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Review

Jingle Cell Rock: Steering Cellular Activity With Low-Intensity Pulsed Ultrasound (LIPUS) to Engineer Functional Tissues in Regenerative Medicine

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ABSTRACT

Acoustic manipulation or perturbation of biological soft matter has emerged as a promising clinical treatment for a number of applications within regenerative medicine, ranging from bone fracture repair to neuromodulation. The potential of ultrasound (US) endures in imparting mechanical stimuli that are able to trigger a cascade of molecular signals within unscathed cells. Particularly, low-intensity pulsed ultrasound (LIPUS) has been associated with bio-effects such as activation of specific cellular pathways and alteration of cell morphology and gene expression, the extent of which can be modulated by fine tuning of LIPUS parameters including intensity, frequency and exposure time. Although the molecular mechanisms underlying LIPUS are not yet fully elucidated, a number of studies clearly define the modulation of specific ultrasonic parameters as a means to guide the differentiation of a specific set of stem cells towards adult and fully differentiated cell types. Herein, we outline the applications of LIPUS in regenerative medicine and the *in vivo* and *in vitro* studies that have confirmed the unbounded clinical potential of this platform. We highlight the latest developments aimed at investigating the physical and biological mechanisms of action of LIPUS, outlining the most recent efforts in using this technology to aid tissue engineering strategies for repairing tissue or modelling specific diseases. Ultimately, we detail tissue-specific applications harnessing LIPUS stimuli, offering insights over the engineering of new constructs and therapeutic modalities. Overall, we aim to lay the foundation for a deeper understanding of the mechanisms governing LIPUS-based therapy, to inform the development of safer and more effective tissue regeneration strategies in the field of regenerative medicine.

Introduction

Low-intensity pulsed ultrasound (LIPUS) consists of sound waves with a characteristic frequency greater than 20 kHz. Recently, LIPUS stimulation has been used as a non-invasive method for therapeutic applications in regenerative medicine [1]. Notably, LIPUS has been used for the clinical treatment of a wide range of skeletal pathologies such as osteoarthritis [2], tendinitis [3], carpal tunnel syndrome [4], fracture healing [5], but also ischemic stroke [6], obesity [7], hepatic insufficiency [8], and other conditions.

Novel LIPUS-based applications are coming to the fore to guide the stimulation of cellular compartments as well as to probe the mechanical properties of biomaterials. LIPUS waves have been found to selectively influence cell fate, conditioning their behavior and ultimately their functionality [9]. In particular, LIPUS has demonstrated the ability to influence cell morphology through the application of mechanical pressure

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fields, consequently activating specific cellular pathways and altering gene expression [10–12].

To date, the underlying mechanisms of LIPUS are not fully understood. However, a number of studies have shown that following LIPUS-based stimulation of cells, a series of biochemical events are triggered at the molecular level driving cell proliferation, adhesion, migration, differentiation and the production of extracellular matrix (ECM) [11–15]. More specifically, LIPUS has been demonstrated to foster the release of a gradient of cytokines, growth factors and adhesion molecules that enhance the homing of cells [16].

Among the most promising applications of LIPUS is the modulation of the physical properties of biomaterials, such as cell membrane permeability, for enhanced drug delivery and more. Notably, LIPUS can modulate the release kinetics of molecules from specific biopolymers and this approach has been shown to be effective in delivering chemotherapeutics to tumors without damaging the surrounding healthy tissues [17]. LIPUS has also been harnessed to induce cell differentiation to repair a variety of tissue defects. In a number of studies, the use of specific LIPUS parameters including frequency ranging from 1 to 1.5 MHz, intensity between 30 and 100 mW/cm^2 and duty cycle between 5% and 50%, has been reported to direct the differentiation of stem cells towards skeletal [18], chondrogenic [19], and adipose [20] lineages.

In this review, the use of LIPUS technology in the context of regenerative medicine will be discussed, reporting the latest findings on the physical and biological mechanisms of action and the direct and indirect effects of LIPUS on the cellular activity and functionality, for the engineering of human tissues.

LIPUS in medicine as a therapeutic modality

Fundamental principles of LIPUS

Before discussing the therapeutic applications of LIPUS, it is crucial to comprehend the basic principles of this technology to better interpret the physical parameters that will be mentioned throughout this review. Furthermore, understanding the physics underlying LIPUS generation and propagation is essential for designing meaningful applications in the medical field. LIPUS belongs to the category of sound waves with a frequency above the range of human hearing, that is, typically >20 kHz. Therefore, LIPUS waves have a much shorter wavelength (λ) when compared to audible sound waves [21]. Particularly, the acoustic spectrum is comprised of three main frequency ranges (subsonic, audible, and ultrasonic), with ultrasound (US) waves further divided into three subgroups: low frequency (20–200 kHz), conventional (200 kHz–100 MHz) and high frequency (>100 MHz) [22,23]. In the range between 20 kHz and 2 MHz are power US and therapeutic US, while between 2 and 10 MHz are diagnostic US (Fig. 1a).

Since US and LIPUS waves share the same basic physical characteristics, they propagates as a pressure wave in space and time, and can be characterized using parameters such as the wavelength (λ), period (T), and amplitude (Fig. 1b). The time to complete a wave cycle corresponds to the period (T). The frequency (f), that is, the inverse of the period (T), represents the number of cycles completed in one second and is measured in Hertz (Hz). LIPUS exclusively travels through liquids or solids, and not through a vacuum. The speed at which a LIPUS wave travels

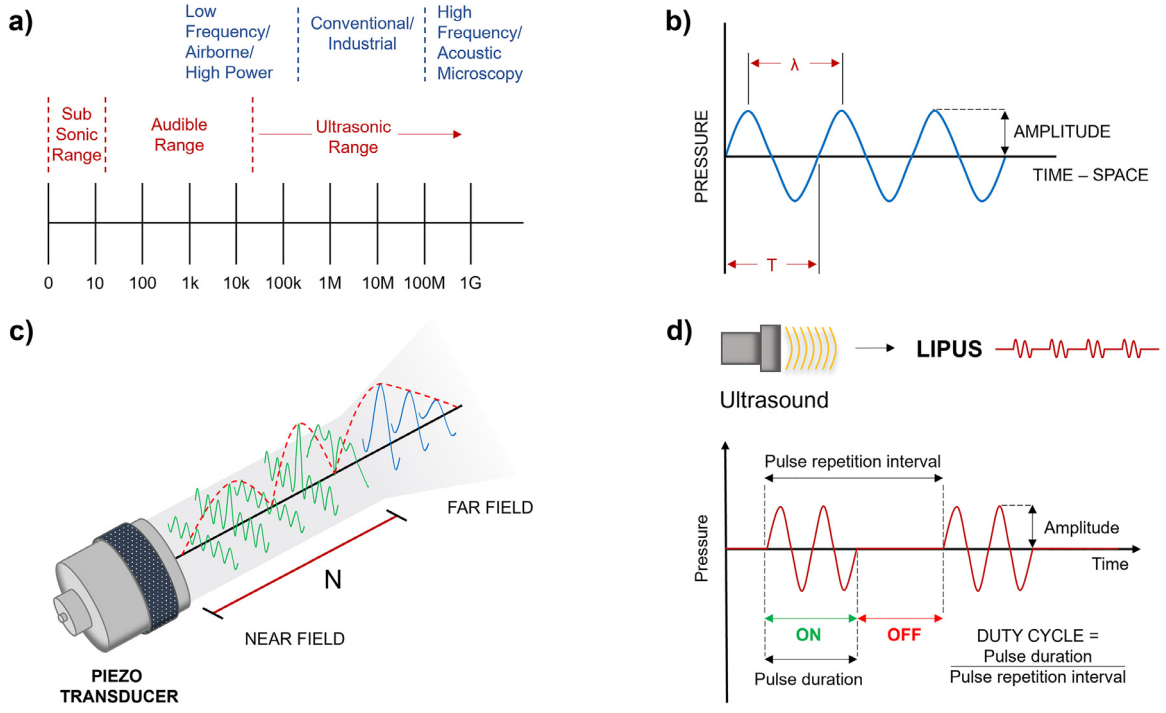

Figure 1. Key properties of LIPUS waves and typical application regime in therapeutic applications. (a) The acoustic spectrum is divided into three main frequency ranges: subsonic, audible, and ultrasonic. Ultrasound can be further classified into three frequency sub-domains: low frequency, conventional, and high frequency. (b) Representation of an ultrasonic wave. The key parameters describing the wave are indicated: the wavelength (λ) is the distance between two maxima or minima of the wave, the period (T) is the time required to complete one cycle and is the inverse of the frequency (f). A piezoelectric ("piezo") transducer (c) is typically employed to generate an ultrasound field consisting of two spatial zones: the near field and the far field. In the area closest to the transducer surface (near field), the generated ultrasound waves are characterised by peaks of amplitude oscillating with a lack of uniformity. In the far field, the wave pressure amplitude gradually decreases away from the transducer surface. At this given distance from the transducer, the pressure distribution is more uniform, due to the higher stability and predictability of the acoustic field. (d) Low-Intensity Pulsed Ultrasound (LIPUS) (and pulsed ultrasound in general) comprises of pulses of US waves, characterised by alternating periods where the US wave is ON and OFF, respectively. The pulse duration corresponds to the ON time, while the ON + OFF time corresponds to the pulse repetition interval. The number of pulses of a repeating signal in a specific time unit corresponds to the PRF. The duty cycle is the ratio between the pulse duration and the pulse repetition interval, and is expressed as a percentage.

through an elastic material (c) at a given environmental temperature and pressure is constant, and is expressed by eqns (1) and (2) [21,24]:

$$c = \lambda \cdot f \quad (1)$$

$$c = \lambda / T \quad (2)$$

Ultrasonic waves propagating through a medium induce an oscillatory motion of the particles that constitute the medium. LIPUS fields can be classified in different categories depending on the direction of particle's oscillation with respect to the direction of pressure wave propagation. The wave type most commonly used are longitudinal waves (particles travel in the same direction as the direction of wave propagation), shear waves (particles travel perpendicular to the direction of wave propagation) and surface waves (particles travel elliptically over the surface of the material) [24].

