The spherical probe shape minimizes stress concentrations and plastic deformation compared to Berkovich tips, thus allowing extraction of elastic modulus and indentation yield strength before the damage.[43] Data of the initial loading portion of the stress-strain curve is fitted with the Hertz contact model till the appearance of inelastic deformation and also, initial unloading indentation data is used for extraction of elastic property. In this approach, the plastic deformation induced during the loading segment is likely to influence extracted properties and a successful analysis of the unloading segment using Hertz's theory needs calibrated area functions and effective indenter shapes.[39] Paietta R C *et al*., investigated the role of the contact depth, the contact area, and the tip radius using a spherical indenter.[44] It was noted that in the case of a tip with a smaller radius, indentation modulus increases with contact depth and plastic deformation. With the increase in tip radius, the surface area of contact increases and provides the average property over the region of contact. So specific contributions of features smaller than the contact area will be averaged out. A tip with a relatively smaller radius is best suited to study the underlying lamellar bone structure. The majority of the literature shows wide acceptance of Berkovich indenters and is commonly used for indenting hard biomaterials like bone. The sharp Berkovich indenter does not evoke the viscosity significantly but causes plastic deformation at the place of indentation. It is preferred in the study of trabecular bone where the thickness sample in the testing zone is limited. A more detailed analysis of the proper tip selection and its effect on mechanical properties can be found in the reference.[17,29]

In the case of a biological sample, extracted properties do alter depending on the anatomical location and direction of the sample. It is seen that anisotropy influences are more significant in the extracted properties when tested along a transverse direction compared to longitudinal.[45-47] It is hypothesized that the presence of collagen in the form of bundles is a cause for this. Also, it can be due to homogeneous mineralization along the length of the bone.[45] Reigigner A G *et al*., proposed a novel method to capture the orthotropic elastic properties of human secondary osteon lamella assembly.[48] The tests were conducted on three distinct planes of bone lamellae of the same osteon (dehydrated). It was found that osteons are stiffer in the longitudinal direction than circumferential. Maximum stiffness was observed on a plane slightly rotated to the osteon axis indicating the presence of moderate helical winding in the osteon development.[48] Similarly, in another study on porcine femoral bone differing elastic modulus and hardness were obtained in proximal, central, and distal parts. Also, noted significantly higher values of properties along the longitudinal direction than transverse.[27] These studies indicate the presence of non-uniform development in bone microstructure attributing to a varied level of mineralization or differences in collagen fibril orientation.

Apart from this, the state of tissue hydration also plays a crucial role in tissue response to mechanical testing. Such as a study by Bushby A J *et al*., (2004).[48] on a sample made from a cortical region of the third metacarpal bone of a horse, a considerable increase in modulus was noted when the sample was tested in wet, dehydrated (in ethanol), and embedded condition (in PMMA). In general, noted a significantly higher magnitude of properties in a dehydrated state compared to a hydrated one.[9,29,38] But these procedures will not affect the comparative trends.[45]

Similarly, the temperature is also known to affect the extracted properties. A three-point bending test over specimens from the mid-diaphysis region of human cadaveric femurs (in dehydrated condition) showed an increase the bending stiffness and strength with an increase in temperature.[40] This is attributed to the loss of water with an increase in temperature but significantly decreases bone toughness due to loss of plasticity. In another study, it was noted that the creep behavior of bovine cortical bones gets affected by changes in temperature.[34] It will be interesting to see the effect of temperature on the bone while testing under a physiological environment. The nanoindenter may provide critical insight into variations in the viscoelastic nature of bone. Another important parameter that has a substantial influence on indentation response is surface roughness. The large-scale corrugations affect the outcome of the nanoindentation test, as the test demands that a flat surface is required to have well-defined contact between the tip and specimen surface. These corrugations or roughness have to be brought under a certain level relative to indentation depth.[50] This can be attained by adopting a polishing on silicon carbide papers of increasing grit size under deionized water. Further slurries with different particle dimensions can be used to attain the required surface roughness. The RMS value of roughness can be evaluated using an AFM-based imaging technique or using the nanoindenter itself. In the study of irradiated human bone samples, the ratio of indentation depth to roughness was maintained in the order of 31.[9] In general, as per the ISO14577-2 standard, surface roughness has to be less than 5% of the maximum indentation depth.[51] Another crucial factor that may influence the indentation response is subsurface voids. In their presence, the specimen may show a significantly softer response. A focused ion beam-scanning electron microscope (FIB-SEM) can be used to identify subsurface cavities or lacunae in the testing zone. This process helps in reducing the scatter of nanoindentation data and tissue-specific properties.[46] Clarity on all these factors is necessary for successful experimental conduction, data analysis, and result interpretation. It helps obtain meaningful outcomes and increases the possibility of validation with published literature.

