Review

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Men who stare at bone: multimodal monitoring of bone healing

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Abstract: Knowledge of the physiological and pathological processes, taking place in bone during fracture healing or defect regeneration, is essential in order to develop strategies to enhance bone healing under normal and critical conditions. Preclinical testing allows a wide range of imaging modalities that may be applied both simultaneously and longitudinally, which will in turn lower the number of animals needed to allow a comprehensive assessment of the

healing process. This work provides an up-to-date review on morphological, functional, optical, biochemical, and biophysical imaging techniques including their advantages, disadvantages and potential for combining them in a multimodal and multiscale manner. The focus lies on pre-clinical testing of biomaterials modified with artificial extracellular matrices in various animal models to enhance bone remodeling and regeneration.

Keywords: bone; *ex vivo*; *in vivo*; matrix engineering; remodeling; soft tissue.

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Introduction

The skeleton performs its supporting function inside the body, well protected from external influences, but therefore not accessible to the observing eye. This prompts the need for adequate techniques that allow monitoring the healing of fractures and bone defects.

Of particular interest are critical size bone defects, i.e. defects that do not heal spontaneously during lifetime (Bosch et al. 1998). Those occur after tumor resection, infection, or trauma and require treatment strategies that induce osteogenesis (Cianciosi et al. 2019; Rammelt et al. 2020; Zhang et al. 2018). Autologous bone grafting combined with internal fixation represents the current clinical gold standard (Rammelt and Marx 2020). Other treatment regimens focus on guided bone formation around an intramedullary nail, membrane-induced or distraction osteogenesis with the use of external fixation (Andrzejowski et al. 2020; Bezstarosti et al. 2020).

Monitoring bone regeneration in critical situations is necessary also to diagnose and treat complications such as non-union, infection, or implant failure. The most widely applied standard radiography allows only incomplete assessment of bone healing (Claes and Cunningham 2009; Hammer et al. 1985) and does not distinguish between infection and implant loosening while symptoms are similar (Sanderson 1991). A further drawback of X-rays including computed tomography scanning is radiation exposure (Rammelt and Boszczyk 2018).

Over the past decade, numerous techniques have been developed and refined that allow non-invasive bone imaging. This review provides an overview of selected methods including their advantages and drawbacks regarding their value in monitoring of bone regeneration. The focus lies on the development of novel preclinical techniques in small and large animal models reflecting the work within a collaborative research center focusing on improving skin and bone regeneration with the use of organic extracellular matrix (ECM) components (Förster et al. 2013). A PubMed database search was performed in February 2021 using key words and phrases ‘acoustic emission’, ‘biomechanical testing’, ‘CT’, ‘fluorescence’, ‘imaging’, ‘microCT’, ‘microdialysis’, ‘MRI’, ‘PET’, ‘resonant frequency analysis’, ‘SPECT’, ‘tracer’, ‘ultrasound’ or ‘wave propagation’ linked to the key words ‘bone defect’, ‘fracture healing’ and ‘*in vivo*’ by AND as Boolean function.

Morphological imaging

Radiographs, CT, and μ CT

Bone formation or fracture healing is a process that takes several weeks depending on the site and location of the lesion. Bone remodeling on a microscopic level and bone modeling on a macroscopic level following full weight-bearing last about a year (Rentsch et al. 2014b). Therefore, techniques that allow non-invasive assessment of bone formation over its long course are desirable. Plain radiographs allow for a rapid and cost-effective overview of bone healing and at least semi-quantitative measurements (Förster et al. 2020; Rentsch et al. 2014b). However, standard X-rays have a limited resolution in small animal models and do not allow for a reliable assessment of bone bridging (Dorsey et al. 2009). Computed tomography (CT) approaches have become established for detection of bone tissue *in vivo* (Li et al. 2008; Stock et al. 2003). CT as well as micro-computed tomography (μ CT) are based on the differential absorption of ionizing radiation by calcified tissue (Babatunde et al. 2010) revealing both the architecture of bone and the progress of defect bridging (Kallai et al. 2011). CT/ μ CT can be performed rapidly, is non-destructive to tissue and biomaterials (Jones et al. 2007b) and can be used to quantify the amount and density of mineralized tissue (Dudeck et al. 2014; Förster et al. 2017; Picke et al. 2016). Due to three-dimensional rotation, CT scans delineate overlapping bone structures and the images are not affected by surrounding soft tissue (Wong et al. 2012).

The preclinical application of serial radiographs, CT and μ CT (Figure 1A) facilitates longitudinal non-invasive imaging leading to a reduction of animal numbers and improves

quality of the data obtained, as the regeneration process can be followed in the same individual (Förster et al. 2020; Rentsch et al. 2014b). Quantitative synchrotron radiation micro-computed tomography (SR μ CT) allows analyses of bone adjacent to metallic implants with high resolution (Figure 1B) but due to size limitations has to be performed *post mortem* (Dudeck et al. 2014; Li et al. 2008). μ CT was used to quantify bone matrix deposition in calvarial defects in rabbits and revealed both a more homogenous bone formation and higher bone volume in animals treated with implants that were functionalized with collagen/chondroitin sulfate (Rentsch et al. 2014a). Another calvarial defect model evaluated the bone mineral density (BMD) in rats after implantation of β -tricalcium phosphate (β -TCP) or granular deproteinized bovine bone (GDPB) grafts with dental pulp stem cells (DPSC) seeded in them (Annibali et al. 2013). By using μ CT, the authors found increased BMD in the GDPB group, especially when DPSC were seeded in those grafts.