Typically, LIPUS is generated by piezoelectric transducers made of lead zirconate titanate (PZT) that convert electrical energy into mechanical energy. Ultrasonic transducers are a combination of a damping material (which dissipates energy) placed under an active, vibrating material (which stores energy). Their combinatorial response causes a reduction in amplitude of the transmitted wave and reduced pulse length [25,26]. The diffraction pattern of the ultrasonic wave generated by PZT elements is divided into two characteristic zones: the near field (*i.e.*, located near the transducer's surface) and the far field (*i.e.*, located distally from the transducer's surface) (Fig. 1c). The complexity of the spatial characteristics of the ultrasonic field is higher in the near field. After a series of successive axial oscillations between maximum and minimum acoustic pressure, the last pressure peak is located at a distance *N* from the transducer. This distance represents the boundary between the near and the far fields, beyond which the pressure peak of the transmitted wave gradually decreases to zero [27] with an attenuation rate that depends on the milieu in which LIPUS propagates.

LIPUS is often applied to targets that are located in the far field, due to the higher predictability of the acoustic field in this region compared to the near field. Knowing the maximum pressure peak in the far field area and the attenuation coefficient of the medium, the wave intensity at a given distance from the source can be predicted. Approaches such as acoustically produced luminescence (APL) [28] can be exploited for this purpose to map the ultrasonic pressure field.

The amount of attenuation of the ultrasonic wave through a material plays a key role in the selection of a transducer (and the frequency it operates at) for a given medical application. Attenuation can be caused by diffraction, scattering and absorption of the incident LIPUS wave. Acoustic impedance represents another important property and it is a measure of the resistance that a material offers to the propagation of LIPUS through it [29]. This parameter is related to the density of a material and to the speed of sound propagating through the material. Acoustic impedance is used to predict the amount of energy reflected and/or transmitted when the wave travels across an interface between two different materials. For instance, the higher the impedance mismatch between neighbouring materials the larger the proportion of wave reflected.

The understanding of the fundamentals of LIPUS physics is an important pre-requisite, not only to facilitate development of medical technologies but also to gain insights on the principles behind their therapeutic efficacy. This knowledge provides the basis for the many therapeutic uses of LIPUS, which holds promise in accelerating the healing of biological defects and treating a number of pathological conditions.

LIPUS for therapeutic applications

Typically, the majority of minor injuries are able to heal on their own in a short period of time, while major defects and damaged tissues require specific treatment [30]. Several therapeutic technologies have been proposed to regenerate damaged tissues, including the use of LIPUS [31]. Already used for diagnostic imaging, US was introduced as a medical treatment in the 1950s [9]. Further clinical investigations [32] resulted in the approval of the use of EXOGEN (Ultrasound Bone Healing System) in 1994 by the U.S. Food and Drug Administration (FDA). This approach used an ultrasonic beam directly focused on the specific defect of interest, in order to accelerate the repair process of a lesion such as a fractured bone tissue [9]. More broadly, therapeutic US has been classified into high-intensity focused ultrasound (HIFU, 100 and 10000 W/cm^2 [33]) and low-intensity pulsed ultrasound (LIPUS, 0.03–0.1 W/cm^2 [34]). HIFU is employed for disrupting diseased tissues and ablating tumours [35], as well as for lithotripsy (treatment of kidney stones) [36]. Kidney stones are selectively disintegrated due to cavitation triggered by HIFU waves, without affecting the surrounding tissues [37]. On the other hand, LIPUS is mostly applied for the regeneration and healing of bone fractures [9], but at the same time to also treat conditions such as obesity [7,38], hepatic failure [8] or even brain injury [39,40] (Table 1). Although many studies [41–44] have used acoustic intensities of about 30–100 mW/cm² and frequencies between 1 and 1.5 MHz in LIPUS applications, it is important to note that this is not a rigid definition. Various studies have reported LIPUS applications with parameters outside this range [45–48]. Therefore, while this review will focus on the commonly used parameter range, it should be recognized that LIPUS can be applied under different conditions depending on the specific medical application and desired therapeutic outcome. It has been previously demonstrated that LIPUS is able to promote the healing of bone defects both in animal models and in clinical studies [49,50] by using mechanical stimulation produced by ultrasonic waves. Bone defect healing has been observed to occur with a 40% reduction in healing time and has been associated with increased osteogenic activity, protein synthesis and cell proliferation [18,51]. Heckman and co-workers reported a 20-min LIPUS treatment that demonstrated a 38% acceleration in the healing of fresh fractures [52], while Nolte and colleagues demonstrated a 86% improvement in pseudoarthrosis [53]. The LIPUS parameters USED, including frequency, duty cycle, energy and intensity [54,55], may vary depending on the therapeutic applications. Thus, the selection of parameter values is essential to determine the mode of transmission of the ultrasonic waves through the tissues and to obtain specific biological responses [56]. A brief introduction to basic concepts relating to LIPUS parameters and corresponding properties is therefore provided below.

LIPUS in medical treatments

LIPUS parameters including frequency, pulse repetition frequency (PRF), duty cycle, and intensity significantly influence cellular responses. The choice of these parameter values determines the biological effects observed in different tissues. As mentioned above, typical frequencies used in LIPUS treatments vary between 1 and 1.5 MHz. Higher frequencies are often associated with enhanced bone healing and osteogenic differentiation. On the other hand, frequencies below 1 MHz can be used for targeting deeper tissues or in specific applications like neurostimulation (Table 1). A further key parameter in LIPUS is the PRF, which represents the number of US pulses transmitted per second expressed in Hertz (Hz). Higher PRF values have been shown to enhance cellular responses in some cases, such as through increased production of growth factors and cytokines 9. Due to their pulsed nature, LIPUS is characterised by ON/OFF cycles of sound waves; the ratio between the time during which a transducer is switched ON and the total time between the start of consecutive pulses is called duty cycle. A duty cycle of 20% means that the transducer is 20% ON and 80% OFF during a LIPUS pulse (Fig. 1d). The most commonly used values of duty cycle in LIPUS treatments range from 5% to 50%. Higher duty cycles have been shown to promote processes of cell proliferation and differentiation 9. For example, a 20% duty cycle has been effective in promoting chondrogenic differentiation and cartilage tissue regeneration [71]. The LIPUS intensity (mW/cm^2) is also a key parameter to be controlled in order to modulate and generate cellular responses. The intensity of LIPUS waves

Table 1
Low-intensity pulsed ultrasound (LIPUS) treatment in different tissues and for a range of different applications

Tissue	US parameters				Applications	References
	Frequency	Intensity	DC	Treatment time		
Bone	1.5 MHz	30 mW/cm^2 SATA	20%	20 min/d	Bone fracture	[57]
					Osteogenic differentiation	[58]
		100 mW/cm^2 SATA			Osteoporotic fracture	[18]
					Femoral fracture	[5]
				3 min/d	Bone regeneration therapies	[59]
	1.0 MHz	100 mW/cm^2	10%	10 min/d		[12]
		50 mW/cm^2	20% – 50%		Periodontal healing and regeneration	[60]
Cartilage	1.5 MHz	100 mW/cm^2 SATA	20%	3 min/d	Regeneration of damaged cartilage tissues	[19]
		30 mW/cm^2 SATA		20 min/d	Osteoarthritis	[61]
						[62]
					Osteochondral defects	[63]
Brain	1.0 MHz	528 mW/cm^2 SPTA	5%	5 min/d	Brain injury	[39,64]
		100 mW/cm^2	–			[40]
		110 mW/cm^2 SPTA	50%	15 min/d	Neurodegenerative diseases	[65]
	0.04 MHz	50 mW/cm^2	–	60 min/d	Ischemic stroke	6
Fat	1.0 MHz	100 mW/cm^2 SATA	20%	3 min/d	Regeneration of adipose tissues	[20]
	0.5 MHz	–	–	10 min/d	Clinical obesity control	[7]
	3.0 MHz	60 mW/cm^2	–	20 min/d		[38]
Liver	1.0 MHz	500–1500 mW/cm^2	20%	30–120 sec/d	Regeneration of hepatic tissues	[8]
		1000 mW/cm^2		60 sec/d		[66]
Nerve	1.0 MHz	50 mW/cm^2 SPTA	20%	5 min/d	Nerve regeneration	[67]
		30 mW/cm^2	–	20 min/d		[68]
		250 – 750 mW/cm^2	20%	5 min/d	Peripheral nerve regeneration	[69]
		300 mW/cm^2			Repair of sciatic nerve	[70]

