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Ultrasound-driven mechanosensing and the parameters shaping cellular response in LIPUS

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Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy



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Abstract

Low intensity pulsed ultrasound (LIPUS) therapy is a widely used and approved by regulatory bodies such as the FDA and NICE for bone repair however, reported outcomes remain variable across both clinical and experimental studies. Despite its widespread use, the mechanisms governing cellular responses remain poorly defined, in part due to limitations in *in vitro* experimental design and in the reporting of ultrasound parameters. Standard cell culture platforms introduce reflections and standing-wave artefacts, resulting in poorly controlled, spatially non-uniform exposure conditions that can also cause heating of the culture plate. Furthermore, commonly reported metrics such as spatial-average temporal-average intensity (I_{SATA}) do not represent the mechanical stimulus experienced at the cell-substrate interface, and the absence of direct *in situ* field characterisation further limits reproducibility and the interpretation of results.

This thesis addresses these limitations by developing an acoustically and optically transparent cell culture vessel that enables the controlled delivery of ultrasound, fluorescence imaging, and the direct measurement of substrate motion using laser Doppler vibrometry. Ultrasound exposures were systematically varied in pressure, duty cycle, pulse repetition frequency, and temporal envelope, including conditions where I_{SATA} was maintained constant across distinct waveform configurations. This approach allowed the decoupling of acoustic intensity from the resulting mechanical displacement at the cell substrate. Biological responses were assessed using MG63s which is a human-derived osteoblast-like cell line. Cell area, nuclear area, and immunofluorescence quantification of actin and vinculin intensities were used as indicators of the cell mechanosensitive response.

Across all parameter studies, cellular responses were maximised under specific waveform conditions defined by pressure amplitude, duty cycle, and pulse repetition frequency. Exposure conditions matched in I_{SATA} produced different substrate displacements and corresponding cellular responses, demonstrating that I_{SATA} alone is insufficient to describe the mechanical stimulus experienced by the cells. Temporal features of the waveform, particularly strain rate, influenced mechanosensitive outcomes, indicating that the rate of force application is equally important in determining cellular response.

LDV measurements provided frequency-resolved quantification of substrate motion demonstrating nanoscale displacements not only at the 1 MHz carrier frequency, but also at the 1 kHz pulse repetition frequency, with the latter varying significantly across waveform conditions. This work establishes a relevant framework for ultrasound exposure and interpretation, supporting the design of more reproducible and physiologically relevant LIPUS studies.

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Outputs

Peer-reviewed Journal Publications

Orthodoxou, A., Lucas, M., & Mulvana, H. (2026). Pressure and not spatial average temporal average intensity governs mechanosensitive responses of osteoblast-like cells exposed to low intensity pulsed ultrasound. *Ultrasonics*, 161, 107945.

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Lingamgunta, S., Yadav, C., **Orthodoxou, A.,** Gilmour, L., Ellis, M., Metzger, H., Bistrovic Popov, A., Mulvana, H., & Fruk, L. (2026). Hollow-core polydopamine nanocarriers for ultrasound-enhanced drug delivery. *Nanoscale Horizons*, 11, p211-224.
<https://doi.org/10.1039/d5nh00297d>

Conference Proceedings

Orthodoxou, A., Lucas, M., & Mulvana, H. (2024). Optimizing ultrasound dosimetry for bone fracture healing: Unveiling cellular response mechanisms. In 2024 IEEE Ultrasonics, Ferroelectrics, and Frequency Control Joint Symposium (UFFC-JS). IEEE.

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Oral Presentations and Posters

Orthodoxou, A., Lucas, M., & Mulvana, H. (2026). The rate of applied ultrasound pulse may be as important as ultrasound pressure in predicting osteoblast cell responses to LIPUS [Abstract]. International Symposium on Mechanobiology, Glasgow, United Kingdom.

Orthodoxou, A., Lucas, M., & Mulvana, H. (2025). Nanoscale measurement of vibration of *in-vitro* systems can provide new information to understand cellular responses to pulsed ultrasound [Poster presentation]. 2025 IEEE International Ultrasonics Symposium (IUS), Utrecht, Netherlands.

Orthodoxou, A., Lucas, M., & Mulvana, H. (2025). Investigating bone cell mechanobiology with ultrasound [Oral presentation]. Artimino Conference on Medical Ultrasound, Lyon, France.

Orthodoxou, A., Lucas, M., & Mulvana, H. (2024). Controlled in-vitro ultrasound stimulation enhances actin and vinculin expression in osteoblast-like cells [Oral presentation]. Mechanobiology: Shaping Life, London, United Kingdom.

Orthodoxou, A., Lucas, M., & Mulvana, H. (2024). Optimising ultrasound dosage to enhance cell mechanosensitivity for bone regeneration [Poster presentation]. FUSE Annual Scientific Meeting, University of Glasgow, Glasgow, United Kingdom. Awarded Best Poster.

Orthodoxou, A., Lucas, M., & Mulvana, H. (2023). Design and optimisation of in-vitro experimental set-up for the investigation of LIPUS on MG63 cells [Poster presentation]. FUSE Annual Scientific Meeting, University of Strathclyde, Glasgow, United Kingdom.

Supplementary academic contributions

Orthodoxou, A., Mulvana, H., & Lucas, M. (2025, December). Can mechanosensitive osteoblast response guide optimisation of LIPUS treatments for bone healing? [Invited talk presented by Dr. Helen Mulvana]. Therapeutic Ultrasound Session (ThUNDDAR). British Medical Ultrasound Society 56th Annual Scientific Meeting (Ultrasound 2025), Harrogate Convention Centre, Harrogate, United Kingdom.

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Author declaration

I hereby declare that this thesis is my own work and has not been submitted, in whole or in part, for any other degree or professional qualification.

Andrea Orthodoxou

Dedication

This thesis is dedicated to my biggest supporters, Antonis and Myroulla.

List of Abbreviations

2D	Two dimensional
3D	Three dimensional
ANOVA	Analysis of variance
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAD	Computer assisted design
COX2	Cyclooxygenase-2
CTCF	Corrected total cell fluorescence
DAPI	4',6-diamidino-2-phenylindole
DC	Duty Cycle
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ERK	Extracellular related kinase
FA	Focal adhesion
F-actin	Filament of actin
FAK	Focal adhesion kinase
FBS	Foetal bovine serum

FFT	Fast Fourier Transform
FITC	Fluorescein Isothiocyanate
FWHM	Full-width half maximum
hASC	Human adipose-derived stem cells
hMSC	Human mesenchymal stem cells
Hz	Hertz
IL-1	Interleukin-1
IL-6	Interleukin-6
iPSCs	induced pluripotent stem cells
I _{SATA}	Spatial-average temporal-average intensity
I _{SATP}	Spatial-average temporal-peak intensity
I _{SPPA}	Spatial-peak pulse-average intensity
I _{SPTA}	Spatial-peak temporal-average intensity
JNK	c-Jun N-terminal Kinase
LDV	Laser Doppler Vibrometry
LINC	Linker of nucleoskeleton and cytoskeleton
MAPK	Mitogen-activated protein kinase
MEM NEAA	Minimum essential medium non-essential amino acid solution
MMP	Matrix metalloproteinase

mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
OCN	osteocalcin
PGE ₂	Prostaglandin-2
pk-pk	Peak-to-peak
PRF	Pulse Repetition Frequency
PW	Pulse width
RANKL	Receptor activator of nuclear factor κ B Ligand
RhoA	Ras homolog family member A
ROCK	Rho-associated coiled-coil containing protein kinase
Runx2	Runt-related transcription factor 2
SD	Standard deviation
TAZ	Transcriptional Co-activator with PDZ-binding motif
TBG- β	Transforming growth factor β
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRITC	Tetramethyl rhodamine Isothiocyanate
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site

YAP Yes-Associated Protein

Z Acoustic impedance

Chapter 1: Introduction

1.1 Background and clinical motivation

Bone fractures represent a rising global health concern, with approximately 178 million new cases recorded in 2019 alone [1]. The current incident rate stands at 11.6 per 1000 individuals annually, with a 33.4% increase in new fractures observed over the past decade [1], [2]. This trend is strongly linked to increasing life expectancy and the resulting growth of an ageing population, in which individuals are more prone to fractures due to age-related and post-menopausal bone loss. Compounding factors such as low physical activity, inadequate nutrition, smoking, and excessive consumption of alcohol further elevate the risk of impaired bone healing [3], [4].

Although most fractures undergo spontaneous repair, a significant proportion fail to heal effectively. Approximately 1.8% progress to non-union, which is clinically defined as the failure of a fracture to unite within 6 to 9 months [1]. More broadly, the global incidence of bone non-union or delayed healing is estimated to range from 5% to 10%, with data indicating an annual increase [5]. The implications of prolonged healing span clinical, economic, and psychosocial dimensions, including elevated healthcare costs, surgical morbidity, and extended periods away from work and daily activity.

Fracture healing proceeds through a tightly orchestrated sequence of events, beginning with inflammation, and is mediated by complex cellular processes such as proliferation, migration, differentiation and matrix deposition. Disruption at any of these stages, through impaired vascularisation, defective osteogenic recruitment, or excessive inflammation, can compromise outcomes, necessitating surgical intervention.

Current treatment methods for bone healing include surgical or pharmacological treatment, or biophysical stimulation. Pharmacological treatments are widely used and generally effective, but their utility is often limited by adverse side effects, high costs, poor patient compliance, and potential drug interactions [6]. In the context of fracture and non-union management, pharmacological intervention alone is often insufficient to achieve healing. In those cases, surgical methods remain the standard of care, including closed or open reduction with internal fixation, external fixation, bone grafting (autografts, allografts,

synthetic substitutes) or reoperation with revision fixation [7]. However, these approaches are not without drawbacks, including surgical site infection, donor site morbidity, immune system rejection, suboptimal graft integration, and challenges associated with rehabilitation adherence or premature loading [8]. Such interventions typically involve major surgical procedures that require hospital admission, thereby increasing healthcare costs and the overall patient burden.

Consequently, there is growing interest in non-invasive biophysical stimulation modalities, such as shockwave therapy, electrical stimulation, and low-intensity pulsed ultrasound, which offer promising adjuncts or alternatives due to their safety, simplicity, cost-effectiveness, and ability to promote healing without surgical intervention [9]. Among these, low-intensity pulsed ultrasound (LIPUS) has gained particular attention for stimulating bone healing because of its safety, significant cost savings and a reduced risk of surgical complications.

LIPUS is typically delivered transcutaneous with a spatial-average temporal-average intensity (I_{SATA}) between 30 and 100 mW/cm² [10]. Preclinical and clinical trials have demonstrated its efficacy as an adjunctive treatment for bone repair in fresh fractures and delayed unions [11], [12]. Furthermore, many *in vitro* studies report beneficial effects across a range of cellular processes relevant to bone regeneration, including bone cell differentiation, and signalling pathways associated with osteogenesis [13], [14], [15]. LIPUS was approved by the U.S. (FDA) and the U.K. (NICE) for fracture and non-union treatment [16], [17]. Clinical evidence summarised by NICE reports pooled healing rates of 82-84% in non-union cases. However, substantial heterogeneity between studies, driven by variations in the diagnostic definition of non-union, which often encompass varying degrees of delayed healing, diverse fracture types and differing patient comorbidities [17].

Despite decades of clinical use, the efficacy of LIPUS remains variable across patient populations, with a systematic review excluding high-bias trials concluding that LIPUS is not effective, partly due to patient compliance, fracture characteristics, fixation stability, treatment protocols, and patient-specific variables (e.g. BMI) [18]. A further potential contributor to this inconsistency is the lack of standardised dosimetry and limited understanding of how treatment conditions relate to the mechanical stimulus experienced at the tissue level.

Although widely adopted, the mechanism by which LIPUS enhances repair is not fully understood. Investigations suggest that LIPUS exerts its beneficial effects via mechanical stimulation, while any temperature rise is considered an accompanying effect rather than the main driver of biological response [19], [20]. At the cellular level, LIPUS has been reported to activate mechanosensitive signalling pathways, including integrin-mediated focal adhesion signalling and downstream pathways associated with increased cell proliferation, differentiation, and matrix production all of which are crucial for effective bone healing [9], [21], [22], [23], [24], [25]. However, the relationship between the applied ultrasound field and the resulting cellular effects remains incompletely understood.

Most commercial devices (e.g. Exogen) employ fixed settings including 1.5 MHz carrier frequency, 1 kHz pulse repetition frequency, 200 μ s pulse duration, 20% duty cycle, and 30 mW/cm² I_{SATA} . These parameters were largely inherited from early preclinical studies and have remained unchanged despite considerable biological variability across fracture types and patient populations. In the LIPUS literature, I_{SATA} is used to describe the intended dose delivered at the fracture site and while it is a useful descriptor of the ultrasound output and for ensuring safe operation, it does not uniquely define the ultrasound pressure field at the treatment site, and it may not adequately capture the mechanical stimulus experienced at a cellular level. From a clinical standpoint, there is a need to clarify and optimise the therapeutic parameters of LIPUS, to improve treatment consistency, enhance patient outcomes, and realise its potential as a non-invasive therapy for bone healing.

1.2 Bone structure and composition

Bone is a dynamic, mineralised connective tissue that performs essential structural and physiological functions. Beyond providing mechanical support, facilitating movement, and protecting internal organs, bone is actively involved in mineral homeostasis, haematopoiesis (blood cell formation), and endocrine regulation through the release of signalling molecules, including cytokines and growth factors [26]. The adult human skeleton comprises 206 bones, representing roughly 15% of total body weight, and is broadly classified into four categories: long, short, flat, and irregular bones, each serving specialised biomechanical roles [27].

There are two types of bone tissue, namely cortical (compact) bone and trabecular (spongy) bone. Cortical bone is dense, forms the outer shell of bones, and provides mechanical strength. It contains functional units called Haversian systems (osteons), which house blood vessels and nerves, enabling communication between bone cells [27], [28]. Trabecular bone has a porous, honeycomb-like structure composed of a network of trabecular plates and rods. This architecture dissipates multidirectional loads and accommodates bone marrow.

Bone tissue is a composite material, consisting of both inorganic and organic components. The inorganic phase (roughly 60-70%) is mainly composed of hydroxyapatite crystals, a mineral that confers rigidity and compressive strength. The organic matrix is predominantly composed of type I collagen, which serves as a scaffold for mineral deposition and imparts tensile strength. Additional organic components include glycosylated proteins, such as small leucine-rich proteoglycans, which regulate cell proliferation and mineral deposition, and non-collagenous proteins, such as osteocalcin (OCN), osteopontin, and osteonectin, which mediate cell-matrix interactions and calcium binding [8], [27]. The matrix also contains numerous growth factors, including transforming growth factor (TGF), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF), which are crucial signalling markers for bone homeostasis, remodelling, and matrix synthesis [29], [30].

Bone tissue includes several cell types with distinct roles in formation and remodelling. Osteoblasts are responsible for new bone formation and the secretion of extracellular matrix proteins, including alkaline phosphatase (ALP), OCN, osteopontin, and collagen I [31]. Osteoblasts are derived from the differentiation of mesenchymal stem cells (MSCs), a process regulated by signalling molecules such as Runx2 and BMPs [32]. Osteoclasts differentiate from hematopoietic stem cells, and their function is to resorb old, non-functional bone by releasing receptor activator of nuclear factor kappa-B ligand (RANKL) [33]. Under physiological conditions of bone homeostasis, a balance is maintained between osteoclast-mediated bone resorption and osteoblast-mediated bone formation, ensuring skeletal integrity and mineral homeostasis. Osteocytes, which are terminally differentiated osteoblasts embedded within the matrix, are the most abundant type of bone cells. They form extensive communication networks that are critical for mechanosensation and the regulation of bone turnover [34]. Bone lining cells cover inactive bone surfaces and

contribute to bone maintenance and homeostasis by facilitating communication between surface activity and deeper bone cells [35].

In essence, bone is not a passive structural material but a highly dynamic tissue. Its integrity is actively maintained by specialised cells operating within a complex extracellular matrix, constantly adapting to mechanical and biochemical stimuli. This innate responsiveness supports bone remodelling and regeneration and also provides the foundation for therapeutic interventions.

1.3 Stages of healing

Several interrelated biological processes govern bone maintenance and repair. The idea that bone is a dynamic tissue was first described by the anatomist and surgeon Julius Wolff, who postulated that bone architecture evolves in response to functional stress [36]. This concept was later expanded into the mechanostat hypothesis, which introduces microstrain ($\mu\epsilon$) thresholds that determine the biological response of bone [36]. Microstrain ($\mu\epsilon$) is a dimensionless measure of the deformation experienced by bone tissue under load relative to its original length, expressed in units of 10^{-6} . Disuse scenarios with low strain (50 $\mu\epsilon$ or 0.005% strain), such as in spaceflight or bed rest, lead to bone resorption. Standing or light activity generates $\mu\epsilon$ in the range of 50-1000 (0.005-0.1% strain) sufficient to maintain or stimulate bone mass. When mechanical strain exceeds the tissue's adaptive capacity, generally above 25000 $\mu\epsilon$ (0.25% strain), it can lead to macrofractures and trauma. It is important to distinguish that these strains arise from low-frequency, bulk mechanical loading, whereas ultrasound stimulation, although mechanical in nature, is delivered as high-frequency, dynamic, and spatially complex oscillatory deformation that cannot be characterised by a single strain value.

Bone modelling and remodelling are continuous physiological mechanisms that regulate bone shape, mass, and material quality throughout life in response to mechanical and metabolic demands. In contrast, bone healing is a repair process specifically initiated following injury. During fracture repair, mechanical cues support structural alignment and actively modulate biological pathways that drive healing [9]. The presence and magnitude of mechanical forces can influence inflammation, progenitor cell recruitment,

angiogenesis, and matrix deposition, thereby affecting both the rate and quality of bone regeneration.

Bone healing is a complex, tightly regulated physiological process that restores mechanical integrity through two main pathways, depending on the mechanical environment. Primary (direct) bone healing occurs when the fracture site is stable (i.e. surgical plates) or when bone fragments are perfectly aligned. Secondary (indirect) bone healing is a more time-consuming process, which involves the formation of a soft callus due to motion at the fracture site [26].

1.3.1 Bone modelling and remodelling

Healthy bone is a mechanosensitive tissue that adapts to mechanical demands via modelling and remodelling. Bone modelling alters bone shape and structure in response to mechanical strain. On the contrary, bone remodelling is a lifelong process of bone turnover that maintains bone strength and mineral composition (i.e., calcium and phosphate levels). It progresses through four phases, namely, (1) activation of osteoclast precursors, (2) resorption of old bone, (3) reversal wherein osteoprogenitors are recruited and (4) formation of new bone by osteoblasts. These coupled cellular activities are orchestrated by systemic hormones and local signals to resorb old bone and deposit a new matrix, which eventually becomes calcified bone [8].

In the context of fracture healing, the local mechanical environment, particularly the degree of fracture stabilisation, is key in determining the pathway of repair. Primary bone healing typically occurs under conditions of high stability with minimal interfragmentary motion, usually through external rigid fixation, whereas secondary bone healing is associated with lower stability where limited interfragmentary motion is present and proceeds through callus formation. In cases where stability is not achieved, normal healing does not proceed effectively, resulting in impaired healing and non-union. The FDA defines a non-union as a fracture where healing has failed to progress, such that no signs of recovery are observed over three months, following a minimum of nine months post-injury [37].

1.3.2 Primary (direct) bone healing

Primary bone healing (Figure 1A) occurs when the fracture fragments are anatomically aligned, allowing for direct physical and mechanical continuity across the fracture site. This is rare but the ideal form of healing, characterised by the absence of callus formation and proceeding through direct remodelling of existing bone structures, particularly the Haversian systems [26]. Osteoclasts form longitudinal cutting cavities across the fracture line, which are subsequently filled by osteoblasts with new matrix. This coordinated cellular activity restores vascular channels, enabling the delivery of nutrients and the maintenance of intercellular communication, both essential for complete structural integration [38].

1.3.3 Secondary (indirect) bone healing

Secondary bone healing (Figure 1B) is the most prevalent form of repair, particularly in fractures managed without compression. It mainly involves four overlapping biological stages: (1) inflammation, (2) soft callus formation, (3) hard callus formation, and (4) bone remodelling.

The first stage begins immediately after fracture, when disruption of the vasculature triggers hematoma formation, which provides a provisional matrix and an inflammatory microenvironment for callus formation [8]. Several cells, including macrophages, migrate to the site and release cytokines, which are small proteins that mediate inflammation and cell communication. Such cytokines include tumour necrosis factor-alpha (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6), which initiate the recruitment of MSCs. In parallel, growth factors, including vascular endothelial growth factor (VEGF), BMPs, and TGF- β , are secreted to promote angiogenesis and support tissue regeneration [39], [40]. Within days, MSCs differentiate into fibroblasts, chondrocytes, and immature osteoblasts, forming a fibrocartilaginous callus. This soft tissue bridges the fracture gap, stabilising the site. Key regulators promote cell proliferation, matrix deposition and early chondrogenesis.

Hard callus formation follows as osteoblast-mediated mineralisation replaces the cartilaginous scaffold with woven bone, restoring increased mechanical stability [38]. Over weeks to months, the woven bone is resorbed and replaced by lamellar bone through

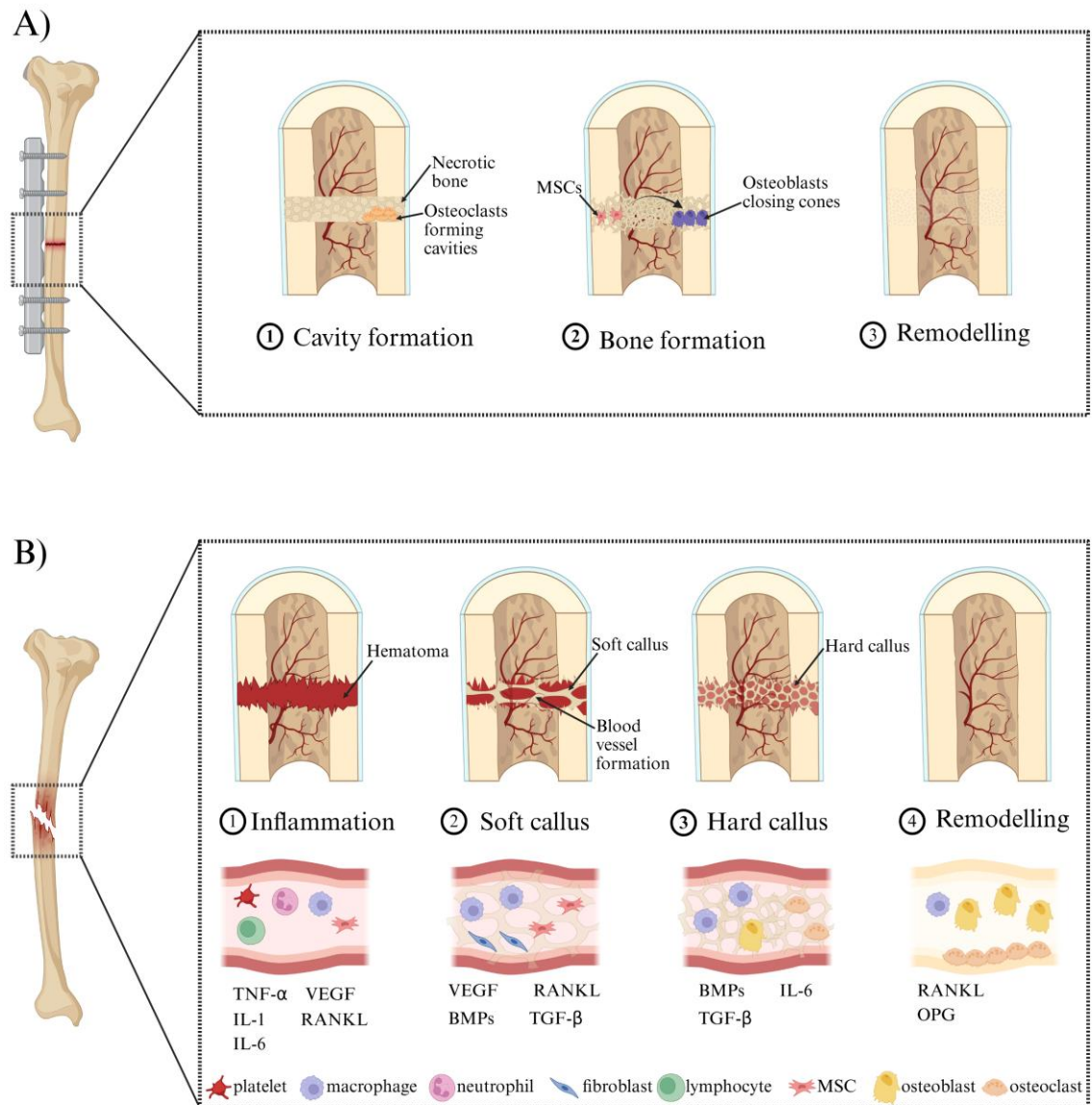


Figure 1. Schematic illustration of (A) primary bone healing and (B) secondary bone healing. Primary bone healing occurs under rigid fixation, or when the gap is small, where osteoclasts form cavities, followed by osteoblast-driven bone formation and subsequent remodelling. In secondary bone healing, which proceeds through sequential stages of inflammation, soft callus formation, hard callus formation, and remodelling. Key cells and signalling molecules associated with each stage are indicated. Created using Biorender.

balanced osteoclast and osteoblast activity regulated by RANKL/osteoprotegerin balance. This final phase restores normal bone architecture, strength, and load-bearing capacity. Remodelling also reestablishes Haversian systems, which are necessary for vascularisation and communication [8].

1.3.4 Biological and mechanical interdependence

The outcome of bone healing depends on the synergy between biological activity and mechanical stability. Factors such as fracture gap size, vascular supply, infection, and systemic conditions (e.g., diabetes and smoking) markedly influence healing rates and outcomes [41]. Inadequate mechanical support or biological function can lead to non-union. Hypertrophic non-union is characterised by an adequate blood supply but insufficient mechanical stability. Conversely, atrophic non-union is defined by both poor vascularity and inadequate cellular response [42]. Understanding the interplay between the cellular and molecular events involved provides the rationale for developing therapies that modulate the mechanical environment.

Clinical fixation strategies have long leveraged the principle that mechanical conditions at the fracture site determine the healing trajectory. For example, rigid internal fixation promotes primary healing, while controlled micromotion facilitates secondary healing through callus formation. These intervention methods serve as instructing signals to guide tissue regeneration. Non-invasive modalities such as LIPUS offer a means to introduce low-magnitude, controlled mechanical stimuli in cases where surgical intervention is either inadequate or ineffective. Optimising the timing and parameters of these interventions is critical to achieving favourable outcomes, particularly in compromised healing environments such as in diabetic patients or those with poor vasculature.

Given the evolving cellular and structural characteristics of the fracture site during healing, the mechanical environment and thus the tissue responsiveness to mechanical stimuli change significantly. It is plausible that different stages of bone healing may require distinct LIPUS parameters, such as frequency, intensity, or duration, to achieve equivalent mechanical stimulation and biological effects. This may reflect not only differences in the cell populations present at each stage, and thereby their sensitivity to mechanical stimulation, but also changes in the local mechanical properties of the tissue, such that, a stiffer environment may require greater energy input to produce comparable levels of deformation effective for bone healing. To understand how LIPUS-induced mechanical

cues can influence these healing stages, it is essential to examine how bone cells perceive, sense, and respond to mechanical forces at different stages of healing.

1.4 Cellular mechanotransduction

Mechanotransduction is the process by which extracellular mechanical forces are converted into biochemical signals that direct cellular behaviour, ultimately influencing tissue formation, adaptation, and repair [43]. This process is not a single pathway but a multi-step process involving diverse mechanosensors, structural proteins, intertwined signalling cascades, and transcriptional factors. Conceptually, it is divided into three key stages. The first stage is mechanosensing, the detection of mechanical cues by specialised components within cells. Signal transduction involves the relay and amplification of these forces through intracellular pathways, and nuclear mechanotransduction ultimately influences cell fate, where mechanical forces reach the nuclear envelope and influence chromatin organisation and gene expression [44], [45].

A variety of plasma membrane mechanosensors contribute to this process including integrins, ion channels, primary cilia, and cadherins [44]. Among these, integrins or clusters of integrins that form focal adhesions are the most widely studied and play a central role in both sensing and transmitting the mechanical forces from the extracellular matrix to the intracellular environment and ultimately the nucleus. Integrins operate through bidirectional signalling mechanisms, commonly described as outside-in or inside-out signalling [46]. In the former, mechanical or biochemical cues from the ECM induce conformational changes in integrins that promote focal adhesion assembly and activation of intracellular pathways. In the latter, intracellular adaptor proteins such as talin, focal adhesion kinases, and YAP regulate integrin affinity and clustering, thereby modulating how cells interact with and exert forces on the ECM [47]. This dual function as both a sensor and a transmitter of cues makes integrins a critical component of the osteogenic response to mechanical stimulation [9], [47].

1.5 Focal adhesions and force transmission

Focal adhesions are complex, macromolecular assemblies located at the cell membrane that physically link the extracellular matrix to the intracellular actin cytoskeleton and serve

as central hubs for sensing and transmitting mechanical forces [48]. A cell actively senses its microenvironment through structural rigidity, defined biophysically as the resistance force experienced per unit of substrate displacement [49].

The process of focal adhesion formation, begins when integrins which are heterodimeric transmembrane receptors composed of α and β subunits, undergo conformational changes upon activation (Figure 2). The changes expose binding sites that allow integrins to interact with extracellular matrix (ECM) ligands such as collagen and fibronectin [44]. Integrins then cluster at the cell periphery and initiate the formation of focal complexes, which are small, transient anchoring points, mapping out a mechanical cycle of activation and catch-bond maturation [49]. These early responses are associated with cell spreading and migration and eventually cell fate, differentiation and survival [50].

As cells adhere to and spread across a substrate, actin polymerisation generates forces that drive membrane extension and the formation of new sites [51]. This, in turn, promotes actomyosin contractility through the RhoA/Rho-associated coiled-coil-containing protein kinase (ROCK) pathway, generating internal tension that is transmitted to focal complexes via actin filaments [52]. RhoA (Ras homolog gene family member A) is a small molecule that activates ROCK to control the actin cytoskeleton [53]. The transmission of internal actomyosin tension through the growing adhesion complex relies heavily on the physical link between the actin cytoskeleton and vinculin, which is critical for sensing and transducing mechanical stimuli [54], [55].