Applying radiographs and μ CT simultaneously, bone regeneration was assessed in critical size bone defects in rat femora filled with 3D plotted biphasic scaffolds consisting of calcium phosphate cement and alginate/gellan gum (Ahlfeld et al. 2019). New bone formation from the proximal and distal host bone ends was detected with radiographs and quantified with μ CT. The same was seen in 5 mm defects filled with PCL (polycaprolactone) scaffolds coated with an artificial extracellular matrix (aECM) containing collagen/chondroitin sulfate or collagen/hyaluronic acid of different sulfation grades (Förster et al. 2020). Rentsch et al. (2014b) used radiographs and CT imaging at different time points to reveal increased bone formation in critical size defects in the sheep tibia filled with embroidered PCL scaffolds coated with an aECM consisting of collagen and chondroitin sulfate (Rentsch et al. 2014b).

Another study quantified bone regeneration after implantation of hydroxyapatite specimens with varying pore sizes in drill hole defects in sheep tibiae (Jones et al. 2007a). The authors established a segmentation algorithm for μ CT data, which distinguishes between scaffold, host bone, new formed bone matrix, and soft tissue and, thus, enables a quantification of these structures and tissues.

The disadvantages of CT imaging compared to plain radiographs are the significantly higher radiation exposure (Wong et al. 2012), the size and price of the device, and the more demanding handling. New developments like cone beam CT, ultralow dose protocols, and automatic exposure control have the potential to significantly reduce radiation exposure at the extremities (Konda et al. 2018; Rammelt and Boszczyk 2018). Both conventional X-ray and CT imaging are only useful in the later stages of

Figure 1: (A) Radiographs (left panel) and mCT imaging (right panel) for monitoring bone regeneration in a critical size defect in the rat femur. Longitudinal imaging at two weeks (top) and eight weeks (bottom) reveals bone growth from the fracture ends into the PCL scaffolds containing an aECM of collagen and highly sulfated hyaluronic acid. (B) Quantitative synchrotron radiation micro-computed tomography (SR μ CT) showing newly formed bone adjacent to a titanium rod in the rat tibia coated with an aECM of collagen and RGD peptide.

bone repair when calcified tissue has been formed and do not allow for early identification of fractures at risk of non-union (Mathavan et al. 2019).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a non-invasive and non-radiation imaging technique, which enables morphological, functional, and molecular analysis of bone regeneration with high spatial resolution and unlimited depth (Kim et al. 2016). The technique is based on the excitation of hydrogen protons in tissues by strong magnetic fields. MRI signal intensities are dependent on different longitudinal (T_1) or transverse (T_2) relaxation times of the individual tissues, as well as on their varying water content and environment. In this respect, MR imaging of solid tissues like bone or teeth remains challenging due to their low content of unbound water and a resulting much faster ^1H signal decay than in soft tissues (Korn et al. 2015). For this reason, ^1H MRI mainly visualizes bone as a dark structure with poor signal. To overcome this substantial limitation, several alternative MRI approaches to assess bone repair have been tested, of which the combination of various sequences with ^{31}P nuclear magnetic resonance (NMR) spectroscopy and the use of contrast agents are most promising (Dyke and Aaron 2010; Seifert and Wehrli 2016).

A minipig study evaluated conventional MRI regarding its applicability for evaluating osseointegration of titanium-coated PEEK implants in the jaw (Korn et al. 2015). Comparative analyses of MRI and

histomorphometry revealed that MRI could distinguish between bone, fatty and soft tissue (Figure 2A and B) and can be used to evaluate osseointegration. In contrast to X-ray-based techniques, MRI enables to follow the bone formation process from the beginning, when the tissue contains water and unmineralized structures. The combination of *ex vivo* MRI and μ CT techniques was advantageous in simultaneously rating new bone formation and implant degradation in a rabbit long bone defect filled with polylactide sponges (Kłodowski et al. 2014).

Like MRI, NMR spectroscopy is based on the excitation of protons by magnetic fields, whereas it is able to detect the frequency of the emitted signal. Thereby, this technique can identify and quantify organic as well as inorganic substances as has been done for cartilage (Duer et al. 2009), dentine (Huang et al. 2009; Tseng et al. 2007) and bone composition (Jaeger et al. 2005; Vyalikh et al. 2017). Different mineralization stages as well as tissue compositions/collagen content can be investigated using ^{13}C and ^{31}P NMR to obtain information on the molecular properties and healing state of the tissue (Figure 2C and D) (Penk et al. 2013). Quantitative solid-state NMR found accumulations of collagen and calcium phosphate compounds within critical size bone defects in rats that were treated with biodegradable PCL scaffolds functionalized with sulfated glycosaminoglycans indicating progressive bone regeneration (Förster et al. 2020). μ CT-based bone volume measurements confirmed bone matrix deposition within the defects.