As an instrument, the nanoindenter inherently has a thermal drift issue. Calibration of the device, indenter tip shape, and adoption of standard operating procedures are required to achieve reproducibility of material properties.[40] Thermal drift can influence the creep test results, which can be minimized by including the 20-30 min time gap between setting up the instrument and the experiment. This will allow the instrument to reach the same temperature as the material. Another issue in data analysis is the evaluation of contact area due to piling up or sink-in around the indentation zone.[39] This needs the adoption of correction factors and a proper selection of analysis methods to address the issue.[9,15] In case not done, pile-up can lead to overestimation in calculated hardness and elastic modulus (apparent). The errors can be as high as 60%.[23] The AFM-based imaging method can be used to visualize the presence of pile-up or sink-in and in evaluating the contact area. Overall, it is important to have clarity on experimental and all other influencing parameters for the extraction of more meaningful and reproducible biomechanical properties from the bone sample.

## 3. Nanoindentation Applications

Nanoindentation has evolved as a powerful technique for studying variations in biomechanical properties at the submicron level. It has been used in various studies on the effect of aging, cancer, and osteoporosis, analysis of treatment effects, the development of biomaterials, etc. Some of its applications are discussed here to highlight nanoindenter utility.

### Aging

The nanoindentation plays a greater role in studying the consequence of age on bone mechanical behavior and its anisotropic microstructure. A study of the porcine femoral cortical bone of different ages (6, 12, and 42 months) under hydrated conditions showed an increase in elastic modulus and hardness of individual lamellae within bone's microstructure (laminar, interstitial bone, and osteons).[27] A similar study by Feng L., 2012[52] noted a similar outcome but it was observed that the magnitude of increment was different for each microstructural component indicating the presence of different rates of development. During the growth period, the bone tissue tends to remodel itself regularly. But in the later stage of normal life, the balance in bone remodeling is lost in favor of bone resorption leading to significant changes in bone quality, mineral mass, and mechanical properties.[53] This increases the risk of osteoporotic fractures.[54] It was noted that nano-mechanical properties of bone do not vary with aging after bone maturation or with osteoporosis.[40] Another study on human femora of various ages (35-95 years) shows that recorded variations in elastic modulus ($E_R$) at bone matrix level are slight and observed no apparent difference in nanomechanical properties of younger and older bones.[55] The outcome of these studies indicates that the effects of resorption are localized and cannot be found across the bone. Ojanen *et al.* 2015[51] conducted a creep test (250 mN force and 60s hold time) on a hydrated trabecular bone sample extracted from the femoral neck of male cadavers aged between 17-82 years, the test was conducted in the longitudinal direction of trabeculae and data was analyzed using Burgers model (Table 2). The study showed no age-related changes in elastic moduli ($E_1$ and $E_2$), viscosities $\eta_1$ and $\eta_2$, and creep time constant. Instead, the crystallinity determined using Raman spectroscopy was found to be significantly related to nanoindentation properties. So it can be concluded that there is no specific association between nanomechanical properties and age or that the mineral crystallinity that contributes to the viscoelastic properties of trabecular bones may be independent of human age.[51]

### Osteoporosis

It is a degenerative disease that leads to high bone turnover due to adverse remodeling, resulting in reduced bone quality.[56] This disease is caused by disproportionate activity between osteoblast and osteoclast cells.[57] In the recent past, variation in bone mineral density was accepted as an accurate indicator of this disease. With the advent of nanoindenters and other material characterization tools and techniques, the focus is shifted toward assessing the variation in microstructural bone parameters. The static nanoindentation tests showed increased elastic modulus and hardness.[57] This is due to increased hydroxyapatite crystallite growth and was ascribed to modification in the organic matrix. The XRD and Raman spectrometer confirmed changes in the organic matrix at the microstructural level. This increased mineralization of osteoporotic trabeculae leads brittle nature and reduced macroscopic compressive strengths.

Bisphosphonates (BP), an anti-resorptive agent, have been a standard drug for the treatment of osteoporotic patients. A detailed study on BP treatment for 1.1 to 20 years shows an increase in the elastic modulus ($E_R$) of bone in the first six years and remains unchanged later. Also, hardness peaked at 12.4 years and remained constant for the next 7.6 years.[58] Here the nanoindentation tests were conducted on anterior iliac crest bone collected from a Caucasian woman using a Berkovich tip (peak load 8mN, loading rate 0.4 mN/s, 10s hold time). In another study on the effect of pharmacologic agents on bone mechanical behavior at the osteocyte level, OVX rats treated for 270 days (9 months) period with a combination of Raloxifene, parathyroid hormone (hPTH), and alendronate was considered.[59] A nanoindentation study (peak load 1mN, holding time 3 sec, and rate 250 μN/s) on the bone specimen from a cortical region of the tibia was conducted along with SEM imaging to explore the relationship of structure difference in hard tissues. Through these tests, it was found that osteoporosis treatment agents do not affect the mechanical properties near osteocyte lacunae and the perilacunar matrix near cortical osteocyte lacuna is less stiff than the bone matrix further away.

