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A review of in-situ mechanical testing combined with X-ray microfocus computed tomography: Application and current challenges for biological tissues

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ABSTRACT

Biological tissues undergo physiological mechanical loading during their functioning *in vivo*. To properly respond to these mechanical signals, tissues have a highly complex microstructural organization. However, there is not yet sufficient knowledge about the link between their microstructural organization and their mechanical behaviour. Therefore, there is a need for methods to dynamically assess how the microstructure of biological tissues changes during mechanical loading. 4D-μCT is an imaging technique combining mechanical testing with X-ray microfocus computed tomography (μCT) imaging. It has been extensively used to visualize, at the microscale and in full 3D, the deformation of the microstructure of non-biological materials during mechanical loading. Additionally, postprocessing of the 4D-μCT datasets allowed 3D strain field calculations. This review aims to provide an overview of the current state of the art of the use of 4D-μCT specifically for the assessment of the mechanical behavior of biological tissue, and this both for mineralized and unmineralized tissues. We highlighted the advancements as well as the current limitations and challenges to overcome, such as the need for complex loading modes, the effect of X-rays on the mechanical behavior and the need to keep the samples hydrated during testing. We finally conclude with some future perspectives.

1. Introduction

The mechanical behavior of biological tissues are intricately linked to their microstructure (i.e. the spatial organization of the cells and their extracellular matrix - ECM). The highly complex arrangement of structural proteins of the ECM, such as collagen or elastin, allows them to withstand physiological stress and effectively fulfill their function *in vivo* [1]. To better understand the intrinsic physiological behavior of biological tissues, it is essential to investigate their microstructure, as well as its link with the tissue's mechanical properties. Moreover, since microstructural changes may be caused by diseases or by ageing [2], it is important to obtain detailed information about disease-related microstructural changes and their consequences on the proper functioning of the tissue.

The most commonly used imaging techniques to study the tissue microstructure are classical 2D histology with brightfield or fluorescent microscopy, scanning electron microscopy and confocal laser scanning microscopy. These are, however, limited by their inability to give complete 3D spatial information. This is a major disadvantage for studying the microstructure of complex anisotropic materials such as most biological tissues [3]. Furthermore, these techniques are often destructive, as most of them require embedding and/or sectioning of the samples for their analysis. Therefore, in recent decades, more and more 3D imaging techniques have been used for the analysis of biological tissue at the microscale. *In vivo*, several 3D imaging techniques allow the observation of biological systems, but they are limited by their achievable spatial resolution and the lack of contrast for unmineralized tissues [4]. One of these is X-ray computed tomography (XCT), a technique widely used in medicine to image the whole human body for anatomical evaluation [5]. It exploits the penetrating power of X-rays to visualize non-destructively the inside of the human body, and allows large field of view imaging [6]. On a laboratory scale, *ex vivo* microfocus XCT (μCT)

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offers a spatial resolution of down to the (sub)micrometer range, and its versatility and non-destructive nature make this technique a relevant method for *ex vivo* 3D analysis of the microstructure of biopsies of biological tissues.

To better understand the relationship between microstructure and the proper functioning of biological tissues, dynamic assessment of the tissue under physiological loading would be of interest. The combination of mechanical testing methods and μCT imaging, named 4D-μCT (where 4D stands for 3D with a temporal component), allows the qualitative 3D analysis of the deformation at the microstructure scale under mechanical loading, and this is in full 3D. For quantitative results, 4D-μCT can be combined with post-processing methods to obtain the local strain field at the microscale. 4D-μCT is a promising technique that has been used for decades to characterize non-biological materials such as metals and composites [3], but has been much less described for its use on biological tissues. Therefore, the aim of this review, schematically represented in Fig. 1, is to provide an overview of the use of *ex vivo* 4D-μCT for the assessment of the mechanical behavior of biological tissues. For this purpose, first a general description of the technical aspects of 4D-μCT and the associated post-processing has been provided and this has then been translated to biological tissues. Then, we aimed to highlight the major breakthroughs and the limitations for the mechanical characterization of biological tissues with 4D-μCT, first for mineralized tissues and then for unmineralized tissues. Finally, the remaining challenges of the use of 4D-μCT for biological tissues have been discussed, mostly because of their complex microstructure, viscoelastic properties and their sensibility to hydration state and influence of X-rays. The review was concluded with future perspectives.

2. From 3D to 4D: technical aspects

Defining the mechanical properties of materials is an important part of their design and application. Standard mechanical testing devices are commercially available to assess the dynamic behavior of materials at the macroscale. Nevertheless, the microstructure of materials has a large impact on the mechanical properties and conventional testing devices do not allow the visualization of the microstructural changes during mechanical deformation. Several *ex situ* μCT experiments have been performed over the years, consisting of post-mortem microstructural characterization of samples after mechanical testing [9]. However, this approach does not allow for the visualization and quantification of microstructural changes over time. Therefore, *in situ* mechanical testing stages have been developed to overcome this limitation and, thus, to define the direct link between microstructural changes and mechanical behavior. Nevertheless, combining mechanical setups and μCT equipment is not straightforward, and there are several constraints on the stage design. Furthermore, a high temporal resolution is needed, especially for highly time-dependent materials such as polymers or biological tissues, which is not always guaranteed with lab-based μCT. Finally, post-processing of the 4D-μCT datasets pose still several challenges.

2.1. Mechanical loading stages

Regardless of the loading mode and the CT facility technology, all mechanical stages must fulfill certain design criteria to be suitable for 4D-μCT. Dall'Ara *et al.* (2022) already addressed this concern more specifically for applications in synchrotron XCT (SR-XCT) facilities [10], but it also applies to lab-based μCT systems. The main constraints are the size, the materials to manufacture the stages and additional technical features for specific environmental conditioning. For lab-based μCT systems, the stages must fit into the μCT chamber, which sometimes limits the range of sample sizes and also loading systems. For systems with a static X-ray source and detector and a rotating sample, the device must be as symmetrical as possible around axis of rotation to avoid