In addition, MRI can provide functional information, for example regarding diffusion and perfusion of tissues (Le Bihan 2013). In terms of tissue perfusion, rapid

Figure 2: (A, B) Magnetic resonance imaging (MRI) of a cylindrical defect in the tibial head of an adult male Wistar rat. Empty defect (A) and new bone formation within a scaffold containing an aECM of collagen and chondroitin sulfate (B). MR images have been acquired on a Bruker Avance NMR spectrometer with a 7T wide-bore magnet (300 MHz Larmor frequency for protons) using a 15 mm birdcage resonator (1 s repetition time, 3 ms echo time, 1 mm slice thickness) and a Bruker Micro2.5 microimaging accessory generating magnetic field gradients of 1T/m on three axes. Images are courtesy of U. Scheler, Dresden, Germany. (C, D) Tissue composition at bone defect sites can be assessed by using nuclear magnetic resonance (NMR). Both, collagen and hydroxyapatite (HAP) were identified in (C) ^{13}C and (D) ^{31}P MAS NMR spectra 12 weeks after osteotomy proving advanced bone formation. Reprinted from Materials Science and Engineering C, 116, Förster Y et al., The influence of different artificial extracellular matrix implant coatings on the regeneration of a critical size femur defect in rats, 111157, Copyright (2020), with permission from Elsevier. The publication is available at <https://doi.org/10.1016/j.msec.2020.111157>.

acquisition of an image series before and after the application of contrast-enhancing agents generate information on tissue microvascularization, capillary permeability and interstitial space enhancement (Singh et al. 2018). One study combined *in vivo* MRI in rats for bone-healing and perfusion measurements (Ribot et al. 2017). Bone regeneration estimations made by MRI could be verified by μCT measurements and histological evaluation suggesting MRI a valuable tool for bone healing monitoring and simultaneous assessment of new vessel formation.

Due to the extremely short transverse relaxation times of bone, especially cortical bone, which range from 0.39 to 0.5 ms, ultrashort measuring sequences, like ultrashort time to echo (UTE) or zero time to echo (zero TE) have been generated that are of either qualitative or quantitative nature (Du and Bydder 2013). Using a 3D UTE sequence, a novel MRI-compatible, ceramic intramedullary implant could be clearly distinguished from fracture site and growing callus tissues without any artifacts in mice (Schmitz et al. 2020).

Furthermore, 3D analysis of the asymmetric callus based on MRI was more reliable and representative than cross-sectional histological analyses. However, MRI does not yield a measure of the osteogenic potential at the fracture site (Mathavan et al. 2019).

Functional imaging

Nuclear imaging techniques

PET

In contrast to X-ray, CT, and MR imaging, positron emission tomography (PET) enables monitoring of local metabolic activity through the uptake of radioactive isotopes or radiolabeled molecules ('radiotracers') in tissue and, therefore, allows for functional imaging.

$[^{18}\text{F}]$ Fluoride is a positron-emitting isotope with high affinity for bone. It rapidly diffuses through bone capillaries

after intravenous injection and when arriving at the bone surface, ionic exchange occurs between [^{18}F]fluoride and hydroxyl groups of hydroxyapatite resulting in [^{18}F]fluoroapatite. Because this process necessitates the presence of exposed bone surfaces, tracer activity was initially presumed to be indicative for active sites of both osteoblastic bone formation and osteoclastic bone resorption (Sorensen and Ullmark 2009). However, more recent studies suggest that [^{18}F]fluoride binds only to bone mineral deposited by osteoblasts (Toegel et al. 2006) and allows for quantitative analysis of bone formation independent of bone resorption (Mathavan et al. 2019). Due to its rapid uptake and blood clearance a high bone-to-background ratio can be achieved with [^{18}F]fluoride. Moreover, [^{18}F]fluoride precedes bone mineralization detected by CT later on and, thereby, constitutes an early prognostic marker for fracture healing (Mathavan et al. 2019). However, [^{18}F]fluoride accumulation may be exaggerating osteoblast activity in fractures or defects treated with bone allografts since [^{18}F]fluoride binds with high(er) affinity to dead bone and calcium phosphate materials (Bernhardsson et al. 2018).