### Osteogenesis imperfecta and Type-1 diabetes mellitus

Osteogenesis imperfecta is a genetic disorder that causes reduced bone quality and excessive skeletal deformations leading to increased fragility. Fiedler *et al.* (2018)[60] used an array of bone quality analysis tools, including nanoindenter, and proposed that Chihuahua Zebrafish is a valid model for studying classical dominant human osteogenesis imperfecta. Cai J *et al.*'s (2018)[61] work on Type-1 diabetes mellitus (TiDM) shows that pulsed electromagnetic field (PEMF) treatment improves the bone microarchitecture, mechanical properties, and osseointegration of porous titanium implanted in a rabbit. Load-controlled nanoindentation studies on re-hydrated specimens were conducted on the cortical and peri-implant bone regions. PEMF induced an increase in elastic modulus and hardness in diabetic rabbits indicating a significant improvement in intrinsic cortical bone material properties. The treatment using external electromagnetic signals enhanced the osseointegration of the implant. Similarly, nanoindentation tests can be instrumental in establishing disease, signal pathways, or genes of interest that influence bone tissue at microscopic levels.[40]

### Cancer and Radiotherapy Treatment

One of the most aggressive cancers known to humankind is malignant melanoma which metastasizes to multiple sites, including the lungs, liver, and bone. The reduction in bone density and altered microanatomy due to metastasis harm the mechanical performance of bone. Studies show that nanoindentation can measure the impact of cancer progression and treatment outcomes quantitatively.[9,10,62] In a study of female athymic rats inoculated with HeLa Cancer cells for 21 days, nanoindentation was used to evaluate the modifications in bone mineral density and biomechanical properties on sagittal L5 vertebrae. The study showed reduced mineral crystal growth, diminished values of hardness, and elastic modulus compared to the control specimen.[62] Similarly, in another study on cancer-induced rat tibias, lower values of elastic modulus were noted compared to sham-operated ones. Creep data extracted during the 30 sec hold period at depth of 500 nm and analyzed using Voigt Model showed lower values of viscosity compared to sham-operated ones. Cancer metastasis increases the risk of fragility fractures in the bone.[63] In another study on B16F10 cell inoculated C57BL/6 mice bone, nanoindentation tests were conducted in the longitudinal axis of the rehydrated bone and noted significantly lower values of Young's modulus compared to controlled bone tissue. These lower values were attributed to disorganization in bone microstructure.[64]

Radiotherapy intends to improve clinical outcomes in cancer patients with soft tissue sarcomas. But irradiation affects the collagen network and leads to decreased osteoblasts and changes the bone matrix composition.[9,65] This further influences the bone's mechanical properties negatively. Nanoindentation studies on irradiated human femur bones showed a reduction in elastic modulus and hardness. The time-dependent creep properties studies showed more pronounced creep behavior in irradiated samples compared to pre-irradiated ones.[9,50] Such investigations help in gaining better insight into the radiotherapy treatment effects and its potential role in post-radiotherapy fragility fractures. Given that two-thirds of cancer patients undergo radiotherapy treatment, there is a necessity to identify optimal parameters to mitigate the negative consequence of this treatment procedure.

### Biomaterial and Orthopedic Applications

The continuous advancement of experimental and computational techniques has contributed to understanding underlying mechanisms related to age, disease, and treatment. So, the focus of studies has shifted to extracting the type of data needed to develop novel biomaterials and treatments. The design and synthesis of multi-functional biomaterials (bionics) have improved substantially and enabled the regeneration of damaged tissues.[66] One of the prime challenges an orthopedic surgeon face is the fixation of fractures as they tend to produce subtle progressive inflammation at the bone-implant interface. Bioceramic like calcium phosphate is most adopted for a variety of orthopedic applications, but their brittleness and low toughness may lead to fractures, and limit their applications. Hence more suitable alternatives are under exploration. Zhao R *et al.* (2020)[56] used the nanoindentation technique to understand the mechanical configuration of an SrWCP bioceramic scaffold used in repairing large bone defects due to osteoporotic conditions. The mechanical properties of newly formed bone and the remaining material in the healing area were tested by applying a load of 10 mN at a loading/unloading rate of 20 mN/min using the Berkovich indenter and data was analyzed using the OP model. The study showed SrWCP bio-ceramic as a promising and safe substitute for the treatment of osteoporotic bone defects promotes local bone regeneration and implant osseointegration from the fractured bone itself.

Nanoindenter is also used in assessing the evolution of material properties of woven bone during the regeneration process and fracture healing,[31] Fig. 3. One of the factors that contribute to the macroscopic stiffness of distraction callus is continuous improvements in the microscopic properties of woven bone during distraction osteogenesis. The samples harvested from sheep intervened bone (35, 161, and 525 days after surgery) from cortical, distraction callus, and docking sites were tested using the nanoindentation technique (Berkovich diamond indenter, Peak Load 5 mN, Rate 0.5 mN/s, hold time 40 sec). The study showed a continuous increase in the elastic modulus ($E_R$) of woven bone during the bone transport process. The rate of increase in $E_R$ reduced with time in both distraction and docking site calluses. These outcomes indicate the presence of a similar evolution pattern as in fracture healing. Magnesium alloy-based degradable bone substitutes are another alternative to biological bone grafts. In a study conducted in in-vivo and in-vitro environments, it was noted that both magnesium concentration and the average modulus of the degrading layer were significantly lower than that of the base material.[67] The application of Nanoindenter and Scanning electron microscopy (SEM) showed variation in localized mechanical and material properties in magnesium implants with degradation. It is possible to develop an alternative to biological bone grafts to address patient-specific issues with the help of knowledge of mechanical properties variation during the degradation process and can be used as input to develop suitable computational models and Finite Element Simulations.[23]