The importance of cytoskeletal tension in mechanotransduction has been demonstrated in MSCs exposed to oscillatory fluid flow [56]. The authors demonstrated that RhoA and its effector protein ROCK regulate osteogenic differentiation. Furthermore, an intact and dynamic actin cytoskeleton under tension was necessary for the expression of osteogenic genes, highlighting the central role of cytoskeletal force transmission in mechanosensitive signalling. This intracellular tension exposes cryptic binding sites on force-sensitive proteins such as talin, enabling the recruitment of vinculin, which stabilises and reinforces the linkage between integrins and actin filaments [57]. This feedback loop marks the maturation of focal complexes into stable, force-bearing focal adhesions that structurally integrate the contractile cytoskeleton with cell matrix boundaries (Figure 3) [58].

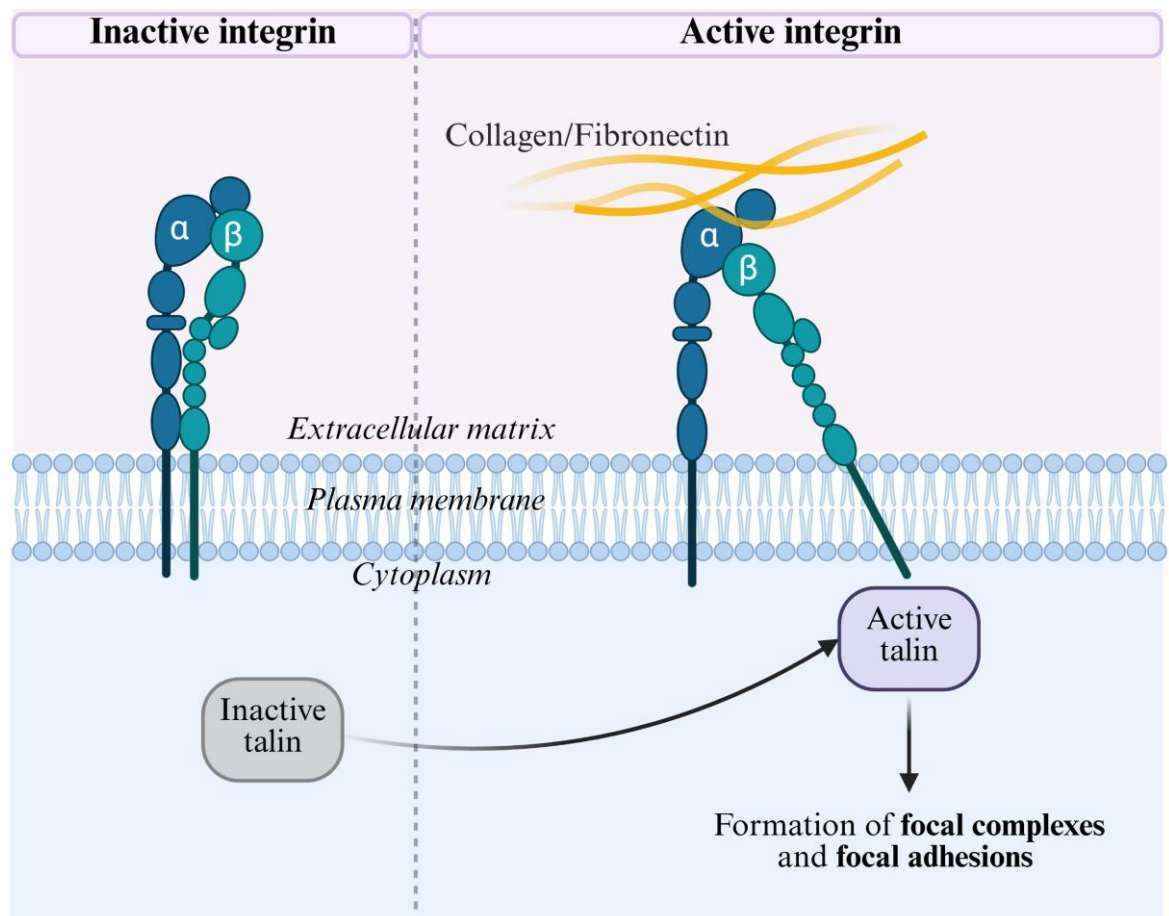


Figure 2. Schematic representation of integrin activation at the cell membrane. In the inactive stage, integrins remain in a closed conformation within the plasma membrane. Activation occurs through talin binding to the cytoplasmic tail of the integrin β subunit, inducing a conformational change that allows the integrin to bind to collagen or fibronectin in the ECM. This activation promotes the formation of focal complexes and focal adhesions. Created using Biorender.

Vinculin occupies a central position in focal adhesions. As mechanical tension increases, more vinculin is recruited to adhesion sites, where it binds to actin filaments, thereby transmitting intracellular forces to the ECM. Simultaneously, vinculin senses the resisting forces (from the ECM) and amplifies the recruitment of intracellular signalling molecules such as focal adhesion kinases (FAKs) which are part of the non-receptor protein tyrosine kinases, the mitogen-activated protein kinase (MAPK) superfamily consisting of three main families, all of which are crucial for skeletal repair, osteogenic lineage commitment, matrix production, and cell migration [59], [60]. The most widely known MAPKs are extracellular signal-regulated kinase (ERK), which is directly related to osteoblast differentiation by activating runt-related transcription factor 2 (Runx2), p38 MAPK, which is involved in bone cell homeostasis and differentiation of chondrocytes, and c-Jun N-terminal kinase (JNK), which regulates bone resorption through osteoclasts [61], [62].

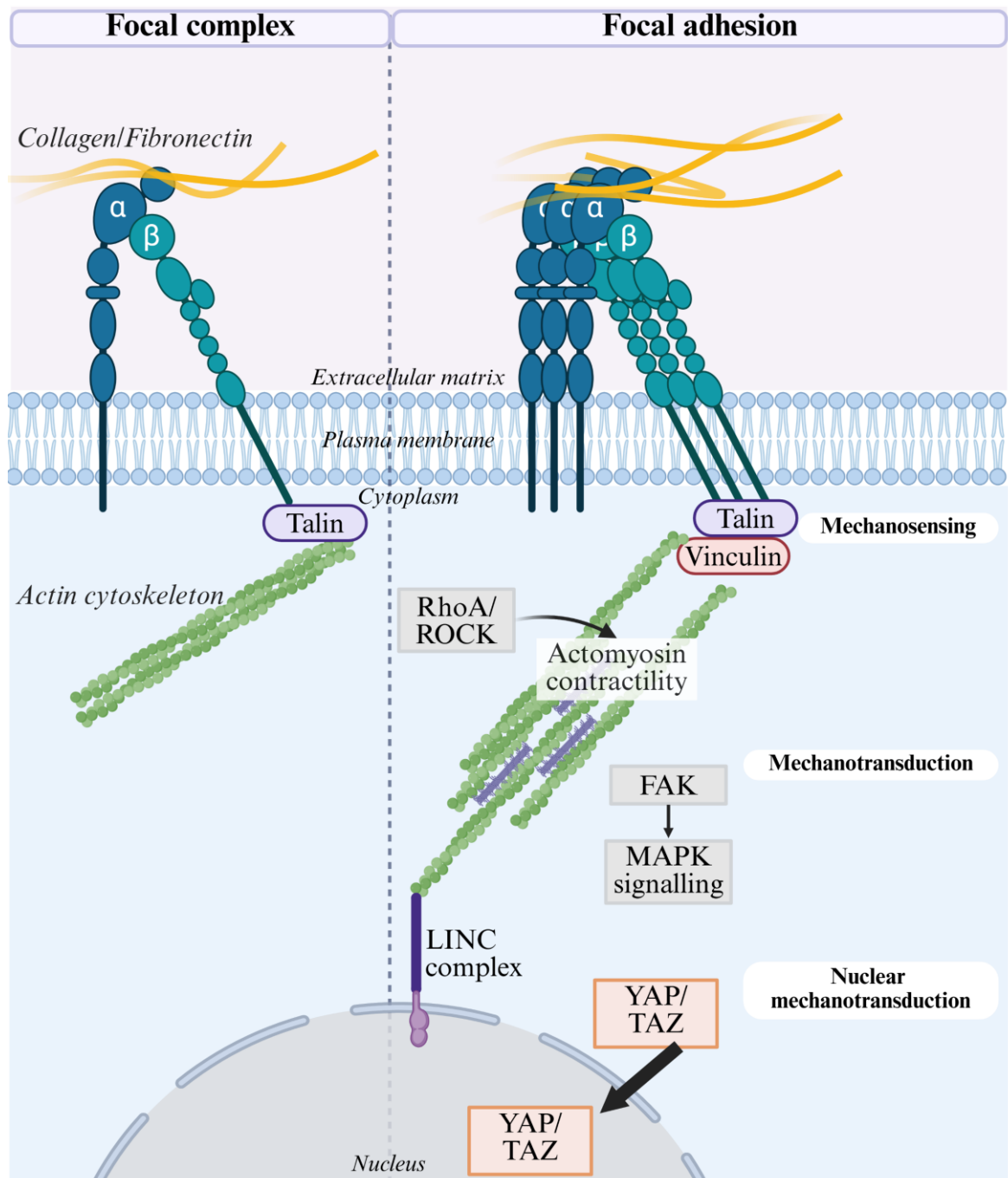


Figure 3. Schematic illustration of the formation of focal complexes and focal adhesions in mechanotransduction. Initial integrin engagement with ECM proteins (i.e. collagen, fibronectin) leads to the formation of focal complexes through talin-mediated linkage to the actin cytoskeleton. With increasing cytoskeletal tension and integrin clustering, focal adhesions form, recruiting downstream proteins and signalling pathways. Mechanical forces are then transmitted through the actin cytoskeleton and the LINC complex to the nucleus, contributing to downstream nuclear mechanotransduction. Created using Biorender.

Additionally, mechanical cues transmitted through focal adhesions activate yes-associated protein and transcriptional coactivator with PDZ-binding motif (YAP/TAZ), key transcriptional coactivators that translocate to the nucleus in response to cytoskeletal tension. As the actin cytoskeleton pulls on the nucleus, the nucleus expands, stretching its

pores and allowing gene-altering proteins such as YAP/TAZ to enter. Once in the nucleus, YAP/TAZ regulates the expression of genes involved in cell proliferation and differentiation [63].

Mechanical forces that originate at focal adhesions, travel through the actin cytoskeleton and are conveyed to the nucleus, through the LINC (linker of nucleoskeleton and cytoskeleton) complex, where they can influence nuclear architecture, chromatin organisation, and gene expression [64]. Nuclear mechanotransduction may manifest as an increased nuclear area, reflecting mechanical stretching of the nucleus mediated by cytoskeletal force transmission [65]. Together, focal adhesions, the actin cytoskeleton, and the LINC complex constitute a mechanically coupled pathway linking the extracellular matrix to the nucleus, through which externally applied mechanical cues may influence nuclear organisation and gene regulatory processes all of which are essential for mechanotransduction.

1.6 The molecular clutch model

The molecular clutch model provides a framework for understanding how cells mechanically couple the actin cytoskeleton to the ECM through focal adhesions. In this framework, integrins and associated adaptor proteins form a dynamic linkage between the ECM and the actin cytoskeleton. Contractile forces generated by actomyosin drive retrograde flow of actin filaments, which continuously pull inward from their site of assembly at the leading edge toward the cell centre [66]. When integrins bind to the ECM ligands, adaptor proteins such as talin connect the integrin to actin, allowing this linkage or clutch to engage, enabling the cell to actively probe and respond to the stiffness of its microenvironment [67], [68]. As force is transmitted through talin, the protein can unfold, exposing previously hidden vinculin-binding sites [67], [68]. Vinculin binding then strengthens the connection between integrins and the actin cytoskeleton, promoting focal adhesion maturation and stabilising force transmission [50], [59], [69].

Interactions between integrins and the ECM are force-dependent. While classical physical bonds break predictably under increasing mechanical load, the integrin-ECM interface acts as a catch-bond that counterintuitively strengthens as tension increases [49]. At moderate levels of tension, the bond between integrins and their ligands becomes more stable,

whereas excessive force can lead to bond rupture [69]. Because the protein components within these force-regulated adhesion sites turn over rapidly to facilitate cell spreading and migration, the overall strength and spatial configuration of the clutch are continuously remodelled under force [49].

Consequently, the outcome of clutch engagement depends strongly on the stiffness and mechanical resistance of the ECM [70]. In soft matrices that deform easily, integrins unbind and release the force before substantial tension can be transmitted to the intracellular components. In contrast, substrates with moderate tension provide sufficient resistance to oppose cellular traction, allowing mechanical tension to build across the clutch. This force drives talin to unfold and expose cryptic binding sites for vinculin recruitment, stabilising the focal adhesion and triggering downstream mechanotransduction pathways. At very high tension the clutch violently snaps so the protein bonds break before they can stabilise and form focal adhesions.

The dynamics of the molecular clutch model operate along a distinct chronological timeline. Initial engagement and subsequent folding of talin occur within milliseconds to seconds after application of stimuli, whereas the physical recruitment and accumulation of vinculin and the active rearrangement and polymerisation of actin filaments required to reinforce focal complexes to unfold require several minutes [71], [72]. When mechanical stimulation is sustained for minutes to hours, downstream signalling and protein synthesis can be triggered.

The molecular clutch model provides a framework to interpret how cytoskeletal and adhesion-related proteins may vary under different mechanical loading regimes. Within this context, reductions in expression observed under certain conditions may reflect suboptimal force transmission at the cell-substrate interface. This suggests that the mechanotransductive responses may occur within an optimal mechanical regime, beyond which force transmission becomes less efficient.

1.7 Relevance of externally applied mechanical stimuli

Cells are exposed to various mechanical cues within the body that shape their behaviour and fate. For example, ECM stiffness has been shown to regulate integrin clustering and downstream signalling, thereby directing differentiation of MSCs. In particular, soft

matrices designed to mimic brain tissue have been shown to promote neurogenic differentiation, whereas rigid matrices resembling bone have been shown to promote osteogenesis in MSCs [73]. Fluid shear stress generated by fluid flow in the vasculature or tissue interstitium engages mechanosensors and activates pathways that regulate cell migration, alignment, and survival. Turbulent flow or low fluid flow in blood vessels alters the shape of endothelial cells, gradually leading to atherosclerosis [74]. Another example of *in vivo* mechanical cues is the tensile force generated by muscle contractions, joint movement, or external loading. The magnitude, frequency, and duration of the tensile forces experienced ultimately alter cellular migration proliferation, differentiation and fate as a result of sensing through integrins and ion channels [75], [76].

The potential to stimulate mechanotransductive pathways has created an opportunity to develop non-invasive therapies such as LIPUS that can promote bone regeneration. LIPUS is a safe and widely researched modality that delivers low-amplitude pressure waves that may mimic physiological strain, engaging focal adhesion-mediated signalling pathways without inducing structural damage [9], [77]. In principle, its application parameters (e.g. frequency, intensity, ultrasound pressure) can be adapted to modulate the delivered mechanical stimulus however, such flexibility is not yet routinely implemented in practice. This positions LIPUS as a particularly attractive modality for delivering controlled mechanical stimulation, with the potential to modulate tissue behaviour for therapeutic applications, including fracture repair.

1.8 Therapeutic ultrasound as a modality

Therapeutic ultrasound refers to the delivery of acoustic waves at frequencies above 20 kHz, up to several MHz, that can be used to induce biological responses or ablate tissue. While it has been demonstrated at both high and low ultrasound intensities, depending on the application, LIPUS has gained attention for its potential to stimulate tissues at low intensity, sometimes requiring less energy than is typically used during diagnostic ultrasound imaging. The most investigated application of LIPUS is in stimulating bone regeneration. It delivers low-energy short bursts that minimise thermal effects while still exerting mechanical forces on target tissues.

1.8.1 Ultrasound definition and physical principles

In water and tissues, ultrasound typically travels as a longitudinal wave, with propagation supported by an oscillatory motion of particles in the direction of travel. Depending on the wave intensity, frequency, and delivery mode, ultrasound can serve either as a diagnostic or therapeutic tool [78]. Diagnostic ultrasound, such as B-mode imaging or elastography, is undertaken at low intensities ($< 100 \text{ mW/cm}^2$) to visualise tissue architecture or assess mechanical properties without inducing permanent biological changes. Therapeutic ultrasound is designed to interact directly with tissues through thermal or non-thermal (i.e. cavitation, radiation force) mechanisms.

1.8.2 LIPUS clinical adaptation and parameters

LIPUS is defined by the delivery of ultrasound at intensities typically below 100 mW/cm^2 in a pulsed manner, optimised to minimise thermal effects while exerting mechanical perturbations on cells and tissues. The first therapeutic LIPUS devices were approved by the U.S. Food and Drug Administration (FDA) in the mid-1990s, initially for enhancing the healing of fresh fractures (1995) and subsequently for treating bone non-unions (2000) [16]. In the UK, the National Institute for Health and Care Excellence (NICE) has also supported its use in selected cases, particularly for accelerating healing in delayed union [17]. These approvals reflect the capacity of LIPUS to influence bone repair processes without the tissue heating associated with high-intensity regimes.

Early non-invasive ultrasound applications for bone repair date back to the 1950s, with the first clinical study beginning in the late 1970s by Xavier and Duarte, reporting positive effects of ultrasound on delayed healing and pseudoarthrosis [11], [79]. A later study by Heckman *et al.* showed that applying LIPUS at 30 mW/cm^2 accelerated healing in tibial fractures [11]. These parameters were subsequently adopted across a range of *in vitro* and *in vivo* studies and have been retained as the operational parameters applied in LIPUS treatments today.

Current devices such as Exogen (Bioventus) employ a spatial-average temporal-average intensity (I_{SATA}) of 30 mW/cm^2 , a carrier frequency (f_c) of 1.5 MHz , a pulse repetition frequency (PRF) of 1 kHz , and a pulse width (PW) of approximately $200 \text{ }\mu\text{s}$ resulting in 20% duty cycle (DC), applied for around 20 minutes daily at the fracture site [80], [81]. These commercial devices typically deliver standard rectangle pulse envelopes without variable waveform shaping. A schematic representation of this pulsed waveform and its defining parameters is shown in Figure 4. These parameters were established early in the development of LIPUS and remain the basis for most human and animal studies. However, numerous experimental studies have explored a broader range of settings, including frequencies from 45 kHz to 3 MHz , intensities from 5 to 3000 mW/cm^2 , varied duty cycles up to 50%, and a daily exposure duration of 1-30 minutes [19], [82].

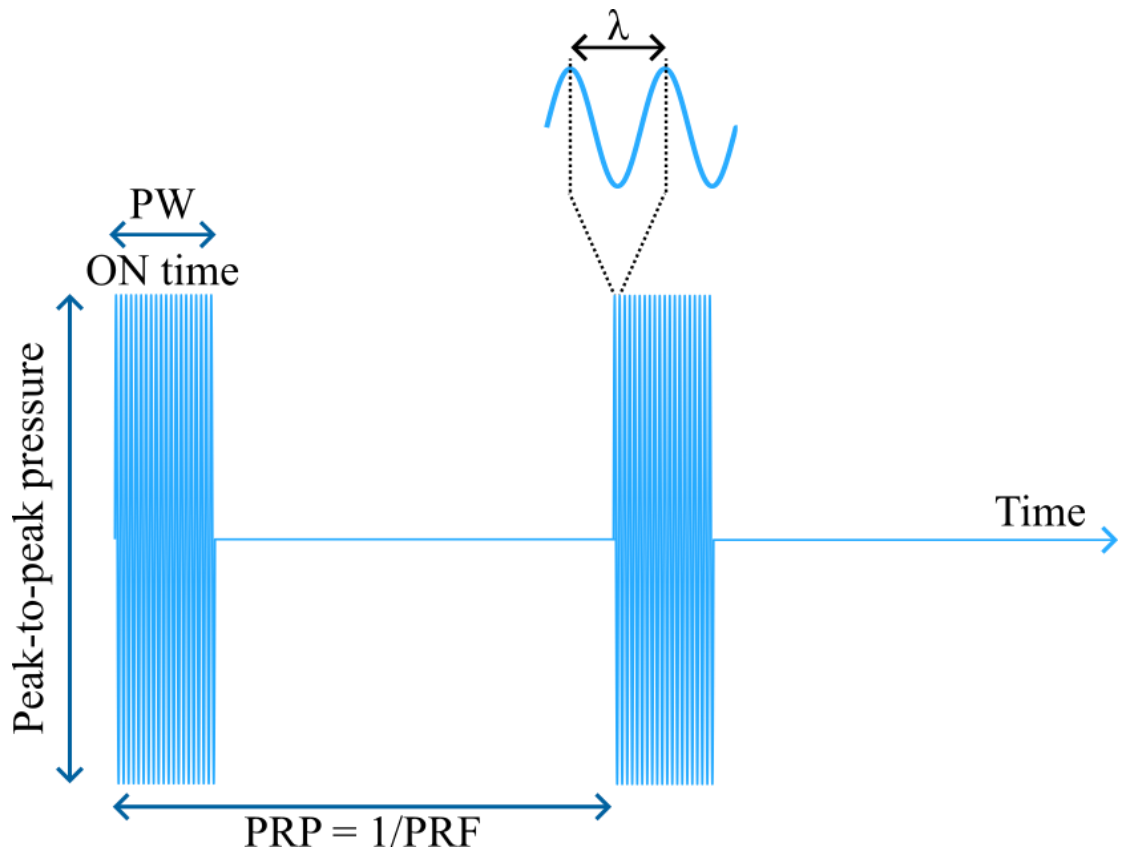


Figure 4. Schematic representation of a pulsed sinusoidal ultrasound waveform with wavelength = λ . The pulse width (PW) defines the ON time of the burst, while the pulse repetition period (PRR) is the time between consecutive bursts and it's the inverse of pulse repetition frequency (PRF). The vertical axis represents the peak-to-peak pressure.

Attenuation

Attenuation refers to the reduction in amplitude (or intensity) of the ultrasound beam with propagation distance as it travels through a medium. It is primarily caused by absorption, scattering, and reflection, and it is commonly measured in dB. It is determined by the material properties of the medium, including its viscous and elastic behaviour and structural heterogeneity. A more viscous material creates greater internal friction, thereby converting sound to heat more quickly, while particle size (microstructure) determines scattering. The effects of attenuation on LIPUS wave propagation have not been widely investigated within the LIPUS literature but could have a significant impact on the eventual dose experienced at the fracture site.

This is highlighted in a recent study by Panalisamy *et al.*, in which ultrasound was applied at 0.5 and 1.5 MHz while compensating for tissue attenuation to maintain an equivalent intensity of 30 mW/cm^2 at the fracture site [78]. *Ex vivo* hydrophone measurements using human cadaver and porcine tissues revealed that 1.5 MHz ultrasound experienced $\sim 6 \text{ dB}$ of attenuation over 5 cm of tissue, reducing the effective intensity to around 6.26 mW/cm^2 while the 0.5 MHz frequency indicated better transmission through the soft tissue with only $\sim 2 \text{ dB}$ attenuation.

The authors developed a custom LIPUS system calibrated to deliver a consistent I_{SATA} of 30 mW/cm^2 at the fracture site, based on estimated soft-tissue thicknesses obtained via ultrasound imaging. Indeed, both compensated groups outperformed the commercial device, with the 1.5 MHz showing moderate improvements in bone mineral density, mechanical strength, and osteocyte density. However, the 0.5 MHz consistently achieved the most favourable outcomes across all evaluated parameters, which the authors attribute to the better capacity of 0.5 MHz to reach deeper parts of the bone. This suggests that, while correcting for attenuation is necessary to ensure adequate energy delivery at the fracture site, frequency itself plays an important role in determining the biological response. As such, both attenuation and frequency should be considered when defining LIPUS exposure conditions.

Frequency

Frequency refers to the number of cycles or oscillations of an ultrasound wave per second and is expressed in MHz. As ultrasound propagates through biological tissue, its amplitude decreases due to attenuation, which arises from energy loss due to absorption and scattering of the wave. Attenuation is strongly dependent on the frequency of the ultrasound wave as well as the acoustic properties of the medium (e.g. density, bulk modulus, structural heterogeneity of the tissue). In LIPUS, the frequencies range from 45 kHz to 3 MHz, and the penetration depth in biological tissue is therefore frequency dependent. Lower frequencies penetrate more deeply, which is important when targeting deeper structures such as bone [78]. However, the actual penetration also depends on the acoustic properties of the tissue. For tissues with higher density and attenuation ultrasound is absorbed more readily. At 1 MHz, ultrasound can penetrate approximately 30 mm in muscle and just 7 mm in bone [83].

Pulse width

Pulse width is the duration within each burst for which ultrasound is actively emitting a pulse and is measured in microseconds (μs). It determines how long tissues are exposed to acoustic energy in each pulse, influencing both mechanical and thermal effects. A pulse width of 200 μs is commonly used in LIPUS and has been shown to enhance bone healing outcomes [11], [84]. However, some studies suggest that increasing pulse width (up to 2000 μs) may further enhance outcomes such as bone mineral density and recovery in rat models, though this warrants further investigation as increased pulse width alters both the duration for which acoustic radiation force is applied, and the energy delivered per pulse, making it difficult to distinguish whether observed effects arise from changes in mechanical stimulation, intensity, thermal contribution, or their combined influence.

Pulse repetition frequency

Pulse repetition frequency (PRF) refers to how many ultrasound pulses, comprising a series of sinusoid cycles, are delivered each second, measured in Hz. In LIPUS, PRFs of 1-1000 Hz have been explored, but devices generally employ 1 kHz [85], [86]. PRF together with pulse duration (or duty cycle) define the temporal structure of the ultrasound signal, dictating how frequently and for how long mechanical stimulation is applied. This spacing

between pulses minimises thermal effects by allowing tissue to cool between consecutive bursts.

Importantly, PRF governs how often cells experience the onset of acoustic radiation force, which arises from the transfer of momentum from the propagating ultrasound wave to the medium, resulting in a net force and associated tissue displacement. While the oscillatory motion of the wave occurs at the carrier frequency (typically MHz) and is governed by the acoustic properties of the medium, PRF determines the rate at which these force-generating events are delivered. As such, PRF does not directly define the amplitude of oscillation, but rather the temporal pattern of mechanical loading experienced by the tissue.

Duty cycle

Duty cycle refers to the percentage of time that the ultrasound is active during each pulse. It is calculated as the ratio of pulse width (PW) in seconds, to the total duration of one pulse period, which is the inverse of PRF (Equation 1). In LIPUS, a 20% duty cycle is the most commonly used, meaning that ultrasound is on for 20% and off for 80% of each burst.

$$Duty\ cycle\ (\%) = PW \times PRF \times 100 \quad \text{Equation 1}$$

Acoustic impedance

Acoustic impedance (Z) is a physical property of a medium that describes its opposition to motion when an ultrasound wave propagates through it. It is defined as $Z = \rho c$, where ρ is the density (kg/m^3), and c is the speed of sound (m/s) in the medium.

Intensity

Intensity in ultrasound refers to the amount of acoustic power delivered per unit area and is expressed in mW/cm^2 . It determines the total energy transferred into the tissue and is a key factor in both safety and therapeutic efficacy. In LIPUS, intensities are kept low to minimise thermal effects to allow mechanical bioeffects to be induced without thermal damage. Various measures of intensity are used in the field, depending on the application or safety concern, such as spatial-peak pulse-average (I_{SPPA}), spatial-peak temporal-average (I_{SPTA}), spatial-average temporal-average (I_{SATA}), and spatial-average temporal-peak (I_{SATP}),

each representing different ways of averaging power over space and time, described in the following sections [87].

Spatial peak pulse average intensity (I_{SPPA})

I_{SPPA} represents the highest intensity measured at a specific point in the beam during the actual on-time of the pulse and is directly related to the peak pressure amplitude. It does not account for the off-time between pulses and so does not provide a useful indicator of the potential for thermal effects. Physically, I_{SPPA} can be estimated from ultrasound pressure assuming a plane wave and a rectangular pulse using *Equation 2*.

$$I_{SPPA} = \frac{p^2}{2Z} \quad \text{Equation 2}$$

Where p is the peak ultrasound pressure (Pa), and Z is the acoustic impedance of the tissue (Rayl). This relationship shows how both pressure amplitude and tissue properties influence the energy delivered during each pulse.

Spatial peak temporal average intensity (I_{SPTA})

I_{SPTA} measures the peak intensity at a specific point in the ultrasound beam, averaged over both the on and off times of each burst. It accounts for the duty cycle, making it a useful estimate of thermal exposure at the point of highest intensity and can be calculated from I_{SPPA} using *Equation 3*.

$$I_{SPTA} = I_{SPPA} \times DC \quad \text{Equation 3}$$

Spatial average temporal average intensity (I_{SATA})

I_{SATA} refers to the spatial-average, temporal-average intensity across the beam area, reflecting the total energy delivered to tissue during treatment. It is the primary parameter used to define dosage in LIPUS (*Equation 4*).

$$I_{SATA} = \frac{\text{Total output power (Watts)}}{\text{Effective radiating area (m}^2\text{)}} \quad \text{Equation 4}$$

Where total output power is the output power of the device, which can be measured using a radiation force balance or estimated from a pressure field map that encompasses the entire beam, as shown in *Equation 5*.

$$\text{Total acoustic power} = \frac{PRF \times PW}{\rho c} \sum_k^N p_k^2 \times a_k \quad \text{Equation 5}$$

Where ρ is the density of the medium in kg/m³, c is the speed of sound in water in m/s, k is the scan point index, N is the number of scan points, p_k is the root mean square pressure measured at the scan point k in Pascals, and a_k is the area of each scan point k -6 dB (FWHM) in m². In water, $\rho = 1000$ kg/m³ and c in water at 37 °C is 1525 m/s [88].

Equation 5 was used throughout the thesis to estimate the total acoustic power from 2D hydrophone raster scans. I_{SATA} was subsequently determined using *Equation 4*.

1.9 LIPUS mechanism of action

LIPUS is a form of mechanical stimulation that interacts with tissues through multiple overlapping physical phenomena, making it difficult to isolate a single mechanism of action. In practise, LIPUS could generate mechanical, piezoelectric, or thermal effects, that together influence cellular function and tissue repair. Understanding how these physical quantities interact with tissues and cells is essential for interpreting experimental results and optimising the therapeutic efficacy of LIPUS.