[^{18}F]fluoride PET has been used in various preclinical studies investigating biomaterial-assisted bone healing in rats (Annibali et al. 2014; Cheng et al. 2014; Hsu et al. 2007; Hulsart-Billström et al. 2018; Lohmann et al. 2017; Neuber et al. 2019; Ventura et al. 2014b) and mice (Hayer et al. 2019; Lee et al. 2009). Bone regeneration of a rat calvarial defect model grafted with either granular deproteinized bovine bone or β -TCP was monitored over 12 weeks by utilizing [^{18}F]fluoride PET/CT (Annibali et al. 2014). The [^{18}F]fluoride tracer uptake as well as the bone mineral density detected by μ CT was highest in granular deproteinized bovine bone suggesting benefits of this graft for bone regeneration. Ventura et al. (2014b) also applied [^{18}F]fluoride PET/CT to validate bone matrix deposition in a rat calvarial defect model grafted with BMP-2-functionalized calcium phosphate cements (Ventura et al. 2014b). [^{18}F]Fluoride PET/CT scans were performed every second week for eight weeks after surgery and the healing state was additionally analyzed by histological analyses. In the same model, a 3D architecture hydrogel ('ArcGel') was found to be superior to the clinical standard autologous bone and Bio-Oss[®] collagen by longitudinal [^{18}F]fluoride PET/CT (Lohmann et al. 2017). Quantitative analysis of [^{18}F]fluoride PET/CT was also found to be useful and sensitive for longitudinal observation of osteoporotic bone healing after replacement with various biomaterials (Cheng et al. 2014), appropriate to identify early nonunions in a rat femoral fracture model (Hsu et al. 2007) and to monitor bone healing in a rat femur defect model after implantation of a BMP-2 releasing hyaluronan hydrogel-hydroxyapatite biomaterial (Hulsart-Billström et al. 2018). A recent study from our group

used longitudinal PET/CT measurements to investigate the impact of PCL scaffolds coated with different aECM on bone healing in a rat femur defect model (Neuber et al. 2019). Over eight weeks, the bridging of a critical size femoral defect was monitored using [^{18}F]fluoride and [^{18}F]FDG radiotracers for mineral matrix deposition and metabolic activity, respectively (Figure 3). The study showed both positive effects of chondroitin sulfate coating on bone regeneration and the advantage of PET/CT as non-invasive functional technique for bone healing observation (Neuber et al. 2019). In another study, [^{18}F]Fluoride PET/CT revealed increased bone healing in mice injected with adipose-derived mesenchymal stem cells (Lee et al. 2009). However, in a mouse model of chronic inflammatory erosive polyarthritis treated with an anti-TNF antibody, [^{18}F]fluoride PET/CT analysis was unable to quantify the severity of bone damage. Although a majority of preclinical studies suggest [^{18}F]fluoride PET as a predictive diagnostic tool to identify fractures at risk for delayed healing or non-union, it has only been used sparsely in clinical applications, probably due to high costs and limited availability (Lundblad et al. 2017; Petri et al. 2013).

2-Deoxy-2-[^{18}F]fluoroglucose ([^{18}F]FDG) is another positron-emitting radiotracer predominantly used in oncology due to the Warburg effect of tumors. [^{18}F]FDG can be used conditionally as a marker for metabolic activity of bone cells as well as a marker for inflammation associated with bone loss or healing in animal models. Therefore, [^{18}F]FDG should be considered as a surrogate parameter and has been used in various preclinical rat or mouse studies in combination with [^{18}F]fluoride PET/CT (Hayer et al. 2019; Hsu et al. 2007; Lohmann et al. 2017; Neuber et al. 2019). In contrast to the significant difference in [^{18}F]fluoride tracer accumulation in the defect between successful and delayed bone healing in a rat femur fracture model, [^{18}F]FDG PET was not helpful to differentiate metabolic activity between the two groups (Hsu et al. 2007). In a rat calvarial defect model filled with a 3D architecture hydrogel ('ArcGel') or the clinical standard autologous bone and Bio-Oss[®] collagen, [^{18}F]FDG PET signal continuously decreased after an initial increase in the early phase after implantation (day 1–3) indicating that ArcGel and the clinical standard autologous bone and Bio-Oss[®] collagen induce the same short-term inflammatory response after implantation (Lohmann et al. 2017). In a mouse model of chronic inflammatory erosive polyarthritis, longitudinal PET/CT scans revealed a significant decrease in [^{18}F]FDG standard uptake value (SUV) in the affected joints demonstrating a complete remission of inflammatory processes due to the anti-TNF antibody induced TNF blockade (Hayer et al. 2019). Thus, even in a mouse model – a challenging model with regard to spatial resolution of PET imaging – [^{18}F]FDG PET is appropriate to quantify and monitor inflammation-mediated

Figure 3: (A) Surgery of a rat resulting in a 5 mm critical size bone defect at the right femur. (B–E) Representative PET/CT images of a rat (pelvic region) after i.v. injection of $[^{18}\text{F}]\text{FDG}$ (left panel) and $[^{18}\text{F}]\text{fluoride}$ (right panel). (B/C) Rotated maximum intensity projection (MIP) of PET data (each left) fused to CT image (each right). B – bladder, E – epiphysis (D/E) Two-dimensional view of femur defect fixed by 5-hole plate and four screws visualized by CT (upper panel), PET (lower panel), and fused PET/CT image (mid panel). Reprinted from *Clinical Hemorheology and Microcirculation*, vol. 73, no. 1, pp. 177–194, 2019, with permission from IOS Press. The publication is available at <https://doi.org/10.3233/CH-199208>.

bone damage. In clinical application, $[^{18}\text{F}]\text{FDG}$ PET imaging allowed for detection of hip and knee endoprosthesis loosening in 76.4% of patients and of periprosthetic infection in 100 and 45.5% for septic and aseptic cases, respectively, which is of importance in decision-making for surgical revision (Delank et al. 2006).