## 4. Advantages of combined nanomechanical and compositional studies

In the case of bone studies, composition plays a crucial role in bone integrity.[15] So along with bone nanomechanical properties, its material characterization is equally essential. Many different techniques can be selected depending on the physio-chemical characteristics to be studied.[68] These techniques help to evaluate bone quality in the form of a mineral-to-matrix ratio, the carbonate-to-phosphate ratio, collagen quality, and crystallinity. These properties are vital as they allude to the organization and design of bone tissue. Together decide the capacity to perform mechanical functionalities. So, a combination of material characterization techniques and nanomechanical investigation using a nanoindenter can be a better approach in the studies. The link-up between compositional and mechanical changes will deepen understanding of the overall bone functionality changes.[9,39,68] Using co-localized Raman spectroscopy and nanoindentation is advantageous and it has helped in the understanding of the age-dependent patterns in mechanical and compositional properties at the tissue scale in murine femora.[15] In another study on the effect of vitamin D deficiency on growing rats, the author used the Micro-CT scan attenuation values for the evaluation of weighted section moduli, co-localized tissue-level mechanical and compositional information using nanoindentation, and Raman spectroscopy technique.

### 4.1 Future perspectives

Mechanical properties in the nanostructure of bone differ among age, sample direction, diseases, anatomical sites, and individuals. Evaluating such mechanical changes and corresponding structural changes has been important in our understanding of bone's mechanotransduction. This review tried to link the role of bone quantity & quality has to play in the mechanical behavior of bone tissue. Also, it gives insight into the unexplored areas of bone research and opens up various orthopedic treatment advancements & evolvement of various new treatment techniques to treat bone disorders.

## 5. Conclusions

The review highlights its successful application in the study of bone material mechanics to extract the properties like hardness and elastic modulus using static indentation and viscoelastic modulus using creep and nano-DMA tests. But as a tool, nanoindenter only provides the sample response to an applied loading condition and the analysis of obtained response (force-indentation data) using relevant mathematical models yields the biomechanical properties. Its successful application as an experimental tool needs a clear understanding of various influencing parameters coming from setup, selection of indenter, experimental parameters, surface roughness, mathematical models, etc. The review provides the necessary information to the researchers in the interdisciplinary domain about nanoindenter mode, major influencing parameters, and recent applications. In the past few years, a combination of material characterization techniques along with nanomechanical investigation has gained importance. This helps in identifying the changes in response to mechanical loading and the reasons for it. Overall information gathered from nanoindentation experiments will be useful in understanding the mechanism of bone formation, bone transport, healing, the effect of disease progression, improving the treatment procedures, and even developing suitable computation models for inference purposes.

## Acknowledgment

The authors thank Dr. Nagamani Jaya Balila, Metallurgical Engineering, and Materials Science, Indian Institute of Technology Bombay, Mumbai, India, for providing conceptual suggestions for improving the manuscript. The authors would like to acknowledge the financial support for this project from the TARE fellowship (Grant no: TAR/2018/00133) offered by the Science and Engineering Research Board (SERB), Government of India.

## Conflict of Interest

There is no conflict of interest.

## Supporting Information

Not applicable.

## References

[1] P. K. Zysset, Indentation of bone tissue: a short review, *Osteoporosis International*, 2009, **20**, 1049-1055, doi: 10.1007/s00198-009-0854-9.

[2] N. Reznikov, R. Shahar, S. Weiner, Bone hierarchical structure in three dimensions, *Acta Biomaterialia*, 2014, **10**, 3815-3826, doi: 10.1016/j.actbio.2014.05.024.

[3] J. Scheinpflug, M. Pfeiffenberger, A. Damerau, Journey into Bone Models: A Review. *Genes (Basel),* 2018, **9**, 247, doi: 10.3390/genes9050247.

[4] P. Lemoine, J. Acheson, S. McKillop, J. J. van den Beucken, J. Ward, A. Boyd, B. J. Meenan, Nanoindentation and nano-scratching of hydroxyapatite coatings for resorbable magnesium alloy bone implant applications, *Journal of the Mechanical Behavior of Biomedical Materials*, 2022, **133**, 105306, doi: 10.1016/j.jmbbm.2022.105306.

[5] C. Gao, S. Peng, P. Feng, C. Shuai, Bone biomaterials and interactions with stem cells, *Bone Research*, 2017, **5**, 17059, doi: 10.1038/boneres.2017.59.