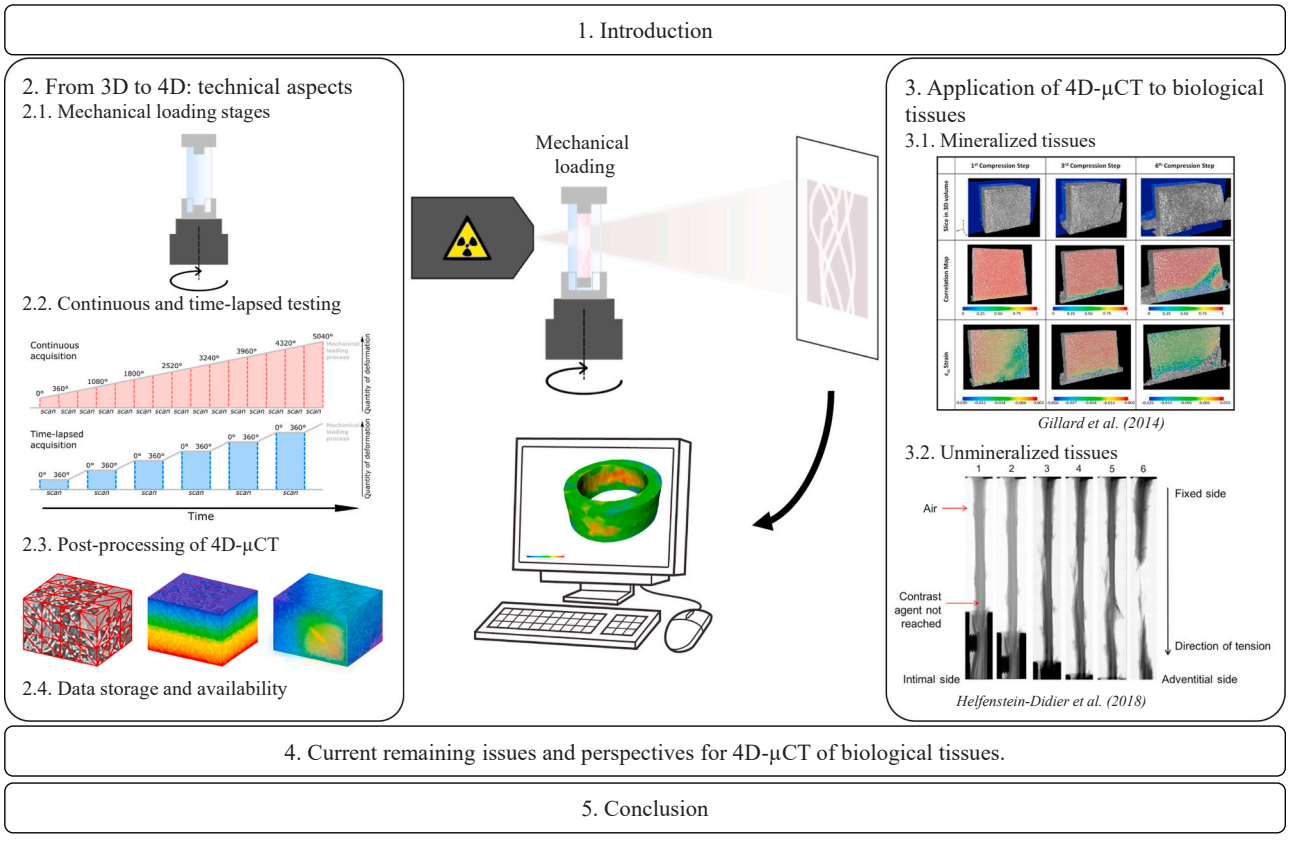

Fig. 1. Schematic representation of the structure of this review. The different images were reused and modified with permission of Elsevier from References [7,8], with our acknowledgments.

movement because of unbalanced weight. Also, the parts that are in the field of view (FOV) must remain in the FOV during the entire acquisition (360°) [9]. Furthermore, if the stage is too bulky, the sample cannot be close enough to the source to obtain sufficient spatial resolution [11]. Another complexity regarding the design is the compromise between stiffness and X-ray transparency. The stage frame that is within the field of view during the acquisition must be X-ray transparent to avoid loss of contrast and to reduce artifacts such as beam hardening and scattering. At the same time, the stage must be stiff enough to avoid frame deformation during loading. Specifically for biological tissues, this last limitation is more pronounced for mineralized tissues since they have a high elastic modulus while unmineralized tissues have a lower elastic modulus. Control measures, such as temperature and humidity control can be added specifically for biological tissues, but must be kept out of the FOV to avoid artifacts or loss of contrast, or, to prevent damage to the electronics over time.

Several loading modes such as uniaxial tension [9,11–13], compression [11,14–21], 3-points bending [22,23], indentation [24,25] and cyclic loading [9,26,27] have been used *in situ* on conventional materials and mineralized biological tissues. However, for biological tissues, whether mineralized or unmineralized, the actual loading mode within these types of tissues *in vivo* cannot be reduced to simple uniaxial loading, especially at the organ level. For example, for unmineralized tissues, setups should be adapted to biaxial loading, such as planar biaxial testing or extension-inflation, the latter being especially useful to mimic the stresses encountered in the vascular system [28]. This makes the loading stages even more complex than the classical uniaxial ones. Furthermore, mechanical loading stages dedicated to the testing of biological tissues must also allow the control of loading or displacement to perform creep and relaxation, since they are viscoelastic. Therefore, although several commercial *in situ* loading stages exist for both synchrotron and lab-based μCT set-ups, due to the complexity of their use specifically for biological tissues, a lot of in-house designed non-commercialized stages have been developed. They specifically fit with the application needs, as the requirements might differ a lot between tissue type, type of loading, research question, etc. [9,15,29,30].

2.2. Time-lapsed and continuous testing

There are two acquisition protocols available for 4D-μCT: time-lapsed and continuous acquisitions (Fig. 2). Time-lapsed testing is the oldest and most described approach, and it allows high spatial resolution imaging [16,31,32]. This acquisition approach consists of loading the sample in discrete steps and imaging between each loading phase (Fig. 2). The continuous acquisition method consists of imaging the sample during continuous mechanical loading to obtain complete information about the mechanical behavior of the sample over time (Fig. 2). For this method, high temporal resolution is needed, which is mostly available on SR-XCT systems [33].

The continuous acquisition method has been used to assess the microstructural changes during loading for materials such as metals [17,19], composites [18], but also bone samples [27,33]. Despite the lower temporal resolution of lab-based μCT, Kytýř *et al.* (2017) performed continuous *in situ* compression test on hydrogel-based scaffolds to avoid relaxation effects, but at very low deformation rate to avoid motion blurring [34]. Vopalensky *et al.* (2021) performed fast continuous compression tests on foam with an in-house lab-based μCT system [35]. It is interesting to note that this method has also been used to investigate other fast physical behavior of materials such as sintering [36,37] or even to better understand the physics behind 3D-printing manufacturing processes [38,39]. Dewanckele *et al.* (2020) validated continuous acquisition with lab-based μCT by studying the collapse of beer foam [40]. Although the time resolution was high enough to perform continuous acquisition, the spatial resolution was low in return. More recently, Dejea *et al.* (2024) performed in-situ synchrotron 4D-μCT on connective tissues in the knee joint with continuous acquisition to validate a new loading setup [41]. Peña Fernández *et al.* (2021) compared time-lapsed and continuous acquisition on foam samples to assess the influence of sample relaxation on strain accumulation [33]. Although these results showed similar elastic behavior and comparable 3D strain distribution for samples tested with both acquisition modes, the strain magnitudes were lower in time-lapsed tested samples. This difference was a consequence of the waiting time between two loading steps, during which local strains are redistributed due to relaxation, altering the elastic-plastic transition of samples [33].