The thermal effects of LIPUS in biological tissues arise from the absorption of energy due to molecular friction [82]. At clinical intensities (< 500 mW/cm²), these effects are generally negligible however, at much higher intensities of up to 5000 mW/cm², thermal effects dominate and could lead to adverse effects such as fibrosis or necrosis [89]. In *in vitro* experimental investigations, temperature increases are typically reported as less than 0.2 °C, insufficient to drive major thermal signalling or damage [90].

Acoustic radiation force results in tissue deformation. Though small in magnitude (micro- to milli-newtons) in the context of LIPUS, this persistent force is sufficient to generate

cellular displacement or strain the cytoskeleton and potentially engage mechanosensitive pathways involved in bone remodelling or structural adaptation of the *ex vivo* retina [91], [92], [93].

Acoustic streaming refers to steady fluid motion that enhances local circulation and nutrient redistribution *in vitro*, potentially triggering biological effects. Streaming forces can apply shear stresses and activate mechanosensitive components on the cell membranes [94]. Acoustic cavitation refers to the formation, oscillation, and collapse of gas or vapour bubbles in a liquid under an acoustic field [95]. In LIPUS, inertial cavitation is unlikely due to the low acoustic pressures employed, but stable cavitation (without collapse) is still plausible in *in vitro* and *in vivo* settings where free gas bubbles (i.e. in the cell culture media) can oscillate periodically, producing local microstreaming and shear forces. Both acoustic streaming and stable cavitation may affect diffusion dynamics and transiently increase membrane permeability, potentially enhancing cellular sensitivity to mechanical cues [96].

Bone is a piezoelectric material due to the non-symmetric arrangement of minerals within the ECM [97]. Bioelectricity across the body is essential for cellular and tissue homeostasis as well as for cell migration during healing [98]. Hosokawa *et al.* established that the application of mechanical stresses using ultrasound results in a measurable electrical response in cancellous bone, thereby contributing to bone regeneration [99].

1.10 LIPUS in bone healing

1.10.1 Clinical use and broad efficacy reports

LIPUS has been evaluated in both animal models and clinical studies for its effects on bone healing. *In vivo* animal studies and clinical trials have shown that LIPUS can enhance structural aspects of repair, including increased callus formation, bone volume, and mineralisation in fracture and delayed-union models [100], [101], [102]. These findings support its biological activity at the tissue level and provide a rationale for clinical investigation.

Clinical trials suggest that LIPUS can modulate the coordinated formation and resorption of bone, thereby promoting healing of non-union and delayed-healing fractures. In delayed

union of osteotomised fibulae treated over 2-4 months, LIPUS treatment did not change the number of blood vessels but increased blood vessel volume and perfusion, indicating enhanced local blood supply [103]. Inflammation is a critical early phase of bone healing that enables the delivery of oxygen, nutrients, and cells at the injury site to initiate tissue repair. The study was conducted on a relatively small cohort ($n = 13$), which inherently limits the extent to which the findings can be generalised beyond the study population. The variation in treatment duration introduces an additional uncontrolled parameter, which may confound the observed outcomes and limit the ability to attribute the reported effects.

In a randomised, sham-controlled, multi-centre trial of delayed union tibial shaft fractures, LIPUS significantly improved bone mineral density, as measured by CT, and reduced the fracture gap area, with statistically significant healing observed after 112 days of treatment [101]. While the study design is robust ($n=101$), healing progression was assessed via CT imaging, which can directly capture mineralisation and geometric closure of the fracture site but cannot directly evaluate mechanical competence or the bone's ability to sustain physiological loading.

In another randomised, double-blind, placebo-controlled trial, histological and histomorphometric analyses were used to assess tissue-level bone healing in 13 patients with delayed union of the osteotomised fibula following 2-4 months of treatment with or without LIPUS [102]. LIPUS-treated patients exhibited both direct and indirect bone healing, whereas placebo samples showed only indirect healing. This was accompanied by significant increases in osteoid thickness, mineral apposition rate, and bone volume at sites of new bone formation, indicating enhanced osteoblast-driven activity at the fracture front. The authors state that the effects are likely attributed to increased blood flow although angiogenesis was not reported. However, the small cohort size, together with the variation in treatment duration, introduces variability in cumulative exposure and may limit the extent to which these findings can be generalised.

LIPUS has also been reported to stimulate endochondral ossification in mice with femur delayed union fractures, by promoting endothelial cell migration and neovascularisation around the fracture sites [104]. In another animal study, LIPUS was reported to enhance healing by acting on inflammation, angiogenesis, chondrogenesis, endochondral ossification and bone remodelling in rat closed femoral fractures [105]. However, as these findings are derived from murine models, their direct translation to human fractures

remains uncertain, due to the smaller size of rodent bones and overlying soft tissues, the acoustic beam penetrates proportionately deeper and covers a larger volume of the target tissue compared to human clinical applications.

Despite these positive findings, results across clinical trials do not represent a uniformly clear picture. A double-blind, placebo-controlled study following Chevron osteotomy found no significant benefit of LIPUS on clinical outcomes or radiographic healing at 6 weeks or 1 year, which may reflect limited follow-up intervals that missed early transient effects and high innate healing capacity of the participants [105]. This suggests that LIPUS may have limited influence in mechanically stable, uncomplicated healing environments, with effects more likely to be observed in cases of impaired or delayed healing. Similarly, a randomised, placebo-controlled trial of lower-limb stress injuries reported no significant advantage of LIPUS, potentially owing to non-standardised treatment parameters, a small and predominantly elderly sample, and lack of control over device compliance and patient activity during the study [106].

Animal studies have also reported mixed outcomes across various species, including dogs and horses [107], [108]. In a prospective, randomised, double-blinded, placebo-controlled *in vivo* study, fifty dogs undergoing tibial plateau levelling osteotomy were assigned to either LIPUS or sham treatment groups. LIPUS was delivered daily for 20 minutes (1.5 MHz, 1 kHz PRF, 20% DC, 30 mW/cm²) over a 12-week period. Bone healing was assessed radiographically at 4, 8, 10, and 12 weeks post-operation, while limb function was evaluated using gait analysis. Both groups demonstrated progressive improvement in radiographic healing and limb use over time however, no significant differences were observed between LIPUS-treated and control groups at any time point. These findings indicate no measurable benefit of LIPUS under these conditions.

These inconsistencies likely reflect variation in study design, fracture type and treatment parameters, as well as variability in patient characteristics and species-specific bone physiology. However, a key underlying factor is that identical ultrasound settings at the device level do not translate to equivalent mechanical exposure at the fracture site. Variations in soft tissue thickness, bone depth, and anatomical geometry alter acoustic propagation, resulting in substantial differences in the pressure field and cumulative dose delivered locally. The variability in biological characteristics within and between cohorts, including age, body composition, and baseline healing capacity, may further influence both

the tissue response and the effective mechanical stimulus experienced at the fracture site. As such, discrepancies in reported outcomes may reflect differences in the actual mechanical stimulus experienced by the tissue, rather than inconsistencies in LIPUS efficacy alone. Finally, the timing of LIPUS application, as well as the complex relationship between radiographic indicators of healing and functional recovery, limits the extent to which outcomes can be directly interpreted.

1.10.2 Mechanotransduction pathways and mechanosensors stimulated by LIPUS

A growing body of evidence is beginning to identify the molecular mechanisms by which LIPUS transduces mechanical signals into cellular responses. Mechanotransductive pathways mediated by integrins, cytoskeletal elements, focal adhesion proteins, and ion channels are likely implicated, with responses varying across cell types and experimental contexts (Figure 5).

Yang *et al.* used continuous wave ultrasound (1 MHz, 125 mW/cm² I_{SATA}, 10 min) delivered through water coupling in Petri dishes to stimulate MC3T3-E1 pre-osteoblasts and primary rat osteoblasts [109]. They showed that LIPUS transiently upregulates integrin expression on the cell surface, peaking at 3-6 hours post-stimulation and returning to baseline by 24 h. FITC-phalloidin staining revealed enhanced actin filament organisation, especially at the cell periphery, 1h after stimulation, which declined by 3h. These changes suggest that integrin upregulation may drive actin cytoskeleton remodelling in response to ultrasound.

Similarly, Tang *et al.* demonstrated that LIPUS transiently increases surface integrin expression in osteoblasts [110]. Using the Exogen device (1.5 MHz, 30 mW/cm² I_{SATA}, pulsed at 1 kHz), applied via vertical immersion of the transducer into each culture well. LIPUS stimulation elevated Cyclooxygenase-2 (COX-2) mRNA and protein levels as well as prostaglandin-E₂ (PGE₂) production, which are key mediators of inflammation and mechanically induced early-stage bone repair [111]. These effects were attenuated by anti-integrin antibodies or Phosphoinositide 3-kinase (PI3K) inhibitors, indicating the involvement of the integrin-FAK-PI3K-Akt (protein kinase B) and ERK pathways.

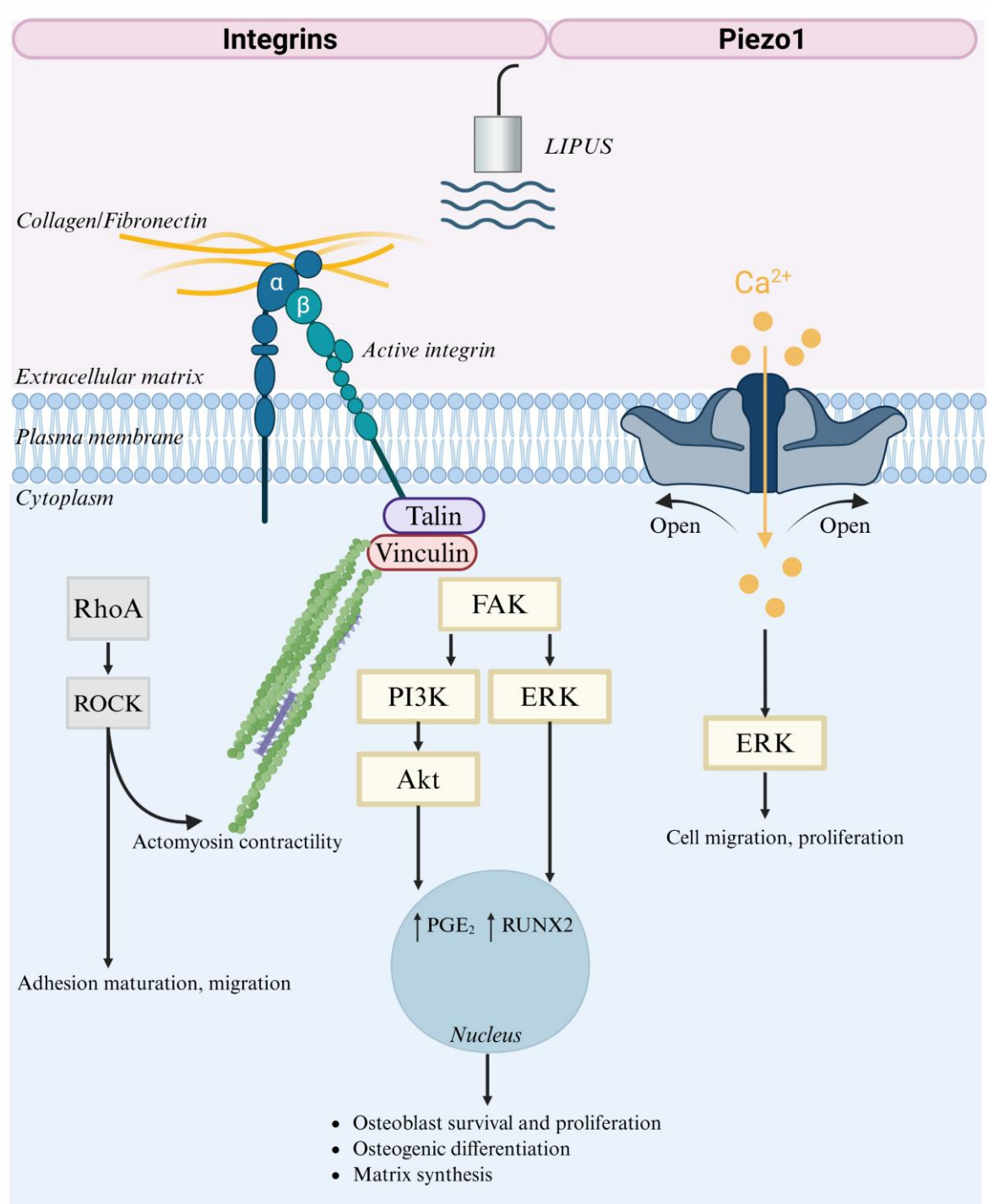


Figure 5. Schematic representation of proposed mechanosensors and intracellular pathways activated upon exposure of cells to LIPUS. Mechanical stimulation is transmitted through integrin-mediated adhesions, linking the ECM to the cytoskeleton via talin and vinculin, and activating downstream signalling pathways including FAK, PI3K/Akt, and ERK. In parallel, mechanosensitive ion channels such as Piezo1 facilitate Ca^{2+} influx, contributing to ERK activation. These pathways are associated with cellular processes including adhesion, migration, proliferation, and osteogenic differentiation. Created using Biorender.

Focal adhesion kinases (FAKs) act as vital links between the extracellular mechanical cues and intracellular signal transduction. Once activated by integrins, FAKs recruit and

phosphorylate downstream effectors, among these, the PI3K/Akt pathway supports osteoblast survival and proliferation, while ERK promotes osteogenic differentiation and matrix synthesis [9], [23]. The involvement of ERK in LIPUS-induced osteogenic response was further confirmed by Kusuyama *et al.* who showed that LIPUS stimulation increased Runx2 expression via the ROCK-ERK pathway in MSCs [112]. ROCK, a downstream effector of RhoA that regulates cytoskeletal tension, cell migration, differentiation, and survival, and can also promote ERK activation. ERK signalling promotes gene transcription and cell motility in response to mechanostimulation [45]. This pathway operates in parallel to the FAK-PI3K-Akt pathway, with both contributing to LIPUS-induced osteogenesis through distinct but complementary mechanisms.

Interestingly, a similar integrin-FAK-PI3K-Akt pathway was identified by Cheng *et al.* in osteoarthritic chondrocytes, where LIPUS modulated ECM-related gene expression of collagen II and aggrecan, and suppressed MMPs expression, key enzymes in driving cartilage degradation in osteoarthritis [113], [114]. These changes were linked to upregulation of integrin $\beta 1$ and increased phosphorylation of FAK, PI3K, and Akt signalling. Xie *et al.* confirmed the involvement of the PI3K/Akt pathway in LIPUS-induced proliferation of human bone marrow-derived MSCs [115]. Daily exposure to LIPUS (Exogen) increased cell proliferation significantly. This response was abolished by PI3K inhibition, confirming the pathway dependency. These results highlight the cell-type-specific functional outcomes of integrin-FAK-PI3K-Akt signalling.

Atherton *et al.* identified vinculin as a critical component of LIPUS-induced mechanotransduction [77]. While earlier studies focused on integrins and FAK as key transducers, this group showed that the connection of vinculin to the actin cytoskeleton is required for cells to sense and respond to pulsed ultrasound. When this link was disrupted, LIPUS-induced signalling was blocked. They also found that Rab5 and Ras-related C3 botulinum toxin substrate 1 (Rac1) are activated downstream, establishing a mechanistic link between LIPUS stimulation and both cytoskeletal remodelling and endocytic activity. Specifically, Rab5, a key regulator of early endosome formation, facilitates receptor-mediated endocytosis and intracellular trafficking, processes essential for sustaining signal transduction during bone repair. Meanwhile, Rac1, a Rho GTPase, orchestrates actin cytoskeleton organisation, promoting membrane ruffling and cell migration, all of which are vital for cell recruitment and osteogenic differentiation at the injury site [116]. These

findings add to previous work showing that effective mechanotransduction depends not just on pathway activation, but also on how the mechanical signal is physically transmitted through structural connections inside the cells.

More recent investigations highlighted Piezo1 as a key mechanosensor involved in LIPUS-induced cellular responses [117], [118], [119]. Piezo1 is a mechanosensitive ion channel located in the plasma membrane involved in maintaining bone homeostasis and promoting osteogenesis. Upon activation, it undergoes conformational changes that open its pores and allow Ca^{2+} influx, which can trigger downstream pathways that can affect cytoskeletal remodelling, gene expression, and ultimately skeletal remodelling [120]. Piezo1 deficiency in osteoblasts has been reported to reduce bone mass, elevate bone resorption and even lead to spontaneous bone fractures [121].

Zhang *et al.* showed that LIPUS activates Piezo1 in MC3T3-E1 osteoblasts, leading to increased cell migration, proliferation, ERK phosphorylation, and perinuclear F-actin accumulation [122]. Knockdown of Piezo1 suppressed these effects, indicating its central role in transducing mechanical signals into calcium influx and downstream signalling. Inoue *et al.* further confirmed the role of Piezo-1 in accelerating fracture repair in mice. They further showed that Piezo1 inhibition reduced chondrogenesis and osteogenesis both *in vivo* and in a 3D co-culture system [123].

1.11 Limitations of present LIPUS literature

Despite extensive investigations of LIPUS for bone healing, several limitations constrain the interpretation of the existing literature and hinder progress towards optimising its therapeutic use. The inconsistencies in reported outcomes stem from a combination of biological variability such as differences in fracture types, anatomical sites, or *in vitro* models (discussed in Chapter 2) and the lack of physical standardisation. Crucially, most investigations report only nominal transducer output rather than the actual mechanical cues experienced at the fracture site or cell monolayer. Without *in situ* characterisation of the ultrasound pressure field and its spatial distribution, the true mechanical stimulus transmitted to the cells remains undefined, making it impossible to accurately reproduce reported outcomes (Discussed in Chapter 3).

Furthermore, the physical behaviour of ultrasound waves introduces confounding variables depending on the experimental environment. In *in vitro* setups or preclinical trials, unwanted secondary effects such as standing waves, acoustic streaming and localised heating can significantly alter biological responses, masking the true effects of the primary ultrasound stimulus [124], [125]. This issue is compounded *in vivo* by tissue attenuation, which can reduce the effective dose delivered to the fracture site by up to 80% depending on tissue thickness and frequency. Palanisamy *et al.* directly demonstrated this by comparing 0.5 and 1.5 MHz LIPUS in a rabbit hip fracture model [126]. The group showed that a nominal I_{SATA} of 30 mW/cm² dropped to 6.16 mW/cm² after passing through 5 cm of soft tissue at 1.5 MHz.

Finally, the concept of dose in LIPUS remains insufficiently standardised. While I_{SATA} is a useful metric for regulatory purposes, it fails to describe the magnitude or temporal characteristics of the mechanical stimulus at the cellular level. Researchers frequently report I_{SATA} as a standalone measure of dose yet simultaneously modify interdependent parameters such as duty cycle or pulse repetition frequency. Because different combinations of these parameters can yield the exact same I_{SATA} , reporting this value alone creates an ambiguous definition of the mechanical stimulus. Ultimately, the absence of controlled, directly measured acoustic conditions and standardised dosing protocols hinders the clinical optimisation of LIPUS therapy (examined further in Chapters 3 and 4).

A clearer understanding of the mechanisms involved is needed to optimise LIPUS protocols. Current literature focuses on downstream biological effects, tracking the expression of late-stage markers or proteins without mapping the initial upstream mechanosensation events that trigger them. Because the exact mechanical forces experienced by the cells remain undefined, variations in cell type or culture environments lead to conflicting outcomes. *In vitro* apparatus for systematic, controlled and repeatable investigation is therefore foundational. This need is addressed in Chapter 2.

Translational limitations are also evident. While *in vitro* experiments are low-cost and easier to control, they lack systemic interactions and do not always translate to real biological systems. In the case of ultrasound-induced biological effects, micro-scale fluid motion, or acoustic microstreaming may not occur to the same extent in living bone tissue. While this may further modulate LIPUS effects *in vivo*, it may only be effectively investigated once the exact basis of LIPUS activated cellular mechanosensing are

established. For this reason, micro-scale fluid motion has not been directly investigated in this thesis.

In addition, clinical application of LIPUS and indeed the clinical studies reported here are subject to additional confounding effects, including patient compliance, anatomical variability, fracture stability, and differences in healing capacity [18]. It is important to clarify that bone formation does not necessarily translate to meaningful clinical benefit, but it remains the case that these important aspects of translation can only really be addressed once a more thorough mechanistic understanding of LIPUS and its potential therapeutic effects has been established.

Taken together, these limitations highlight the need for improved reporting of acoustic conditions, greater standardisation of dosing metrics, and experimental models that more closely reflect the mechanical environment experienced by one tissue. Addressing these gaps is essential for resolving inconsistencies in the literature and for establishing clear mechanistic and translational foundations for LIPUS-based therapies.

1.12 Aims, research questions, and thesis structure

This thesis addresses key limitations in the mechanistic understanding and dosimetry of LIPUS in bone healing, with a focus on improving experimental control, measurement precision, and clinical translatability.

The first aim was to develop a reliable *in vitro* platform for studying LIPUS under physiologically relevant conditions. To achieve this, an acoustically transparent cell culture vessel that minimises secondary acoustic effects was developed and employed. *In situ* ultrasound field characterisation enabled the reporting of key variables such as pressure amplitude, spatial distribution, and waveform characteristics at the cellular interface. This experimental apparatus allowed the accurate and controlled delivery of ultrasound to adherent cells.

The second aim was to systematically examine how changes in ultrasound parameters, including pressure, duty cycle, pulse repetition frequency, exposure duration, and strain rate, affect cellular responses. Importantly, these parameters were varied while acknowledging their interdependence through ISATA. The validity of ISATA as a meaningful

metric for cellular mechanostimulation is challenged by achieving identical I_{SATA} values using different waveform configurations.

The final aim was to introduce and validate laser Doppler vibrometry (LDV) as an effective means of quantifying mechanical dose in the context of LIPUS. LDV can measure the displacement induced in the cell substrate by LIPUS with high precision, providing a more direct and interpretable mechanical input. By identifying displacement thresholds that trigger osteogenic responses *in vitro*, this approach may support the optimisation of existing LIPUS protocols, particularly in complex *in vivo* or 3D environments, by enabling the delivery of a consistent effective mechanical stimulus, even if I_{SATA} or other device parameters must be altered to achieve it.

The thesis is structured as follows:

- Chapter 2 focuses on the development of the *in vitro* platform and ultrasound field characterisation for LIPUS investigation
- Chapters 3 and 4 present experimental findings on cellular responses to varying LIPUS parameters
- Chapter 5 explores how rise time may influence the mechanosensitivity of cells subjected to LIPUS, and employs LDV to explore its potential for translational mechanobiology
- Chapter 6 discusses the implications of these findings and outlines future directions of LIPUS research

Chapter 2: *In vitro* experimental setup design

2.1 Introduction

This chapter presents the *in vitro* LIPUS exposure system designed to investigate the biological effects of LIPUS under controlled and measured conditions. *In vitro* methodologies offer a distinct advantage by isolating specific cellular responses. However, most exposure systems reported in the literature, often suffer from limitations such as acoustic heterogeneity, standing-wave artefacts, poor acoustic matching, and the absence of *in situ* acoustic field measurements [94], [124]. These elements compromise the reproducibility of *in vitro* data and ultimately hinder the clinical translation of LIPUS into an effective therapy. The setup developed herein addresses these limitations and provides a robust, well-characterised experimental platform for studying LIPUS bioeffects *in vitro*.

The experimental configuration consists of a single-element transducer submerged in a water tank, driven via a custom-built matching circuit, a signal generator, and a power amplifier. Voltage sweeps and field-mapping measurements were performed to identify the linear operating regime of the transducer and to design experiments within its operating limits. These measurements informed the parameter space explored throughout this thesis. The calibrated transducer allows independent modulation of ultrasound pressure output, duty cycle, and pulse repetition frequency within its operational limits.

A custom cell exposure chamber, the Biocell was developed to accommodate cells for exploring LIPUS bioeffects while minimising impedance mismatch and standing-wave formation. The Biocell is acoustically and optically transparent, allowing consistent, measured stimulation of a cell monolayer with LIPUS while also enabling microscopy imaging of the cells post-exposure to assess bioeffects. The cytocompatibility of the vessel was confirmed using Alamar Blue assay. Coupling of the transducer to the cell monolayer is achieved through degassed deionised water held at 37 °C. Due to the vessel being made in-house, LDV was used to assess batch-to-batch variation between samples and to confirm its acoustic transparency.

While 2D culture systems represent a simplification of the *in vivo* environment, the setup presented here enables precise control and quantification of ultrasound exposure, serving

as a platform for systematically investigating the mechanobiological effects of LIPUS under well-defined conditions.

2.2 Ultrasound field complexity *in vitro*

It is important to consider the complexity of the ultrasound field as it traverses the various boundaries before reaching the cell monolayer. The presence of boundaries with different acoustic impedances (Z) may introduce unwanted reflections or transmission losses ultimately altering the effective dose delivered to the cell monolayer and potentially leading to standing waves or resonance effects that can significantly distort the mechanical cues experienced by the cells.

Standing waves can form on the culture dish as a result of uncontrolled reflections when the distance between the transducer and the culture plate is a multiple of half the acoustic wavelength ($\lambda/2$). Constructive and destructive interference between the incident and reflected waves can manifest as local pressure nodes and antinodes, which, when incident on the cell layer, can significantly alter the ultrasound pressure at the cell surface and could also lead to the accumulation of heat.

Resonance effects are determined by the thickness and the properties of the culture dish and occur when the ultrasound carrier frequency (or its harmonics) coincides with the natural frequency of the culture plate. At resonance, the reflection coefficient at the plate-medium interface approaches zero, allowing near complete acoustic energy transmission to the cells. As a result, the plate couples directly into the surrounding medium (water or air) leading to amplified unstable oscillations which alter the acoustic dosimetry and elevate the risk of localised heating.

Furthermore, transmission losses through the culture material due to impedance mismatch and reflections can significantly attenuate the signal when using culture plates with rigid bases, even if conditions for resonance effects and standing waves are not met [13], [127]. Therefore, the pressure distribution at the cell monolayer can differ substantially from the nominal intended device intensity, making it almost impossible to reproduce the experiments unless the exact conditions are replicated.

These phenomena are highly sensitive to physical parameters such as plate geometry, transducer distance, and ultrasound frequency. Even minor variations in experimental approach, such as ultrasound transducer orientation (i.e. whether ultrasound is delivered from above or below the culture plate), can lead to substantial differences in acoustic dosimetry and field distribution. Additionally, several investigations employ multiwell plates, which have been shown to be prone to crosstalk between adjacent wells due to lateral propagation of acoustic energy, thus confounding the integrity of experimental results [128].

Gupta *et al.* used identical transducers and acoustic inputs and compared the effects of ultrasound on cells adhered onto multiwell plates versus Petri dishes [124]. They concluded that pressure distribution can vary significantly depending on the type of plate employed, as evidenced by statistically significant differences in cell metabolic activity. In some cases, this variability may explain differences in results observed across studies, as the biological response depends not only on the acoustic input but also on the experimental configuration employed. Their findings underscore the limitations of conventional culture ware for *in vitro* ultrasound investigations and reinforce the need for purpose-built exposure systems that ensure spatially uniform and reproducible acoustic fields.

Collectively, these insights emphasise the necessity for rigorous control and documentation of physical parameters in ultrasound *in vitro* studies to improve reproducibility and ensure that the observed biological effects are attributable to the intended acoustic stimulus.

Nominal parameters such as manufacturer-specified intensities, peak voltages, or transducer output values alone are insufficient to describe exposure dose *in vitro* as they do not represent the acoustic conditions experienced at the cell layer.

2.3 Exposure systems in previous studies

A range of *in vitro* LIPUS exposure configurations have been reported in the literature (Figure 6), each with distinct advantages and limitations in terms of acoustic control, reproducibility, and biological relevance.

The benchtop direct immersion method (Figure 6A), in which the transducer is vertically immersed in the culture medium in a Petri dish or multiwell plate, is widely adopted for its

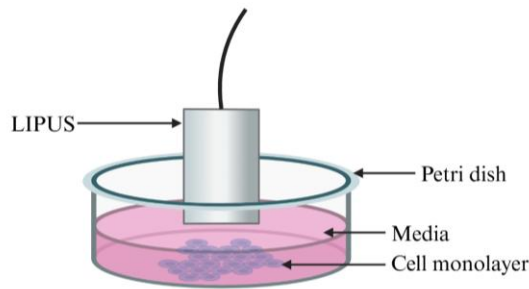
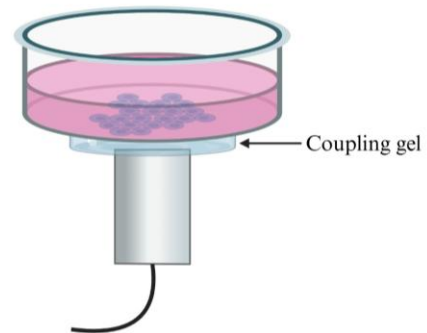
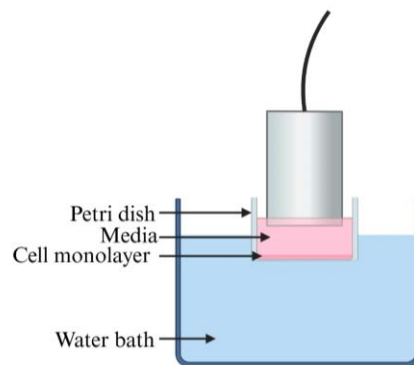
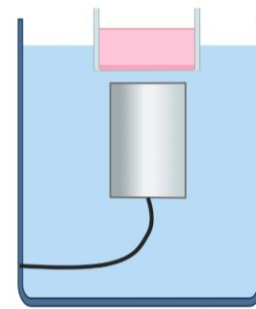
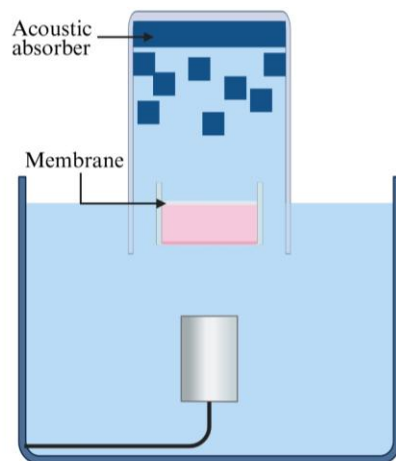
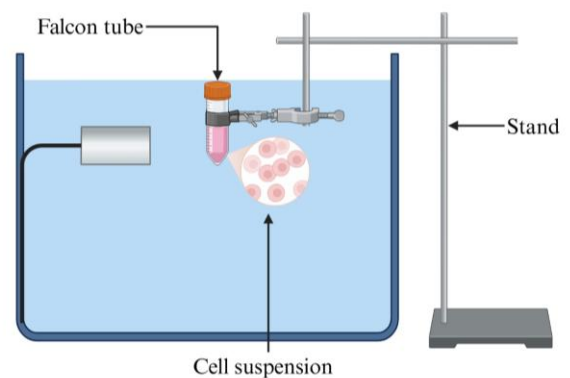
A) Benchtop direct immersion**B) Direct coupling****C) Tank dip****D) Inverted tank dip****E) Absorption tube****F) Custom tank**

Figure 6. Reported experimental configurations. (A) Benchtop direct immersion where the transducer is directly immersed into the culture medium above a cell monolayer in a Petri dish. (B) Direct coupling in which ultrasound is transmitted through a coupling gel layer at the base of the culture vessel. (C) Tank dip where the culture dish is partially immersed in a water bath and isonified from above. (D) Inverted tank dip where the transducer is positioned beneath the culture vessel and water is the coupling medium. (E) Absorption tube incorporates acoustic absorbers to minimise reflections and standing wave formation while isonifying cells through a membrane interface. (F) Custom tank-controlled ultrasound delivery to cells in suspension using a mounted Falcon tube within a water bath.

configuration is likely susceptible to acoustic reflections due to impedance mismatches at material boundaries or within the dish (Appendix A). It is also sensitive to variations in equipment positioning that can lead to inconsistent pressure fields and reduced experimental reproducibility across laboratories.