[6] S. Xie, K. Manda, R. J. Wallace, F. Levrero-Florencio, A. H. R. W. Simpson, P. Pankaj, Time dependent behaviour of trabecular bone at multiple load levels, *Annals of Biomedical Engineering*, 2017, **45**, 1219-1226, doi: 10.1007/s10439-017-1800-1.

[7] D. Sundh, M. Nilsson, M. Zoulakis, C. Pasco, M. Yilmaz, G. J. Kazakia, M. Hellgren, M. Lorentzon, High-impact mechanical loading increases bone material strength in postmenopausal women—a 3-month intervention study, *Journal of Bone and Mineral Research*, 2018, **33**, 1242-1251, doi: 10.1002/jbmr.3431.

[8] R. Oftadeh, M. Perez-Viloria, J. C. Villa-Camacho, A. Vaziri, A. Nazarian, Biomechanics and mechanobiology of trabecular bone: a review, *Journal of Biomechanical Engineering*, 2015, **137**, 010802, doi: 10.1115/1.4029176.

[9] S. Chauhan, S. A. Khan, A. Prasad, Irradiation-induced compositional effects on human bone after extracorporeal therapy for bone sarcoma, *Calcified Tissue International*, 2018, **103**, 175-188, doi: 10.1007/s00223-018-0408-2.

[10] S. Chauhan, K. Manoj, S. Rastogi, S. A. Khan, A. Prasad, Biomechanical investigation of the effect of extracorporeal irradiation on resected human bone, *Journal of the Mechanical Behavior of Biomedical Materials*, 2017, **65**, 791-800, doi: 10.1016/j.jmbbm.2016.09.032.

[11] A. A. Abdel-Wahab, K. Alam, V/ V. Silberschmidt, Analysis of anisotropic viscoelastoplastic properties of cortical bone tissues, *Journal of the Mechanical Behavior of Biomedical Materials,* 2011, **4,** 807-820, doi: 10.1016/j.jmbbm.2010.10.001

[12] T. N. Shepherd, J. Zhang, T. C. Ovaert, R. K. Roeder, G. L. Niebur, Direct comparison of nanoindentation and macroscopic measurements of bone viscoelasticity, *Journal of the Mechanical Behavior of Biomedical Materials,* 2011, **4**, 2055-62, doi: 10.1016/j.jmbbm.2011.07.004.

[13] D. Wu, P. Isaksson, S. J. Ferguson, C. Persson, Young's modulus of trabecular bone at the tissue level: A review, *Acta Biomaterialia,* 2018, **78**, 1-12, doi: 10.1016/j.actbio.2018.08.001.

[14] D. B. Kimmel, S. Vennin, A. Desyatova, J. A. Turner, M. P. Akhter, J. M. Lappe, R. R. Recker, Bone architecture, bone material properties, and bone turnover in non-osteoporotic post-menopausal women with fragility fracture, *Osteoporosis International*, 2022, **33**, 1125-1136, doi: 10.1007/s00198-022-06308-y.

[15] M. Raghavan, N. D. Sahar, D. H. Kohn, M. D. Morris, Age-specific profiles of tissue-level composition and mechanical properties in murine cortical bone, *Bone,* 2012, **50**, 942-953, doi: 10.1016/j.bone.2011.12.026.

[16] Y. T. Chang, C. M. Chen, M. Y. Tu, H. L. Chen, S. Y. Chang, T. C. Tsai, Y. T. Wang, H. L. Hsiao, Effects of osteoporosis and nutrition supplements on structures and nanomechanical properties of bone tissue, *Journal of the Mechanical Behavior of Biomedical Materials,* 2011, **4**, 1412-20, doi: 10.1016/j.jmbbm.2011.05.011.

[17] D. M. Ebenstein, L. A. Pruitt, Nanoindentation of biological materials, *Nano Today*, 2006, **1**, 26-33, doi: 10.1016/s1748-0132(06)70077-9.

[18] E. Broitman, Indentation hardness measurements at macro-, micro-, and nanoscale: a critical overview, *Tribology Letters*, 2017, **65**, 1-18, doi: 10.1007/s11249-016-0805-5.

[19] A. L. Boskey, L. Imbert, Bone quality changes associated with aging and disease: a review, *Annals of the New York Academy of Sciences journal,* 2017, **1410**, 93-106, doi: 10.1111/nyas.13572.

[20] H. Isaksson, S. Nagao, M. Małkiewicz, P. Julkunen, R. Nowak,J. S. Jurvelin, Precision of nanoindentation protocols for measurement of viscoelasticity in cortical and trabecular bone, *Journal of Biomechanics,* 2010, **43**, 2410-2417, doi: 10.1016/j.jbiomech.2010.04.017.

[21] M. Boi, G. Marchiori, M. Sartori, F. Salamanna, G. Graziani, A. Russo, A Nanomechanical investigation of engineered bone tissue comparing elastoplastic and viscoelastoplastic modeling, *Advances in Materials Science and Engineering,* 2017, **2017,** 7472513, doi: 10.1155/2017/7472513.