These studies confirm the relevance of the continuous acquisition mode to study materials with strong time-dependent mechanical behavior, which is the case for biological tissues. This behavior is due to the presence of water trapped in the protein network and the high presence of collagen, resulting in an important viscoelastic behavior [42,43]. For unmineralized biological tissues, continuous synchrotron-based 4D-μCT can be a relevant technique as it allows to obtain full tomographic volumes in less than a minute, which could prevent too much creep between loading steps, in contrast to lab-based μCT systems [44]. However, working with low loading rates can also decrease the creep rate during mechanical testing, which can make time-lapsed testing suitable for biological tissues.

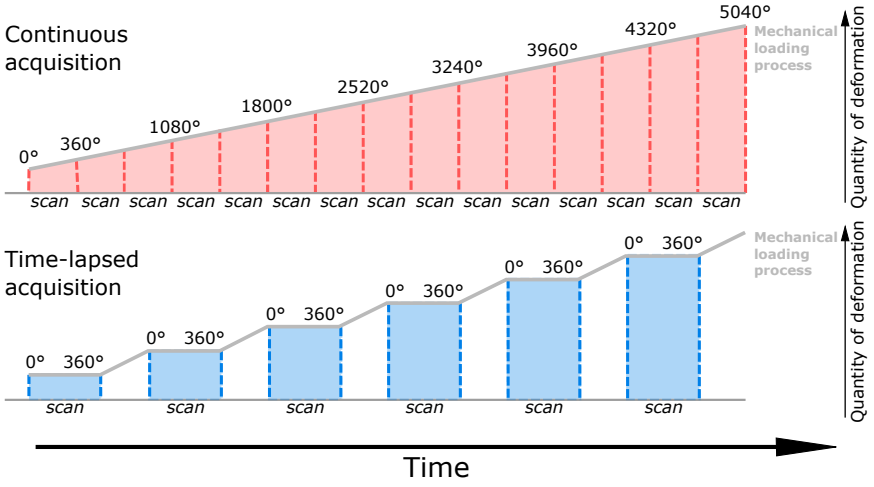

Fig. 2. Schematic overview of continuous and time-lapsed acquisition for 4D-μCT.

2.3. Post-processing of 4D-μCT

Apart from visual inspection of the change of the sample microstructure under mechanical loading, it is also possible to quantitatively assess the mechanical behavior of samples with 4D-μCT by generating 3D volumetric strain fields. For this, post-processing methods are required. Advanced tools have been developed and applied to quantify the local 3D deformation as a function of strain inside loaded samples [41,45–47]. Although other techniques exist such as particles tracking or image registration [41,48], digital volume correlation (DVC) is the most popular method for this purpose. Therefore, in the following we will mainly focus on this technique. A detailed description of the DVC principle can be found in references [49–53]. Briefly, the DVC principle involves the tracking of microstructural features within two datasets in different loading states and from this, computing the deformation pattern between them (Fig. 3) [51]. The correlation between the two μCT datasets is done by optimizing an objective function in smaller subvolumes of the images, created by meshing the dataset. The DVC post-processing method can be divided into two categories: the local method and the global method (Fig. 3). The main difference between the two methods comes from the registration method. In the case of the local DVC, all the subvolumes are registered individually, while in the case of global DVC, the correlation is performed on the entire volume at once. Each method has its advantages and disadvantages and to date, they are complementary and often used together. In fact, although the global DVC method is more accurate than the local DVC because of the loss of displacement continuity between subvolumes for the local approach, the latter is more robust for large displacements and less time consuming. Therefore, the local DVC approach is often used as pre-step to initialize the displacement during the global approach.

When processing 4D-μCT volumes with DVC, whether using the local or global approach, the DVC measurement uncertainties must not be neglected. Uncertainties reflect the accuracy and precision of the calculation. To measure DVC uncertainties, the standard procedure is to acquire two consecutive μCT datasets without deforming the sample and to perform DVC on them. The DVC results obtained from these repeated acquisitions show the systematic errors, referred to as DVC uncertainties. It needs to be mentioned, though, that even when the DVC shows small uncertainties, it does not mean that it will work systematically for non-zero strain deformation calculations. Several parameters can affect the uncertainties of the DVC measurements, and they are not all dependent on the DVC algorithm, e.g. image quality and the spatial image resolution, the subvolume size, the size of the tracked features, the load or deformation range, etc. [51]. Dall'Ara *et al.* (2017) showed that higher image quality (i.e. spatial resolution and parallel geometry beam), for example obtained with SR-XCT technology, showed systematically smaller errors. They also evaluated the influence of subvolume size on accuracy and demonstrated the correlation between them, since uncertainties increased with subvolume size [54]. Regarding the microstructure, Roberts *et al.* (2014) highlighted that the bone microstructural properties such as the bone volume fraction, trabecular number or separation strongly influence the accuracy of the strain calculations [51]. It can be assumed that the same issues can be transferred to the structural proteins constituting the unmineralized tissues. Furthermore, since the features in the structural network of unmineralized tissues are typically smaller than those of bone, a higher image spatial resolution is required to detect these features and to minimize the errors, which can significantly increase the acquisition, the post-processing and the computation time. Concerning the load or deformation range, since DVC is based on grayscale values and the internal pattern of the sample, if the latter is excessively deformed, the correlation between subvolumes fails [7]. Lastly, also important to consider is the increase in errors at the edges of the volume, which is a key concern in the field of DVC. Therefore, it is always advisable to look at the bulk of the volume for strain values rather than the edges.

Finally, DVC applied to 4D-μCT data is not only useful for quantifying the strain field within samples, but also for validating μCT-based finite element models (FEMs), for which experimental validation is crucial [30,55–64]. As highlighted by Chen *et al.* (2017), one of the main benefits of DVC outputs is the accuracy of the full field displacement information for FEMs validation. DVC outputs can also be useful for defining the boundary conditions of FEMs [54]. However, the lack of accuracy of DVC and the risk of error at the edges must still be taken into consideration even for this type of application.

2.4. Data storage and availability

Another key technical concern with 4D-μCT that is not always mentioned in the literature is data storage and availability. A single high-resolution μCT volume can require up to 50 Gb of storage. For 4D-μCT experiments where multiple μCT volumes are often acquired to follow the evolution of strain distribution during loading, the challenges