DC: duty cycle; SATA: spatial average-temporal average; SPTA: spatial peak-temporal average
Although the definition of LIPUS assumes the use of intensities between 30 and 100 mW/cm^2 and frequencies between 1 and 1.5 MHz, there are some exceptions reported in this table. These were still classified as LIPUS, even if the parameter values deviate from the ranges provided by the conventional definition.

is the velocity at which the energy is deposited per unit area and can vary temporally and spatially. Spatial average (SA) and spatial peak (SP) are measurements of spatial intensity while temporal average (TA), temporal peak (TP) and pulse average (PA) are related to temporal intensity. Consequently, combinations of spatial-temporal intensities such as SATA, SAPA, SATP, SPTA, SPPA and SPTP can be determined and have been utilized as LIPUS field descriptors in various applications [9]. Understanding the spatial and temporal distribution of US intensity is crucial for interpreting LIPUS effects. SATA represents the average intensity over the area of US beam and the duration of the pulsing cycle, and is expressed by eqn (3):

$$SATA = \frac{Total\ energy\ delivered}{Area \times Total\ time} \tag{3}$$

SAPA is the average intensity over the US beam area during the pulse's active period. SAPA is less commonly reported but is important for understanding peak intensities during pulsing, and is expressed by eqn (4):

$$SAPA = \frac{Energy\ delivered\ during\ pulse}{Area \times Pulse\ duration} \tag{4}$$

SATP is relative to peak intensity averaged over the area of the beam during the highest point of the pulse and is expressed by eqn (5):

$$SATP = \frac{Peak\ power}{Area} \tag{5}$$

SPTA represents the peak intensity at the focal point of the US beam averaged over time. Is often used to describe the maximum exposure level and is expressed by eqn (6):

$$SPTA = \frac{Peak\ intensity \times Duty\ cycle}{Total\ time} \tag{6}$$

SPPA is the peak intensity at the focal point during the pulse's active period; it is relevant for understanding the maximum instantaneous exposure during pulsing and is defined by eqn (7):

$$SPPA = \frac{Peak\ intensity}{Area} \tag{7}$$

Lastly, SPPA is related to the highest possible intensity at the focal point at the peak of the pulse. It is a useful parameter in applications requiring precise targeting of high intensities and is defined by eqn (8):

$$SPTP = \frac{Peak\ intensity}{Area \times Pulse\ duration} \tag{8}$$

While there are various definitions of LIPUS intensity, the scientific literature commonly employs both SATA and SPTA as intensity metrics. Although SATA has been used frequently over the years [5,71–73], SPTA is also widely discussed and applied in therapeutic US studies [74–77]. Consequently, it is useful to consider both metrics when discussing LIPUS applications.

Each of the explained parameters can be combined to achieve specific cellular responses. For instance, a number of studies used a SATA of 30 mW/cm^2, ultrasonic center frequency of 1.5 MHz, PRF of 1 kHz, and exposure time of 20 min per day to induce osteogenic differentiation and bone healing both *in vivo* [78] (rodent) and *in vitro* [79,58]. Other studies have employed the same parameters with a stimulation of 3 min per day, resulting in an increase in total protein content, calcium deposition and alkaline phosphatase activity *in vitro* [19,59,80].

It has also been proved that different parameters can direct cell differentiation more preferentially towards chondrocytes than osteoblasts. Aliabouzar *et al.* [19] reported that a frequency of 1.5 MHz, intensity of 100 mW/cm^2 and 20% duty cycle are optimal LIPUS parameters for promoting tissue regeneration and maintaining cartilage tissue health. Stimulation increased cell proliferation by 60% after 24 h and glycosaminoglycans (GAGs) synthesis by chondrocytes up to 16% after 2 wk.

Furthermore, an intensity (SATA) greater than 1000 mW/cm^2 has been demonstrated to be detrimental for tissue viability [72]. In this context, the authors observed a negative effect on viability following stimulated fracture healing in a rabbit fibulae model.

Although most of the applications of LIPUS focus on bone healing and regeneration, there are many other conditions and tissues that are treated with LIPUS therapies. This aspect will be discussed in detail in the paragraph "LIPUS effects on tissues" below. However, before

exploring this further, it is essential to understand the mechanisms underlying the biological effects of LIPUS on tissues. Although these are not yet fully elucidated, numerous studies have attempted to gain a more pervasive understanding of the biophysical effects and pathways involved in LIPUS.

LIPUS to stimulate biological tissues

LIPUS has already been used in a variety of therapeutic applications and has been extensively studied. This paragraph reports on recent studies that have focused on the mechanisms of action of LIPUS on living cells and biomaterials.

Biological effects of LIPUS stimuli on cells

LIPUS has demonstrated significant effects on various cellular processes, particularly in skeletal stem cells (SSCS). Notably, LIPUS has been reported to impart a pressure field onto living cells, mechanically stimulating and triggering a series of signaling cascades ultimately altering gene expression and resulting in specific protein synthesis [13,31]. SSCs are multipotent stromal cells with trilineage potential, which are able to differentiate into bone, cartilage or fat cells [9,81], thus assisting in tissue regeneration. Proliferation and differentiation of MSCs following LIPUS stimulation has been previously demonstrated [82,83]. Although studies have attempted to explore the precise mechanisms by which LIPUS exerts bio-effects, these are only partially understood. A reported effect of LIPUS is the generation of a local gradient of cytokines, growth factors and adhesion molecules, which is postulated to facilitate the homing of SSCs [9]. This cascade of events is directly connected to initial stages of inflammatory response following bone injury. Indeed, during the inflammatory phase of the healing process [84], cytokines (such as interleukin-6 (IL-6)) and growth factors (such as stromal cell-derived factor 1 (SDF-1)) released from the damaged tissue, facilitate attraction of skeletal precursors to the injury site, consequently initiating the bone regeneration process [81,85,86]. Furthermore, a significant influence over cell adhesion has been shown for LIPUS stimulation via signaling proteins such as integrins and fibronectins [87]. LIPUS is also able to trigger the release of adenosine triphosphate (ATP) within the extracellular space following the opening of connexin 43 (Cx43) channels on the plasma membrane, with consequent intracellular of Ca^{2+} deposits [88].

There is considerable evidence that different parameters of LIPUS can guide SSCs towards different cellular targets [89,90]. LIPUS drives osteogenic differentiation by increasing gene expression of collagen type I alpha 1 chain (COL1A1), collagen type X alpha 1 chain (COL10A1), osteocalcin (OCN), osteopontin (OPN), osteoprotegerin (OPG), osterix (OSX), alkaline phosphatase (ALP), and bone morphogenetic protein 2 (BMP-2) [58,91–93]. In contrast, when stimulated with chondrogenic media, bone marrow stromal cells (BMSCs) exposed to LIPUS have been shown to overexpress transcription factor SOX-9 (SOX9), collagen type II alpha 1 chain (COL2A1), aggrecan (ACAN), and tissue inhibitor of metalloproteinases 2 (TIMP2) [89,90,92]. In neuronal regeneration, LIPUS increases the expression of brain-derived neurotrophic factor (BDNF) by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via the tropomyosin-related kinase B/phosphoinositide 3-kinase/protein kinase B (TrkB/PI3K/Akt) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) signaling pathways [65]. Moreover, in the process of adipogenesis, LIPUS stimulation increases the expression of adipogenic peroxisome proliferator-activated receptor gamma (PPAR-γ1) and adiponectin (APN) genes in adipose stem cells [20], while in hepatocytes it activates the Wnt/β-catenin signaling pathway, increasing the expression of α-fetoprotein (αFP/AFP), cytokeratin 18 (CK18), albumin (ALB) and glycogen [94]. In contrast, there is a lack of data evaluating effects on nervous tissue, where LIPUS is known to increase neurotrophic factors through focal adhesion kinase/extracellular signal-regulated kinase 1/2 (FAK/ERK1/2) signaling pathway [67] (Fig. 2).