An alternative approach involves direct coupling (Figure 6B) of the transducer to the base of a culture vessel using ultrasound gel [85], [132], [133]. This method reduces contamination risk and facilitates acoustic transmission, but is prone to thermal effects, particularly when the transducer is positioned directly beneath the cell layer [134]. However, this configuration is likely more susceptible to unwanted secondary effects as the media-air interface is no longer mitigated by the relatively low-reflective front face of the transducer (Appendix A).

Improved configurations include the tank dip and inverted tank dip methods (Figure 6C-D), where heated water is used as the coupling medium. In the former, the plate is partially immersed in the water, and the transducer is dipped into the plate from above, while in the latter, the transducer is placed at the bottom of the water tank, and ultrasound is delivered from below the plate [90], [135], [136]. Although the impedance mismatch between water and the culture plate is insignificant (Appendix A), the media-air interface remains a significant source of reflections that could likely introduce spatial heterogeneity in pressure distribution within the plate.

The absorption tube method (Figure 6E) is a more controlled alternative wherein the transducer is positioned at the bottom of a heated water tank, directing energy upward towards the culture plate [14], [15]. To mitigate for the air-water interface, a tube sealed with a thin membrane is placed above the culture plate and water or acoustic absorbers are installed within, to mitigate unwanted secondary reflections. However, resonance effects are still plausible with this configuration. Notably, immersing the transducer in water enhances heat dissipation, resulting in significantly lower temperature increases during exposure. In direct-coupling configurations, temperature increases of up to 2.7 °C have been reported, whereas a water-immersed setup limited the temperature rise to 0.7 °C [14], [134]. This reduction in thermal loading is critical for minimising confounding thermal effects and ensuring that observed cellular responses are attributable to mechanical stimulation alone.

The custom tank method (Figure 6F) represents a more sophisticated and less commonly employed configuration [13], [79], [127]. In this arrangement, both the transducer and sample holder are submerged in a water tank, allowing for controlled delivery of acoustic energy. Mukai *et al.* used a Falcon tube with cell aggregates, Harle *et al.* employed a standard plastic T75 culture flask, both of which may introduce acoustic discontinuities and partial reflections that can generate local interference patterns [13], [127]. In fact, Harle *et al.* report 14% transmission losses due to the culture flask and Mukai *et al.* reported 30% transmission losses due to the Falcon tube however, the pulsed ultrasound wave pressure at the cell interface was not directly assessed. Webster & Dyson constructed a custom sample holder using a 0.13 mm thick mylar film and directly assessed its impact on the acoustic field, showing no measurable evidence of acoustic distortion within the vessel at 3 MHz, indicating their setup effectively minimised artefacts and maintained field uniformity [79].

Although this method is more labour-intensive and requires bespoke protocols for sample handling, staining, and analysis, it provides superior control and reproducibility and has been used throughout this work. Building on this approach, the Biocell cassette used herein integrates similar principles, including acoustically transparent interfaces to minimise internal reflections and enable precise field characterisation while addressing additional practical and experimental constraints.

2.4 Chapter outline

This chapter begins with the characterisation of the acoustic output of the ultrasound transducer using a hydrophone and spatial field mapping to quantify pressure distribution and assess field uniformity. Next, it details the development and validation of the Biocell for acoustic and cellular compatibility. The setup for in vitro ultrasound exposure is then described, covering transducer configuration and impedance matching to optimise power transfer and ensure consistent acoustic output across experiments. Finally, the chapter outlines the cell culture and immunofluorescence protocols used to quantify actin expression, vinculin, cell area, and nuclear area alongside the statistical approaches applied.

2.5 Transducer calibration

2.5.1 Electrical impedance matching

The transducer used for the field measurements was a legacy model A302S (Serial No. 718360, Panametrics, UK) designed for immersion testing. The transducer features a flat, circular active element with a diameter of 1.0 in (25.4 mm) and a nominal central frequency of 1 MHz. According to the manufacturer's calibration certificate, the measured peak frequency is 0.97 MHz.

To optimise power transfer and reduce signal reflection and heating, a custom-built matching transformer was designed and placed between the power amplifier and the transducer. The design was based on the transducer's impedance at the operating frequency of 1 MHz. The impedance magnitude (Z_{load}) and phase angle ($^{\circ}$) were measured using an impedance analyser (HHP 4194A, HP) across 0.5-2 MHz, and the raw data was post-processed in MATLAB (v.R2023a). A 100-mV peak-to-peak (mV_{pp}) sine wave was applied, and the transducer was immersed in water at 37 °C to replicate the conditions used during cell exposure experiments. The resistance at 1 MHz measured at 236.64 Ω with a phase angle of -0.35° , indicating the presence of reactive components (Figure 7A). This deviates substantially from the desired 50 Ω , which is standard for RF signal sources (Z_{source}) and is critical for ensuring maximum power transfer to the transducer (Z_{load}).

A closed-core transformer was designed using a TN9/6/3 core in 3F3 material (Ferroxcube, UK), with its material properties summarised in Table 1. The primary and secondary windings were manually wound externally around the core using a 0.1 mm diameter polyurethane-insulated copper wire (Block Transformatoren, DE). This fine wire was selected to minimise bulk, allowing for tight, uniform windings without overall. To ensure good magnetic coupling and minimise leakage inductance, the turns were applied with consistent tension and even spacing. The complete transformer was terminated with a BNC connector for easy integration into the signal path.

The primary turns number (N_I) is given by Equation 6 below where V_{rms} is the root mean square voltage and f is the operating frequency of 1 MHz and B_{ac} is the peak flux density excursion.

Table 1. TN9/6/3-3F3 core ring properties.

Effective volume (V_e)	102 mm ³
Effective length (L_e)	22.9 mm
Effective area (A_e)	4.44 mm ²
Relative permeability (μ_r)	1800
Peak flux density excursion (B_{ac})	440 mT

$$N_1 = \frac{V_{rms}}{4.44fA_eB_{ac}} \quad \text{Equation 6}$$

$B(t)$ describes the instantaneous magnetic flux density within the transformer core as a function of time. B_{ac} refers to the peak magnetic flux density reached during each cycle of sinusoidal excitation. In this context, B_{ac} represents the maximum magnetic loading of the core under normal operating conditions. For a sine wave centred around 0, $B(t)$ is calculated using *Equation 7*.

$$B(t) = B_{ac}\sin(2\pi ft) \quad \text{Equation 7}$$

To minimise core losses, saturation, heating, and maintain waveform fidelity at high frequencies, the operating flux density (B_{ac}) was deliberately limited to 25 mT. Operating at low flux density minimises hysteresis and eddy-current losses in the 3F3 ferrite core while ensuring clean waveform transmission and consistent acoustic output.

To achieve this, the number of primary turns (N_1) was calculated based on a peak voltage of 15 V which corresponds to a V_{rms} of 10.6 V. Substituting into *Equation 8* yielded a required N_1 of 21.5 turns, which was rounded up to 22. To match the transducer electrical

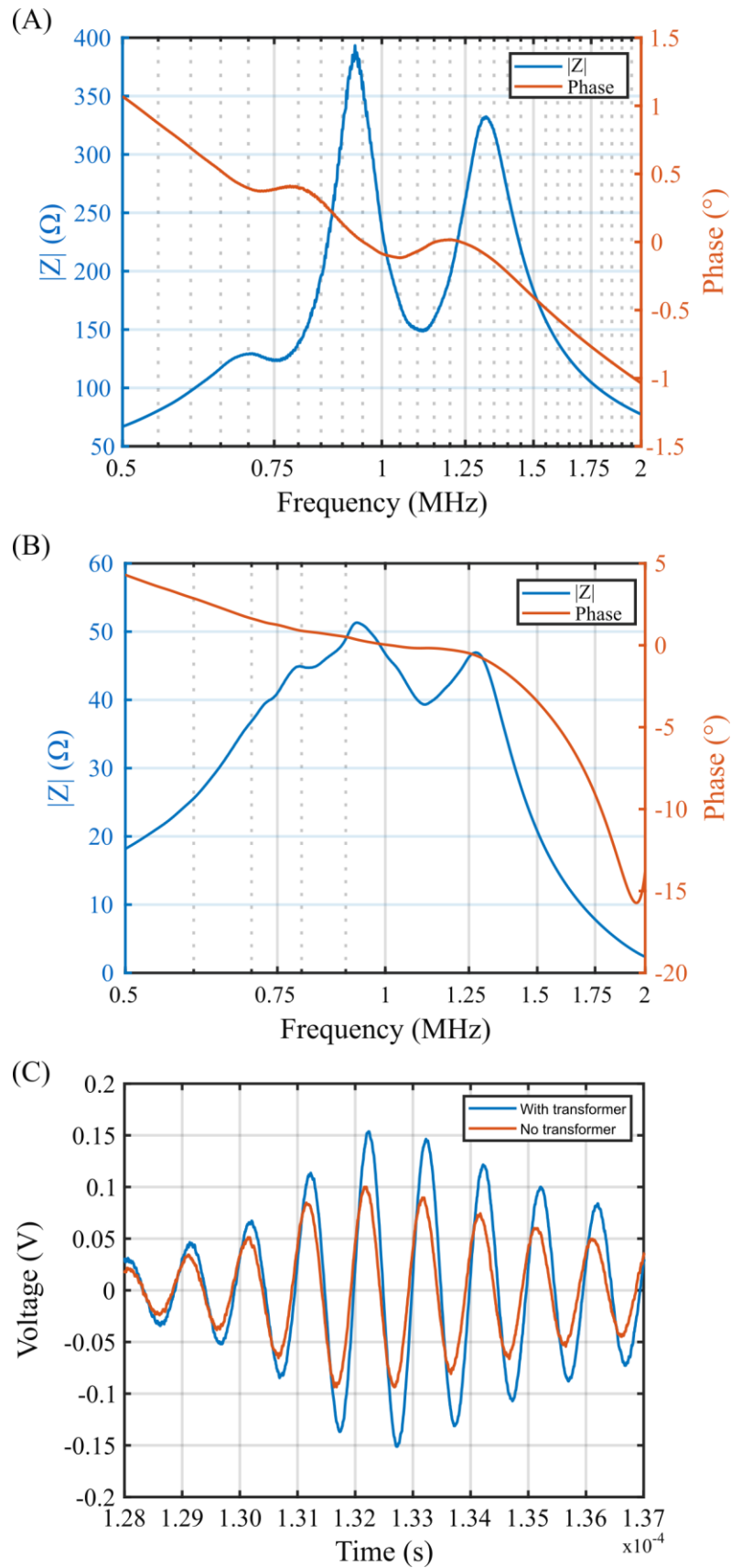


Figure 7. Impedance magnitude (Ω) and phase ($^\circ$) in water plotted against frequency on a logarithmic x-axis. (A) Without a matching transformer, the impedance and phase at 1 MHz are 236.64 Ω and -0.35° respectively. (B) With a matching transformer, the impedance dropped to 46.76 Ω , which is much closer to 50 Ω and the phase dropped to 0° improving power transfer. (C) Represents the sine wave output from the transducer with and without the matching transformer, demonstrating a 56.9 % increase in voltage amplitude following impedance matching. Waveforms were measured under identical conditions to highlight the effect of the transformer on power transfer efficiency.

impedance relationship in *Equation 8*, N_2 is the number of secondary turns.

$$\left(\frac{N_1}{N_2}\right)^2 = \frac{Z_{load}}{Z_{source}} \quad \text{Equation 8}$$

This yields 48 turns for N_2 .

Following the addition of the transformer to the circuitry, impedance measurements were repeated under otherwise identical conditions to verify the effectiveness of the matching. Figure 7B shows the measured impedance at 1 MHz which shifted to 46.76 Ω , which is significantly closer to 50 Ω , with a notable reduction in reactance and a phase angle of approximately 0°, indicating a nearly purely resistive load.

Transducer output was measured with and without the transformer under otherwise identical conditions at the focus in degassed water at 37 °C. As shown on Figure 7C, integrating the transformer resulted in a 56.9% increase in peak pressure under identical driving conditions, confirming improved power transfer through impedance matching.

2.5.2 Ultrasound field mapping and output calibration

The output of the transducer used throughout this thesis was characterised using a calibrated 0.5 mm needle hydrophone (Serial No. 2714, NH0500, Precision Acoustics, Dorchester, UK). The hydrophone was calibrated by the manufacturer (Precision Acoustics Ltd) using a substitution technique [137] with reference hydrophones independently calibrated at the National Physical Laboratory (NPL, Teddington, UK), providing direct metrological traceability to national standards.

The transducer and hydrophone were submerged in a water tank (60 x 90 x 60 cm) filled with degassed, deionised water at room temperature to identify the linear regime and map the spatial pressure field (Figure 8). The transducer was driven using a function generator (RSPRO RSDG 22082X; RS Components, UK) and RF power amplifier (75 W; A075, E&I, MCS Test, Denbighshire, UK) to produce sinusoidal bursts at 1 MHz.

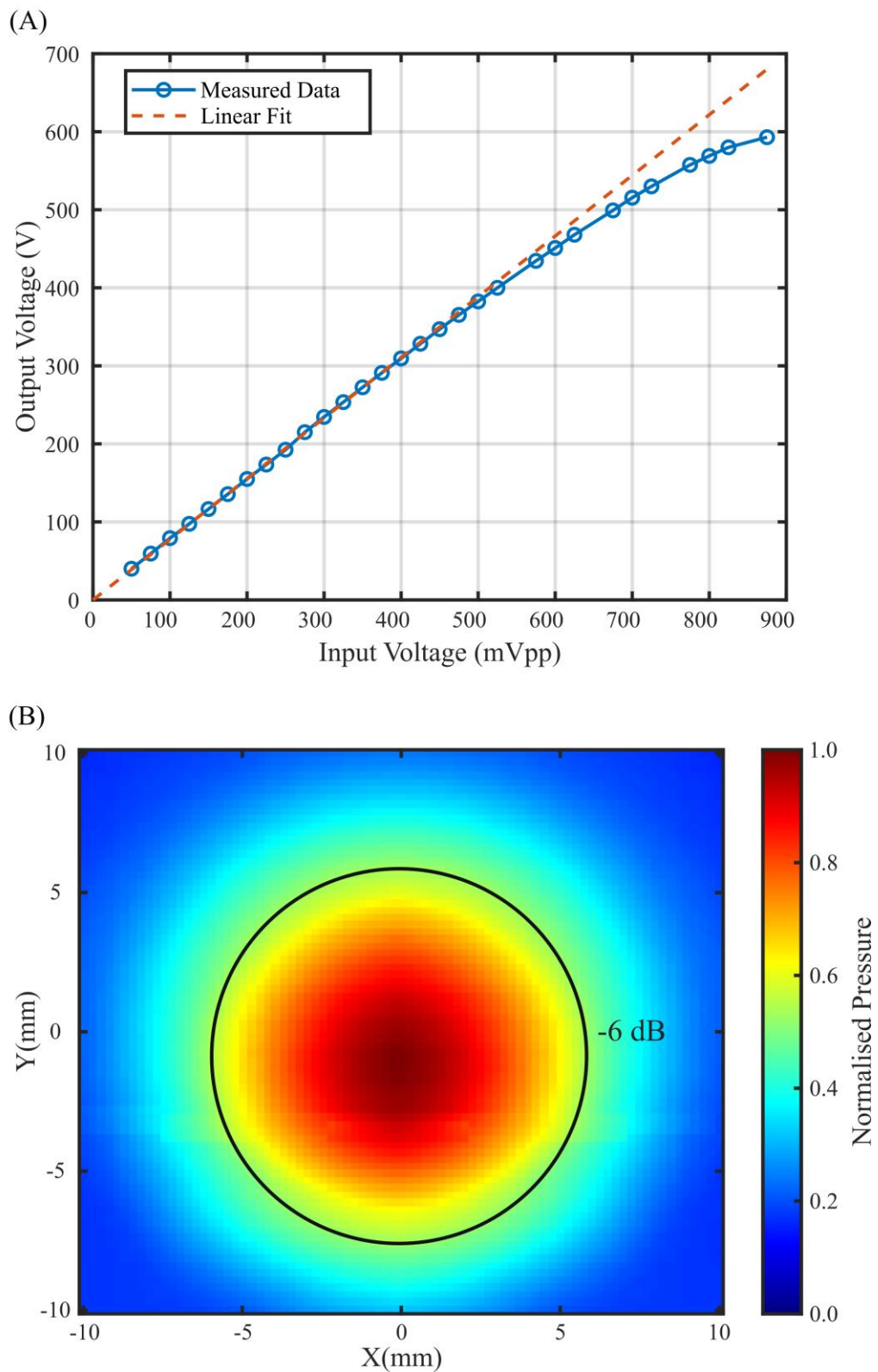


Figure 8. Characterisation of linear response range and spatial mapping of acoustic output. (A) Input-output response of the transducer. The measured data (blue solid line) show the transducer's voltage output as a function of input voltage. A linear fit (orange dotted line) was applied to the data to identify the linear region. (B) Normalised pressure field map of the transducer output mapped in the x-y plane (± 10 mm). *Figure continued on next page*

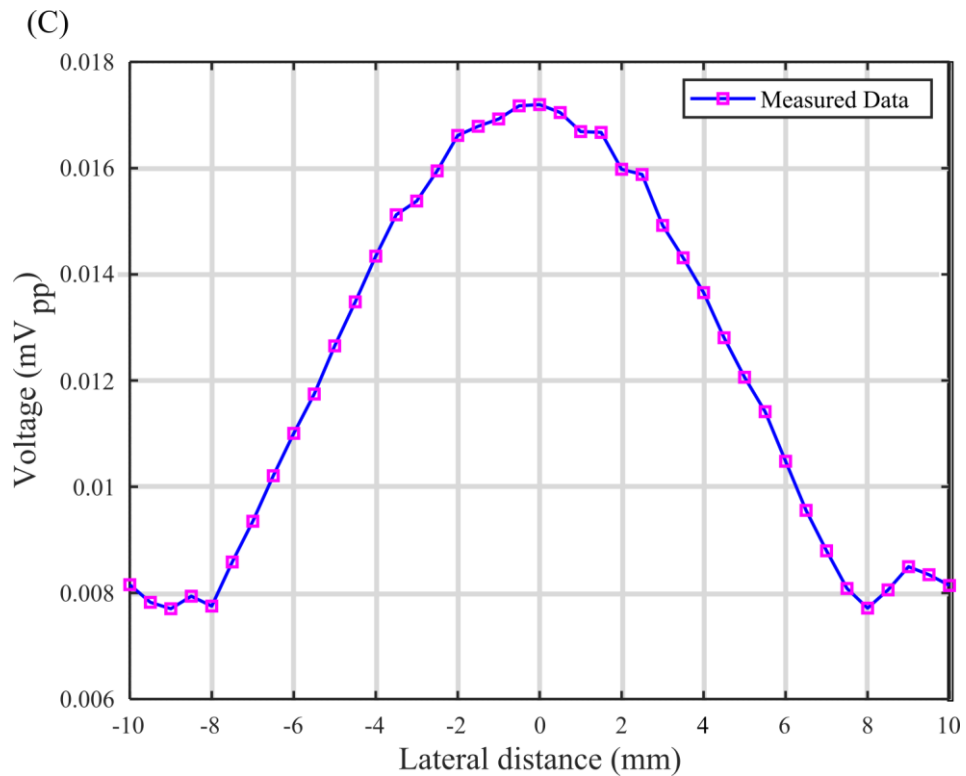


Figure 8. (Continued) (C) Peak-to-peak output voltage along the lateral axis ($Y = 0$ mm) spanning -10 mm to +10 mm. The profile shows a symmetrical main beam response peaking at the centre ($X = 0$ mm) and decaying smoothly to a background noise floor at the boundaries (± 10 mm).

To identify transducer linearity, a voltage sweep was conducted between 50 and 900 mV_{pp}, and the resulting output was measured using the hydrophone. A linear response was confirmed between 100 and 500 mV_{pp}, with linear regression fitted to the data (Figure 8A). Based on this analysis, a drive voltage of 300 mV_{pp} was selected for subsequent pressure mapping, ensuring operation well within the linear regime.

Field mapping was performed by scanning the hydrophone across a raster grid in the XY plane, centred 100 mm from the transducer front face. Prior to spatial mapping, axial and angular alignment between the transducer beam axis and the hydrophone scanning stages was established using a two-point beam alignment procedure. The transverse beam centre (XY-peak) was identified near the transducer face in the near field and again at the far-field. The transducer orientation was iteratively adjusted until the measured acoustic centres coincided across both axial distances, ensuring normal incidence and eliminating angular tilt relative to the scan axes.

Measurements were taken within ± 10 mm of the beam axis at 0.5 mm intervals. The transducer was driven with sinusoidal tone bursts of 10 cycles at 1 MHz. Hydrophone signals were recorded at a sampling rate of 800 MHz. The signal was passed through a pre-amplifier and DC coupler (Precision Acoustics, Dorchester, UK) before being transmitted to a PC via a GPIB interface. To improve signal fidelity and reduce noise, each measurement was averaged over 128 acquisitions. Data acquisition was synchronised with the transducer drive signal to ensure phase-locked measurements.

Hydrophone voltage waveforms recorded at each scan point were initially processed using a custom MATLAB script (version r2024A; MathWorks Inc., USA) originally developed by Dr Alex Muldovan and adapted for the purposes of this study. The complete, temporal voltage waveform was digitised and saved to preserve the full content of the acoustic signal, allowing post-hoc extraction of pressure parameters (including peak negative pressure and peak positive pressure) as well as the spatial coordinates. The script imported tab-delimited text files, removed DC offset, and calculated pressure over an integer number of steady-state cycles while deliberately excluding the initial transient ring-up and trailing ring-down phases. These voltages were converted to pressure using the hydrophone calibrated sensitivity of 297 mV/MPa, following the relationship:

$$p = \frac{V}{S} \quad \text{Equation 9}$$

Where S is the hydrophone sensitivity measured in V/Pa.

The processed pressure data was used to produce 2D colourmaps to illustrate the beam profile (Figure 8B). The -6 dB region of the normalised pressure amplitude was used to define the effective focal area of the field, corresponding to the FWHM region. Within this confined -6 dB region, the script further calculated I_{SATA} using *Equations 4 and 5*. Mapping the specific footprint directly guided the Biocell design, ensuring the cell seeding area aligned with the calibrated acoustic zone and to minimise interference of the Biocell ring with the main field. Additionally, a lateral line scan (Figure 8C) was extracted from the centre of the beam, confirming that the beam profile is symmetrical.

Field mapping was conducted at room temperature (~ 20 °C), rather than at physiological temperature (37 °C) used during *in vitro* experiments. This choice was primarily due to the

practical challenges of maintaining a large water volume of 320 L at a stable and elevated temperature, without introducing convection currents or thermal gradients from heater cycling and water circulation. It also ensured consistency with the standard calibration conditions of the needle hydrophone. An increase in temperature by 17 °C is known to raise the speed of sound in water from approximately 1482 to 1520 m/s, resulting in a predicted shift of about 3 mm in the final axial maximum of the planar transducer. This shift was considered during all future experiments, and it was noted that the Biocell remained in the far-field, within a 10 mm axial region where pressure distribution was approximately uniform.

2.6 The Biocell

The Biocell, (schematic shown on Figure 9) is a custom in-house culture vessel developed to enable consistent and well-characterised ultrasound delivery to cell cultures. It provides acoustic transparency, optical access for microscopy, and secure handling for transport between the incubator and exposure tank, while maintaining sterility. The initial concept design is based on the work of a previous PhD student in our group and has been adapted and optimised for this work as described below [138].

The frame was designed on SolidWorks 2022 (Dassault Systèmes, France) and fabricated using a stereolithography printer for its superior surface finish and dimensional precision. Preliminary tests using a fuse deposition printer produced coarse surfaces that hindered reliable membrane adhesion. The frame features an internal radius of 35 mm and 3 mm wall thickness, ensuring the cell-seeded area remains within the uniform pressure zone and does not interfere with the ultrasound field. Funnel-shaped ports on either side are sealed with medical-grade rubber septa (Z124559, Suba-Seal®, Sigma) to allow sterile access via syringe. Both openings were sealed with 6 µm thick mylar membranes (Goodfellow, UK) forming a fully enclosed chamber. The mylar membrane thickness was selected to ensure minimal acoustic impedance and attenuation at 1 MHz allowing efficient transmission of the ultrasound wave into the cell culture chamber.

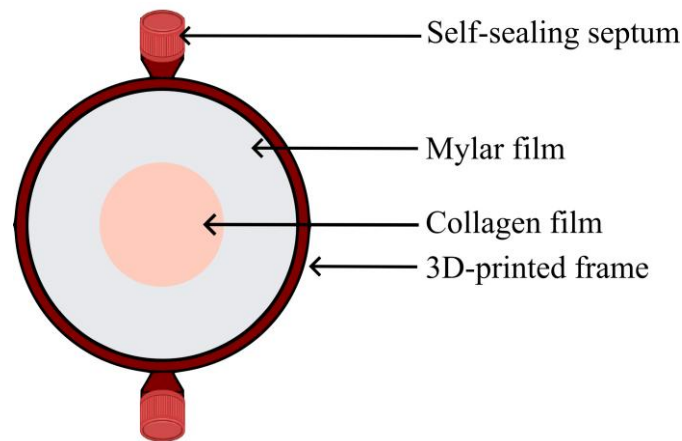


Figure 9. Biocell Schematic. Cross-sectional schematic of the custom Biocell showing the self-sealing septa, 6 μm thick mylar membrane, collagen film seeding region, and supporting frame with an internal diameter of 70 mm.

2.6.1 Fabrication using additive manufacturing

The frame was fabricated using the Prusa SL1S resin printer (Prusa Research, Czech Republic). Models were exported as STL files and sliced in PrusaSlicer (v2.6.1) using a 0.1 mm layer height and default settings for the selected Prusa resins, selected for smoothness and mechanical strength [139]. The printed parts were washed in isopropyl alcohol to remove residual, uncured resin, and UV-cured for an additional hour in the CW1S unit (Prusa Research, Czech Republic) to ensure complete polymerisation and surface stability. After curing, parts were rinsed with isopropyl alcohol, air dried, and stored in containers until use.

2.6.2 Cytotoxicity assessment of candidate materials for Biocell fabrication

To evaluate the biocompatibility of the 3D-printed Biocell assembly, a direct contact cytotoxicity assay was performed in accordance with ISO 10993-5 guidelines [140]. Test discs (10 mm diameter) were printed using Prusament transparent resins (Green, Yellow, and Blue) (Prusa Research, Czech Republic) under identical settings, along with samples of the silicone sealant. Post-processing followed the same the specimens were placed directly onto confluent MG-63 cells cultured in 6-well plates at 2×10^5 cells/well and incubated at 37 °C with 5% CO₂ for 24 hours. After incubation, discs were removed, and

cell viability was assessed using Alamar Blue reduction assay (resazurin-based, Thermo Fisher Scientific) following the manufacturer's protocol.

After incubation, the discs were removed from the well plates, and the media was replaced with medium containing 10% (v/v) Alamar Blue reagent. Control wells containing 10% Alamar blue (AB) in complete medium were included to account for background signal from the reagent. Cells were incubated for 2 hours at 37 °C, 5% CO₂ protected from light. Following incubation, 100 µL of supernatant was transferred to a 96-well plate, and absorbance was measured at 570 nm and 600 nm using a microplate reader (Multiskan, Thermo Fisher Scientific) for reduced and oxidised Alamar Blue, respectively. *Equation 10* was used to calculate the percentage reduction of Alamar Blue.

$$\% AB_{reduction} = \frac{(\epsilon_{oxidised}^{600} \cdot A^{570}) - (\epsilon_{oxidised}^{570} \cdot A^{600})}{(\epsilon_{reduced}^{570} \cdot A_{control}^{600}) - (\epsilon_{reduced}^{600} \cdot A_{control}^{570})} \times 100 \quad \text{Equation 10}$$

Where ϵ is the molar extinction coefficient of oxidised Alamar Blue at 570 nm ($\epsilon_{oxidised}^{570}$) equal to 80586 and at 600 nm ($\epsilon_{oxidised}^{600}$) is equal to 117216. A^{570} and A^{600} are the absorbance of the samples at 570 and 600 nm, respectively. $\epsilon_{reduced}^{570}$ of Alamar Blue at 570 nm is equal to 155677, and at 600 nm $\epsilon_{reduced}^{600}$ is equal to 14652, $A_{control}^{570}$ is the absorbance of the control sample at 570 nm, $A_{control}^{600}$ is the absorbance of the control sample at 600 nm.