[22] R. Singleton, G. Pharr, J. Nyman, Increased tissue-level storage modulus and hardness with age in male cortical bone and its association with decreased fracture toughness, *Bone*, 2021, **148**, 115949, doi: 10.1016/j.bone.2021.115949.

[23] A. C. Fischer-Cripps, Nanoindentation, In: Mechanical Engineering Series, 3rd Edition, Springer New York, doi: 10.1007/978-1-4419-9872-9.

[24] G. Hamdan, T. Granz, M. Bertke, Z. Li, P. Puranto, U. Brand, Nanomechanical traceable metrology of vertically aligned silicon and germanium nanowires by Nanoindentation, In. Multidisciplinary Digital Publishing Institute Proceedings, 2017, **1**, 375, doi:10.3390/proceedings1040375.

[25] V. Managuli, S. Roy, An AFM dynamic contact model with finite thickness correction to study micro-rheology of biological cells, *Experimental Techniques*, 2018, **42**, 551-561, doi: 10.1007/s40799-018-0268-8.

[26] V. Managuli, S. Roy, Influencing Factors in Atomic Force Microscopy Based Mechanical Characterization of Biological Cells. *Experimental Techniques*, 2017, **41**, 673-687, doi.org/10.1007/s40799-017-0199-9.

[27] C. Luo, J. Liao, Z. Zhu, X. Wang, X. Lin, W. Huang, Analysis of Mechanical Properties and Mechanical Anisotropy in Canine Bone Tissues of Various Ages, *BioMed Research International,* 2019, **2019**, 3503152, doi: 10.1155/2019/3503152.

[28] S. Pei, Y. Zhou, Y. Li, T. Azar, W. Wang, K. Do-Gyoon, X. S. Liu, Instrumented nanoindentation in musculoskeletal research. *Progress in Biophysics and Molecular Biology*, 2022, doi: 10.1016/j.pbiomolbio.2022.05.010.

[29] N. Rodriguez-Florez, M. L. Oyen, S. J. Shefelbine, Insight into differences in nanoindentation properties of bone, *Journal of the Mechanical Behavior of Biomedical Materials,* 2013, **18**, 90-99, doi: 10.1016/j.jmbbm.2012.11.005.

[30] J. W. Wang, K. Yu, M. Li, Application of nanoindentation technology in testing the mechanical properties of skull materials. *Scientific Reports,* 2022, **12**, 8717, doi: 10.1038/s41598-022-11216-6.

[31] J. Mora-Macías, A. Pajares, P. Miranda, J. Domínguez, E. Reina-Romo, Mechanical characterization via nanoindentation of the woven bone developed during bone transport, *Journal of the Mechanical Behavior of Biomedical Materials,* 2017, **74**, 236-244, doi: 10.1016/j.jmbbm.2017.05.031.

[32] M. L. Oyen, C. C. Ko, Examination of local variations in viscous, elastic, and plastic indentation responses in healing bone. *Journal of Materials Science: Materials in Medicine,* 2007, **18**, 623-628, doi: 10.1007/s10856-007-2311-7.

[33] Z. Wu, T. A. Baker, T. C. Ovaert, G. L. Niebur, The effect of holding time on nanoindentation measurements of creep in bone. *Journal of Biomechanics,* 2011, **44**, 1066-1072, doi: 10.1016/j.jbiomech.2011.01.039.

[34] H. A. A. Abdalkadum, B. A. Bedaiwi, Temperature Effects on Creep Behaviour of Bovine Cortical Bones, *In IOP Conference Series: Materials Science and Engineering*, 2018, **454**, 012156, doi: 10.1088/1757-899X/454/1/012156.

[35] Y. Bala, B. Depalle, T. Douillard, S. Meille, P. Clément, H. Follet, J. Chevalier, G. Boivin, Respective roles of organic and mineral components of human cortical bone matrix in micromechanical behavior: an instrumented indentation study, *Journal of the Mechanical Behavior of Biomedical Material,* 2011, **4**, 1473-1482, doi: 10.1016/j.jmbbm.2011.05.017.

[36] S. Kumar, I. A. Kumar, L. Marandi, I. Sen, Assessment of small-scale deformation characteristics and stress-strain behavior of NiTi based shape memory alloy using nanoindentation, *Acta Materialia,* 2020, **201,** 303-315, doi: 10.1016/J.ACTAMAT.2020.09.080

[37] I. Sen, S. S. Kumar, Characterizing Stress-Strain Behavior of Materials through Nanoindentation. In A. P. G. A. Evingür, & Ö. Pekcan (Eds.), Elasticity of Materials, *IntechOpen*. 2021, doi: 10.5772/intechopen.98495.