Whether nuclide-based methods for the assessment of bone healing will gain acceptance may depend on the radiation exposure and associated health risks. The application of radiotracers for specific aspects such as the state of arthritis or infection with a high local resolution is getting increasing acceptance in orthopedic surgery (Serino et al. 2020).

SPECT

Single-photon emission computed tomography (SPECT) radionuclides are characterized by nuclear decay that is associated with γ emission. Compared to PET, most radionuclides available for labeling SPECT tracers have relatively long half-lives allowing for longitudinal studies. Furthermore, SPECT provides a slightly higher spatial resolution, which could be advantageous in terms of studying small lesions, especially with regard to preclinical investigations using small animal models.

Bone-affine bisphosphonates labeled with the γ emitter ^{99m}Tc (half-life 6 h) are the most frequently applied SPECT radiotracers used for evaluating primary bone defect repair. [^{99m}Tc]Tc-methylene diphosphonate (MDP) and [^{99m}Tc]Tc-hydroxymethylene diphosphonate (HDP) have been clinically approved for bone imaging (Ventura et al. 2014a). Both radiopharmaceuticals selectively accumulate in bone tissue via chemical adsorption onto the crystalline structure of hydroxyapatite (Ventura et al. 2016). [^{99m}Tc]Tc-HDP has been reported to accumulate at the edges of the bone defect area (Ventura et al. 2016). There has also been evidence for [^{99m}Tc]Tc-MDP uptake in mature osteoblasts (Zhong et al. 2015). These findings suggest that the specificity of ^{99m}Tc -labeled bisphosphonates towards areas of active bone healing is determined through both inorganic and biological mechanisms.

In addition, development of new, bone-selective SPECT tracers continues. For instance, zolendronate derivatives 1-hydroxy-2-(2-ethyl-4-methyl-1H-imidazol-1-yl)ethane-1,1-diylidiphosphonic acid (EMIDP) and 1-hydroxy-2-(2-isopropyl-1H-imidazole-1-yl)ethylidene-1,1-bisphosphonic acid (i-PIDP) were labeled with ^{99m}Tc . Both radiotracers showed selective skeletal uptake *in vivo* with some advantages over [^{99m}Tc]Tc-MDP and [^{99m}Tc]Tc-HDP in regard to chemical stability and pharmacokinetics (Lin et al. 2010; Wang et al. 2011).

For potential theranostic applications, phosphate-containing agents labeled with the β emitter ^{177}Lu (half-life 6.7 days) such as [^{177}Lu]Lu-MDP and [^{177}Lu]Lu-pyrophosphate (PYP) showed high stability and efficient skeletal uptake that has been visualized *in vivo* using planar imaging (Abbasi 2011; Abbasi 2012).

Building on early reports on the use of ^{135m}Ba (half-life 1.2 days) and ^{131}Ba (half-life 11.5 days) as bone-scanning agents (Mahlstedt et al. 1972; Spencer et al. 1971), a recent investigation visualized the efficient skeletal uptake of ^{131}Ba in mice upon intravenous injection of [^{131}Ba]Ba(NO₃)₂ using preclinical SPECT/CT (Reissig et al. 2020). A sufficient cyclotron production route for ^{131}Ba described therein as well as the availability of dedicated multi-pinhole collimators coping with high-energy gamma photons (>400 keV) and recent advances in quantitative image data reconstruction provide the prerequisites to further evaluate the full diagnostic potential of [^{131}Ba]Ba(NO₃)₂ for bone scanning (Crawford et al. 2018).

The release of bioactive molecules such as bone morphogenetic proteins labeled with ^{125}I (half-life 13.2 h) from implanted scaffold materials has been monitored *in vivo* (Hulsart-Billström et al. 2018; Kempen et al. 2009).

Optical imaging

Optical imaging (OI) represents an imaging modality with high sensitivity and a longer signal presence compared to scintigraphy, but low depth (tissue) penetration and spatial resolution (Cowles et al. 2013; Farrell et al. 2018; Lambers et al. 2012). It is based on the detection of photons and currently includes fluorescence imaging, fluorescence molecular tomography, and bioluminescence imaging. Several commercially available bone-affine fluorescent probes are known for OI of bone regeneration *in vivo*. These include tetracycline derivatives and calcium-binding bisphosphonates like risedronate, zoledronate, or alendronate linked to various fluorophores such as carboxy-fluorescein, rhodamine dyes, Alexa Fluor 647, or indocyanine green (Hokugo et al. 2013; Mizrahi et al. 2011; Roelofs et al. 2012). Furthermore, nanoparticles or micelles facilitate the combination of bone-relevant molecules like bisphosphonates or statins and fluorescent dyes obtaining a detectable, highly bone-affine delivery system (Jia et al. 2015; Rudnick-Glick et al. 2015). Near-infrared (NIR) imaging is a rapid and cost-effective non-invasive monitoring method (Jung et al. 2019). A conjugate of the NIR dye IRDye® 800CW and pamidronate enables real-time bone detection *in vivo* based on a deep tissue penetration (Bhushan et al. 2007). Moreover, a conjugate of IRDye® 800CW and a calcium-chelating tetracycline-derivative was used in several murine models including a tissue-engineered construct of a degradable gelatin scaffold and hMSCs to visualize mineralized bone regions (Cowles et al. 2013; Kovar et al. 2011).