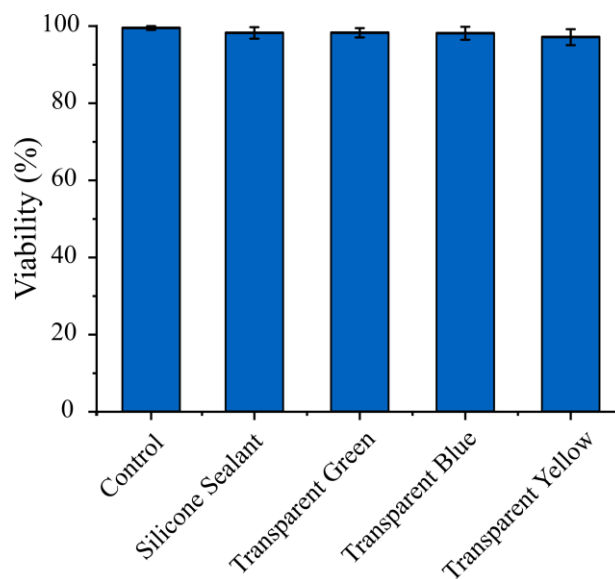


Figure 10. Cytotoxicity assessment of Prusa resins and silicone sealant using Alamar Blue. No significant cytotoxic effects were observed, indicating that the materials are compatible with cell culture conditions.

All three resins of choice as well as the air-dried silicone sealant, supported cell viability well above 70% (Figure 10) which is the ISO 10993-5 threshold for non-toxicity [140]. For subsequent experiments the Prusament Blue or Green resins were used as they appeared to maintain structural integrity better under repeated handling compared to other tested resins.

2.6.3 The Biocell assembly

The Biocell assembly summarised on Figure 11 was performed within a Class II biosafety cabinet under aseptic conditions over a 4-day protocol to ensure consistent curing and sterility. On Day 1, all components, including 3D-printed frames, 10 x 10 cm mylar membrane squares, rubber septa, elastic bands, mylar stretcher rings, and UV-resistant silicone sealant (translucent HA6, Bond It, UK) were washed with soapy water and then submerged in 70% ethanol bath for 30 minutes before being placed into the biosafety cabinet. The parts were then exposed to UV light for 30 minutes and submerged in 70% ethanol overnight to ensure thorough chemical sterilisation (Figure 11A).

The next day, parts were removed from the ethanol bath and left to air-dry. The first mylar membrane square was stretched with a stretcher and secured with an elastic band and adhered to one side of the frame using silicone sealant (Figure 11B). The assembly was then exposed to UV light for 30 minutes and left to dry overnight.

On Day 3, the inner surface of the assembled Biocell (with one mylar in place) was rinsed three times with sterile Dulbecco's Phosphate Buffered Saline (D8537, Sigma) to remove residual ethanol and prepare the membrane surface for further treatment. This step also served as a leak test where Biocells showing signs of leakage were identified, repaired and then set aside to cure for another day. In the meantime, the collagen solution was prepared by diluting Type I collagen (Corning, USA) in sterile DPBS to achieve a final collagen film density of 10 $\mu\text{g}/\text{cm}^2$. The collagen stock was gradually added to the buffer by gently pipetting. Custom-designed positioning rings were placed onto the inner mylar surface to define a confined coating region (Figure 11C). A measured volume of collagen solution was then carefully pipetted within the ring boundary. Biocells were left to air dry overnight within a biosafety cabinet.

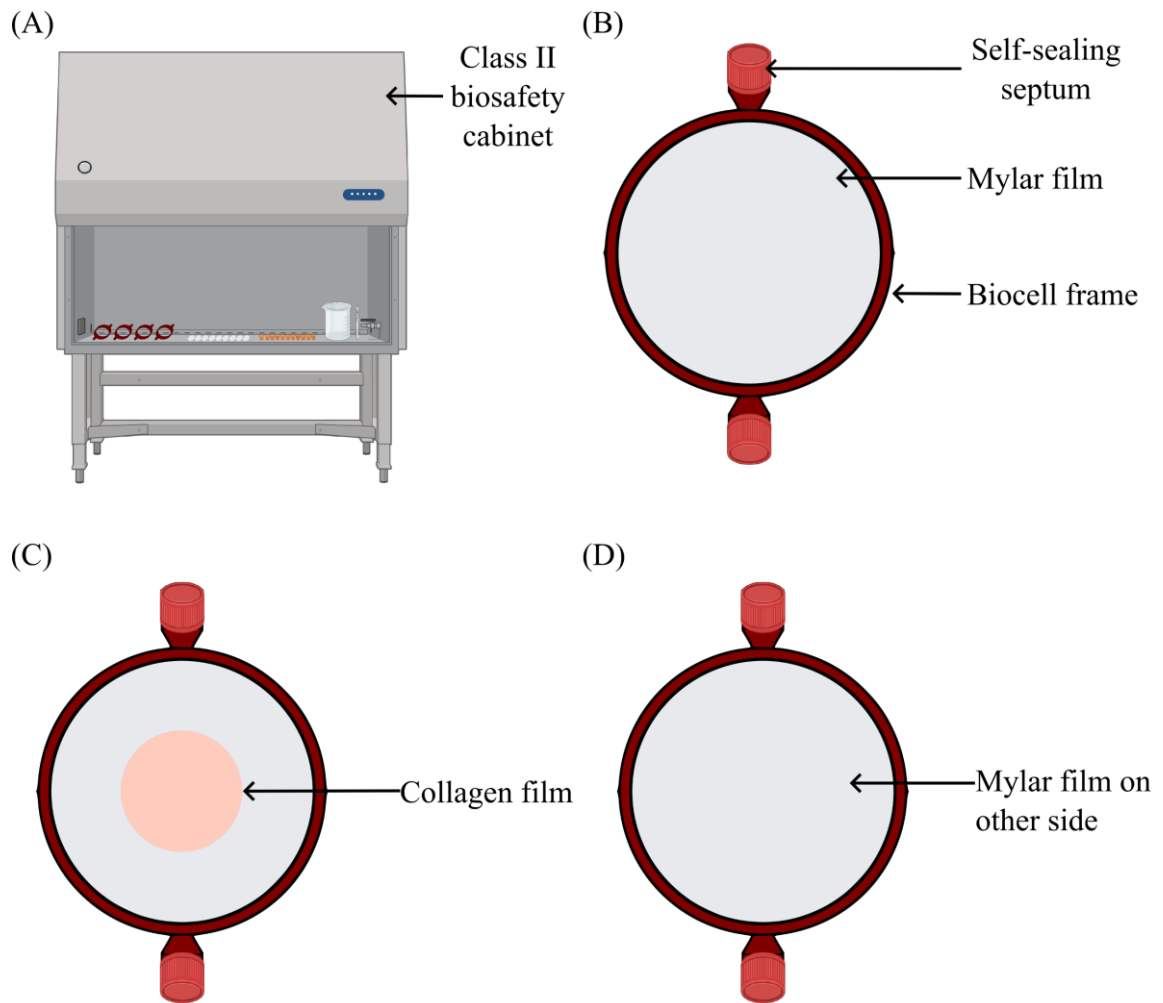


Figure 11. Schematic representation of the Biocell assembly protocol under sterile conditions. (A) All components, including the Biocell frame, mylar membranes, self-sealing septa, are submerged in 70% ethanol inside a Class II biosafety cabinet. (B) On the following day a 6 μm thick mylar film is adhered to one side of the frame to form the base layer and left overnight to dry. (C) On day 3 a thin collagen gel film is prepared and applied onto the mylar surface and left overnight to dry and finally, (D) on day 4 the second mylar membrane is placed on top to enclose the Biocell and complete the assembly.

The sizing of the rings was directly informed by the acoustic hydrophone mapping (Section 2.5.2). A 20 mm diameter ring was selected to align with the -6 dB region of normalised pressure amplitude (FWHM), ensuring cells attached strictly within a bounded acoustic zone. Confining the cell-seeded area prevented steep pressure drops at the beam edge from introducing confounding variables into the biological response data. While a tighter -3 dB threshold would offer greater pressure uniformity, scaling the ring down to a smaller size introduced fabrication challenges. Specifically, carefully pipetting microliter volumes of collagen solution into a smaller footprint was difficult to manage consistently by hand without causing surface spillover. The 10 mm ring provided the optimal trade-off between pressure control and manual fabrication.

On Day 4, the positioning rings were carefully removed using sterile tweezers, and the outer perimeter of the collagen-coated area was marked on the outside of the mylar membrane using a fine-tip permanent marker to ensure consistent identification of the coated region and facilitate imaging later. Care was taken to avoid contact with the coated surface or internal chamber during marking. A second mylar membrane was adhered to the opposite side of the frame, sealing the chamber. To complete the enclosure, sealing septa were press-fitted into both funnel openings (Figure 11D). The fully assembled Biocells were left to dry overnight under before being stored in a sterile container until required for an experiment.

2.6.4 Biocell seeding protocol

Before cell seeding, the inner chamber of each Biocell was rinsed three times with 15 mL of DPBS to remove any residual ethanol and rehydrate the collagen coated surface. This was performed using 20 mL sterile syringes (15889152, Fisher Scientific) fitted with sterile needles (18G, 15321537, Terumo, Fisher Scientific) inserted in both sides of the funnels. One syringe was used to gently inject the buffer, and the other to eject air, preventing internal pressure build-up. An additional rinse was performed with 15 mL of non-supplemented DMEM to neutralise any residuals (Figure 12A). Any Biocells that were damaged during the process, were carefully discarded to avoid contamination.

Cells were harvested at 80-90% confluency using tryp-LE (10718463, Gibco), resuspended in supplemented DMEM and counted using a hemocytometer. Following cell counting, the MG-63 suspension was aliquoted into sterile centrifuge tubes using calibrated pipettes to ensure accurate dosing across all samples. Each aliquot was then diluted with complete DMEM that had been placed in the incubator to absorb gases for 1 hour, to a final volume of 20 mL, corresponding to the full capacity of the Biocell. The resulting suspension was gently injected into the Biocell using 20 mL sterile syringes while gradually ejecting air from the other septum to ensure smooth filling of the Biocell without introducing bubbles or disturbing the collagen-coated surface (Figure 12B). Immediately after seeding at a density of 10 000 cells/cm², each Biocell was placed in the incubator overnight to allow enough time for cell attachment (Figure 12C). Biocells were always maintained at a horizontal position except during the *in vitro* ultrasound exposure.

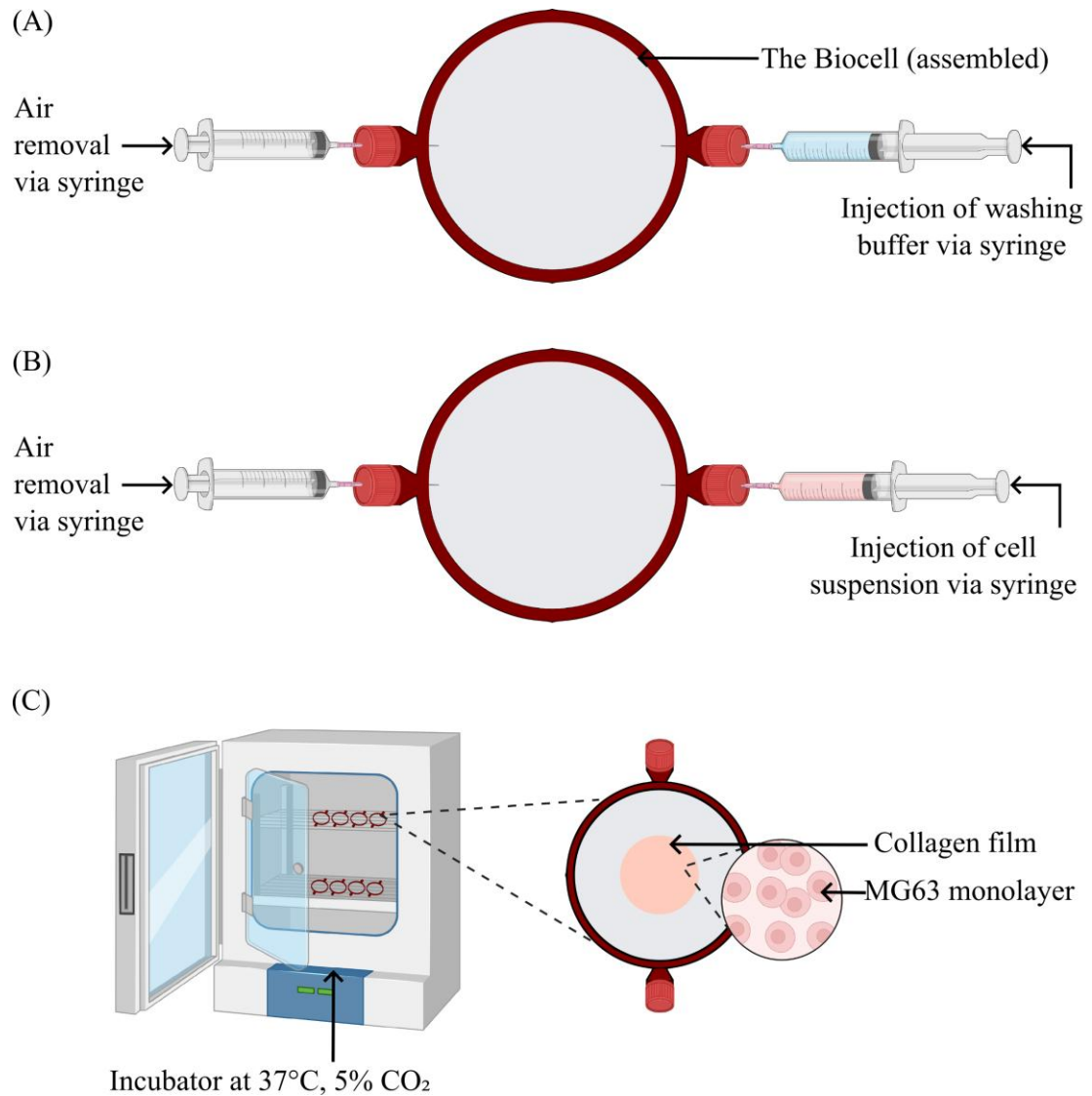


Figure 12. Schematic representation of the Biocell seeding protocol. (A) Air is removed through one septum using a manual syringe fitted with a needle while simultaneously injecting DPBS or non-supplemented media from the opposite septum to wash and neutralised the vessel. (B) cell suspension is introduced in the chamber. (C) Seeded Biocells are placed in the incubator for 24 hours.

2.6.5 Mechanical and acoustic considerations of the Biocell

Given the use of a thin, tensioned mylar membrane, the potential excitation of membrane resonance modes was also considered. For a circular membrane rigidly fixed at its perimeter, the fundamental radial mode of vibration, f_0 , can be approximated using *Equation 11*.

$$f_0 = \frac{c}{2d} \quad \text{Equation 11}$$

Where d is the membrane diameter, and c is the speed of sound of the mylar membrane, which is approximately 2540 m/s [141]. This yields a fundamental frequency of 18.1 kHz. Higher-order modes are expected at integer multiples of the fundamental frequency.

The ultrasound excitation used in this work was centred at a carrier frequency of 1 MHz, which is more than 50 times greater than the fundamental membrane frequency. In addition, the PRFs employed (100, 1000, 10000, 100000 Hz) do not directly coincide with the calculated radial modes. It is therefore unlikely that the applied ultrasound conditions excited membrane resonance, and any measured membrane motion is assumed to reflect the acoustic field rather than intrinsic modal behaviour of the mylar membrane.

The influence of the 6 μm mylar membrane on ultrasound transmission at the operating frequency (1 MHz) was considered minimal. The membrane thickness is substantially smaller than the acoustic wavelength in water at 1 MHz (1.5 mm), meaning that the membrane is expected to be highly acoustically transparent. Previous analysis of the Biocell membrane configuration demonstrated minimal reflection and attenuation of the incident ultrasound field, indicating that the majority of the acoustic energy is transmitted through the membrane [142].

2.6.6 Experimental verification of acoustic transparency of the Biocell

To verify that the Biocell did not alter the applied ultrasound field, hydrophone measurements were taken with the Biocell in place and repeated following its removal. Measurements were obtained using the same experimental setup as in the ultrasound *in vitro* exposure (Chapter 3, Section 3.4.1) with the hydrophone positioned 2 cm downstream of the Biocell. Ultrasound pressure was recorded under identical driving conditions (Figure 13).

Representative lateral ultrasound pressure distribution curves for the two conditions are presented in Figure 13A-B and show complete overlap across the full lateral range. Minor lateral asymmetry observed in the line profiles is attributed to small angular misalignments between the transducer face and the hydrophone scanning plane. Unlike the scanning tank characterisations (Section 2.5.2, Figure 8C) where two-point axial alignment was used to

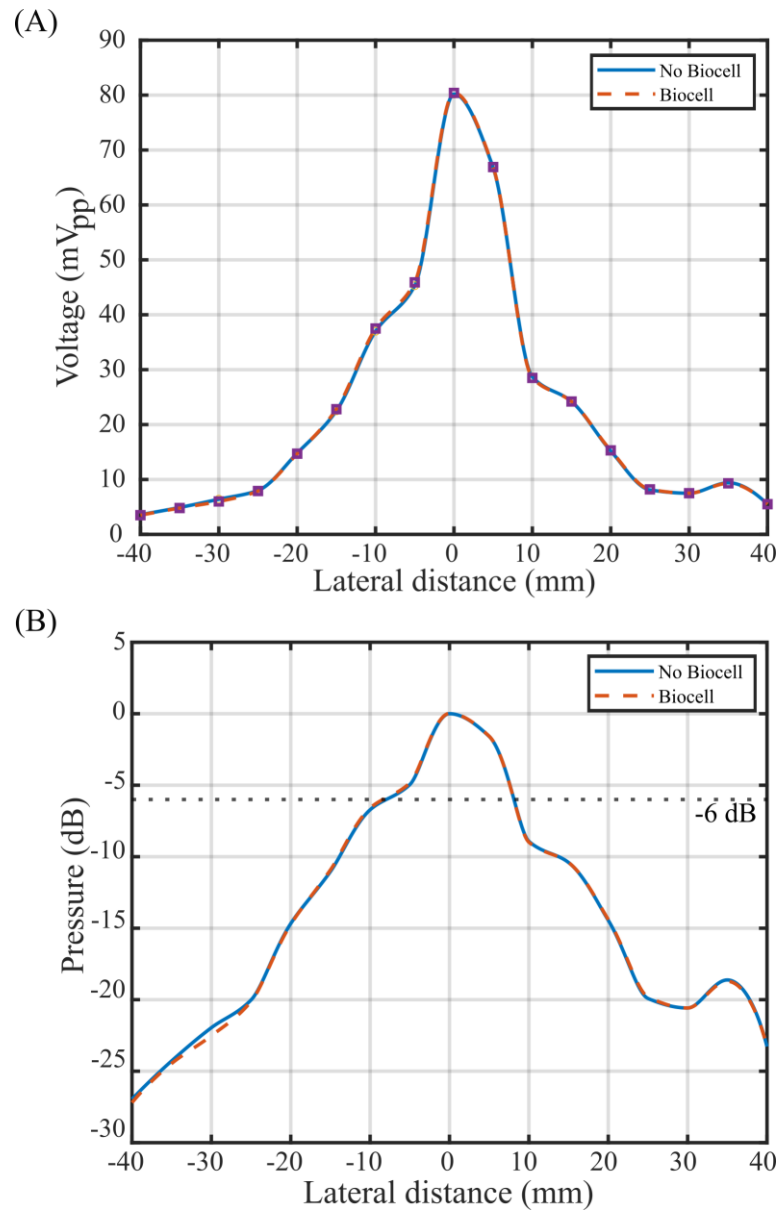


Figure 13. Assessment of acoustic field alteration by the Biocell at 1 MHz. Lateral hydrophone line scales acquired at 1 MHz with (dashed) and without (solid) the Biocell in place shown as (A) peak-to-peak voltage (mV_{pp}) and (B) normalised pressure (dB) relative to peak value. The overlapping profiles indicate that the Biocell does not measurably alter the acoustic field. The dashed -6 dB level corresponds to the defined collagen coated cell-seeding region within the Biocell.

eliminate tilt, the dimensional constraints of the smaller tank used here prevented full far-field alignment, resulting in a minor tilt angle relative to the scan axes.

These results confirm that at 1 MHz, the Biocell vessel is indeed acoustically transparent, consistent with the predicted reflection coefficient of $|R| = 0.01$ from [142]. Therefore, the presence of the membrane does not perturb the ultrasound pressure field delivered to the cells and does not require correction factors during dosimetry.

However, although the membrane does not influence the pressure field, it does not remain stationary when exposed to ultrasound. The displacement of the mylar substrate is governed by the incident pressure, the tension imparted during fabrication, and the boundary conditions of the assembly. These factors cannot be inferred from pressure measurements alone. Therefore, LDV was required to directly quantify the membrane vibration under the stimulation conditions used in this study.

2.6.7 Vibrational analysis of Biocell mylar membrane across batches

Since the Biocells are fabricated in-house, a validation protocol was implemented to assess potential batch-to-batch variability in membrane vibrations. Specifically, to determine whether cells under identical ultrasound parameters would be exposed to comparable mechanical vibration. To evaluate this, the vibrational response of the mylar membrane that the cells were seeded onto, was measured across eight independently fabricated Biocells using a scanning laser Doppler vibrometer (Polytec PSV 500 1D, Polytec, GmbH, Germany). This method was selected for its ability to perform precise, non-contact measurements with sub-nanometre sensitivity in water, providing a reliable means of quantifying the vibrational characteristics of an ultrasound-driven membrane.

LDV measurements were performed using the same experimental configuration employed for the *in vitro* ultrasound stimulation studies, with the only modification being the use of a larger, glass water tank to allow the unobstructed passage of the laser beam. Because the LDV beam must pass through the front wall of the tank, the absorber could not be placed on this surface. To minimise reflections, absorbers were positioned to the remaining tank walls, and the transducer was positioned at the rear of the tank, maximising the propagation distance and reducing the contribution of reflected waves to the measurement region.

The LDV system was equipped with a close-up unit (Polytec, GmbH, Germany) aligned normally to the mylar membrane. The close-up unit reduced the working distance and improved laser spot stability through the water path. A thin reflective layer was applied to the mylar surface using a white marker pen to maximise laser return and improve signal-to-

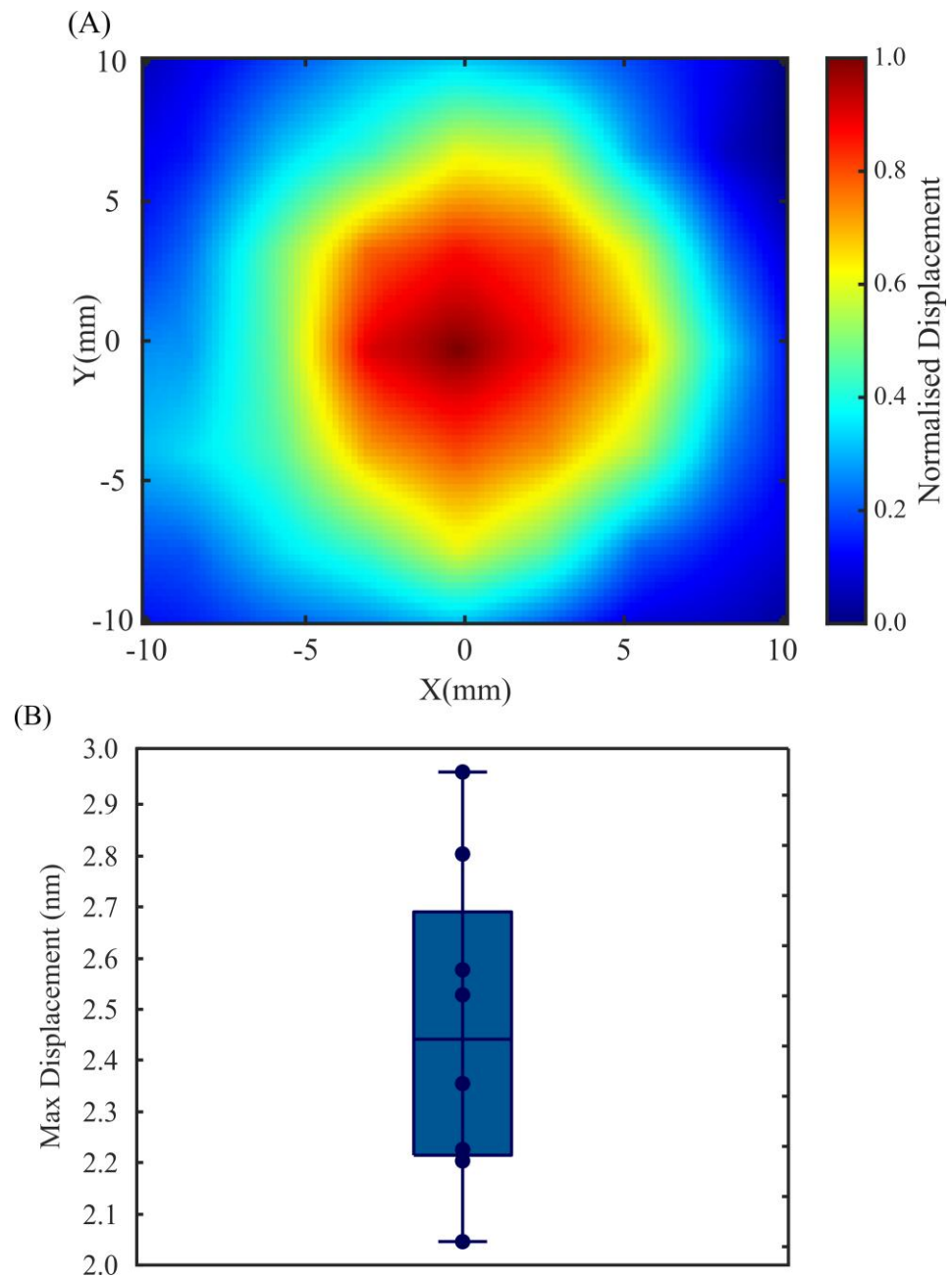


Figure 14. Characterisation of mylar membrane displacement across Biocell samples. (A) Normalised displacement amplitude across the mylar membrane measured using LDV, (B) Maximum displacement amplitude (nm) across eight different Biocell samples with an average displacement of 2.44 nm and a variation of 13.2 % across the samples.

noise ratio. Before scanning, each Biocell was filled with culture medium and all air bubbles removed.

Proprietary PSV Scanning Vibrometry Software (Version 10.2) was used for acquisition and processing. Scans were performed over approximately 20 x 20 mm region centred on the cell seeding area of the membrane corresponding to ± 10 mm from the geometric centre of the Biocell. Approximately 450 measurement points were used per scan. At each point,

the normal-to-surface component of vibration was recorded. The transducer was driven at 1 MHz carrier frequency, I_{SAT} of 60 mW/cm². The resulting velocity signals were subsequently processed in the PSV software to extract the displacement at the carrier frequency using the standard LDV velocity-to-displacement relation (*Equations 12 and 13*).

$$v = A \cos(2\pi ft) \quad \text{Equation 12}$$

$$s = \int v \, dt = \frac{A}{2\pi f} \sin(2\pi ft) \quad \text{Equation 13}$$

Where A is the velocity amplitude.

As seen in Figure 14A, the highest displacement aligns with the centre of the membrane, and gradually decreases towards the periphery, consistent with the lateral pressure distribution profile measured using the hydrophone (Figure 8A). This confirms that the membrane vibrates in a manner that reflects the applied acoustic field, and that, LDV measurements capture the expected spatial pattern of displacement.

Comparing the free-field hydrophone scan (Figure 8B) to the LDV membrane displacement maps (Figure 14A) reveals the structural impact of the Biocell vessel and the fixed boundaries of the mylar membrane. While the free-field scan measures the uninterrupted pressure beam in water, the LDV profile reflects the clamped mylar under exposure to ultrasound, where edge constraints restrict movement and alter the spatial decay of the field. Furthermore, minor asymmetries were revealed in the LDV data when compared to the hydrophone measurements, which may stem from adhesive thickness inconsistencies from in-house fabrication.

Figure 14B shows the maximum displacement measured at the centre point at 1 MHz across eight Biocell samples under identical ultrasound conditions. The values fall within a narrow range with a mean of 2.44 nm and a variability of 13.2%. These results validate the use of the Biocell cassette as a reliable platform to perform these experiments.

2.7 *In vitro* ultrasound exposure

Ultrasound stimulation was performed using a custom *in vitro* setup designed to ensure controlled and reproducible acoustic delivery to the Biocell samples. For all cell experiments, a tank with dimensions 310L x 440W x 280D mm (Really Useful Boxes, UK) was filled with degassed water maintained at 37 ± 1 °C to mimic physiological conditions. While full 2D spatial acoustic field mapping was conducted at room temperature, the location of peak acoustic pressure was re-identified and aligned at physiological temperature prior to cell exposure experiments to account for temperature-dependent shifts (~ 2 mm) in sound speed. A schematic diagram of the experimental setup is shown in Figure 15.

A 1 MHz transducer (A302S, Panametrics, UK) was securely mounted on a translation stage equipped with a manual micromanipulator to ensure precise spatial alignment and consistent acoustic coupling between the transducer and the Biocell assembly. To guarantee reproducible positioning across trials, a custom Biocell holder was designed in SolidWorks 2022 and securely mounted onto an optical breadboard (Thorlabs Inc., UK). The focal zone was then located *in situ* using the hydrophone (NH500, Precision Acoustics) setup described earlier, establishing central coaxial alignment between the Biocell and the transducer. Acoustic absorbers (Aptflex F28, Precision Acoustics Ltd., UK) were positioned around the tank interior, with the rear absorber placed at an angle to

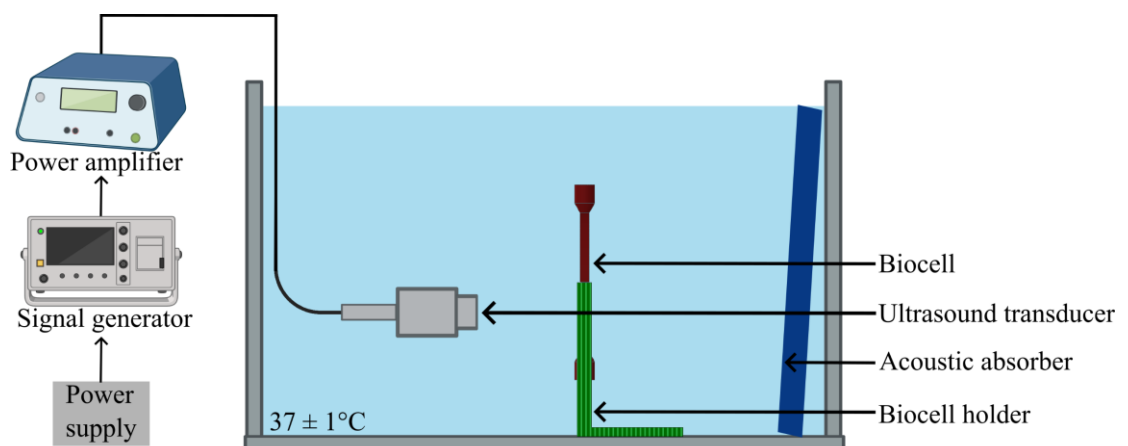


Figure 15. Schematic representation of the *in vitro* experimental setup. The Biocell containing MG63s was positioned within a temperature-controlled water tank maintained at $37 \pm 1^\circ\text{C}$. An acoustic absorber was placed in the tank at an angle to minimise acoustic reflections and deflect any remaining waves away from the test Biocell. Custom holders were used to secure both the Biocell and the transducer (not shown here for clarity) in fixed positions for repeatability.

minimise standing waves and prevent back-reflections into the sample volume. The transducer was driven by a signal generator and connected to a power amplifier via a custom-built matching circuit with the signal generator programmed to output pulsed wave signals.