The biological mechanisms related to osteogenic differentiation are the most widely understood and investigated, and it is accepted that LIPUS further promotes osteogenesis via the Ras homolog family member A (RhoA) protein, which activates the Rho-associated protein kinase (ROCK)–cancer Osaka thyroid oncogene/tumor progression locus 2 (Cot/Tpl2)-mitogen-activated protein kinase (MAPK)- ERK signaling pathway [91,92,95–97]. This in turn phosphorylates Cot/Tpl2 kinases, which is regulated by ERK/MAPK signals [93]. In addition, ERK/MAPK signaling, together with PI3K/Akt is activated by LIPUS, resulting in increased proliferation of MSCs [98–101] (Fig. 3).

Intracellular calcium deposition is also increased as a result of LIPUS exposure. It has recently been reported that the osteogenic differentiation process in stimulated SSCs occurs by activating voltage-dependent Ca^{2+} ion channels (VGCC) [26]. Briefly, mechanical stimulation depolarizes the cell membrane causing activation of L-type VGCC to regulate calcium influx into cells. L-VGCC are activated by physical coupling of the Ca_v 1.1 subunit of the receptor (DHPR) to the receptor (RyR) on the endoplasmic reticulum of cells [102].

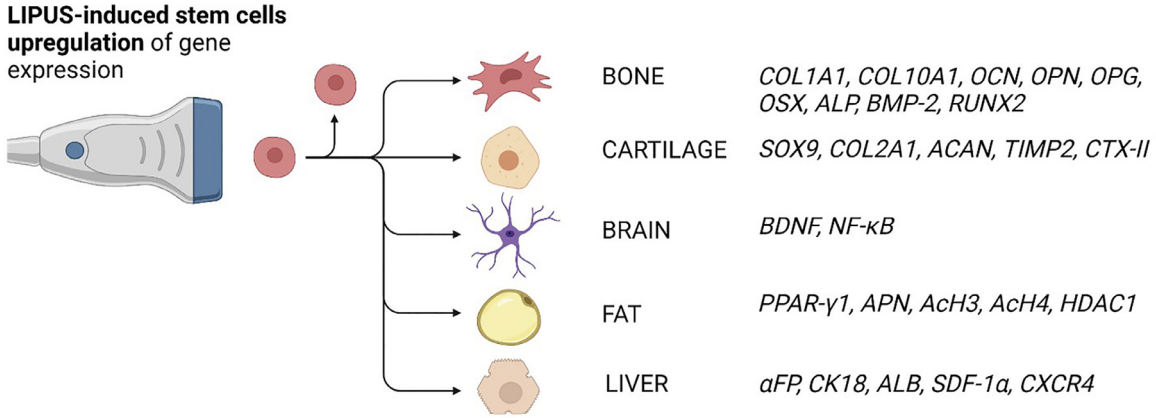

Figure 2. Effects of stimulation with LIPUS on stem cells: upregulation of gene expression in various tissues. LIPUS promotes osteogenic differentiation by increasing gene expression of COL1A1, COL10A1, OCN, OPN, OPG, OSX, ALP, BMP-2, and RUNX2. In chondrogenic differentiation, SSCs exposed to LIPUS overexpress SOX9, COL2A1, ACAN, TIMP2, and CTX-II. In the context of neuronal regeneration, LIPUS increases BDNF expression by activating NF-κB via the TrkB/PI3K/Akt and CaMK signaling pathways. Furthermore, in the process of adipogenesis, stimulation with LIPUS increases the expression of the adipogenic genes PPAR-γ1, APN, AcH3, AcH4, and HDAC1, while in hepatocytes it activates the Wnt/β-catenin signaling pathway, increasing the expression of αFP, CK18, ALB, SDF-1α and CXCR4.

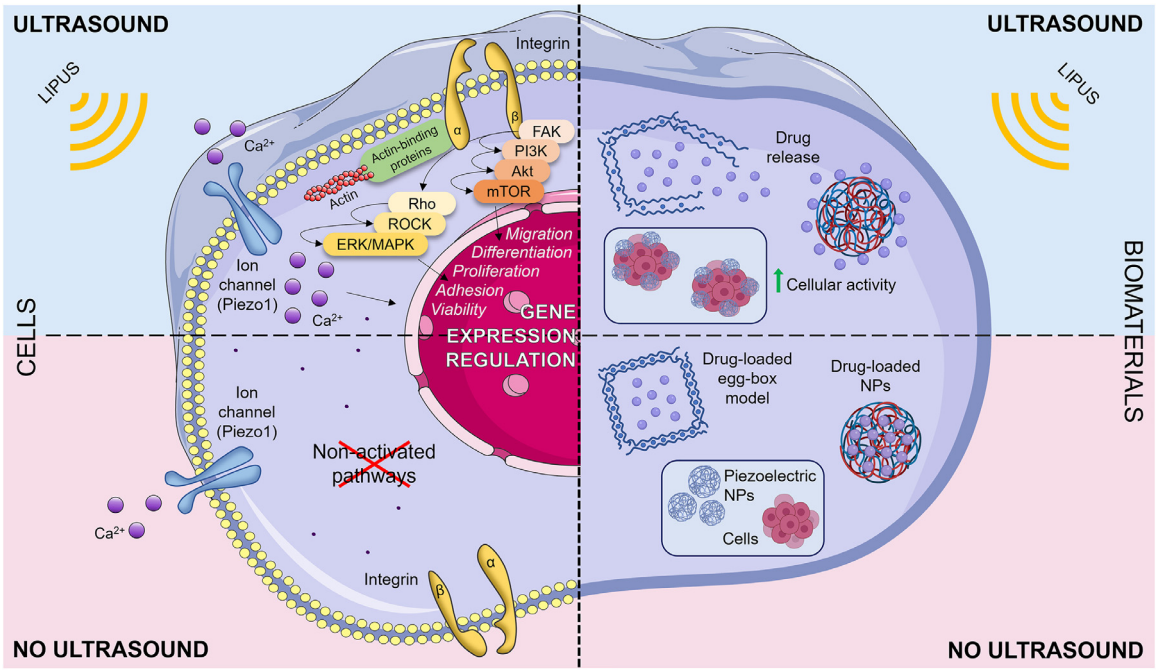

Figure 3. Effects of LIPUS on cells and biomaterials. (Left) The mechanical stimulus provided by LIPUS triggers mechanoreceptors such as integrins, which mediate cell migration, as well as the Piezo1 ion channel, which allows Ca^{2+} influx and activates mammalian target of rapamycin (mTOR) signaling pathways. Subsequently, phosphorylation of signaling proteins occurs, activating Rho/ROCK, which consequently stimulates ERK/MAPK signaling. Other signaling pathways may be activated, including PI3K/Akt. These pathways regulate different cellular functions such as viability, proliferation, adhesion, migration and differentiation. (Right) LIPUS combined with different types of materials. LIPUS activation reversibly breaks the egg-box structure (*e.g.,* alginate) and releases the medicine trapped inside. NPs can be loaded with medicines and stimulated to change their conformation and thus release the payload, such as an anti-cancer drug, in a controlled way. Finally, in an example of LIPUS stimulation of piezoelectric materials, ultrasonic exposure can upregulate the expression of myogenic genes and increase cell aggregation to develop muscle tissue.

LIPUS in biomaterials

Recently, LIPUS has been harnessed to manipulate the mechanical properties of biomaterial-based scaffolds, primarily for drug delivery purposes (Fig. 3). The first studies on the combination of US with biomaterials were conducted by Kost and colleagues [103]. Initially, it was observed how specific biopolymers (polyanhydrides, polyglycolides and polylactides) were degraded by US in order to modulate the release kinetics of substances that were loaded within these matrices. By modulating the intensity of ultrasonic stimulation, a significant increase in the rate of molecule release was observed *in vitro*. Nevertheless, *in vivo* experiments revealed that scaffolds implanted in rats showed no significant differences between normal rat histological samples and US-stimulated samples.

Over the years, LIPUS platforms have proven to be a promising for the modulation of chemotherapeutics dosing at the site of interest, thereby reducing the exposure of non-cancerous tissues. Notably, tumors are known to be highly responsive to greater chemotherapeutic doses during short periods of time compared to sustained low doses over prolonged periods of time [104]. In this regard, Huebsch and co-workers [17] developed injectable alginate scaffolds able to degrade and subsequently self-heal by activation and deactivation with LIPUS stimuli (120 mW/cm^2 SATA intensity, applied for 2.5 min once every 24 h), respectively. Chemotherapeutics were effectively delivered when LIPUS was active causing a temporary opening of the ionic network of the alginate scaffold to allow the release of the encapsulated drugs. Furthermore, following the external LIPUS stimulus, the presence of Ca^{2+} ions resulted in the formation of new ionic bonds thus stimulating further regeneration of the alginic network. In another study, the targeted release of doxorubicin (DOX) from gold nanoparticles (GNPs) driven by LIPUS stimuli in an *ex vivo* tissue model was explored [105]. A numerical model was then created to predict GNPs aggregation and drug release in response to ultrasonic stimulations. Exposure to LIPUS caused significant aggregation of GNPs and thus anti-cancer drug release. The electrical potential of GNPs was found to increase from -30.29 mV to -15.79 mV, indicating lower particle stability and a greater inclination to aggregation. In a number of studies, nanoparticles (NPs) have been engineered to react to US stimuli. For example, the modification of NPs with trigger-responsive polymers that act as a "nano valve" [106], can be used to control the release of molecules from NP carriers. Paris and co-workers [107] synthesized DOX-loaded silica NPs functionalized with a temperature and LIPUS dual trigger-responsive copolymer, which had an open conformation at 4°C and a collapsed conformation at 37°C. Following LIPUS exposure, local hyperthermia was induced, causing a phase transition of the copolymer that changed its hydrophobicity and allowed the release of the encapsulated DOX molecules.