In addition, fluorescent probes for OI of osteoclast activity have been investigated with a cathepsin-K peptide substrate linked to an activatable NIR fluorochrome (Cy5.5) (Jaffer et al. 2007). A strategy for simultaneous OI of bone formation comprises Osteosense750, a far-red fluorescent pamidronate. In an ovariectomized murine model, both fluorescent probes were injected intravenously to successfully detect osteoclast activity and concomitant bone loss by the cathepsin-K-activatable probe, as well as regions of newly formed bone by fluorescently-labeled pamidronate (Kozloff et al. 2009).

In contrast to fluorescence imaging, bioluminescence imaging requires metabolically active organisms for imaging bone repair, for example, by using luciferase-bearing transgenic cells or even transgenic mice (De Boer et al. 2006; Lee et al. 2009; Ventura et al. 2014a). Hence, proliferation of transduced hMSCs seeded on hydrogel implants was analyzed in murine calvarial bone defects (Degano

et al. 2008). In comparison to the 2D fluorescence and bioluminescence imaging, fluorescent molecular tomography enables the detection and 3D quantification of NIR probes *in vivo* (Vonwil et al. 2014; Zilberman et al. 2008).

To overcome the limitations of individual imaging modalities, imaging with multimodal agents, like pamidronate combined with fluorescent (Cy5.5) and MRI (gadolinium) probes, allow more comprehensive predictions regarding bone healing (Cowles et al. 2013; Lambers et al. 2012; Liu et al. 2012).

Microdialysis (biochemical imaging)

Microdialysis is a bioanalytical sampling technique used to collect mediators of the extracellular space initially developed for metabolic and neurobiological research, but applicable to all tissues including bone (Förster et al. 2016). Interstitial or wound fluid is collected continuously by diffusion using a semipermeable catheter. The dialysate is analyzed *ex vivo* regarding its mediator/cytokine composition and growth factor content revealing information about the state and progress of healing in real-time on the molecular level (Förster et al. 2016; Waelgaard et al. 2006).

Furthermore, microdialysis can be used to monitor the pharmacokinetics of antibiotics such as vancomycin (Bue et al. 2015), cefuroxime (Tøttrup et al. 2014) and gentamicin (Stolle et al. 2004) in the bone of patients suffering from osteomyelitis or peri-implant infections. Due to its

minimally-invasive application, microdialysis is less harmful to patients than the traditionally applied bone biopsy and allows temporal tracking of tissue infiltration, the distribution of agents/compounds as well as its clearance (Tøttrup et al. 2014).

In situ microdialysis has been used for quantitative analysis of prostaglandin E_2 (PGE_2) in human bone revealing increased PGE_2 secretion after load bearing exercise (Thorsen et al. 1996).

By comparing cytokine pattern of bone defects and soft tissue wounds, specific mediators were identified, which are characteristic for fracture hematoma but do not appear in soft tissue injuries, including neutrophil cytosol factor (NCF)-2, NCF-4 and cytochrome *b*-245 beta peptide (CYBB), matrix metalloproteinase (MMP)-8 and MMP-9 (Förster et al. 2016). Besides concentration differences in bone versus soft tissue, variations in the temporal release of cytokines were documented for interleukin (IL)-6, transforming growth factor (TGF)- β , chemokine (C-X-C motif) ligand 1, CXCL-2 and stromal cell-derived factor (SDF)-1. The additional use of proteomics and metabolomics allows the identification of dominant metabolic pathways that are activated in the early stages of bone healing (Kalkhof et al. 2014). Preliminary studies suggest specific effects of aECM-based biomaterials on the cytokine composition in the defect region (Figure 4). In particular, inflammation modulating materials are promising to modify mediator patterns, which may have an impact on bone healing (Rothe et al. 2019). Microdialysis allows valuable insights into the processes occurring directly at the defect site, which can

Figure 4: IL-1 β secretion in critical size femoral defects in rats.

(A) The wound fluid was collected by microdialysis for 24 h after osteotomy. IL-1 β content was determined by ELISA. The graph depicts the mean values $\pm$ SEM (control $n = 2$; sHA3 and CS $n = 4$) revealing an increased secretion of IL-1 β into defects filled with scaffolds containing chondroitin sulfate (CS) and highly sulfated hyaluronic acid (sHA3). (B) Proportional depiction of the microdialysis probe.

provide information about local effects of functionalized biomaterials.