To ensure thermal stability throughout ultrasound exposure, water temperature was continuously monitored using a dual-channel thermocouple (Amprobe, USA). The probes were positioned at the transducer end and another one at the absorber end of the tank to detect any spatial temperature variations during exposure. Before each experiment, the system was allowed to reach thermal equilibrium at 37 ± 1 °C. The tank was partially refilled with prewarmed, degassed water between exposures. Experiments were only accepted if the temperature remained stable at both monitoring points for the full duration of the stimulation. Additionally, to evaluate potential acoustic heating within the sample itself, internal Biocell temperatures were recorded immediately prior to exposure and at 5-minute intervals throughout treatment (Appendix B).

To isolate the biological effects of ultrasound from environmental and handling variables, sham control Biocells were subjected to identical mounting, temperature and duration within the tank, but the acoustic signal was turned off.

2.8 Cell culture

MG63 osteoblast-like cells (kindly provided by Dr. Peter Childs, University of Strathclyde) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), 1% (v/v) minimum essential medium non-essential amino acids (MEM NEAA), and 2% (v/v) penicillin/streptomycin (Pen Strep, Sigma). Cells were cultured and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cells were dissociated with Tryp-LE Express (Sigma) when 70-80% confluent or the media was replaced every two days. For experiments, cells were seeded at a density of 10×10^3 cells/cm² and were discarded at passage 30.

2.9 Immunofluorescence staining

Immunofluorescence staining (Figure 16) was performed on fixed cells while the cells were still within the Biocells. Immediately after exposure to ultrasound, the media was removed from each sample using 20 mL syringes, and 10 mL of 4% Formalin solution (Avantor, UK) was injected and left in the Biocells at room temperature for 15 minutes. The fixative was then removed, and cells were thoroughly rinsed three times with DPBS, allowing 5 minutes between washes. Cells were permeabilised using 0.1% Triton X-100 in PBS for 10 minutes initially. This produced low signal-to-noise ratio (SNR) images (Figure

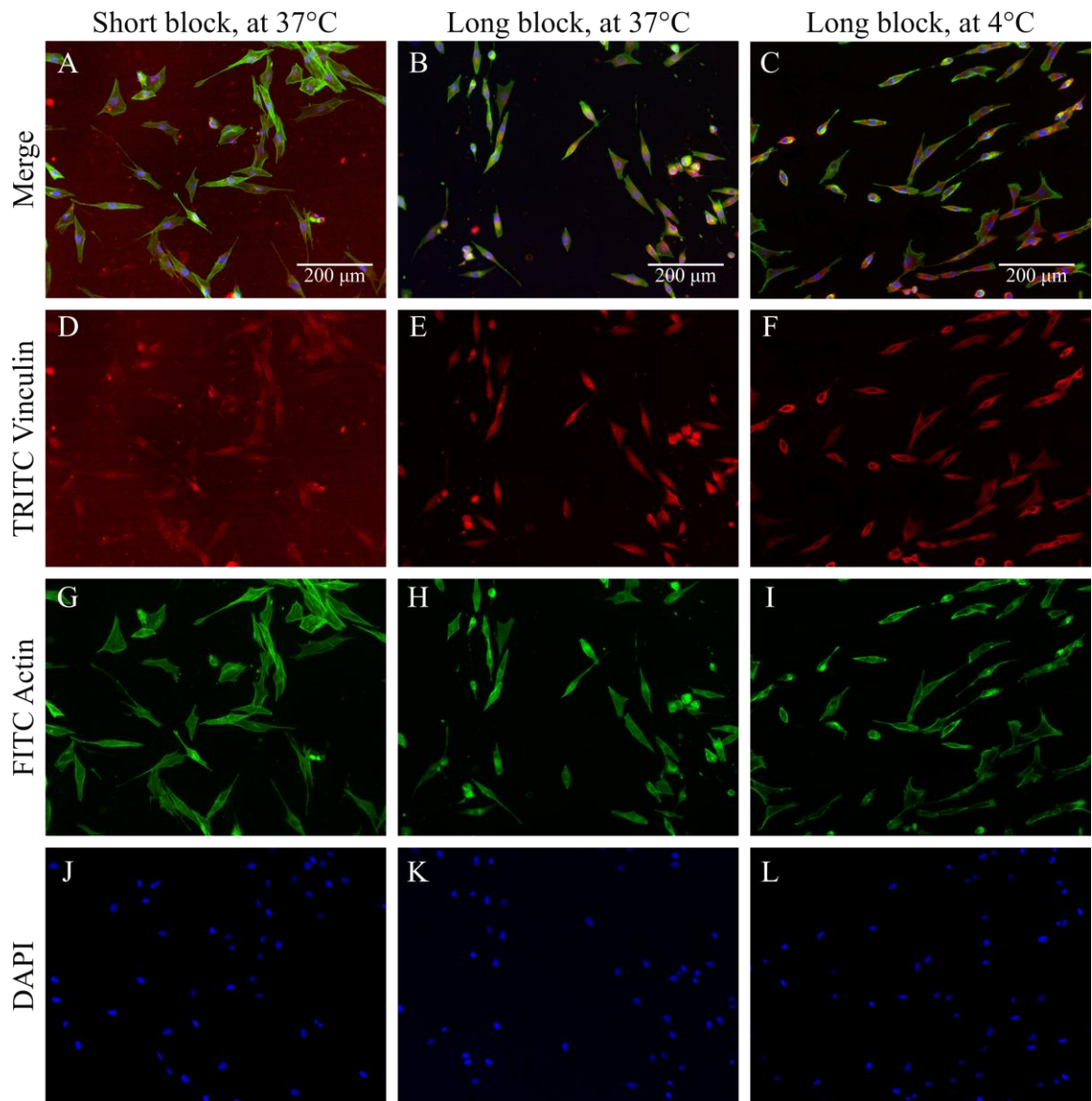


Figure 16. Immunofluorescence images showing the progression of protocol optimisation on vinculin TRITC (D-F), actin FITC (G-I) and DAPI (J-L) staining.

16D) with a diffuse red background and poor visibility of vinculin structures. To improve antibody specificity and imaging the incubation time was increased to 30 minutes.

Following this, the permeabilisation buffer was replaced with a blocking solution made of 10mg/mL bovine serum albumin (BSA) in PBS to block non-specific antibody binding. Blocking was performed for 1 hour at room temperature in early experiments; however, fluorescent aggregates were observed outside cells (Figure 16E), likely attributed to aggregated antibody molecules or non-specific binding. To improve image quality, blocking was extended to overnight incubation at 4 °C (Figure 16F).

The next day, the top mylar membrane was carefully removed using a scalpel under a fume hood for easier access to the cell monolayer during the subsequent antibody staining steps. Vinculin recombinant rabbit monoclonal antibody (REF700062, Thermo Fisher Scientific, UK) was diluted 1:100 in blocking solution, and 1 mL of the antibody solution was added to each sample overnight at 4 °C. The primary antibody was then removed, and samples were washed three times for 5 minutes each with 0.5% Tween-20 (Sigma) in PBS at room temperature. 1 mL of goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody (Thermo Fisher Scientific, Paisley, UK) diluted in blocking solution was added to cells at a concentration of 1:100 and cells were incubated at 4°C for 2 hours. The secondary antibody was then removed, and cells were again washed three times 0.5% Tween-20 in PBS at room temperature.

FITC phalloidin (ab23517, Abcam, UK) was diluted at a concentration of 1:100 in blocking solution. 1 mL of the diluted stain was added to each sample for 30 minutes at room temperature. After the incubation, cells were washed three times with PBS before adding 200 µL NucBlue Live ReadyProbes nuclear stain (Thermo Fisher Scientific, Paisley, UK). Samples were then transferred onto glass-bottom slides (MIC1008, Scientific Laboratory Supplies Ltd, Nottingham, UK) covered with coverslips (Academy, Scientific Laboratory Supplies Ltd, Nottingham, UK), sealed with nail varnish to protect from drying and wrapped in foil to protect from light and stored at 4 °C until imaging.

2.10 Microscopy

Fluorescence imaging was performed using Olympus IX73 (Olympus, UK) brightfield microscope at 10X magnification. The imaging parameters including excitation intensity

and exposure time were determined prior to acquisition by briefly inspecting all samples. Saturation was monitored by enabling the saturation indicator in Olympus CellSens software, which highlights saturated pixels in red during live imaging. This was done to identify settings that produced a clear signal without saturation and that the images are acquired within the dynamic range of the camera for a clear signal allowing for accurate comparisons of the intensities across samples. For each condition tested, three samples were prepared, and ten non-overlapping images were acquired (unless otherwise stated) from each sample to account for technical variability and ensure representative analysis of the cell population. All fluorescence images were acquired on the same day to ensure signal consistency and minimise variability due to potential stain degradation, enabling reliable quantitative comparison.

2.11 Image analysis

To measure nuclear area, images were processed in MATLAB (R2023A) using a custom script (Appendix C). Raw DAPI images were first converted to 8-bit greyscale (Figure 17A), the background signal was removed, and a threshold was applied to segment nuclei (Figure 17B). Following segmentation, nuclear regions were identified, and objects not meeting a circularity threshold (>0.2) were excluded to remove debris, overlapping cells, or segmentation artefacts. The binary masks were refined through morphological

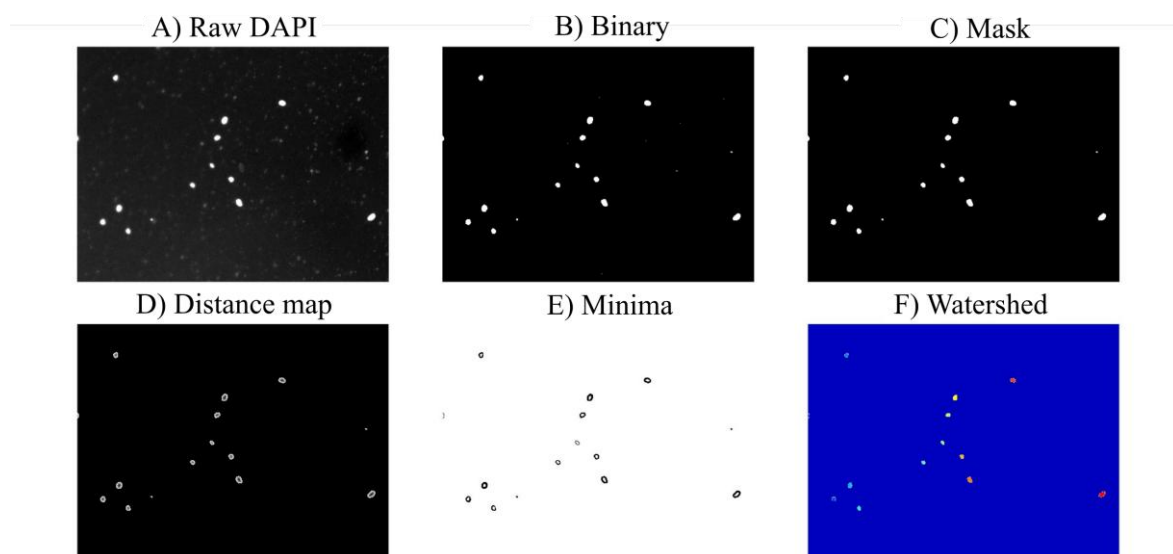


Figure 17. Image processing workflow for nuclear area quantification. (A) Raw DAPI image, (B) Binary image, (C) mask, (D) distance map, (E) identification of local minima and (F) watershed segmentation used to separate individual nuclei.

operations to remove noise and fill holes (Figure 17C). A distance map was computed from the binary mask, where pixel intensity increases toward the centre of each nucleus. This helps apply watershed algorithm to detect boundaries between adjacent nuclei and separate them. The distance map (Figure 17D) was inverted, and local minima (Figure 17E) were detected to serve as seed points for the watershed algorithm. The watershed (Figure 17F) then expanded from these points to accurately separate touching nuclei and define nuclear boundaries. The nuclear area was recorded in pixels and converted to μm^2 using the calibrated pixel-to-micrometre ratio. This automated workflow enabled consistent, unbiased, and high throughput analysis of nuclear morphology across all samples.

Analysis of actin and vinculin images was performed on ImageJ to quantify their intensity. Thresholding followed by the analyse particles function in ImageJ was used to segment individual cells. The resulting cell outlines were then applied to the original fluorescence images in the actin and vinculin channels to extract the mean grey value and integrated density for each cell representing the average brightness per pixel and the total brightness across the region of interest respectively. To correct for background signal, the mean grey value of the background was calculated by averaging values from three background regions in each image. From this analysis, the corrected total cell fluorescence (CTCF), which reflects the overall fluorescence intensity per cell, indicates the total amount of protein present. *CTCF* was calculated using *Equation 14* below.

$$CTCF = Int\ Den - (Cell\ area \times mean\ background\ signal) \quad \text{Equation 14}$$

2.12 Statistical analysis

Statistical analysis was performed using OriginPro 2022 (Version 9.9; OriginLab, USA). For comparisons between two conditions, unpaired two-tailed t-tests were used. For comparisons involving more than two groups, one-way ANOVA followed by Tukey's multiple comparisons test was performed unless otherwise stated. Normality of the data was assessed using Shapiro-Wilk test. If the data were not normally distributed, non-parametric tests were performed. The Mann-Whitney U test was done to compare two groups, and the Kruskal-Wallis test, followed by Dunn's multiple comparisons test was done for comparisons of two or more groups. Statistical significance was defined by p-values indicated with an asterisk (*), and results with $p > 0.05$ were considered non-

significant (ns) while significant differences were denoted as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Biological replicates are samples prepared under identical conditions and treated at the same time are referred to as n. In immunofluorescence imaging analysis, n refers to the number of images analysed per replicate. Error bars represent standard deviation (SD) unless otherwise stated.

Chapter 3: The effects of ultrasound exposure time, pulse width, and ultrasound pressure on cell morphology and mechanosensing in MG63s

3.1 Introduction

Low-intensity pulsed ultrasound has been reported to influence cell behaviour relevant to bone repair, including proliferation, alkaline phosphatase activity, increased collagen synthesis, mineralisation, and extracellular matrix production [84], [102], [104], [105], [143]. These effects have been observed in osteoblasts such as MC3T3-E1 and MG63s and could be linked to mechanical loading at the cell-substrate interface. However, the specific ultrasound parameters responsible for these biological effects have not yet been identified. Due to variations in experimental designs across studies, it is difficult to compare outcomes from different groups or to determine which ultrasound parameters are most effective.

Focal adhesions are key multiprotein complexes that couple the ECM to the intracellular cytoskeleton. Proteins such as vinculin anchor integrins to actin filaments, enabling cells to generate traction forces and detect changes in their mechanical environment. Mechanical stimulation, including that from ultrasound, may influence the cytoskeleton by altering focal adhesion dynamics and actomyosin contractility, making actin and vinculin intensities reliable readouts of mechanosensing [77]. In turn, this LIPUS-induced mechanical stress triggers rapid cytoskeletal remodelling, upregulates vinculin distribution, and may also expand cell area [50]. For osteogenesis, this expanded cell spreading area is an indication of a high-tension cytoskeletal state that favours the osteogenic lineage over adipogenesis [144]. These processes are closely linked to mechanosensitive pathways through RhoA that drive cell migration, matrix production, and osteogenic differentiation and mineralisation, making LIPUS-mediated cell spreading an essential biophysical mechanism for accelerating tissue healing and bone regeneration.

Nuclear morphology is another reliable indicator of cytoskeletal tension transmitted to the cell which is essential for mechanotransduction to take place. The increased actin activity and the associated cell spreading can also lead to larger and flatter nuclei, indicative of nuclear tension. Reduced adhesion, however, is coupled with rounded morphology, which is consequently associated with smaller, compact nuclei [65]. As such, measurements of actin structure, vinculin expression, cell area, and nuclear morphology can serve as indicators of cellular response to mechanically induced stimulation.

In this thesis, MG63 osteoblast-like osteosarcoma-derived cells were selected for their practical advantages as a human-derived, immortalised cell line with functional characteristics of a pre-osteoblastic cell line [145]. These cells are highly responsive to mechanical stimuli, and their integrin subunit profile as well as organisation of internal cellular structures resembles that of primary osteoblasts [146]. Evidence shows that MG63s undergo cytoskeletal reorganisation and nuclear deformation, which are key features of mechanotransduction [147], [148]. Others reported that pulsed ultrasound activates Wnt signalling in MG63 cells, a pathway associated with cytoskeletal organisation and integrin-mediated cell-matrix adhesion essential for osteoblastogenesis [149]. Collectively, these findings reinforce the use of MG63s as a responsive model for examining focal adhesion behaviour and cytoskeletal organisation in response to ultrasound stimulation.

Despite their advantages, MG63s display certain non-physiological characteristics associated with their osteosarcoma origin, including high proliferative capacity, and immature osteoblastic phenotype with limited calcium deposition, reduced expression of extracellular proteins such as type I collagen, and low alkaline phosphatase activity [146], [150]. Prolonged culture and high passage numbers further alter their differentiation potential, emphasizing the need to maintain sub-confluent cultures to preserve consistent behaviour [151].

This chapter investigates how varying ultrasound duty cycle (20-40%), exposure duration (10-20 minutes), and pressure amplitude (33-388 kPa) affect actin and vinculin intensity, as well as cell and nuclear morphology. To contextualise these experiments, a brief review of selected *in vitro* studies is first presented, focusing on those that employed more controlled experimental setups and explicitly addressed or quantified secondary acoustic or thermal effects. Experiments were conducted using the custom-designed, acoustically and optically

transparent Biocell vessel, which enables precise ultrasound stimulation and imaging on the same substrate. Fluorescence microscopy was used to evaluate changes in cytoskeletal organisation (via phalloidin staining), focal adhesion formation (via vinculin), and nuclear shape (DAPI). Together, these measurements were used to characterise the influence of specific acoustic conditions on the mechanical response of osteoblast-like cells.

3.2 LIPUS effects on bone cells in controlled *in vitro* studies

This section summarises the key studies investigating the biological effects of LIPUS. The studies included here were chosen for their attempts to characterise the acoustic field and to account for secondary effects, including thermal contributions, which are often not controlled for in the wider literature. While the *in vitro* experiments present limitations, the following studies demonstrate an effort to account for secondary effects in ultrasound exposure. Although the experimental methodologies and dosimetry approaches vary across studies, the results collectively suggest that ultrasound parameters influence osteogenic activity of cells *in vitro*.

Mukai *et al.* employed the custom tank *in vitro* setup using Falcon tubes to examine the effects of LIPUS on rat chondrocyte aggregates [13]. Cells were exposed to LIPUS (1.5 MHz, 20% DC, 1000 Hz PRF, and I_{SATA} of 30 mW/cm²) for 20 minutes daily over 15 days. LIPUS exposure increased DNA content and chondrocyte proliferation, while sustaining the expression of type II collagen and aggrecan. The responses were attributed to the upregulation of TGF- β signalling, which is a primary orchestrator of bone healing by recruiting stem cells, and stimulating the production of new bone.

Li *et al.* investigated various pulsed ultrasound intensities for stimulating osteoblast proliferation and PGE₂ secretion using the absorption tube method [14]. Osteoblasts were exposed to 1 MHz pulsed ultrasound (Sonopuls 490, Delft Instruments) at five different nominal I_{SATP} of 150, 300, 600, 1200, 2400 mW/cm² (using the relationship: $I_{SATA} = I_{SPTP} \times DC$, this corresponds to $I_{SATA} = 30, 60, 120, 240, 480$ mW/cm², respectively). Ultrasound was delivered at a PRF of 100 Hz and DC 20% for 15 minutes as a one-time exposure. The study identified 240 mW/cm² as the optimal I_{SATA} , which significantly enhanced both cell density and PGE₂ secretion. The results showed a biphasic response, with increased osteoblast proliferation and PGE₂ secretion up to 120 mW/cm², followed by a decline at

higher I_{SATA} . These findings suggest that ultrasound-induced bone cell growth is at least partly mediated by PGE_2 synthesis and support further investigation into the biological mechanisms underlying LIPUS stimulation.

Parvizi *et al.* used rat chondrocytes and employed the inverted tank dip setup in a circulating water bath to mitigate thermal effects and standing waves [136]. Pulsed ultrasound was delivered at 1 MHz, 20% DC, 1000 Hz PRF, and pressures of 88-556 kPa for 10 minutes each day for three consecutive days. The nature of the pressure is not defined, making it unclear whether the value corresponds to peak-to-peak, peak positive or peak negative pressure. At 222 kPa, equivalent to 50 mW/cm^2 I_{SATA} , the strongest Ca^{2+} influx was observed. When calcium channels and internal stores were blocked, the effects of LIPUS were abolished. The authors reported that Ca^{2+} signalling induced proteoglycan synthesis, which are complex molecules involved in cell migration and differentiation, essential for tissue repair. They concluded that intracellular calcium plays a central role in mediating the tissue repair effects of LIPUS.

Lai *et al.* adopted a modified absorption tube configuration to investigate the differentiation of human mesenchymal stem cells towards chondrogenic and osteogenic differentiation under LIPUS stimulation [15]. The transducer was mounted on a rotating platform to minimise standing waves and thermal artefacts within the culture chamber. The system used a 1 MHz planar transducer operating at 100 Hz PRF, 20% DC, and an I_{SATP} of 200 mW/cm^2 ($I_{SATA} = 40 \text{ mW/cm}^2$) for 20 minutes daily for up to 4 weeks to assess time-dependent changes in lineage commitment. The study reported a significant increase in the expression of chondrogenic markers (Sox9, aggrecan, type II collagen), and integrin- $\beta 1$ when LIPUS is combined with TGF- $\beta 1$ and dexamethasone. LIPUS alone significantly increased Runx2 and ALP expression in hMSCs, indicating early osteogenic differentiation.

In summary, LIPUS at 1.5 MHz, 1000 Hz, 20%, 30 mW/cm^2 could promote chondrocyte proliferation through TGF- $\beta 1$ signalling. It can also enhance proteoglycan expression in chondrocytes through Ca^{2+} signalling at 1 MHz, 1000 Hz, 20%, and 222 kPa and I_{SATA} of 50 mW/cm^2 in the presence of chemical messengers, and at the same parameters it can enhance hMSC osteogenic lineage commitment without chemical inducers. LIPUS at 1 MHz, 100 Hz, 20% DC and 240 mW/cm^2 I_{SATA} can enhance PGE_2 expression and proliferation in osteoblasts.

While these findings demonstrate clear biological effects, comparisons across studies remain limited due to differences in ultrasound dosimetry, experimental setups, cell types, as well as the incomplete characterisation of the acoustic field. Notably, the reported optimal ultrasound parameters vary across studies, with different investigations identifying distinct combinations as producing the strongest biological response. This suggests that the observed effects are dependent on the specific experimental configuration and cell type employed in each study.

In contrast to these studies, which predominantly assess downstream outcomes such as proliferation or PGE₂ secretion, this work focuses on the immediate effects of LIPUS. This was achieved by looking at the expression of vinculin and actin, as well as cell and nuclear area, which are early markers of cell mechanosensing and mechanotransduction. Focusing on these early changes allows for more precise definition of how cells sense and respond to LIPUS and helps identify which parameters influence the initial mechanosensitive events. This approach contributes to clarifying the link between LIPUS input and cellular behaviour with reduced influence from secondary biochemical signalling and longer-term adaptive processes.

3.3 Aims and overview

This chapter aims to investigate how acoustic pressure, duty cycle, PRF, and exposure duration independently influence the extent to which MG63 osteoblast-like cells respond to LIPUS stimulation. Observation of morphological, cytoskeletal and adhesion-related responses is used to indicate optimal conditions for each parameter to enhance actin and vinculin expression as well as cell and nuclear spreading. These outcomes are subsequently used in Chapter 4 to inform follow-on work to establish the most effective combination of parameters, ultimately contributing to the development of optimised therapeutic ultrasound protocols for bone healing. Any reference to optimisation here is limited to the specific parameter ranges, cell type, and exposure conditions used in this study. It does not imply that these are the universally optimal settings.

3.4 Chapter methodology and parameter overview

3.4.1 Parameter Overview

Table 2. Summary of ultrasound exposure parameters employed across all experimental sections in this chapter, including peak to peak pressure (kPa), pulse characteristics (PRF, Burst length, DC), the corresponding I_{SATA} as well as the exposure duration.

Experiment Section	Peak to peak pressure (kPa)	PRF (Hz)	Burst length (ms)	DC (%)	I_{SATA} (mW/cm ²)	Exposure duration
3.5.1. Preliminarily acoustic pressure investigation	32., 56-113, 80-160, 113-225, 194-388	1000	200	20	10, 30, 60, 120, 200 respectively	20 minutes
3.5.2. Acoustic pressure study	32-65, 56-113, 80-160, 113-225	1000	200	20	10, 30, 60, 120 respectively	20 minutes
3.5.2. Exposure duration	56 – 113	1000	200	20	30	10, 20 minutes
3.5.2. Acoustic pressure refinement	75-150, 80-160, 85-170	1000	200	20	50, 60, 70, respectively	10 minutes
3.5.2 Acoustic pressure refinement upper limit	80-160, 85-170, 90-180	1000	200	20	60, 70, 80 respectively	10 minutes
4.5.3 Pulse width	80-160	1000	200, 400	20, 40 respectively	60, 120	10 minutes

The experimental exposure system, cell culture protocols, and immunostaining procedures used throughout this chapter are described in detail in Chapter 2. To evaluate the biological response under specific acoustic conditions, a systematically varied set of exposure parameters was investigated. The specific acoustic exposures and treatment durations are summarised in Table 2.

3.4.2 Exposure time affects cellular response to LIPUS

The LIPUS exposure duration was investigated to determine whether stimulation time influences early mechanosensitive responses in osteoblast-like cells. While commercial bone-healing devices typically apply ultrasound for 20 minutes per day over several weeks, this duration is based primarily on tissue-level healing outcomes, and its immediate effects on early cytoskeletal dynamics remain poorly defined. To address this and optimise the conditions for the *in vitro* experiments in this thesis, two stimulation durations of 10- and 20-minutes were explored. Ultrasound was delivered to cells through the Biocells at an I_{SATA} of 60 mW/cm^2 , with pressures ranging from 80 to 160 kPa within the -6 dB region. Nuclear area, cell area, actin intensity, and vinculin intensity were analysed immediately after stimulation to establish an effective exposure time for this *in vitro* model without claiming clinical therapeutic relevance.

Nuclear area and cell area (Figure 18A-B) were significantly higher following both 10- and 20-minute exposures compared to sham controls ($***p<0.001$). For both measures, there was no significant difference between the two exposure durations, though a non-significant decrease was observed at 20 minutes. This shows that nuclear area and cell spreading are enhanced by stimulation at 80-160 kPa, and that a 10-minute exposure is sufficient to achieve this effect.

Actin intensity (Figure 18C) followed a similar trend with both exposure durations yielding significant increases relative to control ($***p<0.001$). However, actin intensity levels were lower at 20 minutes than 10 minutes, indicating a peak in cytoskeletal activation at the shorter duration. Vinculin intensity (Figure 18D) increased significantly following 10 minutes of exposure compared to control ($***p<0.001$) but declined significantly at 20 minutes relative to the 10-minute condition.

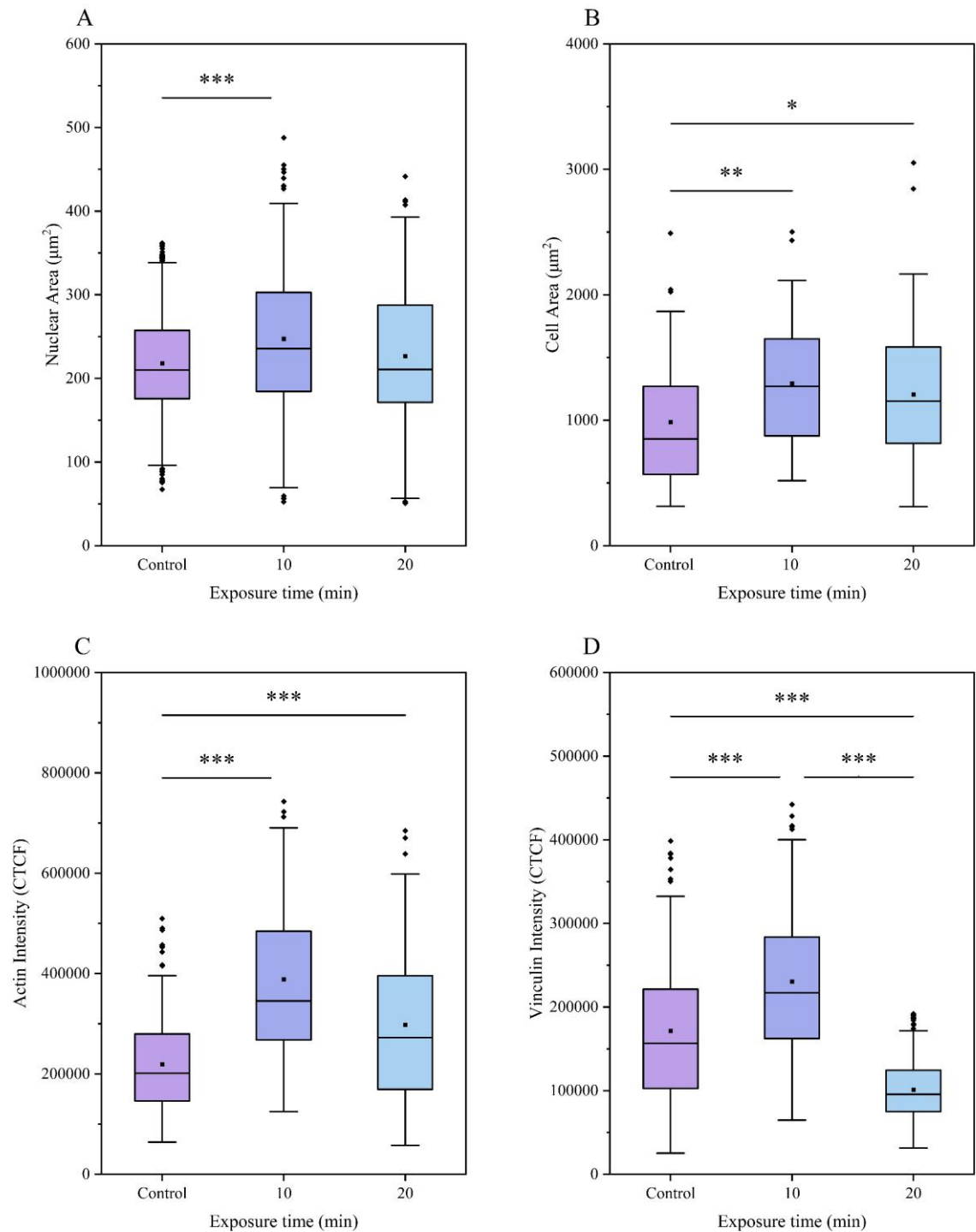


Figure 18. Effects of LIPUS exposure duration on nuclear and cell morphology, actin intensity and vinculin intensity in MG63 cells. (A) Nuclear area, (B) cell area, (C) actin fluorescence intensity (CTCF), and (D) vinculin fluorescence intensity (CTCF) following ultrasound exposure for 10 or 20 minutes at 60 mW/cm^2 I_{SATA} . Boxes indicate interquartile ranges, horizontal lines show medians, and whiskers denote $1.5 \times \text{IQR}$ range. Mean values are shown as black squares. Individual points represent statistical outliers. Statistically significant differences were determined using the Kruskal-Wallis test followed by Dunn's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The simultaneous increases in actin and vinculin intensity at 10 minutes, along with increased cell area and nuclear area, demonstrate that cells are spreading across the substrate and that the actin cytoskeleton is actively polymerising and reorganising. The 20-minute treatment, by contrast, led to a reduction in actin and vinculin signal without promoting further cell spreading. Therefore, within this *in vitro* LIPUS system, a 10-minute treatment produces a more favourable early cytoskeletal response than 20 minutes. Subsequent experiments in this thesis were performed using a 10-minute exposure unless otherwise stated.