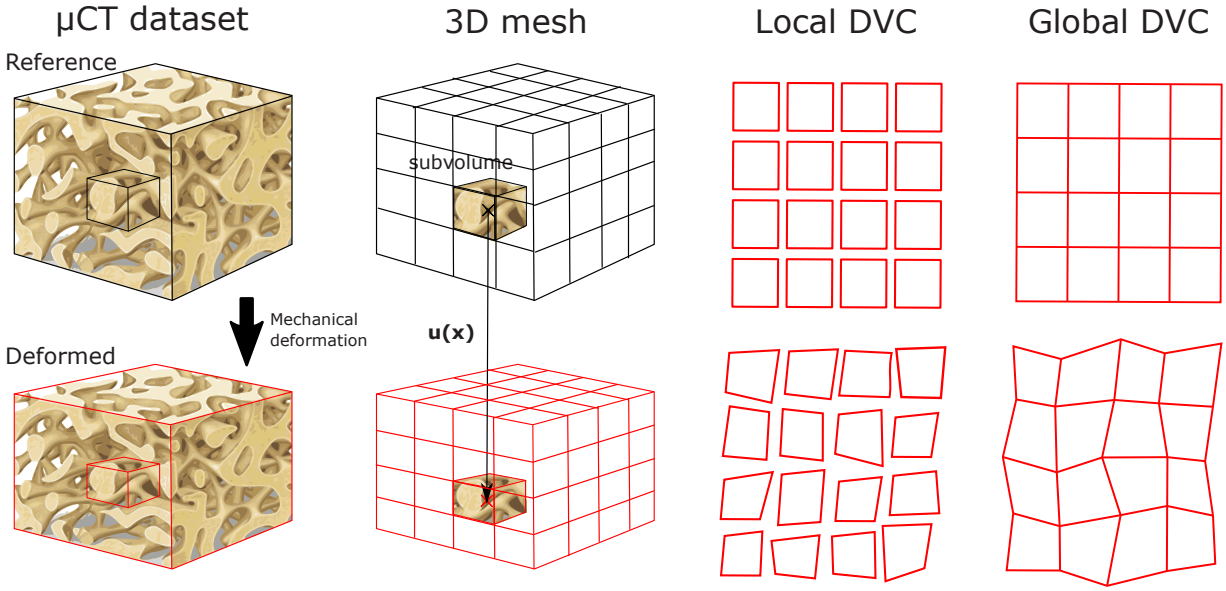

Fig. 3. Schematic representation of the DVC principle, both for local and global DVC.

of data storage, data processing time and computational power become even more pronounced. Furthermore, data sharing is becoming more and more important for the reproducibility of experiments, but no ideal solution has yet been introduced to make it easy and affordable. Dall'Ara *et al.* (2022) also addressed this for data generated from synchrotron facilities, but the concern can be extended to data from lab-based μCT systems [10].

3. Application of 4D-μCT to biological tissues

3.1. Mineralized tissues

Since mineralized tissues have a high X-ray attenuation coefficient and the loads they undergo *in vivo* are often assumed to be simplified to uniaxial compression for small deformations, 4D-μCT has been more extensively applied to study the mechanical behavior of this type of tissue in comparison to unmineralized tissues. Concerning bone fragility, it has long been hypothesized that the bone microstructure plays an important role, but this was poorly understood in the etiology of bone fractures. 4D-μCT has been a valid approach to fill this knowledge gap. Initially, 4D-μCT studies were performed at low spatial resolution and the *in situ* mechanical stages were primitive, only allowing qualitative assessment of the link between mechanical properties and bone microstructure [16,65]. Müller *et al.* (1999) were one of the first to take advantage of the combination of μCT imaging with an in-house micro-compressive device to assess the influence of the bone structure type on the failure mechanism. Although the analysis was only qualitative, they were able to demonstrate the strong link between bone microstructure and failure behavior. Furthermore, with the improvements of testing rigs and the use of the SR-technology, a better qualitative understanding of micro-damage initiation and propagation after monotonic or cyclic loading became possible [20,21,66]. However, the need to obtain quantitative measures of microstructural displacements and local strains remained strong, and thus the combination of compressive *in situ* 4D-μCT with post-processing methods such as DVC started to be used [67–69]. These studies allowed to demonstrate the strong relationship between the microstructural pattern and the displacement field, and the usefulness of DVC for studying mineralized tissues. They were followed by other studies performed on bones [14,25,31,57,58,60,61,70–92].

The most commonly used loading mode for bone is uniaxial compression [14,27,30,31,33,55,57–60,76–81,83,85,88,90,92–96]. However, even though commercial devices exist for uniaxial compression testing, most of these studies use in-house stages due to their application requirements, μCT systems, or because of the complex shape of some samples (e.g. compression on the entire femoral head) [55,57–59,77,79–81,83,85,92]. In fact, in some studies the entire organ has been tested, which requires more space or more complex loading systems than commercial stages. Other loading modes such as bending [76,86], or indentation [25,82] have been used on bones, also using in-house designed systems. As for the loading mode, the pre-conditioning of the sample may affect the results. Peña Fernández *et al.* (2020, 2021) investigated the local residual strain in cortical bone samples (with or without preconditioning compressive cyclic loading) by continuous compression testing [27,33]. They found a significant difference in results between the three loading modes due to the different strain accumulations. This study showed the importance of preconditioning the sample before testing, and the importance to properly choose the number of the cycles to avoid early failure during testing.

4D-μCT showed to be useful not only to assess the mechanical behavior of bone samples, but also to better understand its behavior in the presence of biomaterials such as prosthesis cement or screws [31,78,89,91,97]. This type of study is important because the integrity of bone-cement has a strong influence on the longevity of the prosthesis or implant. For example, Tozzi *et al.* (2012, 2014) investigated embedded bone samples under monotonic and cyclic compressive loading to understand and quantify the accumulation of microdamage at the bone-cement interfaces depending on the cement penetration and the bone structure type [31,97]. Qualitative inspection of the images validated that the DVC algorithm successfully identified the microstructural failure locations. Danesi *et al.* (2016) also studied the behavior of the bone structure in the presence of bio-cement with 4D-μCT and DVC in the case of vertebroplasty. DVC allowed to show that even if the cement locally increased the stiffness of the vertebra, the adjacent "non-cemented" trabecular bone structure was weaker due to the stress shielding induced by the cement. With 4D-μCT and DVC, it was possible to accurately observe the perturbation of the internal strain distribution because of the cement incorporation and hence identify failure locations in a non-destructive manner [78]. This would not have been possible with any other method.

However, the use of DVC as a post-processing method on bone samples is not trivial. Roberts *et al.* (2014) provided an overview of the developments and advances of the DVC method for 4D-μCT of bone and discussed the influence of the experimental design: i.e. the μCT acquisition parameters (voxel size, X-ray beam energy, number of projections and exposure time), the displacement increment value and the DVC algorithm parameters (subvolume size and the robustness of the objective function for the in-house algorithms) [51]. Gillard *et al.* (2014) used 4D-μCT and DVC to assess the influence of noise and subvolume size on the accuracy to study the mechanical behavior of porcine trabecular bone, more specifically the Poisson's coefficient [7]. It was shown that the DVC accuracy decreased with increasing noise. Moreover, they indicated that if the subvolume size is too small, it does not contain a large enough bone portion and is therefore more susceptible to the noise effect. Conversely, if the subvolume size is too large, it will contain multiple whole trabeculae, increasing the risk of incorrect correlation [7]. Hence, a proper balance needs to be found. Dall'Ara *et al.* (2017) collected different results from studies available in the literature and added their own data to characterize the relationship between bone structure, μCT system, DVC approach and spatial image resolution on the DVC outputs [54]. They concluded that, regardless of the microstructure, there is a strong correlation between the accuracy of the DVC outputs and the subvolumes size as for previous mentioned studies. Finally, in addition to noise and subvolume size, unrealistic calculated strains in crushed regions showed the limitation of the DVC algorithm for samples with excessive deformation. According to these results, it is important to define adequate and smaller displacement increments when preparing the experimental design in order to avoid inconsistent results. This last point was also mentioned by Roberts *et al.* [51].