US has also been proven capable of activating piezoelectric biomaterials with potential uses in regenerative medicine. Recently [108], piezoelectric barium titanate NPs (BTNPs) were added to a composite ink made of alginate and Pluronic for the 3D bioprinting of muscle cell-laden hydrogels. The combination of piezoelectric NPs and US stimulation upregulated the expression of myogenic genes and enhanced cell aggregation, a crucial step in muscle tissue development. A further study introduced a new therapeutic paradigm, referred to as piezodynamic therapy [109]. This combines LIPUS stimuli with a titanium alloy (Ti_6Al_4V) substrate coated with piezoelectric $BaTiO_3$. Stimulation of the piezoelectric biomaterial was shown to regulate cell behavior by electrical stimuli. The cells had higher viability than unstimulated controls, increased adhesion and proliferation, as well as increased activation of mitochondria, overall resulting in decreased apoptotic damage. Piezodynamic therapy also increased the expression of osteogenesis-related genes (*i.e*, BMP-2), promoting more efficient bone healing.

LIPUS-stimulated biomaterials can be used as novel platforms for the regeneration of specific tissues. In particular, the combination of LIPUS, biomaterials and cells has demonstrated a consequential increase in cell differentiation and proliferation that can be exploited to repair tissue

defects. Hui and colleagues [110] treated a rabbit spinal deformity by stimulating a porous tricalcium phosphate bioceramic scaffold decorated with BMSCs *in vivo*. The LIPUS-stimulated cell constructs were found to promote spinal fusion between selected vertebral segments compared to unstimulated controls. After 7 wk post-implantation, osseointegration between the LIPUS-treated bone and the implanted tricalcium phosphate scaffold was significantly enhanced in comparison to scaffolds that were not exposed to LIPUS. In a further work [19], the effect of LIPUS on the proliferation and chondrogenic differentiation of BMSCs seeded on polyethylene glycol diacrylate (PEG-DA) scaffolds was investigated. PEG-DA was found to be an excellent cartilage-like material due to its high water content. Following LIPUS stimulation of PEG-DA scaffolds, a cell proliferation of up to 60% was observed after 24 hours. In addition, an increase in GAGs synthesis of 16% and COL2 of 60% was observed after 2 and 3 wk, respectively, demonstrating that cell-loaded and LIPUS-stimulated biomaterials are able to regenerate cartilage damage.

The research concerning the combined use of tissue-specific biomaterials, cells and US has shown that tissue regeneration based on this technology is an efficient method for tissue engineering. Furthermore, this technique has enormous potential with regards to on-demand release of drugs in a controlled manner and thus for the treatment of specific diseases.

LIPUS effects on tissues

LIPUS stimulation has been applied to a number of tissues for engineering purposes, facilitating cell differentiation and growth, and favoring regeneration. We are here reporting the most recent applications of LIPUS for the engineering of new human tissue analogues.

Bone

Bone defects are routinely treated using autologous tissue. However, major skeletal defects can be temporarily filled with custom metal implants. Due to the lack of biodegradability, mechanical mismatch and insufficient quality, research has focused over the last two decades on novel strategies for the repair of bone defects [111]. LIPUS has been proposed as a functional method to promote bone growth in clinical practices [112], stimulating osteogenesis and accelerating bone healing [59].

In recent years, a number of authors have focused on LIPUS-based bone regeneration, both *in vivo* and *in vitro*. BMSCs are widely used for this purpose due to their capacity for multi-directional differentiation. Costa *et al.* [58] reported on the stimulation of human bone marrow stromal cells (HBMSCs) with 20 min/day LIPUS treatments. In this study, LIPUS has been found to be effective in promoting bone growth *in vitro*. The researchers hypothesized that LIPUS would enhance the ability of HMSCs to differentiate into bone cells, as demonstrated by cell viability and proteomic analysis. The results showed that LIPUS induced differentiation through specific signaling pathways (such as RhoA/ROCK) and gene expression (COL1A1, ALP, OCN, Runt-related transcription factor 2 (RUNX2), BMP-2, MAPK1/6, OPN). Overall, these findings suggest that LIPUS may be able to improve the osteogenic commitment of HMSCs and enhance differentiation in the presence of factors that mimic the bone microenvironment [58]. Wei and colleagues [57] reported an enhancement of SDF-1 and CXC chemokine receptor type 4 (CXCR4) in BMSCs. LIPUS treatment using 30 mW/cm^2 induced cell migration at the fracture site to promote the repair process. An additional study confirmed that LIPUS played a key role in cell migration. The use of a focal adhesion kinase (FAK) inhibitor and ERK1/2 demonstrated reduced migration of MSCs that was induced by LIPUS stimulation [113].

In a further work [60], it was reported how LIPUS waves affect the osteogenic differentiation of BMSCs. Researchers focused on the variation of selected ultrasonic parameters, including the duty cycle (varied between 20% and 50%). Cell viability of the stimulated BMSCs was similar between the different duty cycles evaluated but higher in comparison to the unstimulated control. Gene evaluation indicated that after 2 wk, LIPUS increased the expression of the most well-known osteogenic markers, including integrin beta-1 (CD29), CD44 antigen, ALP, COL1A1, and OCN (Fig 4a).

The above-mentioned studies have shown how stem cells treated with LIPUS can be guided towards osteogenic differentiation by modulating a number of ultrasonic parameters. The described technology represents a promising breakthrough therapy for fracture repair and bone regeneration.

Osteochondral tissue interface

Osteoarthritis (OA) is a pathological condition affecting the joints, involving the degeneration of cartilage tissue and, in acute cases, of the underlying bone [114]. Nowadays, OA is managed clinically by surgical and physical therapies or pharmacological treatments [115,116], failing to fully regenerate damaged tissue. Due to the low self-regenerating capacity of cartilage, LIPUS is now considered an effective alternative to promote bone union inducing chondrocyte proliferation [117]. Notably, LIPUS activity has been found to enhance the expression of COL2A1 and COL10A1, ACAN and transforming growth factor (TGF)-β in chondrocytes [118]. Naito *et al.* [62] recently demonstrated the effect of LIPUS in a OA rat model, finding a significant increase in the expression of COL2 mRNA in chondrocytes. The OA defect was established in the rat knee joint and improved in the LIPUS groups (compared to the non-LIPUS group) through increased synthesis of COL2. The levels of C-terminal cross-linked telopeptide of type II collagen (CTX-II) (degradation of COL2) were also assessed, with no degradation of COL2 observed in the OA model in each condition (stimulated and unstimulated). The results suggest that LIPUS has potential as a therapy to treat OA due to chondrocyte activation mediated by COL2 synthesis. A preclinical OA mouse model has been designed to study the timespan of defect repair following LIPUS therapy [63]. Daily LIPUS treatments of 20 minutes improved the morphological and histological characteristics of the cartilage compared to non-stimulated controls. Furthermore, a doubling of the treatment time from 20 to 40 min resulted in significantly improved histological appearance of the cartilage. The results proved that the use of LIPUS can accelerate the healing process of bones by increasing the production and development of cartilage, which is involved in the formation of new bone tissue. Liao and co-workers [61] demonstrated that exosomes derived from SSCs can promote cartilage regeneration in OA defects (Fig. 4b) and improve symptoms in OA. In addition, LIPUS treatment was found to enhance the effects of exosomes by increasing chondrocyte proliferation and ECM synthesis while suppressing inflammation, as well as inhibit inflammation and the activation of the NF-κB pathway. These results suggest that MSCs-derived exosomes and LIPUS may be a promising treatment option for OA.

Brain

Traumatic brain injury (TBI) typically results from an accidental partial disruption of the cerebellar tissue [119]. TBI can often result in the malfunctioning of the blood brain barrier (BBB) due to mechanical disruption with pathological consequences [120]. In a healthy state, the BBB protects the brain from contact with noxious substances that might reach the brain parenchyma, thus damaging the delicate central nervous system (CNS). However, this barrier represents a challenge when drugs have to be delivered to specific regions of the brain tissue. In this regard, the partial and temporary modulation of the BBB permeability is an ideal approach to achieve non-invasive drug therapy, thus modulating cerebral edema that might arise following TBI [39]. LIPUS has been proven to aid the regeneration of the severed axonal appendices following TBI, improving the recovery of the BBB, relieving the edema [40].