[38] S. Pathak, J. G. Swadener, S. R. Kalidindi, H. W. Courtland, K. J. Jepsen, Measuring the dynamic mechanical response of hydrated mouse bone by nanoindentation. *Journal of the Mechanical Behavior of Biomedical Materials,* 2011, **4**, 34-43, doi: 10.1016/j.jmbbm.2010.09.002

[39] S. Pathak, S. R. Kalidindi, Spherical nanoindentation stress–strain curves. *Materials Science and Engineering: R: Reports,* 2015, **91**, 1-36, doi.org/10.1016/j.mser.2015.02.001

[40] J. S. Nyman, M. Granke, R. C. Singleton, G. M. Pharr, Tissue-Level Mechanical Properties of Bone Contributing to Fracture Risk. *Osteoporosis Reports,* 2016, **14**, 138-150, doi:10.1007/s11914-016-0314-3

[41] M. Toledano, M. Toledano-Osorio, E. Guerado, E. Caso, E. Osorio, R. Osorio, Assessing bone quality through mechanical properties in postmenopausal trabecular bone, *Injury,* 2018, **49**, Suppl 2: S3-S10. doi: 10.1016/j.injury.2018.07.035.

[42] J. Zhang, G. L. Niebur, T. C. Ovaert, Mechanical property determination of bone through nano- and micro-indentation testing and finite element simulation. *Journal of Biomechanics,* 2008, **41**, 267-275, doi: 10.1016/j.jbiomech.2007.09.019.

[43] S. Pathak, S. J. Vachhani, K. J. Jepsen, H. M. Goldman, S. R. Kalidindi, Assessment of lamellar level properties in mouse bone utilizing a novel spherical nanoindentation data analysis method, *Journal of the Mechanical Behavior of Biomedical Materials,* 2012, **13**, 102-117, doi: 10.1016/j.jmbbm.2012.03.018

[44] R. C. Paietta, S. E. Campbell, Influences of spherical tip radius, contact depth, and contact area on nanoindentation properties of bone. *Journal of Biomechanics,* 2011, **11**, 285-290, doi: 10.1016/j.jbiomech.2010.10.008.

[45] S. Hengsberger, A. Kulik, P. Zysset, Nanoindentation discriminates the elastic properties of individual human bone lamellae under dry and physiological conditions, *Bone,* 2002, **30**, 178-184, doi: 10.1016/s8756-3282(01)00624-x.

[46] M. Ramezanzadehkoldeh, B. Skallerud, Nanoindentation response of cortical bone: dependency of subsurface voids. *Biomechanics and Modeling in Mechanobiology.* 2017, **16**, 1599-1612, doi: 10.1007/s10237-017-0907-5.

[47] C. Michele, B. Anna, C. Davide, C. Diana, S. Philipp, M. Ralph, Nanoindentation analysis of the micromechanical anisotropy in mouse cortical bone, *Royal Society Open Science,* 2017, **4**, 160971, doi: 10.1098/rsos.160971

[48] A. G. Reisinger, D. H. Pahr, P. K. Zysset, Principal stiffness orientation and degree of anisotropy of human osteons based on nanoindentation in three distinct planes. *Journal of the Mechanical Behavior of Biomedical Materials,* 2011, **4**, 2113-2127, doi: 10.1016/j.jmbbm.2011.07.010.

[49] A. J. Bushby, V. L. Ferguson, and A. Boyde, Nanoindentation of bone: Comparison of specimens tested in liquid and embedded in polymethylmethacrylate, *Journal of Materials Research,* 2004, **19**, 249-259, doi: 10.1557/jmr.2004.19.1.249

[50] E. Donnelly, S. P. Baker, A. L. Boskey, M. C. Van der Meulen, M. C. Effects of surface roughness and maximum load on the mechanical properties of cancellous bone measured by nanoindentation, *Journal of Biomedical Materials Research Part A*, 2006, **77**, 426-435. doi:10.1002/jbm.a.30633

[51] X. Ojanen, H. Isaksson, J. Töyräs, M. J. Turunen, M. K. Malo, A. Halvari, J. S. Jurvelin, Relationships between tissue composition and viscoelastic properties in human trabecular bone, *Journal of Biomechanics,* 2015, **21**, 269-275, doi: 10.1016/j.jbiomech.2014.11.034.

[52] L. Feng, M. Chittenden, J. Schirer, M. Dickinson, I. Jasiuk, Mechanical properties of porcine femoral cortical bone measured by nanoindentation, *Journal of Biomechanics,* 2012, **26**, 1775-1782, doi: 10.1016/j.jbiomech.2012.05.001.

[53] A. Gustafsson, M. Wallin, H. Isaksson, Age-related properties at the microscale affect crack propagation in cortical bone, *Journal of Biomechanics,* 2019, **11**, 95, 109326. doi: 10.1016/j.jbiomech.2019.109326.

[54] A. Bonicelli, T. Tabitha, P. C. Justin, R. B. Oliver, H. Ulrich, L. A. Richard, and Peter Zioupos, Association between nanoscale strains and tissue level nanoindentation properties in age-related hip-fractures, *Journal of the Mechanical Behavior of Biomedical Materials*, 2023, **138**, 105573, doi: 10.1016/j.jmbbm.2022.105573.