Even though μCT is claimed to be a non-destructive technique, the influence of the X-ray exposure and dose on the bone microstructure and mechanical properties cannot be neglected. Since X-rays are known to be damaging to tissues, it is reasonable to assume that the same effects could occur during *ex vivo* 4D-μCT studies and thus, depending on the dose, could affect the mechanical properties of samples, at the molecular level or at the scale of the microstructural features [94,98]. Predicting the effects of the radiation on the mechanical behavior of tissues during 4D-CT testing is complex because it depends on numerous parameters, such as the type and energy of the beam, the system technology, the acquisition time, the nature of the samples, etc. Barth *et al.* (2010, 2011) assessed the effect of X-ray irradiation on the mechanical properties of bone in order to evaluate the actual reliability of *in situ* mechanical testing of bone samples with SR-XCT [99,100]. It was found that with SR-XCT, the level of exposure of the bone to X-rays induced an inability of plastic deformation due to the collagen degradation and thus embrittlement of the material. Peña Fernández *et al.* (2018) also obtained these results, but additionally quantified the irradiation-induced damage at the tissue level [14]. They found no significant drop in elastic properties. Dall'Ara *et al.* (2022) additionally highlighted the importance of considering X-ray damage by showing some examples of failed experiments [10]. Karali *et al.* (2023) investigated the effect of high-resolution μCT radiation on trabecular bone and its mechanical

properties [101]. They performed indentation tests and analyzed them with DVC to visualize crack formation after X-ray exposure. They observed an increasing change in the mechanical properties with the radiation exposure time. Even though the samples did not show the same change in mechanical behavior, they assumed that this difference was due to crack formation and propagation time. With DVC, they observed that samples with increasing stiffness showed cracks formation only after 66 hours of exposure, while those with a drop of stiffness showed crack formation already after 33 hours of exposure. Regarding hardness, there was a systematic decrease with irradiation, and they attributed this change to water radiolysis and collagen network degradation. In this study, the exposure times were 1.5 and 46 seconds for the overview image and high-resolution images, respectively. Therefore, to minimize radiation-induced tissue degradation, one solution could be to reduce the exposure time to X-rays, but with the risk of decreasing the image quality and thus the DVC accuracy [14].

Finally, other parameters that can influence the mechanical behavior of biological tissues are environmental parameters such as the temperature and humidity. Peña Fernández *et al.* (2018) aimed to preserve bone tissue integrity by controlling the temperature during *in situ* SR-XCT testing [94]. In this study, it was assumed that X-ray-induced heating could lead to collagen cross-linking, such as the release of free radicals because of water radiolysis. It was observed that trabecular bone was better preserved from SR-induced microcracks at 0°C than at room temperature. No significant change was observed in compact bone. Therefore, temperature control may be a good method to partially reduce damage during testing by reducing the risk of collagen cross-linking induced by both the beam-induced temperature and high-energy irradiation [94].

In conclusion, 4D-μCT has been successfully used to study mineralized tissues, as summarized in Fig. 4. There are some take-home messages and well as challenges to consider. First, it is important to choose the size of the sample, depending on the mechanical stress to be applied. This will influence the choice of loading mode and sometimes lead to the design of complex mechanical set-ups. During imaging, the loading method might also induce differences in strain distribution due to visco-elasticity, and the in-situ testing protocol must be prepared accordingly. Then, it is also important to have prior knowledge about the studied samples and the size of its microstructural features to better choose an adequate subvolume size for DVC. Furthermore, there is no ideal experimental workflow that perfectly fits for all bone samples due to the high microstructural variation between the biological samples. Therefore, when DVC is used as post-processing method of 4D-μCT data for quantitative analysis of the local strain distribution, it is important to perform a preliminary study to define the best DVC parameters according to the studied microstructure of the samples, the μCT system used, the image quality and the strain range of interest. Finally, the influence of the X-ray irradiation as well as the elevated temperature cannot be neglected since these parameters can significantly affect the mechanical behavior of samples.

3.2. Unmineralized tissues

In contrast to mineralized tissues, the X-ray attenuation of the constituents of unmineralized tissues (i.e. proteins of the ECM and the cells) is too similar and too weak to visualize their microstructure in μCT datasets without contrast-enhancement techniques. Therefore, the grey scale range of the acquired μCT datasets is not large enough to distinguish the different microstructural constituents of unmineralized tissues. To overcome this drawback, several contrast-enhancing techniques have been developed: phase-contrast XCT (PC-XCT) and contrast-enhanced μCT (CECT) imaging.

In contrast to conventional X-ray absorption mode imaging, which is more efficient for elements with high atomic number Z and density, PC-XCT additionally exploits the phase shift (or refraction) of X-rays [102]. Since the X-ray phase cross-section is larger than X-ray attenuation cross-section for the same material and it is also less impacted by X-ray energy, the phase effect is better for imaging low-Z materials [102]. A detailed description of the different phase-contrast methods is given by references [102–105]. PC-XCT was initially only possible with SR-XCT facilities due to the need for a high coherent and monochromatic X-ray beam and complex set-ups. This allows for very fast acquisition times (down to seconds per full acquisition). Using conventional lab-based X-ray tubes, Pfeiffer et *al.* (2006) and T. Zhou *et al.* (2018) for example, demonstrated the possibility of using phase-contrast enhancement methods to visualize unmineralized biological tissues. However, they had to work with long exposure time (40 s and 5 min per radiograph, respectively), which can be an issue for unmineralized biological tissues with regard to tissue dehydration, X-ray damage, etc. [106,107]. 4D-μCT with phase-contrast enhancement (4D-PC-XCT) has been applied on several unmineralized tissues. For example, Arora *et al.* (2017) performed 4D-PC-XCT on injured lung at different pressure states