Su and colleagues [39] investigated the effect of LIPUS in a TBI mouse model. US treatment significantly attenuated brain edema, barrier permeability and neuronal degeneration immediately after the first

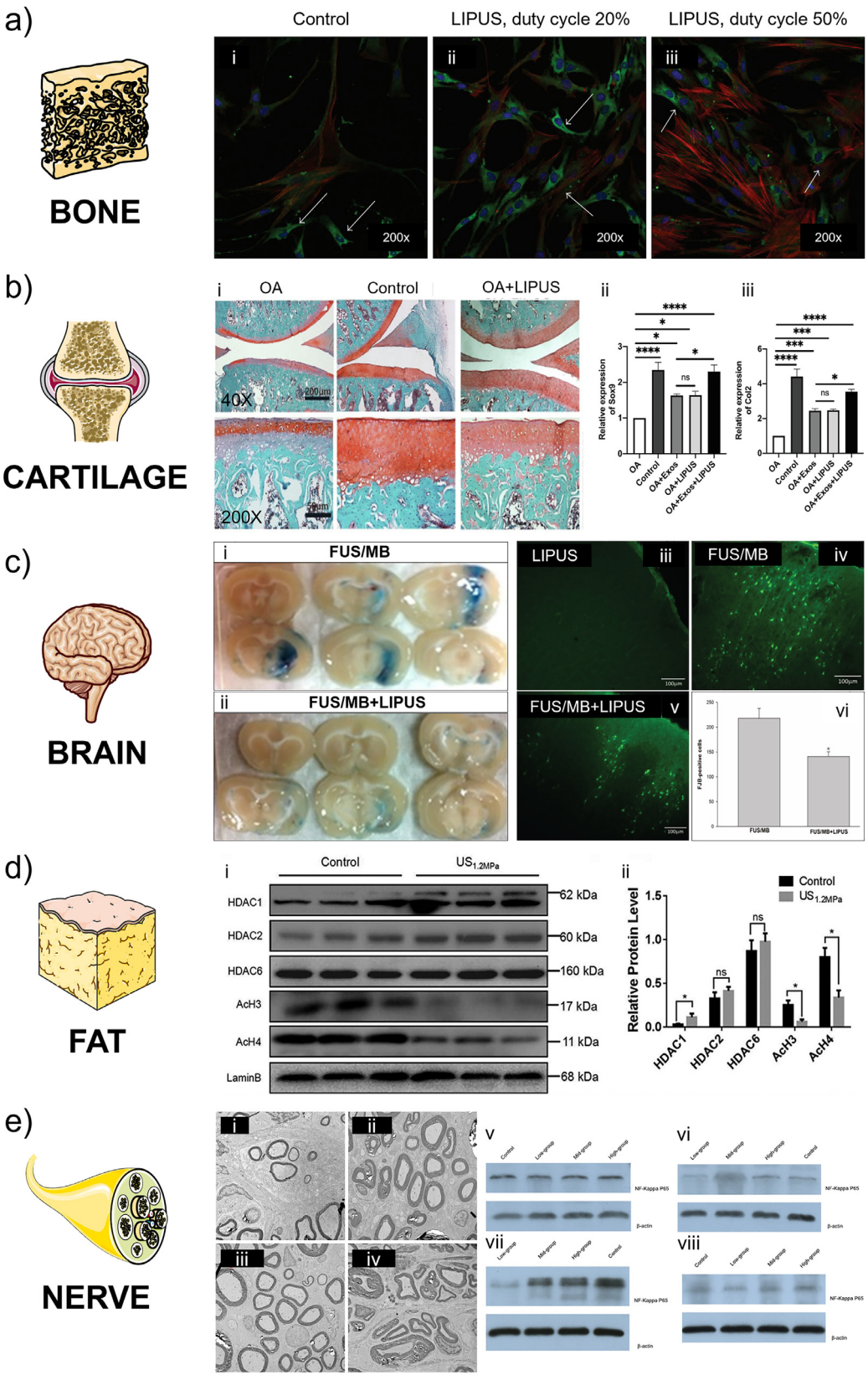

Figure 4. The application of US to different tissues. (a) Confocal laser microscopy images of human alveolar bone-derived mesenchymal stem cells (hABMSCs) cultured for 7 d under different conditions: static (i), with LIPUS at 20% duty cycle (ii), and with LIPUS at 50% duty cycle (iii) after the addition of differentiation media. The merged images show the nuclei of the cells (blue), actin filaments (red), and osteocalcin (green). The confocal laser microscopy images of the cells treated with LIPUS showed more intense staining compared to the control group. Adapted from[60]. Copyright © 2013. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). (b) LIPUS enhances the ability of BMSC-derived exosomes to promote the regeneration of cartilage in osteoarthritis. This was demonstrated by staining sections of knee joints with Safranin O/Fast Green, Toluidine Blue, and H&E (i), and measuring the mRNA expression of Sox9 (ii) and Col2 (iii) using quantitative PCR. The results of statistical analysis are shown in the figure. $^{ns}p > 0.05$, $^{*}p < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

day of treatment (Fig. 4c). The effect of LIPUS was confirmed in a further study carried out in rats with BBB injury [64]. LIPUS was able to modulate local disruption of the BBB, reducing hemorrhage, neuronal damage, and cell death. Results showed that LIPUS could be used for the controlled release of drugs within the brain. The influence of LIPUS on brain development and regeneration has been recently explored by Liu and colleagues [65], demonstrating the increase in BDNF expression both *in vivo* and *in vitro* following treatment. The results showed that LIPUS stimulation upregulated BDNF production in astrocytes by activating NF-κB via the TrkB/PI3K/Akt and CaMK signaling pathways.

Recently, a growing number of studies have shown the beneficial effect of LIPUS stimulation on cerebral edema. The neuroprotective effects of LIPUS therefore open the possibility for their usage in the treatment of numerous brain injuries.

Fat

Adipose stem cells (ASCs) are stem cells with the ability to differentiate into adipocytes [121]. Due to their high availability (because of removal of excess adipose tissue) [122] they are widely used and investigated in scientific research. Typically, the differentiation of ASCs is triggered by factors such as dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin and indomethacin [123]. Although studies on LIPUS-treated ASCs are few, some work has focused on US stimulation to increase adipogenic differentiation of ASCs.

Fu and co-workers [20] applied LIPUS stimuli to ASCs *in vitro* for 3 to 5 d to induce adipogenic differentiation. Using real-time quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence analysis, the expression level of the PPAR-γ1 and APN genes was evaluated. In LIPUS-stimulated conditions, a significantly higher up-regulation of both genes was observed compared to unstimulated controls. Thus, the results showed that LIPUS enhanced the adipogenesis of ASCs. Nonetheless, recent studies [7,38] reported that LIPUS is able to suppress adipogenic differentiation through the inhibition of specific pathways. Xu *et al.* [7] exposed visceral preadipocytes to LIPUS to understand the mechanisms associated with primary differentiation. Inhibition of adipogenic differentiation was estimated by studying the levels of histone acetylation-related molecules. Following LIPUS treatment, a reduction in histone 3 (AcH3) and 4 (AcH4) acetylation and an increase in histone deacetylase 1 (HDAC1) were reported (Fig. 4d). Therefore, the authors hypothesised that HDAC1 inhibition could override the suppressive effect of LIPUS on adipocyte differentiation. These results pave the way for the use of LIPUS for the treatment of visceral obesity.

The combination of LIPUS and ASCs could be an outstanding strategy applicable for the regeneration of adipose tissue. LIPUS-induced adipogenic differentiation might provide a useful *in vitro* model to study and treat conditions such as obesity and insulin resistance.

Liver

In vitro hepatic differentiation of human mesenchymal stem cells (hMSCs) has already been demonstrated in previous studies [124,125]. The combination of factors such as HGF, Insulin-Transferrin-Selenium (ITS), dexamethasone, and nicotinamide [124] has emerged as key to manipulating differentiation towards hepatocyte-like cells. The employment of LIPUS for liver regeneration is still poorly investigated in the literature. Nevertheless, a few studies demonstrate that combining LIPUS with HGF can induce hepatic differentiation of hMSCs.

Li and colleagues [8] examined the effect of applying LIPUS on hMSCs in association with the use of HGF to lead the differentiation of cells into hepatocytes. To evaluate the impact of LIPUS, cells with HGF and LIPUS-stimulated were compared to controls (cells in the presence of HGF and non-stimulated). The results revealed that after 5 d, the main hepatocyte-like cell markers, such as αFP, CK18, ALB and glycogen, were significantly expressed in the LIPUS-HGF samples compared to those with HGF exclusively. Furthermore, following ultrasonic stimulation, the involvement and activation of the Wnt/β-catenin signaling pathway, a molecular mechanism capable of regulating hepatobiliary development [94], was demonstrated. In a recent work, Song *et al.* [66] determined the optimal doses of HGF (10-50 ng/ml) and parameters of LIPUS stimulation (1 MHz frequency, 1000 mW/cm^2 intensity, 20% duty cycle, 60 sec of repeated stimulation 3 times per day) to efficiently differentiate hMSCs. In addition to the expression of the markers already mentioned above, a significantly higher concentration of stromal cell-derived factor 1alpha (SDF-1α) and CXCR4 was observed in LIPUS-stimulated samples in the presence of HGF compared to HGF-only treatment.