[55] J. Y. Rho, P. Zioupos, J. D. Currey, G. M. Pharr, Microstructural elasticity and regional heterogeneity in human femoral bone of various ages examined by nano-indentation, *Journal of Biomechanics,* 2002, **3**, 189-198, doi: 10.1016/s0021-9290(01)00199-3.

[56] R. Zhao, S. Chen, W. Zhao, L. Yang, B. Yuan, V. S. Ioan, A. V. Iulian, X. Yang, X. Zhu, X. Zhang, A bioceramic scaffold composed of strontium-doped three-dimensional hydroxyapatite whiskers for enhanced bone regeneration in osteoporotic defects. *Theranostics,* 2020, **10**, 1572-1589, doi:10.7150/thno.40103.

[57] G. Molino, A. Dalpozzi, G. Ciapetti, M. Lorusso, C. Novara, M. Cavallo, Osteoporosis-related variations of trabecular bone properties of proximal human humeral heads at different scale lengths, *Journal of the Mechanical Behavior of Biomedical Materials,* 2019, **100,** 103373, doi: 10.1016/j.jmbbm.2019.103373.

[58] D. Pienkowski, C. L. Wood, H. H. Malluche, Young's modulus and hardness of human trabecular bone with bisphosphonate treatment durations up to 20 years. *Osteoporosis*

*International,* 2019, **30**, 277-285, doi: 10.1007/s00198-018-4760-x.

[59] A. R. Stern, X. Yao, Y. Wang, Effect of osteoporosis treatment agents on the cortical bone osteocyte microenvironment in adult estrogen-deficient, osteopenic rats. *Bone Reports,* 2018, **8**, 115-124, doi: 10.1016/j.bonr.2018.02.005

[60] I. A. Fiedler, F.N. Schmidt, E.M. Wölfel, C. Plumeyer, P. Milovanovic, R. Gioia, F. Tonelli, H.A. Bale, K. Jähn, R. Besio, and A. Forlino, Severely impaired bone material quality in chihuahua zebrafish resembles classical dominant human osteogenesis imperfecta. *Journal of Bone and Mineral Research*, 2018, **33**, 1489-1499, doi: 10.1002/jbmr.3445

[61] J. Cai, W. Li, T. Sun, X. Li, E. Luo, D. Jing, Pulsed electromagnetic fields preserve bone architecture and mechanical properties and stimulate porous implant osseointegration by promoting bone anabolism in type 1 diabetic rabbits, *Osteoporosis International,* 2018, **29**, 1177-1191, doi: 10.1007/s00198-018-4392-1.

[62] M. Burke, A. Atkins, A. Kiss, M. Akens, A. Yee, C. Whyne, C, The impact of metastasis on the mineral phase of vertebral bone tissue, *Journal of the Mechanical Behavior of Biomedical Materials,* 2017, **69**, 75-84, doi: 10.1016/j.jmbbm.2016.12.017.

[63] K. P. Wong, Y. J. Kim, T. Lee, Measurement of changes in mechanical and viscoelastic properties of cancer-induced rat tibia by using nanoindentation. IFMBE Proceedings. Berlin, Heidelberg: Springer Berlin Heidelberg, 2009, 1900-1903, doi: 10.1007/978-3-540-92841-6_471.

[64] A. Sekita, A. Matsugaki, T. Ishimoto, T. Nakano, Synchronous disruption of anisotropic arrangement of the osteocyte network and collagen/apatite in melanoma bone metastasis, *Journal of Structural Biology,* 2017, **197**, 260-270, doi: 10.1016/j.jsb.2016.12.003.

[65] A. Karali, E.Dall'Ara, J. Zekonyte, A. P. Kao, G. Blunn, & G. Tozzi, Effect of radiation-induced damage of trabecular bone tissue evaluated using indentation and digital volume correlation, *Journal of the Mechanical Behavior of Biomedical Materials*, 2023, **138**, 105636, doi: 10.1016/j.jmbbm.2022.105636.

[66] M. L. Oyen, R. F. Cook, A practical guide for analysis of nanoindentation data, *Journal of the Mechanical Behavior of Biomedical Materials,* 2009, **2**, 396-407, doi: 10.1016/j.jmbbm.2008.10.002.

[67] A. K. Gartzke, S. Julmi, C. Klose, S. Besdo, A. C. Waselau, A. Meyer-Lindenberg, H. J. Maier, P. Wriggers, Investigation of degraded bone substitutes made of magnesium alloy using scanning electron microscope and nanoindentation, *Journal of the Mechanical Behavior of Biomedical Materials,* 2020, **109**, 103825, doi: 10.1016/j.jmbbm.2020.103825.

[68] Z. Mitić, A. Stolić, S. Stojanović, S. Najman, N. Ignjatović, G. Nikolić, M. Trajanović, Instrumental methods and techniques for structural and physicochemical characterization of biomaterials and bone tissue: A review, *Materials Science and Engineering: C,* 2017, **79**, 930-949, doi: 10.1016/j.msec.2017.05.127.