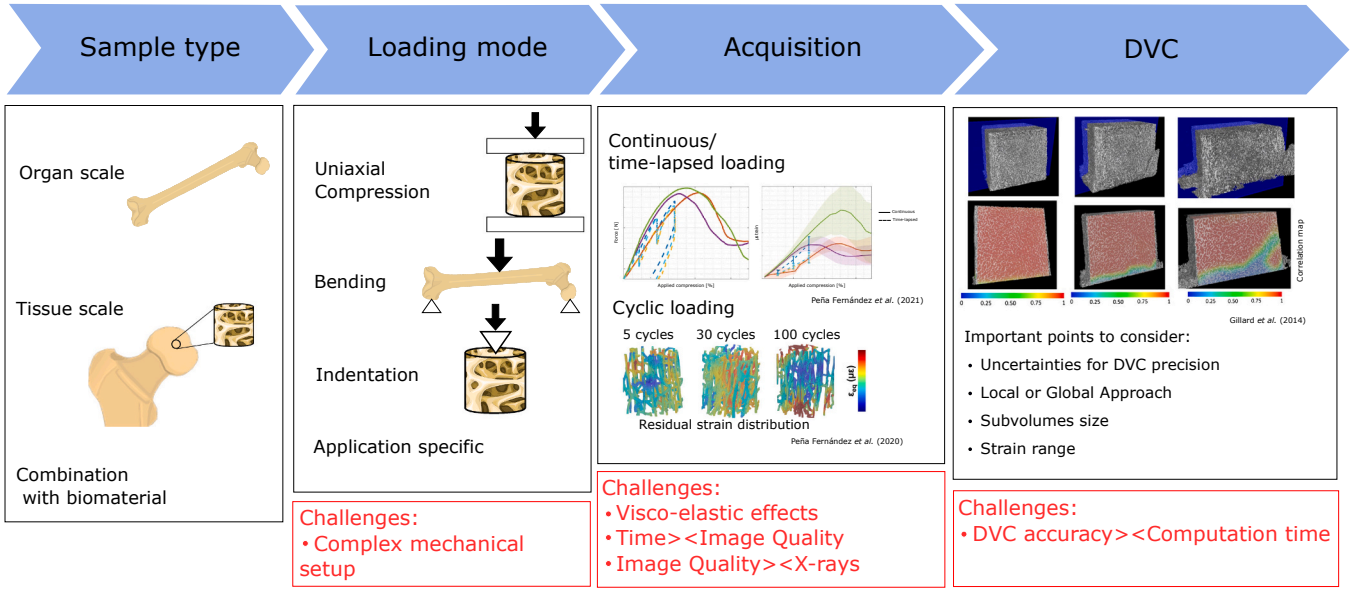

Fig. 4. Graphical summary of the current literature about 4D-μCT for mineralized tissues, including important take-home messages and challenges specifically for mineralized tissues. The different images were reused and modified with permission of Elsevier from [7,27,33], with our acknowledgments.

to assess the effect of the damaged microstructure on mechanical behavior [44]. By using SR-XCT system, the acquisition time for the different datasets could be reduced to 30 s and still sufficiently high-quality images could be obtained to apply DVC at the scale of alveolar walls. However, no concern regarding potential X-ray damage was raised in this study. Trachet *et al.* (2019) presented the use of PC-XCT to visualize the microstructural changes of the tunica media in arterial walls under pressure using SR-XCT [108]. They performed quasi-static pressure inflation testing on murine arteries and used automatic segmentation to distinguish the different constituents in the arterial wall. Their results showed that the elastic lamellae in the media unfold and stretch simultaneously as luminal pressure increases [108]. These results were, however, only qualitative, but we are convinced that their segmentation workflow could have been reliably used to obtain relevant quantitative data of local strain distribution via post-processing methods such as DVC. Dejea *et al.* (2024) used 4D-PC-XCT to simultaneously assess the mechanical properties of the connective tissues in the bovine knee and to validate a new in-situ mechanical setup (i.e. an in-situ rheometer) [41]. With PC-enhancement, they were able to visualize the chondrocytes within the cartilage tissue and quantify the change in their distribution and their orientation during tissue deformation. This allowed to confirm the change in tissue behavior as a function of the strain rate. Regarding X-ray damage, they assessed the change in mechanical properties of the tissue prior to and after X-ray imaging and did not detect any significant drop [41]. Other examples, non-exhaustive, where 4D-PC-XCT was used on unmineralized tissues are: vascular samples [108–111], intervertebral discs (IVDs) [112–114], Achilles tendons [115] and the cartilage-bone interface [116].

PC-enhancement has been shown to be a very useful method for 4D-μCT of unmineralized tissues, and mostly SR-XCT systems have been used for these studies, but SR-XCT is not always readily accessible. Some lab-based μCT systems also allow phase enhancement [109], but to the author's knowledge they have never been used for in-situ 4D-μCT of unmineralized biological tissue due to the high acquisition times needed for obtaining phase enhancement, which leads to a high X-ray dose and potential tissue damage [109]. Therefore, in recent years, several contrast-enhancing staining agents (CESAs) have been developed for CECT imaging of unmineralized biological tissues, as conventional absorption mode imaging can be applied for this and thus conventional lab-based μCT devices can be used. The purpose of the CESAs is to enhance differentiation among tissue constituents by increasing the X-ray attenuation of each of them; this is enabled by associating heavy molecules to the ECM constituents [117]. These chemical compounds, hence, should have a high attenuation coefficient, but should also be specific, non-toxic and sufficiently soluble. Depending on the biological tissue, the CESA must be appropriately selected based on its affinity for specific proteins [4,117] [118]. Furthermore, for 4D-CECT purposes CESAs must be non-invasive, which means tissues must keep function integrity after staining. This is not always the case, as it is known that phosphotungstic acid (PTA) or non-buffered Lugol [119–122] induce tissue shrinkage, which could influence strongly the mechanical properties of the tissue [123]. But, for 4D-CECT, it must be ensured that the CESA does not alter the mechanical properties of the tissue, such as the stiffness or the yield strength, and therefore several studies have been performed in recent years to develop non-invasive CESAs with high attenuation and have already demonstrated their use in the field [4, 118].

Helfenstein-Didier *et al.* (2018) addressed the potential effect of diluted sodium polytungstate (SPT) on the mechanical properties of arterial tissues by comparing samples immersed in saline solution only, and with two different concentrations of SPT [124]. They showed that this CESA had not mechanical effect on the macroscopic mechanical properties of the tissue, but they only tested for short staining times that were not sufficient to fully stain the samples. Pétré *et al.* (2023) tested additional CESAs, and showed that PTA and Lugol iodine with Sorensen's buffer (Lugol) significantly affect the mechanical properties of porcine aortic tissue and should therefore be discarded for 4D-CECT studies (Table 1) [122]. However, other POM-based CESAs, such as Hafnium-substituted 1:2 Wells Dawson POM (Hf-WD 1:2 POM) and Monolacunary Wells Dawson (Mono-WD POM) did not show significant impact on mechanical properties and hence are suitable for 4D-CECT. Finally, Davis *et al.* (2024) compared Hf-WD 1:2 POM and PTA for *ex situ* 4D-CECT to assess residual stress in bone-cartilage interface samples after compression [125]. Also this study demonstrated the importance of the proper selection of CESAs for assessing the mechanical properties of unmineralized biological tissues, and confirmed the results obtained previously by Pétré *et al.* [122].

To the best of our knowledge, only a few studies using lab-based 4D-CECT to assess the mechanical properties of unmineralized tissues are available. Helfenstein-Didier *et al.* (2018) were one of the first to assess the failure behavior of arterial samples under uniaxial tension using 4D-CECT [124]. In this study, loading was applied on SPT stained porcine aortic samples until failure to observe and describe the initiation, delamination, and rupture process in the medial layer of the samples. This study had some limitations. First, fast acquisition was used to avoid too much movement of the arterial sample during imaging, resulting in low quality images. Since they performed continuous loading with lab-based CT system, there was no possibility to do 3D reconstruction of the microstructural changes, and only radiographs were used to assess the failure behavior of the samples. Therefore, only qualitative assessment of the failure pattern of the samples was available. Second, the SPT solution at the concentration they used did not always fully diffuse into the samples, and it is therefore necessary to further define the proper staining protocol (mainly influenced by concentration and staining time). Third, the tests were performed under uniaxial tension, and this loading mode does not fit well with the actual physiological loads that arteries undergo. Finally, the lack of an atmospheric control (i.e., humidity and temperature) could induce unwanted sample dehydration. This impacts the mechanical behavior and reduces reproducibility.