To date, liver failure is a condition that can only be resolved by liver transplantation. This involves a number of problems including limited organ accessibility and post-surgery risks [66]. The combination of LIPUS and HGF represents a promising alternative for the differentiation of stem cells into hepatocytes and thus for liver regeneration.

Nervous tissue

Peripheral nerve injury is a significant clinical challenge with major impact on the healthcare of patients, including through impaired sensory and motor function [126]. Autologous nerve transplantation is currently considered the gold standard repair technique for peripheral nerve injury, but its effectiveness is limited, with only about 49-80% of patients experiencing meaningful recovery [69]. In recent years, LIPUS-based approaches have been studied in an effort to improve peripheral nerve recovery. LIPUS has been found to be effective in promoting nerve regeneration and improving functional outcomes in a range of *in vitro* and *in vivo* animal models.

Chang *et al.* [67] studied LIPUS as a potential method of promoting nerve regeneration in the treatment of peripheral nerve damage. The authors stimulated poly (D,L-lactic acid-co-glycolic acid) (PLGA)

Adapted from reference [61]. Copyright 2021. This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License (CC BY-NC-ND). (c) Evans blue dye (EB) was used to test for extravasation into the brain after using FUS to create a disruption in the BBB (i – ii). The BBB disruption was significantly different between the control group (FUS/MB) and the experimental group (FUS/MB + LIPUS). At one hour after treatment with FUS/MB, the EB dye was more widely distributed and darker in the FUS/MB (i) group compared to the FUS/MB + LIPUS group (ii). Fluoro-Jade B (FJB)-stained brain section of a sonicated region (iii – vi). LIPUS treatment following FUS/MB appears to significantly reduce neuronal degeneration in rats compared to FUS/MB-treated groups. The results of statistical analysis are shown in figure (vi). *$p < 0.05$, n = 3. The results of the work demonstrated a reduction in hemorrhage, neuronal damage, and apoptotic cell death at 1 d after BBBD. Adapted from reference [64]. Copyright 2015. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). (d) LIPUS treatment appears to increase the expression of a HDAC1 and decrease the modification of AcH3 and AcH4 (i – ii). The protein levels of HDAC1, HDAC2, HDAC6, AcH3 and AcH4 were measured using western blotting and compared to a reference protein (Lamin B). The results showed that the levels of HDAC1, HDAC2, HDAC6, AcH3, and AcH4 were significantly different between the treated and control groups. All values are expressed as the mean ± SEM of three independent trials. *P < 0.05. Adapted from[7]. Copyright © 2019. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). (e) Evaluation of the axons growth (i – iv). Electron microscopic photographs of nerves in control (i), low-dose (ii) mid-dose (iii) and high-dose (iv) groups 12 wk after surgery. Expression of NF-κB p65 in four different groups (v – viii): v) 2 wk after operation, (vi) 4 wk after operation, (vii) 8 wk after operation, and (viii) 12 wk after operation. Adapted from reference [69]. Copyright 2016. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).

scaffolds seeded with Schwann cells with LIPUS both *in vivo* and *in vitro*. LIPUS-treated scaffolds showed a significantly increased number and area of regenerating axons in the central part of the implanted PLGA scaffolds compared to control samples. In a further study [69], the authors investigated the effect of LIPUS on the regeneration of autograft nerves in a rat model. The animals received a 10 mm sciatic nerve autograft and were LIPUS-treated with intensities from 250 to 750 mW/cm^2. The stimuli with a lower intensity significantly improved the rate of axonal regeneration in autograft nerve transplants (Fig. 4e). Sato and colleagues [68] evaluated the use of LIPUS as a treatment for sensory loss in the facial skin resulting from injury to the inferior alveolar nerve (IAN). After performing a surgical transection of the IAN in rats and treating the damaged nerve with LIPUS daily, results demonstrated that LIPUS significantly improved the sensitivity of facial skin to mechanical stimulation and increased the number of trigeminal ganglion neurons innervating the area, suggesting that LIPUS may be an effective and novel therapy for IAN injury and the resulting sensory loss in the orofacial region.

LIPUS thus appears to be a promising treatment option for peripheral nerve injury through various mechanisms such as increased neurotrophic factors and cellular signaling activation. While the appropriate intensity of LIPUS for nerve injury repair is still not fully understood, ultrasonic stimulation has the potential to improve recovery from peripheral nerve injury and has the added advantage of being non-invasive. Further research into the use of LIPUS as a treatment for peripheral nerve injury is warranted.

Overall, the use of LIPUS for regenerative therapies may, in the future, represent a potential solution for *in-vivo* tissue repair and could be used for the *in-vitro* preparation of tissue engineering implants. However, further research into the underlying mechanisms of action is required before the full potential of this method is assessed.

Ultrasound for tissue engineering and biofabrication purposes

Although this review has mainly focused on LIPUS use for tissue engineering, it is important to recognize that the available literature on LIPUS in these specific areas is currently limited. However, the most advanced and current US techniques and applications provide a valuable insight into the future potential of LIPUS. The applications discussed in this chapter aim to fill these gaps and pave the way for the integration of existing knowledge with future applications of LIPUS. The US technologies discussed below have enormous potential to be adapted and optimized for exploitation with LIPUS.

US for tissue engineering

Imaging and monitoring

The advent of tissue engineering requires imaging techniques for monitoring implanted scaffolds and characterizing the composition and mechanical properties of tissue constructs. Following the implantation of a purpose-engineered scaffold, it is crucial to evaluate the induced physiological response *in situ*. The most relevant effects to be observed are mechanical (strength, degradation, etc.) and biological (vascularization, cellular infiltration, etc.) and their corresponding changes over time [127]. Ultrasonic imaging is particularly useful as it is non-invasive compared to other imaging methods such as X-ray (danger from ionizing radiation) [128] or positron emission tomography (PET)/ single-photon emission computed tomography (SPECT) (danger from radioactive agents) [129].

Indeed, the tissue integration of newly implanted scaffolds can be monitored using greyscale US imaging. A number of studies have reported on the ability to detect spatial changes in cell density in 3D scaffolds using US imaging. Moreover, this powerful technology has been used to identify the amount of collagen in implanted fibrin-based scaffolds [130], mineralization in collagen constructs [131] and ECM deposition [132]. Doppler US imaging has been extensively used to measure hemodynamic information with high sensitivity, to monitor the formation of the growing vascular system in implanted scaffolds [127]. Kumar and collaborators [133] monitored the bloodstream in acellular vascular grafts modified with collagen-elastin constructs implanted in a rat, using Doppler imaging. Using the same technology, Allen and coworkers [134] studied the vascularization pattern in poly(glycerol sebacate)-based synthetic arterial grafts implanted in a rat model.

An alternative method to track morphological and elastic changes during the gradual degradation of the implanted construct is via US elastography. Ultrasonic elasticity imaging can be used to obtain information on the mechanical properties of biomaterials by exploiting wave propagation in engineered tissue [135]. In a recent report, Winterroth *et al.* [136] used elastography to observe the surface irregularities and properties of engineered oral mucosa tissues by developing deformation maps from acoustic US. Thanks to this technology, it was subsequently possible to verify that cells seeded on the *ex vivo* produced oral mucosa equivalent (EVPOME) surface are able to produce a keratinized top layer that overcomes surface irregularities.

Acoustofluidics

Acoustic manipulation is a non-invasive technology that harness US to spatially trap cells and guide cellular assembly, generating multicellular clusters [137]. US-induced cell aggregation provides numerous advantages, including the absence of materials (scaffolds) required to guide this process, enhanced mechanical stimulation, accelerated mass transfer of chemical species (such as nutrients and waste products), and improved molecular adhesion [138,139].