Cells were fixed immediately following stimulation (at 10 and 20 minutes) to capture the instantaneous cell response right when ultrasound stopped. However, because both exposure duration and total elapsed time prior to fixation varied together, it is worth noting that the lower actin and vinculin levels seen at 20 minutes could reflect either over-stimulation from continuous acoustic energy or time-dependent cytoskeletal disassembly. Incorporating a post-exposure time-matched control in future work such as, fixing 10-minute exposed cells after a 10-minute post treatment rest would help decouple total acoustic energy from temporal cell kinetics.

3.5 Results

3.5.1 Cell response to LIPUS increases with ultrasound pressure up to a threshold

The effects of varying ultrasound pressure on MG63s were explored at five different pressure ranges of 32-65, 56-113, 80-160, 113-225, and 194-288 kPa, corresponding to I_{SATA} of 10, 30, 60, 120, and 200 mW/cm², respectively. These values were selected to span the I_{SATA} range commonly reported in the literature, including nominal intensities employed by commercially available devices. All other parameters were held constant at 1000 Hz PRF, 20 % DC, 1 MHz carrier frequency, and 20-minute exposure time. This preliminary dose-response screen aimed to isolate the impact of acoustic pressure on cellular response by assessing nuclear area and cell area as well as actin corrected total cell fluorescence, which is used to quantify overall marker intensity.

The nuclear area shown in Figure 19A was noticeably modulated by acoustic pressure. A reduction was observed at 32-65 kPa (10 mW/cm²), whereas values at 56-113 kPa (30

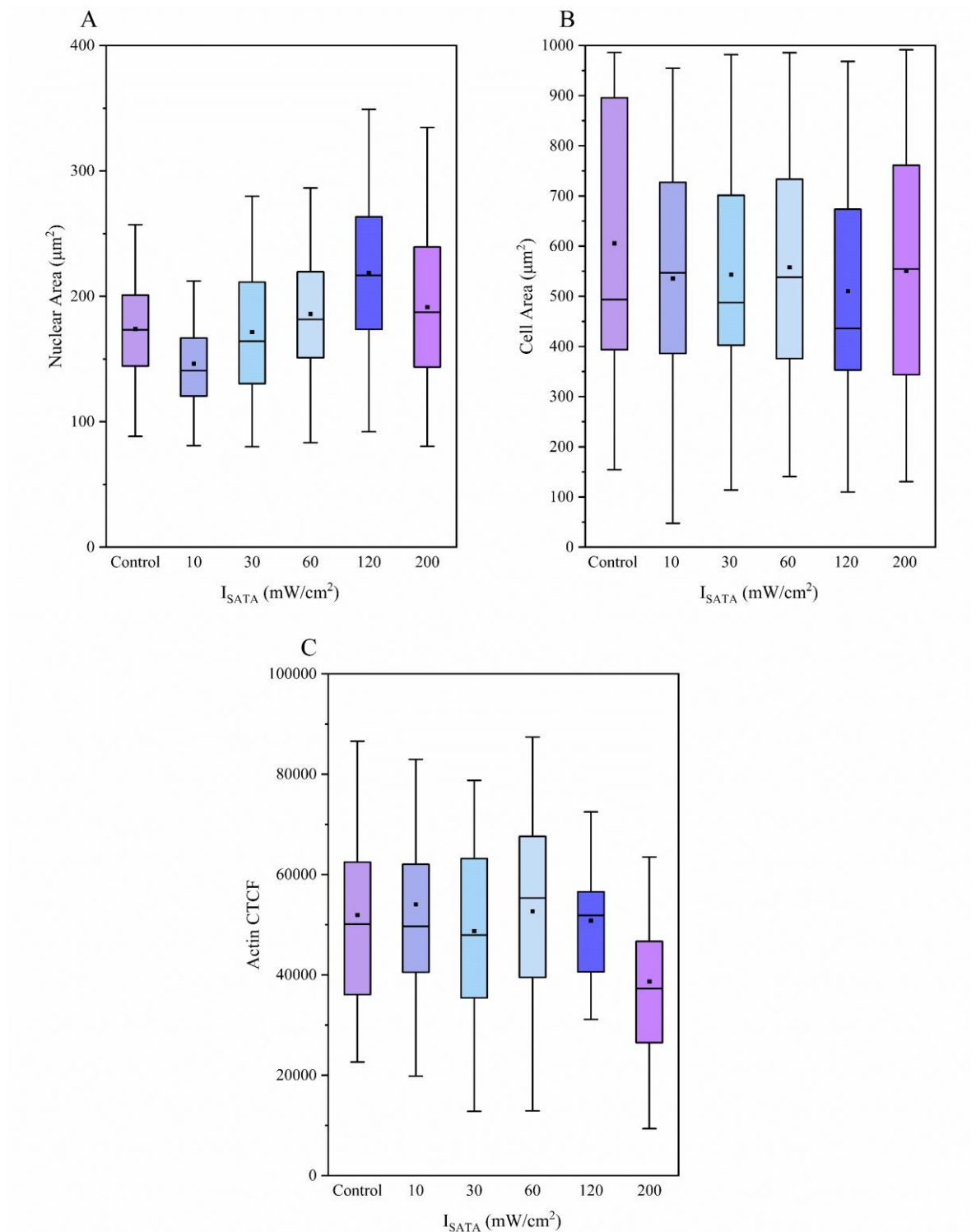


Figure 19. Quantitative comparison of MG63 cell responses to LIPUS at varying I_{SATA} (A) Nuclear area, (B) cell area, (C) corrected total cell actin fluorescence following exposure to LIPUS at I_{SATA} of 10, 30, 60, 120, and 200 mW/cm^2 , resulting in acoustic pressures of 32-65, 56-113, 80-160, 113-225, and 194-388 kPa, respectively. Data are presented as boxplots showing IQR, median (horizontal line), and mean (square), with whiskers representing 1.5 x IQR. Outliers were removed. Due to low number or biological replicates, no statistical tests were performed; these plots are provided for visual interpretation only.

mW/cm²) were comparable to sham controls. The nuclear area increased at intermediate pressures of 80-160 kPa (60 mW/cm²) and 113-225 kPa (120 mW/cm²), before declining again at the highest pressure of 194-288 kPa (200 mW/cm²). This non-monotonic trend suggests that intermediate pressure ranges (80-225 kPa) induce sufficient cytoskeletal tension to deform the nucleus, whereas lower pressures supply insufficient mechanical input and higher pressures over-stimulate the cell, diminishing effective force transmission to the nucleus. Cell spreading area (Figure 19B) revealed a minor elevation at 10 and 60 mW/cm², reflecting enhanced substrate adhesion and intracellular traction force generation, while remaining pressure conditions were comparable to control.

Actin intensity (Figure 19C) remained largely unaffected across the tested I_{SATA} conditions, with an increase at I_{SATA} of 60 mW/cm² and a marked decrease at 200 mW/cm² compared to all other conditions. The observation that this subtle increase in actin intensity at 60 mW/cm² occurred without an expansion in total cell area indicates that early actin polymerisation precedes measurable cell spreading during mechanosensitive remodelling.

It is important to contextualise these findings within the experimental constraints of this initial screen. Due to unexpected leakage of Biocell vessels during cell seeding or mounting, final sample sizes were limited (n=1 for control, 10, 30, and 200 mW/cm² conditions; n=2 samples for 60 and 120 mW/cm² conditions). Consequently, formal statistical testing was omitted, and these data are interpreted strictly as descriptive trends. While these preliminary data suggest that cells are sensitive to ultrasound, the limitations were considered when evaluating the reported trends. These considerations informed the design of the subsequent experiment, which focused on a reduced set of conditions with increased replication to enable a more targeted and statistically robust investigation.

Summary of preliminary findings and refinement of experimental conditions

This investigation aimed to explore the effects of ultrasound pressure on cellular behaviour with I_{SATA} values adjusted because of pressure modulation. Exposure to LIPUS produced differences in early mechanosensitive markers in MG63 cells. Actin intensity and cell area showed an increase at intermediate intensities, most notably at 60 mW/cm². The nuclear area increased at 60 and 120 mW/cm², suggesting enhanced tension at these intensities. At 200 mW/cm² no clear trend emerged. A reduced nuclear area and actin intensity paired with an increase in cell area suggest altered mechanotransduction dynamics. However,

further investigation is required to confirm this. To increase the number of samples per condition, the 200 mW/cm² was not included in subsequent experiments.

Refined investigation of pressure effects within a reduced exposure range

The experiment was repeated with increased biological replicates to offer a more comprehensive and robust data set to obtain statistically meaningful trends. The same I_{SATA} levels (10, 30, 60, 120 mW/cm², excluding 200 mW/cm²) were applied, corresponding to peak-to-peak pressures of 32-65, 56-113, 80-160, and 113-225 kPa, respectively. PRF, DC, carrier frequency, and exposure time were kept identical to the initial experiment (Section 3.5.1). Actin intensity, nuclear area, and cell area were re-assessed to determine whether LIPUS modulated cytoskeletal activation and morphological response. In addition, vinculin was included to assess focal adhesion-associated signalling, which provides a more sensitive indicator of early mechanosensing than morphology alone.

Nuclear area (Figure 20A) decreased significantly (* $p < 0.05$) at 30 mW/cm², whereas 60 mW/cm² produced a non-significant increase compared to the control, indicating that nuclear deformation did not scale uniformly with increasing ultrasound intensity. Cell area (Figure 20B), exhibited a similar trend, with a statistically significant (* $p < 0.05$) decrease at 30 mW/cm². Cell area at 10, 60 and 120 mW/cm² remained comparable to the control.

Actin intensity (Figure 20C) demonstrated a more marked shift, showing a statistically significant increase at an I_{SATA} of 60 mW/cm², whereas intensity across all other conditions remained comparable to the control. Vinculin (Figure 20D) exhibited a response profile similar to actin, peaking in intensity at 60 mW/cm² and dropping at 10 mW/cm², with statistically significant drops at 30, as well as 120 mW/cm² I_{SATA} relative to control (** $p < 0.001$).

Together, these morphological and intensity measurements suggest that ultrasound pressure specifically modulates cytoskeletal and adhesion-related features in MG63s. Actin and vinculin intensity reached maximum levels at 60 mW/cm², whereas further increases or decreases in pressure reduced their signal. The enhanced fluorescence observed at 60 mW/cm², occurring without major changes in cell spreading, suggest that cytoskeletal and adhesion proteins were denser or larger at this intensity. Conversely, the localised

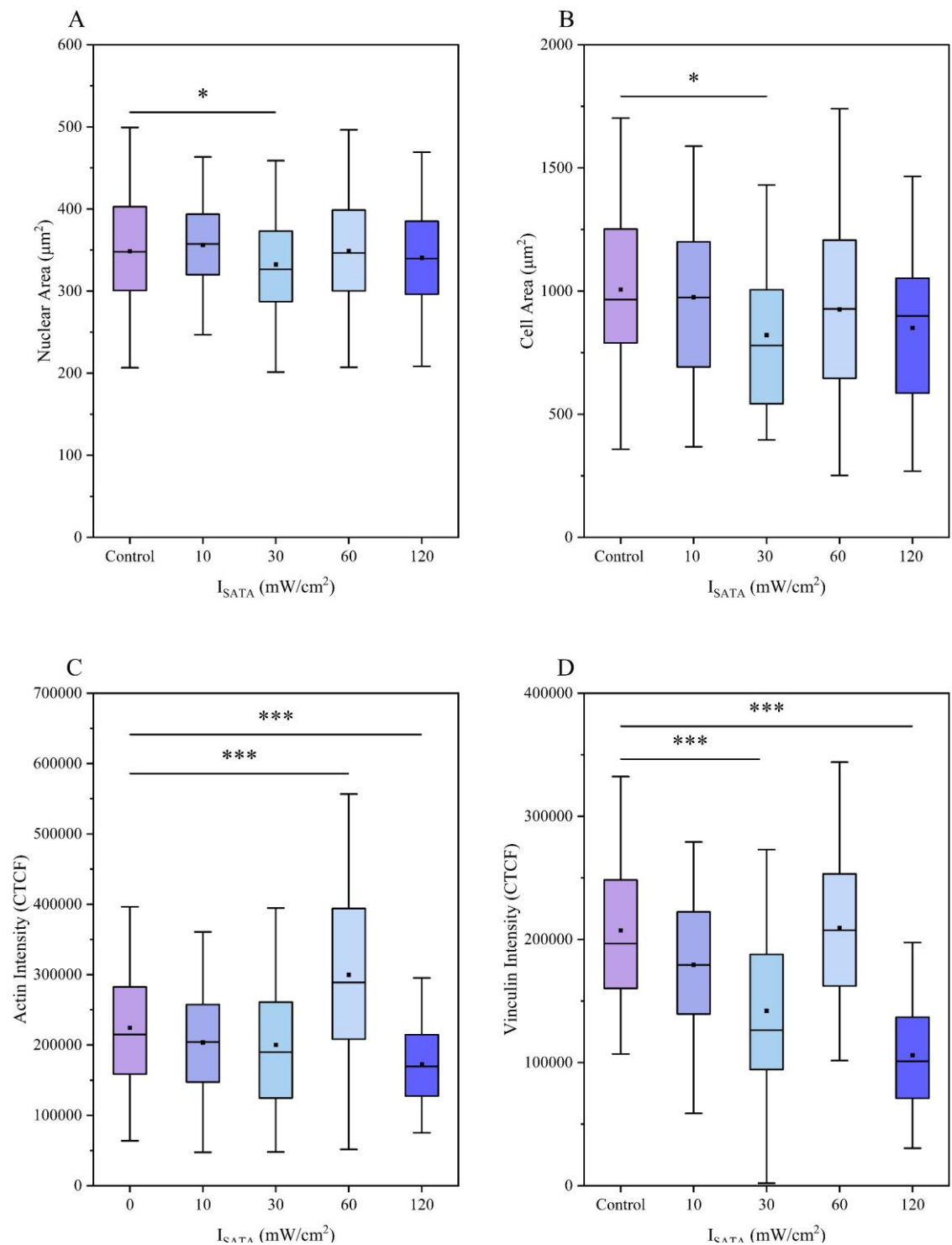


Figure 20. MG63 response to various ultrasound I_{SATA} within a narrower region (30-120 mW/cm²) corresponding to ultrasound pressures of 32-65, 56-113, 80-160, 113-225 respectively. (A) Nuclear area, (B) Cell area, (C) Actin CTCF and (D) Vinculin CTCF. Data are presented as boxplots showing IQR, median (horizontal line), and mean (square), with whiskers representing 1.5 x IQR. Outliers were removed. Mean values are shown as black squares. Individual points represent statistical outliers. Statistical comparisons were determined using the Kruskal-Wallis test followed by Dunn's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

reductions in cell area and nuclear area at 30 mW/cm² align with the concurrent drop in actin and vinculin signal. At these lower pressure levels, cells appear to retain a morphology close to baseline, and the data do not provide evidence for robust mechanosensitive activation.

When interpreting these intensity thresholds, it is important to consider the broad, overlapping acoustic pressure ranges across the Biocell vessel that the cells are exposed to. Because transducer fields are non-uniform, reporting nominal I_{SATA} values means cells across the sample experience a range of local pressures. The peak response at 60 mW/cm² indicates that a critical proportion of cells in the central beam region received pressures within the effective stimulation window. However, a limitation of whole-sample field averaging is that cells located near the edges receive lower pressures than the ones at the centre of the beam. While spatial position was not analysed separately in this dataset, this could be mitigated in future work by using spatial image masking or analysing cells as a function of radial distance from the beam centre.

This investigation focused on a smaller range of intensities to build on the trends established in the preliminary experiment and to provide stronger statistical comparisons. While a greater number of biological replicates (N=3) per condition was initially planned, the unavoidable incidence of Biocell leakage during fabrication and cell seeding restricted biological replication to two samples per condition. To maintain statistical strength, more images were acquired per condition (total number of cells > 500) to maximise data quality and produce a robust dataset for assessing pressure-dependent mechanosensitive responses.

Overall, the broad intensity investigations indicated a moderate response at 60 mW/cm². Considering that the magnitude of this response is also influenced by exposure time (Section 3.4.7), a narrower intensity range was investigated in the next experiments, using 10-minute stimulation to assess whether this enhancement could be further refined.

3.5.2 I_{SATA} of 60 mW/cm² produces the most favourable cell response

This investigation was carried out in two parts. The first set of experiments exposed cells to 50, 60, and 70 mW/cm² I_{SATA} , corresponding to ultrasound pressures of 75-150, 80-160, and 85-170 kPa, respectively. The second part then examined a higher range of 60, 70, and 80 mW/cm² (90-180 kPa) to confirm the trend. In both cases, only the pressure (and

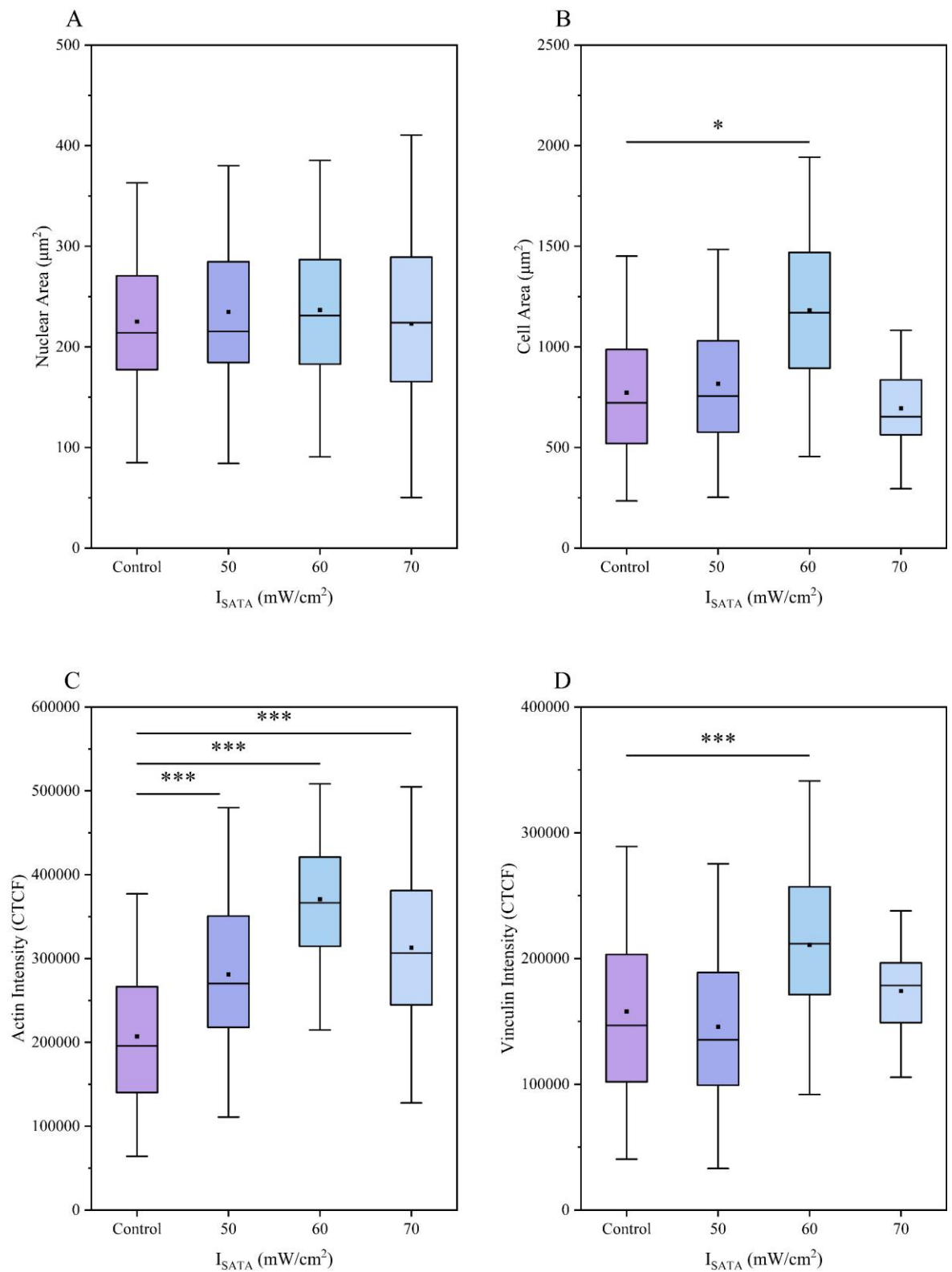


Figure 21. Effects of LIPUS acoustic pressure or I_{SATA} on nuclear and cell morphology, actin intensity and vinculin intensity in MG63 cells. (A) Nuclear area, (B) cell area, (C) actin fluorescence intensity (CTCF), and (D) vinculin fluorescence intensity (CTCF) following ultrasound exposure at I_{SATA} of 50, 60, and 70 mW/cm^2 corresponding to acoustic pressure ranges of approximately 75-150, 80-180, and 85-170 kPa respectively. Boxes indicate interquartile ranges, horizontal lines show medians, and whiskers denote 1.5 x IQR range. Mean values are shown as black squares. Individual points represent statistical outliers. Statistical comparisons were determined using the Kruskal-Wallis test followed by Dunn's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

therefore I_{SATA}) was altered while all other parameters remained unchanged (carrier frequency = 1 MHz, PRF = 1000 Hz, DC = 20%, 10 minutes of exposure).

Cell responses at 50-70 mW/cm² I_{SATA}

Nuclear area remained unaffected across all intensities, with no significant differences relative to the control (Figure 21A). Cell area (Figure 21B) remained unchanged at 50 and 70 mW/cm² compared to the control. At 60 mW/cm² however, cell area increased markedly (**p<0.001) compared to control.

Actin intensity (Figure 21C) followed a more pronounced trend, with statistically significant increases in intensity observed across all LIPUS conditions (50-70 mW/cm²), relative to untreated cells. The highest actin intensity occurred at 60 and 70 mW/cm². Vinculin levels (Figure 21D) at 50 mW/cm² were comparable to control. At 60 mW/cm², vinculin increased significantly, reaching peak intensity within this range. At 70 mW/cm², vinculin remained elevated relative to control, though at lower level than at 60 mW/cm².

Evaluating cellular response across this narrower intensity range confirms that the conditions for mechanosensitivity are optimal at 60 mW/cm², as evidenced by the high fluorescence of actin and vinculin paired with peak cell area expansion.

It is noteworthy that pressure ranges associated with 50, 60, and 70 mW/cm² partially overlap, meaning that cells across different nominal I_{SATA} setting were subjected to varying regional ultrasound pressures. The pronounced response observed at 60 mW/cm² may be explained by a greater proportion of cells experiencing the effective pressure range (80-160 kPa) required to drive focal adhesion recruitment. At 50 mW/cm², peak pressure reaches a maximum of 150 kPa. Consequently, fewer cells cross the upper activation threshold, resulting in a lower overall activation profile. At 70 mW/cm², a portion of the cells were expose to pressures exceeding 160 kPa, which did not further enhance adhesion-related responses. These findings suggest that cellular response is governed primarily by the proportion of cells exposed to a biologically responsive pressure window, rather than nominal intensity alone.

Within the framework of the molecular clutch model, this behaviour aligns with the characteristic biphasic, force-dependent stability of focal adhesions. While suboptimal pressures used to achieve an I_{SATA} of 50 mW/cm² may fail to transmit sufficient tension to

unfold talin and expose cryptic vinculin binding sites, excessive force loading beyond 160 kPa likely accelerates bond dissociation and clutch disruption, preventing further enhancement of adhesion-related proteins. In this experiment, some DAPI images exhibited high background fluorescence and uneven illumination, which compromised nuclear segmentation and reduced sensitivity to subtle morphological shifts, likely contributing to the absence of detectable nuclear area changes. In addition, for the 70 mW/cm² condition, only one sample remained viable through exposure, fixation, and staining, limiting robust assessment of cellular responses at this level. To address these limitations and further explore the relationship between ultrasound pressure and cellular response, a follow-up experiment was conducted at pressures of up to 180 kPa.

Cell responses at 60-80 mW/cm² I_{SATA}

Nuclear size (Figure 22A) decreased statistically across all LIPUS conditions compared to the control. Cell area (Figure 22B), actin (Figure 22C), and vinculin (Figure 22D) intensity increased significantly at 60 mW/cm², and significant reductions at higher I_{SATA} with a stepwise decrease, indicative of cell detachment from the substrate. These data reinforce a narrow optimal window centred around 60 mW/cm² and ultrasound pressures ranging from 80 kPa to a maximum of 160 kPa beyond which biological effects diminish.

Fluorescence imaging reveals cytoplasmic vinculin upon exposure to LIPUS

Representative fluorescent images of cells exposed to ultrasound at 50, 60, 70, and 80 mW/cm², and an untreated control, are presented in Figure 23. Nuclei were stained with DAPI (blue), actin filaments were labelled with phalloidin (green), and vinculin was visualised with antibodies (Red) with merged images illustrating the localisation of these markers.

Across all ultrasound-treated conditions, vinculin fluorescence was notable both at cell periphery and within the cytoplasm, whereas the untreated samples showed vinculin predominantly localised at the edges of spread cells with less pronounced cytoplasmic fluorescence.