Brunet *et al.* (2020) used 4D-CECT for stretching arterial tissue. They investigated the mechanisms leading to intimal tear initiation and propagation using an in-house tension-inflation loading setup and time-lapsed imaging [127]. Similar to Helfenstein-Didier *et al.* (2018), they tried to decrease the acquisition time as much as possible to reduce movement due to relaxation during acquisition, resulting in a total acquisition time of 266 seconds. For comparison, a similar experiment was performed with SR-XCT with a similar acquisition time (240 seconds), but with a higher number of projections, resulting in much higher image quality [110], showing the added value of SR-XCT. Brunet *et al.* (2020) stretched the SPT-stained samples longitudinally and then they performed time-lapsed testing by stepwise increasing internal pressure. A waiting period was required before each acquisition to stabilize the pressure and to avoid image blurring due to sample relaxation. Their approach allowed them to determine the dissection propagation in the medial layer, but without really studying the propagation of damage at the microstructure scale. Also here, no post-processing method was applied to the images to assess and quantify the strain fields inside the samples.

Finally, Iwasaki *et al.* (2024) reported the full-field 3D strain distribution of the muscle-tendon junction using *in situ* 4D-CECT [126]. They first stained their sample with PTA and then performed a uniaxial tensile test. Even though the different samples showed high variability, the DVC post-processing showed that the strain concentration was higher at the junction than in the muscle and the bone. They highlighted the limitation of the use of PTA/ethanol as a CESA solution, as it could lead to tissue dehydration due to the ethanol and could cause change in strain values. Furthermore, they wrapped them in wet gauze and parafilm to prevent dehydration during testing. Although this could reduce the impact of dehydration on the mechanical properties, it could have contributed to the mechanical behavior of the sample, biassing the measured mechanical properties. Finally, another limitation of this study was the long acquisition time (approximately 3 hours per volume)

Table 1
Comparison of the different CESA solutions for their contrast-enhancement and influence on the mechanical properties, + referred to a positive contribution, – referred to a negative contribution. The different studied CESAs are phosphotungstic acid (PTA), Hafnium-Wells 1:2 Dawson POM (Hf-WD 1:2 POM), Monolacunary-Wells Dawson POM (Mono-WD POM), sodium polytungsate (SPT) and Lugol's iodine (Lugol).(Adapted from reference [122,125] under the CC-BY 4.0 license).

CESA solutions	Imaging criteria				Mechanical criteria	
	Penetration Speed	Contrast Enhancement	Ease of Segmentation	Volume Change	Stiffness Change	
PTA	-	+	+	-	-	[125,126]
Hf-WD 1:2 POM	-	+	+	-	+	[125]
Mono-WD POM	+	-	-	+	+	[125]
SPT	+	-	-	+	+	[110,124,125,127]
Lugol	+	+	+	-	-	[122]

to obtain high spatial resolution, which could lead to a change in the microstructure because of the X-rays.

In conclusion, 4D-µCT has already been used for unmineralized tissues, as summarized in Fig. 5, although to a much lesser extent than for mineralized tissues. This is mostly since there are more complex challenges to consider. First, multi-axial mechanical setup need to be designed to fit as close as possible with *in vivo* functional stress application, and stages must include hydration system, since tissue shrinkage is higher than for mineralized tissue. Furthermore, preconditioning of samples is needed to stabilize the tissue before testing because of their strong viscoelastic behavior. As for mineralized tissue, a good trade-off must be done between Image quality for DVC, and exposure time to avoid X-ray damage. While in-situ 4D-µCT with phase-contrast enhancement is already used for different types of unmineralized tissues, 4D-CECT still needs significant optimization to make it consistent. Finally, even with phase-contrast enhancement, there are still only a limited amount studies using DVC to quantify the mechanical behavior of unmineralized tissues during in-situ testing, mostly because of the viscoelastic and non-linear behavior, and their highly complex microstructure.

4. Current remaining issues and perspectives for 4D-µCT of biological tissues

Some challenges still remain for 4D-µCT of biological tissues, and should be considered. First, the effects of X-ray exposure on the tissue integrity must be addressed. In fact, the important water content could increase the risk of free radical production during acquisition, promoting the cross-linking of collagen fibers, which increases the overall stiffness of the tissue [94]. Moreover, some studies have shown that tissue irradiation could reduce the content of proteoglycans for some tissues in the long term by decreasing their synthesis [128]. For *in situ* testing, however, this latter effect can be neglected by assuming that the drop in proteoglycan synthesis does not intervene significantly in the time interval allowed for the 4D-µCT testing. X-rays also affect elastin fibers, which are abundant in unmineralized tissues such as arteries and elastic cartilage [129]. Irradiation of these fibers could induce chain breakage and thus significantly decrease their Young's modulus. Therefore, as already mentioned by Dall'Ara *et al.* (2022), it is crucial for every new 4D-µCT experiment on biological tissues to estimate de X-ray radiation dose, ad to select the proper system and acquisition parameters [10].

Second, biological tissues are sensitive to the level of hydration, which has an important impact on their mechanical properties – this is even more pronounced for unmineralized tissues than for mineralized tissues. For example, as shown in the study of Wang *et al.* (2018), elastin fibers, which are one of the main proteins involved in unmineralized tissues with high extension capacity (skin, elastic arteries, connective tissues, etc.), have elastic and viscoelastic properties that are highly dependent on the water content in their immediate environment [130]. In case *in situ* testing would be done without immersion of the sample in a liquid solution or without keeping high humidity in the environment (>99 %) during loading, this can lead to dehydration during sample imaging. Wang *et al.* (2018) showed that, if the water loss is too important, it can lead to embrittlement of the elastin and can induce incorrect measurement of the mechanical properties [130]. Moreover,