Biophysical imaging

Ultrasound

Ultrasound velocity as well as ultrasound attenuation have been used for quantitative analysis of fracture healing. The technique is based on the principle of signal transduction, which is determined by the characteristics of the tissue between a transmitter and a receiver. Ultrasound velocity varies in different tissues depending on its composition and structure (Goss and O'Brien Jr 1978). It is decreased at fracture sites compared to intact bone because of lower elasticity and less mineralization of the newly formed callus (Protopappas et al. 2008). During the healing process, mineralization results in increased ultrasound

propagation (Wong et al. 2012). Thus, delayed and non-unions can be identified with this technique (Njeh et al. 1999).

Ultrasound signal propagation in bone was evaluated in a bovine femur (Machado et al. 2011). Simulating different states of mineralization, a bone fragment was demineralized in EDTA for increasing time periods and then inserted into the fracture gap. Depending on the degree of tissue mineralization at the defect site, time of flight (TOF) changed over time, revealing a correlation between calcium content and TOF, suggesting ultrasound transmission measurement as an adequate non-invasive method for monitoring bone healing.

The healing of calvarial defects in rabbits filled with PCL scaffolds coated with an aECM of collagen I/chondroitin sulfate was examined with ultrasonography (Rentsch et al. 2014a). New bone formation detected by ultrasound (Figure 5) correlated with the bone volume measured in postmortem μ CT. However, because

Figure 5: Ultrasonographic examination of the rabbit skull and image analysis.

(A) Defect location and ultrasound probe positioning during examination. (B) Schematic drawing (left) and corresponding ultrasound image (right) of an empty defect 24 h post surgery. (C) Schematic drawing (left) and ultrasound image (right) of a PCL Coll I/CS scaffold at 12 weeks post surgery with evident new bone formation. A defined ROI (red square) was used to quantify the tissue formation within the defect zone and both parietal bone ends are marked in green. Reprinted from BioMed Research International, 2014, with permission from Hindawi. The publication is available at <https://doi.org/10.1155/2014/217078>.

mineralized bone matrix reflects the ultrasound signal in the same way as solid connective tissue, these two types of tissue cannot be distinguished from each other, which may lead to misinterpretation of the healing progress.

In segmental defects in sheep tibiae treated with porous hydroxyapatite ceramic cylinders, mineralized tissue could be detected earlier by ultrasonography than with standard radiography (Wefer et al. 2000). A recent study on bone regeneration in a sheep osteotomy model found significantly more new-bone bulk, surface, and contact with ultrasonography than with CT in early stages of bone healing (Tang et al. 2020). However, this technique identified neither axial malalignment nor non-unions of the long bones as reliable as radiographs. Additionally, since mineralized tissue is highly echogenic, ultrasonography only allows conclusions about the mineralized surface.

Ultrasound attenuation has been shown to be more sensitive than ultrasound velocity measurements (Claes and Cunningham 2009). While a fracture causes a significantly reduced signal amplitude and increased attenuation, the formation of a mechanically stable callus leads to less signal attenuation (Dodd et al. 2007; Wong et al. 2012). Ultrasound was applied to determine blood flow, blood volume and microvascular flow rate for monitoring neovascularization in rat calvarial defects (Leu et al. 2009). Attenuation of blood flow imaging through the bone defects due to defect bridging was used to indirectly track bone regeneration. Defects treated with bioactive glass healed faster and were vascularized more densely than untreated controls.

Several authors combined ultrasound and color-coded Doppler measurements to observe bone healing in patients with periapical lesions on maxillary or mandibular teeth (Rajendran and Sundaresan 2007; Tikku et al. 2010). These measurements distinguish arteries from veins, allowing predictions about the oxygen supply and removal of metabolic products at the defect site (Baab et al. 1986; Tikku et al. 2010). Callus is visible with ultrasound up to three weeks earlier than in radiographs (Tikku et al. 2010; Wong et al. 2012).

Major drawbacks of ultrasound-based evaluation of bone healing are (I) the challenging evaluation of bone strength after a critical level of mineralization is exceeded; (II) the fact that ultrasound measurements are affected by the soft tissue cover (Babatunde et al. 2010); and (III) asymmetric defect bridging generating the same signal as does uniform bone healing (Cunningham et al. 1990). Additionally, small alterations in mineralization degree cannot be detected by ultrasound (Machado et al. 2011; Wong et al. 2012).

Acoustic techniques

Acoustic emission is based on acoustic waves that occur when a material is deformed or destroyed and propagate wave-like through the material (Kapur 2016; Nicholls and Berg 1981). Therefore, this non-destructive technique can be applied for monitoring bone healing and for determination of biomechanical strength of bone and other materials (Pacheco-Salazar et al. 2020; Watanabe et al. 2001). Additionally, several studies documented changes in the amplitudes of acoustic emission measurements through osteoarthritis and minor interventions at the knee compared with healthy individuals (Browne et al. 2016; Sarillee et al. 2014). As the dynamic measurements can detect smallest fissures, acoustic emission may be suitable for quality control of biomaterials and implants of all kinds. A clinical study on callus formation in long bone defects (Hirasawa et al. 2002) found that axial load increased proportionally to the healing progress whereas the acoustic emission signal was eliminated with fracture healing.