When subjecting a cell suspension to an ultrasonic standing wave field, the generated acoustic pressure field produces acoustic traps for cells (Fig. 5). The radiation forces that originate from the standing wave field typically direct cells into spatial acoustic pressure nodes where the pressure is the lowest [140]. Thus, US-guided manipulation is able to

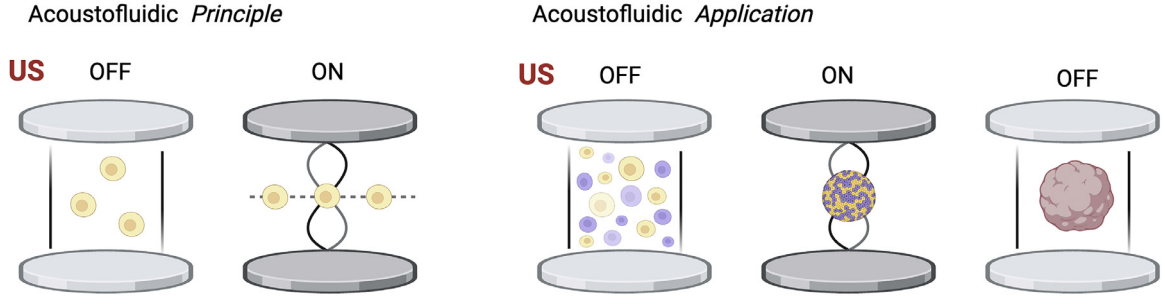

Figure 5. Principles and applications of cell manipulation via acoustofluidics. This technique uses ultrasonic standing waves established within a fluid cavity, to create acoustic traps and guide cell assembly. On the left is the random arrangement of cells in suspension when US is not activated. Upon application of the US, cells are trapped at acoustic pressure nodes of the standing wave field, creating specific configurations. On the right is an example of application of this technology, where US is used to create multicellular clusters, which are stable once the stimulus is removed.

induce cells to migrate to specific positions within a fluid cavity and can organize them spatially into different geometric arrangements depending on the selected ultrasonic parameters [138,141].

Li and colleagues [139] applied microfluidic perfusion bioreactors combined with acoustic wave fields on neocartilage grafts. To overcome some limitations caused by common tissue engineering strategies (low cell viability and inadequate mechanical properties), the authors formed chondrocyte clusters by generating acoustic traps with US frequencies ranging between 890 and 910 kHz. The induced cell agglomeration drastically enhanced the micromechanical properties of the grafts, which had characteristics similar to native hyaline cartilage. Furthermore, the implantation of grafts into an *ex vivo* model was found to promote formation of hyaline-like cartilage tissue.

In another work [138], neural cells were trapped within a ultrasonic standing wave field, creating a multicellular cluster with 1 mm diameter. The US frequency of 1.5 MHz generated a spatial arrangement of the clusters similar to a monolayer of cells with limited effect on cell viability (>99%). The results confirmed that this acoustic manipulation technique increased intercellular adhesion, a key factor in processes such as tissue generation or wound healing [142].

Petta and collaborators [143] reported on a novel method called sound-induced morphogenesis (SIM) to create and control the spatial patterning of cells (endothelial cells and SSCs) within a hydrogel to form vascular networks. This process can be controlled using acoustic surface standing waves, for example, Faraday waves, to form tissue structures under mild culture conditions. The formation of the vascular network by the SIM process initially involved the dispersion of cells in the biopolymer solution, followed by mechanical vibration to generate the cell pattern. At d 0, following hydrogel crosslinking, the cell pattern showed an increase in local density, while at day 5 the vascular network began to organize and self-assemble. It follows from these results that SIM may have potential applications in the fields of regenerative medicine and cell therapy. Currently, acoustofluidics applied to tissue engineering technology is an evolving area of research, with potential for manipulating living cells on demand to increase the effectiveness and application potential of tissue engineering. However, the lack of scalability and high-throughput might limit the translation of acoustofluidics-based tissue engineering to the clinics.

US and bioprinting

To overcome the limitations of acoustofluidics, the combination of US and bioprinting may have the potential to be used in the manufacture of biomimetic tissues for clinical, diagnostic and research-level applications. US-assisted bioprinting (UAB) is a 3D biofabrication approach that uses US waves to preferentially organize cells within 3D bioprinted constructs and to mimic the micro-architectural characteristics of native tissues [144,145]. The accurate alignment of cells and extracellular constituents is crucial for their biological functionality [144]. Hence, a system able to combine acoustic manipulation during cell printing represents a critical improvement in tissue engineering and regenerative medicine.

A number of studies have recently investigated how to spatially manipulate cells following 3D scaffold fabrication to simulate highly complex and organized tissues. Sriphutkiat *et al.* [146] tested acoustic stimulation of cells during 3D bioprinting using a specific structural vibration mode of the device. Using C2C12 cells stimulated with an US frequency of 871 kHz, the authors succeeded in focusing the cells in the central part of the 3D bioprinted construct to mimic the structure of a capillary. In a further work [145], the combination of ultrasonic bulk acoustic waves (BAW) and bioprinting allowed a similar cellular manipulation: a multilayer construct of the knee meniscus was printed with a circumferential organization of hASC cells, recreating a scalable biomimetic tissue. The authors evaluated the effect of varying the US frequency (between 0.7 and 2 MHz), the signal source voltage amplitudes from 200 to 400 mV peak-to-peak (pp), the viscosity of the alginate-based bioink, and the timing of cell alignment. Results revealed a decrease in the space between adjacent cell arrays with increasing US frequency. Furthermore, higher amplitudes, lower bioink viscosities and increased alignment time resulted in a decrease in array width. UAB demonstrated the ability to obtain a custom-made aligned knee construct for generating patient-specific architectural patterns of tissue.

Very recently, a groundbreaking use of US was demonstrated by Kuang and colleagues [147], applying a US-driven material ink polymerization coupled with 3D axis motion. Deep-penetration acoustic volumetric printing (DAVP) represents a remarkable advancement in 3D bioprinting. DAVP overcomes light-guided polymerization techniques, which are limited by the optical properties of the materials, but rather focuses the energy of focused ultrasound (FUS) deep into the substrate to cross-link the printing ink with high accuracy. Using viscoelastic inks based on PEG-DA, poly(N-isopropyl acrylamide) (PNIPAm) and ammonium persulfate (APS) as a thermal initiator, the engineered formulation is able to attenuate the acoustic wave flow, ensuring a uniform temperature distribution and a fast, deep curing. The 3D FUS printer demonstrated ultrasonic wave penetration depth of up to 295.2 mm, in contrast to the limited penetration of only 0.48 mm of light at 405 nm. This technology allows printing to depths of centimeters through biological tissue, opening opportunities for future application in minimally invasive medicine.

Although the synergistic potential of US and 3D bioprinting is still poorly explored, the combination of these platforms holds promise to revolutionize the biofabrication paradigm. Due to their exceptional characteristics, US waves could be used not only following material deposition, but also during 3D printing to obtain complex biological structures with active properties.

Conclusions and future perspectives

The multifaceted applications of LIPUS in medicine and rehabilitation, as well as its crucial role in stimulating biological tissues and promoting regeneration, emphasize the clinical relevance of this technology. The non-invasive nature of ultrasonic waves makes it a milestone in medical diagnostics, providing valuable information on the structure and function of human tissues. Among tissue-specific applications, LIPUS has demonstrated effectiveness in bone regeneration, osteochondral tissue interface repair, neural regeneration and adipose tissue engineering. Modulation of the BBB and therapeutic potential for conditions such as osteoarthritis further emphasize the broad impact of LIPUS in a variety of biomedical scenarios.

Moreover, other emerging areas of application include acoustofluidic manipulation of cells for tissue engineering and the integration with bioprinting technologies, to guide the spatial organization of cells into three-dimensional constructs for customized tissue fabrication.

Looking ahead, the future possibilities in the synergistic use of 3D bioprinting and LIPUS are promising. The integration of LIPUS during the bioprinting process, from bioink deposition to construct polymerization, could generate complex biological structures with active properties. In particular, recent studies [148,149] have shown how microfluidic technologies integrated with biological 3D printing can lead to transformative developments in personalized medicine. The easy customization of microfluidic printing devices can lead to the simple integration of PZT elements, enabling the precise fabrication of microfibers creating complex, biomimetic human tissues with LIPUS-driven functional properties.

The application of LIPUS in tissue engineering is promising, but has several limitations that may not be underestimated. LIPUS can penetrate deeply into tissues, but precise control of the effects is not easy due to several parameters involved, including frequency, intensity and duty cycle. Tuning the parameters to achieve the desired therapeutic effect can vary significantly between different tissue types [150]. In addition, LIPUS provides a relatively weak mechanical stimulation compared to the forces typically required for bone regeneration. This may limit its potential to stimulate appropriate bone healing in cases of severe bone

injury [151]. Moreover, the mechanical forces applied by LIPUS are often modulated through external inputs to enhance its benefits, such as microbubbles, targeted biomaterials, and culture media supported by specific factors. This makes LIPUS a technology that may not be powerful enough to be used alone 91,152,153. As mentioned throughout this review, the precise molecular and cellular mechanisms by which LIPUS exert their effects are not fully understood. This results in challenges in fine-tuning LIPUS protocols for clinical use.

Ongoing interdisciplinary research and technological innovations, together with a more pervasive understanding of the underlying mechanisms of action, may pave the way for the use of LIPUS in personalized and effective healthcare solutions. The continued exploration of the influence of LIPUS on various tissues and the fine-tuning of protocols for specific applications will contribute to the possible future adoption in clinical contexts for the benefit of patients.

Conflict of interest

The authors declare no competing interests.

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