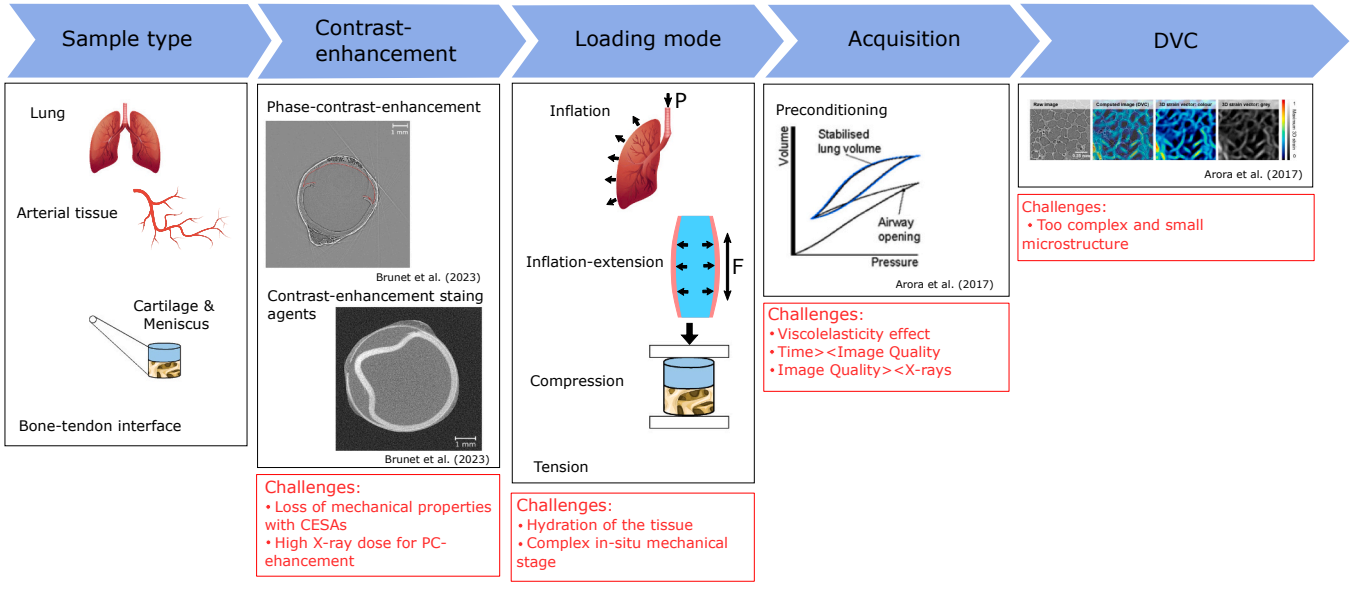

Fig. 5. Graphical summary of the current literature about 4D-µCT for unmineralized tissues, including important take-home messages and challenges specifically for unmineralized tissues. The different images were reused and adapted with permission from Elsevier from references [44,110], with our acknowledgments.

since water is one of the main constituent of biological tissues, it also plays crucial role in their viscoelastic behavior [42]. Therefore, with dehydration, some relevant information about the *in vivo* mechanical behavior of biological tissues could be lost (e.g., quantification of energy storage, transmission and dissipation). Another importance of humidity control is to mimic some of the symptoms of aging or diabetes, which are strongly suspected to cause water loss in unmineralized tissues [130].

Third, for hyperelastic unmineralized biological tissues such as arteries, the loading stage must be sensitive enough in load and displacement to detect small changes during testing and the displacement range must be large enough to avoid saturation before reaching the load range of interest. However, the space inside the lab-based μCT systems might limit the extension. Moreover, most of the unmineralized biological tissues do not only undergo uniaxial load *in vivo,* but much more complex loading conditions. Mechanical testing stages therefore need to be upgraded to allow biaxiality to mimic the physiological loading conditions. This kind of loading mode is already available for *ex situ* experiments [131], but the adaptation of the design for *in situ* testing is not always trivial because of the space limitations and a loading system that cannot interfere with X-rays.

Fourth, unmineralized tissues present significant non-linear and viscoelastic, creep and relaxation behavior [132], which can distort the computed mechanical properties if the time period between displacement steps for time-lapsed 4D-μCT is too long. Moreover, if the sample relaxes during acquisition, tissue rearrangement at high resolution can blur the acquired images. Time resolution with SR-XCT or the newest lab-based μCT devices could be high enough to counteract this behavior by using continuous acquisition mode. Current DVC techniques may also be limited to study highly viscoelastic materials because they require high spatial resolution and signal-to-noise ratio, which decreases with increase of the temporal resolution. Therefore, further optimization of the reconstruction algorithms and DVC protocols may be required.

Finally, for lab-based 4D-CECT of unmineralized tissues, the CESAs might impact the mechanical properties of the unmineralized biological tissues as mentioned earlier. Some CESAs lead to shrinkage, which is partially assumed to be water loss, and thus could affect mechanical properties [122]. Furthermore, some CESAs stiffen unmineralized tissues, which must be avoided in 4D-CECT studies. Since the effect of CESAs on tissues is not always properly understood, experiments to assess the influence of new CESAs on the mechanical properties of unmineralized biological tissues must be performed when a new tissue is tested with a known CESA or when new CESAs are used [122].

5. Conclusion

μCT imaging combined with *in situ* mechanical loading, i.e. 4D-μCT, allows to better understand the dynamic mechanical behavior of materials and to investigate the interplay of the microstructure with the mechanical properties. This review aimed to show the potential of 4D-μCT to study the mechanical behavior of biological tissues, and this using both synchrotron and lab-based μCT systems. This review showed that this technique has been used extensively to study the failure behavior and mechanical properties of mineralized biological tissues, although there are still some challenges to overcome such as the potential X-ray damage and the effect of dehydration and temperature on the mechanical behavior. We also showed the current limitations and challenges for unmineralized biological tissues, for which much less literature is available on the use of 4D-μCT to study their mechanical behavior. SR-XCT has been the most widely used system for both tissues, due to its high spatial and time resolution and the phase contrast imaging mode, despite its limited accessibility. This review also shows the importance of wisely choosing the imaging and testing parameters to obtain consistent results, whether for mineralized or unmineralized tissues, and implies a good prior knowledge of the microstructural specificities of tissue of interest.

In contrast to mineralized tissues, for the limited number of studies using lab-based μCT to study unmineralized tissues, the spatial resolution was not optimized, as the main objective was the assessment of the failure pattern at the macroscale without analyzing the microstructure. Therefore, there is still room for further improvement. Also, with the development of non-invasive CESAs that promote microstructure visualization without distorting the mechanical properties of the tissue, there are many future opportunities to better understand the link between the microstructure and the mechanical behavior of unmineralized tissues. Finally, with ongoing improvements in the image processing methods, the signal-to-noise ratio could be increased with lower acquisition time on lab-based CT systems, potentially allowing more accurate assessment of unmineralized tissues on this type of μCT system.

Even though it is clear that still important improvements must be performed to make 4D-μCT a reliable technique to study biological tissues, it could bring breakthroughs in the biomedical field for all types of biological tissues. In general, 4D-μCT could be useful to better understand the impact of different diseases on the mechanical properties of mineralized and unmineralized biological tissues at different stages of the pathology of interest. It could also be useful to assess the potential impact of invasive treatments on mechanical behavior of tissues, such as balloon angioplasty on arteries. Finally, 4D-μCT could also be used to further improve scaffold design for tissue engineering purposes and the design of synthetic grafts.

CRediT authorship contribution statement

Kerckhofs Greet: Writing – review & editing, Supervision, Funding acquisition. **Mazy Lara:** Writing – original draft, Resources, Investigation, Conceptualization.

Statement

During the preparation of this work, the authors used ChatGPT and Deepl AI in order to check language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

No data was generated for this work.

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