Advantages of using acoustic emission in monitoring bone healing and detecting osteoarthritis are low equipment costs, portable gear and non-invasive measurements (Teague et al. 2016).

Visualization of mechanical properties

Vibration is utilized for both stimulation of bone regeneration (Jafarabadi et al. 2016) and monitoring of bone healing. It is based on the transduction of mechanically induced excitation, which may vary depending on composition, density, and stiffness of a certain tissue (Nokes 1999; Wong et al. 2012). Vibrational techniques such as wave propagation or resonant frequency analysis are primarily utilized in evaluating the stability of dental implants in preclinical models (Stadlinger et al. 2012). In clinical applications, this non-invasive and non-destructive method has been used to detect changes in bone mass and stiffness in osteoporosis, fractures, and endoprosthesis loosening (Cunningham et al. 1990; Nikiforidis et al. 1990; Nokes 1999). The resonant frequency of an object is directly proportional to its stiffness (Tower et al. 1993). Hence, with increasing fracture bridging, the resonant frequency of the injured bone approaches that of the healthy contralateral side (Claes and Cunningham 2009; Cunningham et al. 1990).

Vibrational spectroscopic techniques such as Fourier transform infrared microspectroscopy (FTIRM) and imaging (FTIRI) and Raman spectroscopy provide simultaneous,

quantitative, and qualitative information on liquid bone tissue components, mineral and organic matrix with good spatial resolution (Paschalis et al. 2017). These analyses may be combined with other techniques such as histology/histomorphometry, small angle X-ray scattering, quantitative backscattered electron imaging, and nano-indentation (Dudeck et al. 2014; Paschalis et al. 2017). In an osteoporotic rat model, there was a good correlation between synchrotron radiation μ CT, histomorphometry, the indentation modulus, and microhardness of the newly formed bone, measured with scanning nano-indentation suggesting that coatings with collagen and chondroitin sulfate improve both the quantity and quality of bone formed around titanium implants in ovariectomized rats (Figure 6). A major drawback of vibrational analysis is soft tissue interference (Nokes 1999; Wong et al. 2012).

A study on rabbits dealt with injectable calcium phosphate ceramics of different particle size and their effects on critical size defects in the femur (Gauthier et al. 2005). Micro-indentation tests revealed a much higher yield strength for the operated bone after six weeks than for native bone. Another study combined micro-hardness measurements and push-out tests on osteoporotic rats with cylindrical bone defects, which were treated with either titanium or collagen-coated titanium screws (Sartori et al. 2015). Micro-hardness was rather influenced by the

osteoporotic state than by implant coating, but push-out tests revealed a better osseointegration of the collagen-coated titanium implants compared to uncoated titanium. Classical biomaterial testing including pull-out, push-out, torsional or axial compression, and three- or four-point-bending tests are beyond the scope of this review. They are nevertheless important amendments to imaging techniques as detailed above because the mechanical load capacity of bone does not always correlate well with bone volume but is an independent parameter for assessing bone regeneration (Förster et al. 2020). The major drawback of biomechanical testing is the destruction of the tested specimen that is not available for further investigations and precludes clinical application. Although *in vivo* biomechanical testing has been documented, these analyses are not well established, because they are thought to affect bone healing compared to non-biomechanically stimulated controls (Wulsten et al. 2011).

Summary and outlook

Adequate longitudinal imaging is essential in understanding and modifying the processes taking place during the various stages of bone healing. Refinement of these methods will substantially reduce preclinical methods that require

Figure 6: Synopsis of synchrotron radiation mCT, scanning electron microscopy (SEM), indentation modulus (Er) and microhardness (H) of the newly formed bone around a titanium implant in the rat tibia coated with an aECM of collagen and chondroitin sulfate.

Measurements were performed with scanning nano-indentation in the enlarged area depicted on the left panel. Reprinted from Acta Biomaterialia, 10, Increased bone remodeling around titanium implants coated with chondroitin sulfate in ovariectomized rats, 2855–2865, Copyright (2014), with permission from Elsevier. The publication is available at <https://doi.org/10.1016/j.actbio.2014.01.034>.

Figure 7: Synopsis of multimodal imaging for the monitoring of bone healing.

explanation of the specimens, which in turn reduces the number of animals required for preclinical research. Computational-based methods replaced the traditionally performed 2D radiographic analysis in research and amend histological techniques. Qualitative information is thus complemented by quantifying evaluations. In the future, smart materials, which not only serve to track bone healing, but also enable sensing as well as computer-based and individualized communication, are likely to gain importance in the course of personalized therapy.

To assess the efficacy of new biomaterials, analytical techniques must be tailored individually. For example, PET scans are more suitable than X-ray or CT for functional aspects, whereas the latter are usually sufficient to assess defect bridging. A synopsis of imaging techniques for the monitoring of bone healing is provided in Figure 7.

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