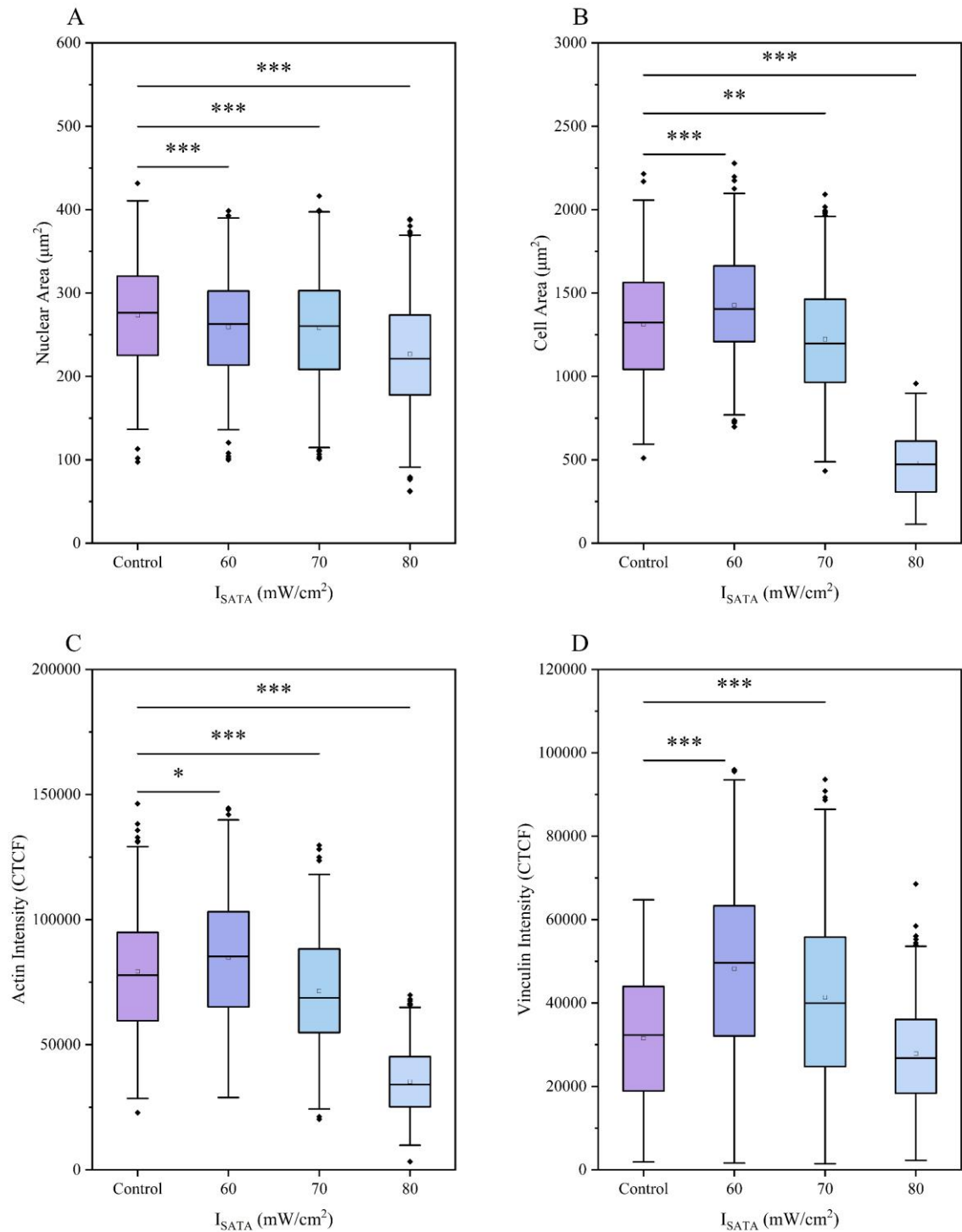


Figure 22. Effects of LIPUS acoustic pressure or I_{SATA} on nuclear and cell morphology, actin intensity and vinculin intensity in MG63 cells. (A) Nuclear area, (B) cell area, (C) actin fluorescence intensity (CTCF), and (D) vinculin fluorescence intensity (CTCF) following ultrasound exposure at I_{SATA} of 60, 70, and 80 mW/cm² corresponding to acoustic pressure ranges of approximately 80-180, 85-170, and 90-180 kPa respectively. Boxes indicate interquartile ranges, horizontal lines show medians, and whiskers denote 1.5 x IQR range. Mean values are shown as black squares. Individual points represent statistical outliers. Statistical comparisons were determined using the Kruskal-Wallis test followed by Dunn's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

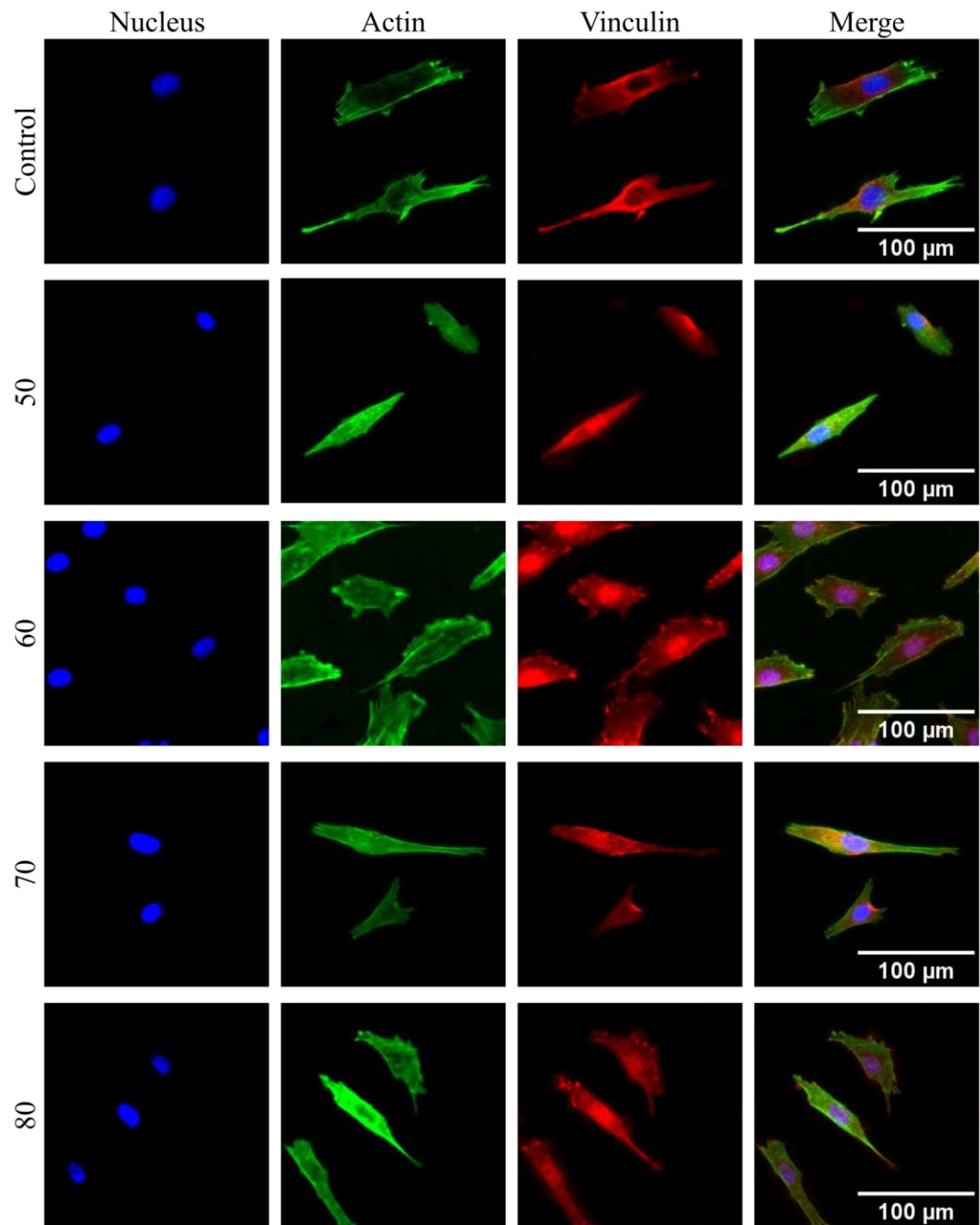


Figure 23. Fluorescence images of MG63 cells exposed to pulsed ultrasound at 0 (control), 50, 60, 70, and 80 mW/cm^2 I_{SATA} , showing nuclei (blue, DAPI), actin (green, phalloidin) and vinculin (red, anti-vinculin). Individual channels are shown with the corresponding merged images. Visual inspection indicates that cells exposed to 60 mW/cm^2 appear more spread, with denser actin cytoskeleton and more vinculin attachment points compared to all other conditions. At 70 and 80 mW/cm^2 the cells remain spread, but the attachment points decrease with increasing I_{SATA} . The 50 mW/cm^2 condition shows a weaker fluorescence signal with fewer visible adhesions and reduced spreading compared with higher stimulation levels. Scale bar applies to all panels (100 μm).

At 60 mW/cm², cells appeared more spread with brighter actin signal compared with untreated control and other stimulation conditions. The vinculin channel showed more pronounced vinculin adhesion sites at the cell perimeter, with distinct and bright adhesion points visible at the edges. The spatial co-localisation of these vinculin adhesion points with actin stress fibres is indicative of mature focal adhesions, representing functional mechanical linkages between the cytoskeleton and the extracellular matrix. In comparison, the 50 mW/cm² condition exhibited weaker actin fluorescence and fewer visible vinculin attachment points. The 70, and 80 mW/cm² conditions showed spread morphology however, the number of vinculin-rich attachment sites visibly decreased with increasing I_{SATA} relative to 60 mW/cm².

3.5.3 LIPUS at 20% DC enhances cell response

Duty cycle defines the proportion of time that the transducer is active within a burst, which in turn reduces the off time and influences the overall energy delivery in terms of I_{SATA} . In this experiment, duty cycles of 20% and 40% were examined resulting in I_{SATA} values of 60 and 120 mW/cm², respectively. All other exposure settings remained the same as previously, with an ultrasound pressure range of 80-160 kPa, PRF of 1000 Hz, and 1 MHz carrier frequency. The effects of extending DC were assessed to determine whether longer stimulation increases cellular indicators of mechanosensing in MG63 cells.

Nuclear area, cell area, and vinculin levels increased significantly at 20% DC compared with the untreated control, whereas actin levels increased only moderately. At 40% DC, all measures of mechanosensing and cell attachment to the substrate decreased significantly. Representative fluorescence images for the 40% DC reveal smaller, rounded cells, consistent with the reduced levels of all other markers under this condition. This change likely reflects reduced cytoskeletal organisation and engagement of the cells with the substrate.

Because cell rounding and surface area reduction can mimic thermal stress responses, bulk fluid temperature was continuously monitored throughout exposure using a calibrated thermocouple positioned directly adjacent to the cell monolayer inside the Biocell chamber. Temperature variations remained within 37±0.2 °C, confirming that bulk heating did not occur. Furthermore, given the low time-averaged intensity (I_{SATA} = 120 mW/cm²) and high fluid volume relative to the monolayer, significant thermal accumulation at the

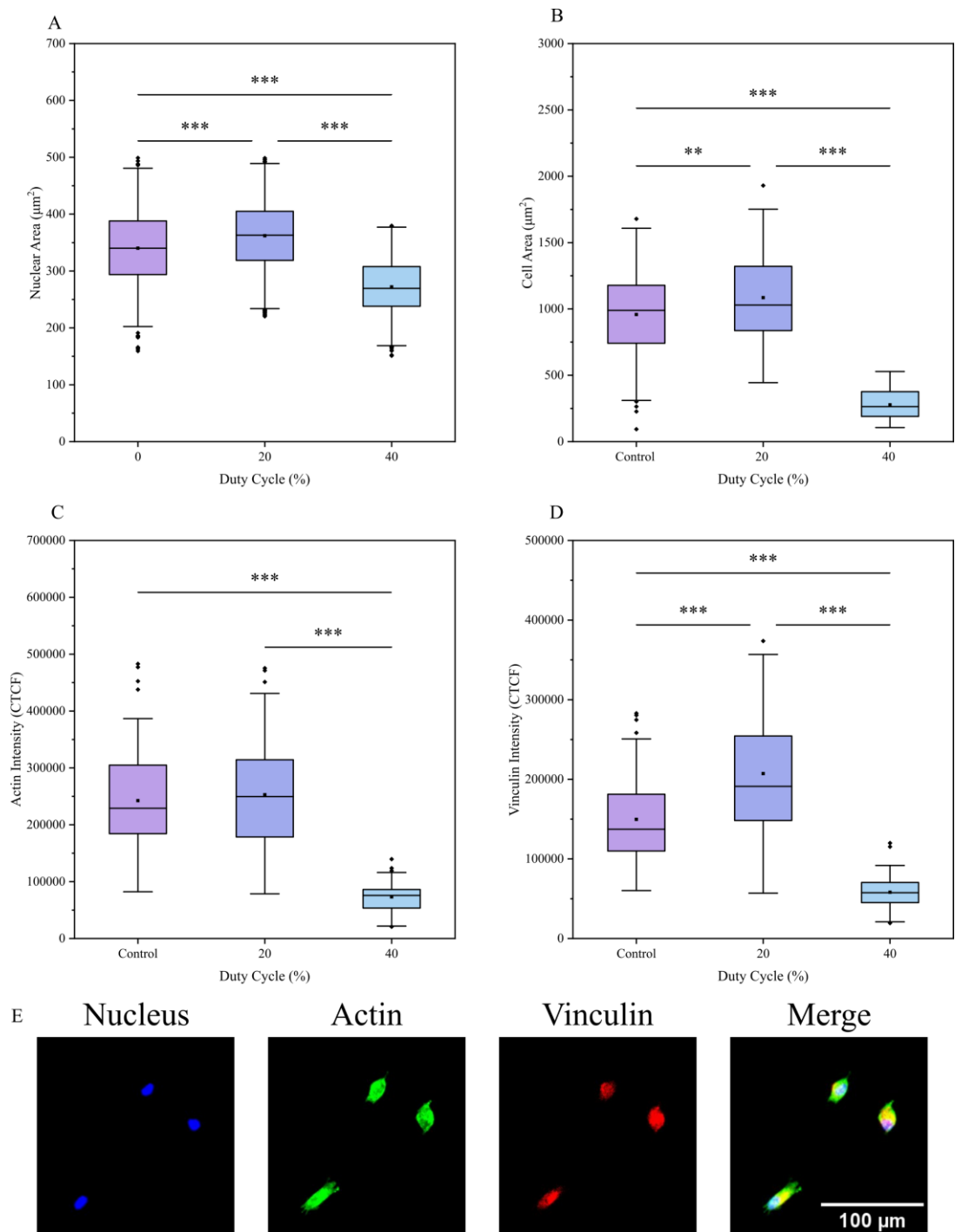


Figure 24. Effects of duty cycle on nuclear and cell morphology, actin intensity, and vinculin expression in MG63 cells. (A) Nuclear area, (B) cell area, (C) actin fluorescence intensity (CTCF), and (D) vinculin fluorescence intensity (CTCF) following LIPUS exposure at 0% (control), 20%, and 40% duty cycle ($I_{\text{SATA}} = 0, 60, 120 \text{ mW/cm}^2$ respectively). Boxplots show interquartile ranges, with medians (horizontal lines), means (squares), whiskers representing $1.5 \times \text{IQR}$, and individual points denoting outliers. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test (** $p < 0.001$, ** $p < 0.01$). (E) Representative fluorescence images of MG63 cells showing nucleus (DAPI, blue), actin filaments (FITC-phalloidin, green), vinculin (TRITC, red) and merged channels at 40% DC. Scale bar: 100 μm applies to all panels.

cell level is highly unlikely under these exposure conditions. Consequently, the cell rounding and area reduction observed at 40% DC are attributed to mechanical over-stimulation rather than thermal effects.

3.6 Discussion

The cellular ability to sense mechanical stimulation can be recognised through a range of early-onset events, including changes in cytoskeletal organisation, focal adhesion composition, and spreading behaviour. This makes it difficult to predict exactly how cells will respond to a given stimulation regime, meaning that the effect of vibration cannot be assumed to scale with increasing energy delivery. Before LIPUS can be reliably used for non-union bone healing, it is necessary to understand how cells respond to individual ultrasound parameters. This chapter addressed this challenge by systematically examining how early mechanosensitive markers in MG63 cells respond to a range of ultrasound pressures (32-388 kPa) and duty cycles (20-40%).

Across all investigations in this chapter, a consistent trend emerged: cells exposed to ultrasound at 60 mW/cm², with a 20% DC and a pressure range of 80-160 kPa, showed the strongest cellular response. This was reflected in more spread cell and in elevated actin and vinculin intensities. Actin polymerisation drives cell protrusion, and vinculin is a key component of cell adhesions to the substrate. An increase in cell area may correspond to cell flattening and spreading out. The simultaneous increase in these markers strongly shows that the cells flatten and spread on the substrate which would also indicate the formation of stronger, more stable attachments to the substrate. Furthermore, an increase in nuclear area under these parameters is consistent with mechanotransduction driven morphological changes, as effective mechanosensing relies on mechanical signals propagating through the actin cytoskeleton directly to the nucleus. These observations reinforce the findings of Atherton *et al.*, who demonstrated that LIPUS stimulation leads to rapid cytoskeleton rearrangement and that the sensing of LIPUS is mediated through vinculin and its link to actin [77].

Fluorescence images revealed that cells exposed to 50-80 mW/cm² expressed more noticeable cytoplasmic vinculin than the untreated controls, and that vinculin-rich sites were most clearly concentrated at the cell edges at 60 mW/cm². Vinculin is not exclusively located within focal adhesions in the cell periphery. It resides in the cytoplasm in an

inactive conformation before being recruited and activated at adhesion sites [152]. The increased peripheral intensity suggests an elevation in focal adhesion assembly, which is strongly associated with enhanced cellular mechanosensing. The elevated cytoplasmic signal implies that adhesion components are actively turning over in response to stimulation rather than remaining static. While the exact kinetic mechanism cannot be determined from fixed fluorescence imaging alone, these observations are consistent with reports showing that mechanical loading regulates vinculin recruitment and localisation within focal adhesions at the cell periphery [153].

Notably, when the DC was increased to 40% ($I_{\text{SATA}} = 120 \text{ mW/cm}^2$) the cells were smaller, and more rounded, indicating that mechanosensing and adhesion to the substrate may have decreased. Extended pulse duration most likely disrupts actin filaments and impairs cell attachment to the substrate. The observation that a 20% DC elicits a more favourable mechanosensitive response than a 40% DC aligns with the findings by Hsu *et al.* [154]. In their study on human chondrocytes, a 20% DC at 67 mW/cm^2 maximised extracellular matrix production, whereas increasing the DC at a matched intensity induced cellular stress, as evidenced by elevated nitric oxide levels, which are associated with inflammatory responses. This supports the hypothesis that extended pulsed durations may surpass a beneficial mechanical threshold, potentially inducing cytoskeletal disruption or cell stress.

The effective stimulation range identified in this chapter closely aligns with intensities reported in previous *in vitro* LIPUS studies. Xie *et al.* demonstrated that stimulating hMSCs within a $30\text{-}80 \text{ mW/cm}^2$ at 20% maximised cellular proliferation at $50\text{-}60 \text{ mW/cm}^2$ after a brief, 5-minute exposure, though prolonged exposure diminished this effect [115]. A similar intensity window has been shown by Xia *et al.* to activate the p38/MAPK mechanosensitive signalling pathway, which regulates inflammation, cell differentiation and growth [143], [155]. However, while the nominal intensities between these literature reports and the current study are in agreement, a direct comparison requires careful nuance. The use of a direct coupling method without characterising the ultrasound field at the cell substrate introduces experimental uncertainty. This is because it is not possible to know exactly how much energy reached the cells or whether thermal and mechanical effects could be separated.

Temperature monitoring during ultrasound exposure showed no measurable increase within the Biocell vessel across all conditions in this chapter. In fact, the highest

temperature rise recorded was $0.2\text{ }^{\circ}\text{C}$ at $I_{\text{SATA}} = 120\text{ mW/cm}^2$. For comparison, there is a $\pm 0.2\text{ }^{\circ}\text{C}$ variation in the temperature control of a standard cell culture incubator. Under the same conditions, the highest mechanical index (MI), an indicator of the likelihood of cavitation-related mechanical damage, was 0.07. The clinically accepted upper safety value for MI, below which cavitation effects are considered negligible, is 0.7 [156]. These observations suggest that the cellular responses observed here arise from the primary ultrasound field rather than thermal or other cavitation effects.

A key aspect of this work is the precise control of acoustic energy at the cell layer through the careful design of the tank setup and Biocell vessel. This gives greater confidence that the changes observed here arise from the primary ultrasound field rather than thermal or other secondary effects.

3.7 Conclusions

This chapter examined how MG63 cells respond to changes in ultrasound pressure and duty cycle at fixed carrier frequency and PRF. Across all investigations, cells showed a strongly enhanced mechanosensitive response at 60 mW/cm^2 delivered at 80-160 kPa and 20% DC. At higher intensities or duty cycles, marker expression decreased, showing that stronger exposure does not continuously increase response.

While these findings defined a reproducible stimulation window under well-characterised conditions, the results indicate that I_{SATA} alone cannot fully describe the response. This was evident when comparing two separate experiments that both produced a nominal I_{SATA} of 120 mW/cm^2 but achieved it through different parameter combinations. In one case, (Section 3.5.1), the higher I_{SATA} was reached by increasing ultrasound pressure (20% DC, 113-225 kPa), whereas in the other (Section 3.5.3), it was obtained by raising the duty cycle to 40% at a pressure of 80-160 kPa. Despite the same cumulative dosage, cells exposed to 40% DC exhibited a markedly different response, characterised by rounded morphology, and apparent loss of adhesion, indicative of cell detachment or reduced viability. In contrast, when 120 mW/cm^2 was achieved by ultrasound pressure increase, cells remained relatively spread, and although actin and vinculin expression were reduced relative to 60 mW/cm^2 condition, the decrease was less pronounced. These observations suggest that the mode of energy delivery, specifically, how pressure and duty cycle

combine to produce a given I_{SATA} can substantially influence cellular outcomes and formed the basis for the investigations carried out in the next Chapter.

Building on the fact that changing a single parameter independently, such as ultrasound pressure, duty cycle, or PRF, alters both the overall I_{SATA} delivered to cells and the delivered mechanical stimulation, a different approach has been employed in Chapter 4. To investigate the interdependent relationship between ultrasound parameters under controlled I_{SATA} , two parameters are investigated in coordination so that the total dose remains unchanged, allowing the influence of waveform structure to be explored independently of dose itself. This approach is intended to aid in the identification of the parameter that cells are most responsive to.

Chapter 4: The effect of I_{SATA} on MG63s

4.1 Introduction

Ultrasound can produce a range of biological effects depending on the exposure parameters, and no single dosimetry quantity can capture the full spectrum of possible outcomes. This is particularly relevant in ultrasound-stimulated bioeffects, where cell response is governed by distinct waveform characteristics such as pressure, duty cycle, and pulse repetition frequency. Nevertheless, much of the literature continues to report dosage and compare exposure conditions primarily using I_{SATA} . Because the same I_{SATA} can be achieved by using entirely different combinations of ultrasound parameters, studies that report a constant I_{SATA} while altering parameters such as duty cycle or PRF, may expose cells to fundamentally different stimulation conditions. This oversight limits our understanding of ultrasound-induced biological effects.

Furthermore, I_{SATA} is a time-averaged measure, and it does not reflect the peak pressures and pulse durations that cells experience during LIPUS stimulation. As a consequence, it may obscure the specific waveform features responsible for driving biological responses. Clarifying which of these waveform parameters most strongly influence biological outcomes is essential for understanding and optimising ultrasound therapy for tissue healing.

To investigate whether I_{SATA} alone is sufficient to predict cellular responses or identify the most influential parameter to cellular mechanosensitivity, two separate experiments were designed in which I_{SATA} was held constant while different ultrasound parameters were varied. In the first approach, pulse repetition frequency and burst duration were adjusted while maintaining a fixed duty cycle and I_{SATA} . In the second, ultrasound pressure and duty cycle were adjusted to maintain a constant I_{SATA} of 60 mW/cm^2 , which had previously been shown to be the most effective for MG63 mechanosensitivity.

4.2 Literature review

Very few investigations have acknowledged the dependence of I_{SATA} on other ultrasound parameters and have explicitly examined how variations in parameters such as duty cycle and pulse repetition frequency affect cellular response.

Hsu *et al.* conducted a systematic investigation into the effects of ultrasound I_{SPTA} (33, 67, and 100 mW/cm²), DC (20, 50, 100%), and PRF (100 Hz or continuous), on human chondrocytes [154]. The study employed the inverted tank-dip configuration, in which a 24-well plate was placed above a 1 MHz transducer mounted at the base of a thermostatically controlled, degassed water bath lined with antireflective materials. Ultrasound was delivered and compared across nine different conditions with matched intensities but varying DCs and *vice versa*. The results demonstrated that 67 mW/cm² at 20% DC produced the most favourable outcomes by significantly enhancing cell proliferation and glycosaminoglycan (GAG) production while minimising nitric oxide levels, which are associated with inflammatory responses. Interestingly, increasing the DC at the same I_{SPTA} led to elevated nitric oxide levels, suggesting that shorter pulse durations may be more effective at stimulating chondrocyte activity without inducing stress. Similarly, increasing I_{SPTA} did not yield additional benefits. These findings demonstrate that maximising favourable cellular outcomes depends on specific parameter combinations rather than the total intensity delivered, emphasising the need for careful parameter tuning.

Hua *et al.* employed a similar experimental setup in which the ultrasound transducer was coupled to the multiwell plates using acoustic gel [157]. This study systematically investigated nine combinations of LIPUS intensities (20, 40, 60 mW/cm²) and DC (20, 50, 80%) at fixed PRF (100 Hz) and 1.5 MHz carrier frequency in induced pluripotent stem cells (iPSCs). Their findings revealed that 40 mW/cm² and 50% DC (LIPUS40/50) produced the most consistent enhancement of osteogenic markers, including ALP activity, mineralisation, and the expression of Runx2, collagen I, and OCN, via activation of the Wnt/ β -catenin signalling pathway. Because the study maintained a fixed PRF, altering the DC directly manipulated the pulse duration (50% DC = 5 ms ON, 5 ms OFF). Therefore, the observed optimal effects may be specific to this PRF setting. This highlights the need to consider interactions between DC and PRF, to truly understand which individual waveform parameter drives the cellular responses.

LIPUS has been shown to influence both proliferation and osteogenic differentiation of human adipose-derived stem cells (hASCs) and bone marrow-derived mesenchymal stem cells (hMSCs) with PRF emerging as a key parameter [85]. The study employed the direct coupling method without the coupling gel by positioning a 1 MHz transducer in the near field beneath BioFlex culture plates that have low transmission loss (0.6 dB) measured using a hydrophone. An overall dose of 30 mW/cm^2 I_{SATA} was administered at PRFs of 1, 100, and 1000 Hz while keeping DC at 20%, inevitably this will have required there to be an adjustment in the number of cycles to keep I_{SATA} constant across conditions.

The authors reported reduced proliferation in all LIPUS-treated groups by day 7 for hASCs and day 14 for both hMSC donors compared to untreated controls. Calcium accretion normalised to DNA content was assessed as an indicator of osteogenic differentiation and showed significant increase in hASCs by day 7, particularly at 1000 Hz PRF. hMSCs from two donors showed variability, with donor 2 producing more calcium overall, but both followed a similar trend of increased calcium accretion with increasing PRF with the highest accretion reported at 1000 Hz. These findings suggest that LIPUS, particularly at 1000 Hz PRF, may promote early osteogenic commitment in stem cells, highlighting the importance of optimising PRF for regenerative applications.

Monici *et al.* investigated the effects of LIPUS on osteoclast precursor cell line FLG 29.1, which plays a key role in bone resorption associated with osteoporosis [86]. The study employed a 10 MHz immersion transducer delivering pulses at PRFs of 1, 100, and 1000 Hz, with exposure durations ranging from 5 to 60 minutes, I_{SPTA} of 0.1, 10, and 100 mW/cm^2 , while the peak negative pressure was kept constant at 250 kPa across all conditions. Their study demonstrated that these cells are highly responsive to PRF of ultrasound with stronger inhibitory responses at higher PRFs and longer exposure durations, as evident by the dose-dependent suppression of proliferation and differentiation, and disruption of actin. These findings underscore the importance of PRF in modulating cellular responses to mechanical stimuli and support the potential of ultrasound in suppressing osteoclastogenesis. However, the investigation employed a modified benchtop dip method where the cell suspension was gently stirred to minimise the formation of standing waves. While this approach may reduce standing wave formation, the absence of direct field characterisation means that their presence cannot be excluded, which may have influenced the observed cellular responses.

In summary, controlled investigations that examine the interplay between ultrasound I_{SATA} , pulse repetition frequency, duty cycle, and pressure have demonstrated that LIPUS can significantly enhance mineralisation and the expression of osteogenic markers in iPSCs, promote proliferation in human chondrocytes, facilitate early osteogenic commitment in both adipose-derived and bone marrow-derived stem cells, and inhibit osteoclast activity at elevated frequencies, PRFs, and intensities. Furthermore, improved regenerative outcomes have been observed in cranial bone defects, and hip fracture models.

Despite the documented biological effects that LIPUS can induce, the reported optimal conditions remain inconsistent across studies. Although ultrasound parameters such as pressure, duty cycle, PRF, and nominal intensity are reported, the studies employ different experimental configurations, cell types, and specific parameter combinations, making direct comparison difficult. As a result, the extent to which I_{SATA} as a standalone metric is sufficient to describe the effective dose experienced by the cells or to reliably predict biological outcomes remain unclear. Notably, findings from the previous chapter suggest that maintaining a constant I_{SATA} while varying waveform parameters may lead to different cellular responses; however, this observation remains indicative and requires a systematic and controlled investigation.

The investigations presented in this chapter were designed to control and decouple key ultrasound parameters that are often confounded in the literature. Specifically, the effects of varying duty cycle and ultrasound pressure while maintaining a constant I_{SATA} were explored, enabling a more precise evaluation of their individual effects on cellular responses and the adequacy of I_{SATA} as a dosing metric. A second experimental design isolates the independent effects of PRF by holding duty cycle constant and adjusting the number of cycles to preserve an I_{SATA} of 30 mW/cm². Together, these experiments aim to clarify the extent to which specific waveform parameters, beyond I_{SATA} , govern the mechanobiological effects of LIPUS.

4.3 Chapter methodology and parameter overview

All experiments described in this chapter were performed using the Biocell exposure system, cell culture conditions, immunofluorescence protocols, and statistical analysis

detailed in Chapter 2. Only the procedures specific to the parameter variations investigated here are described below.

4.3.1 Varying pulse repetition frequency and pulse width at a constant I_{SATA} of 30 mW/cm²

PRF determines how often pulses occur, while pulse width defines the duration of each pulse. Together, these parameters dictate the proportion of on-time (or duty cycle) and the temporal spacing between pulses. This approach isolates the effects of pulse timing and repetition on cellular responses from those of total acoustic dose.

Although duty cycle and pulse width are mathematically linked through PRF, they describe distinct aspects of pulsed ultrasound. DC represents the overall proportion of transmission as a percentage, whereas pulse width specifies the duration of a single acoustic burst, measured in seconds. Different combinations of PRF and pulse width can therefore yield the same DC. Because ultrasound intensity depends on the total active time, modifying any of these parameters alters the total energy delivered.

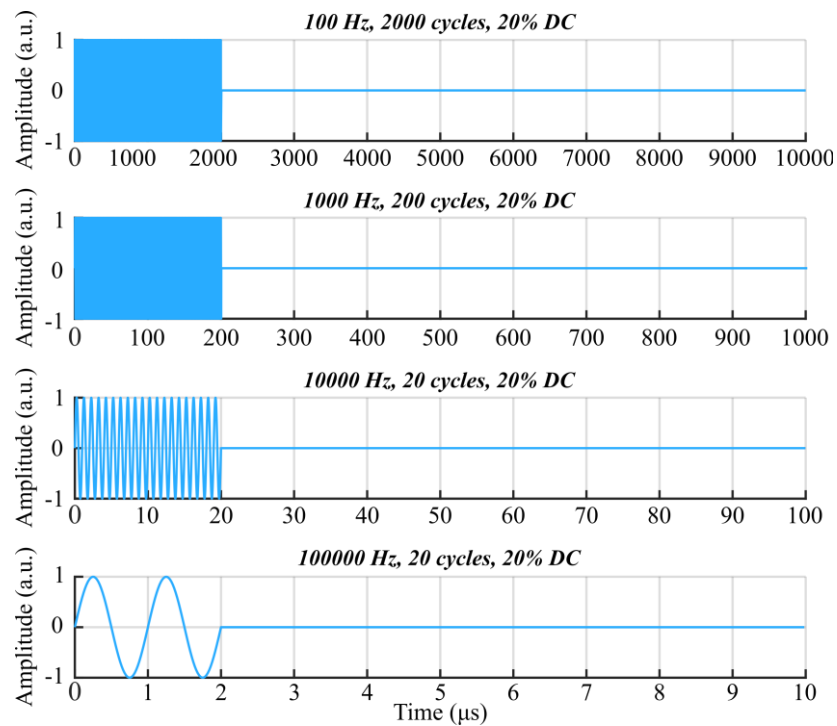


Figure 25. Schematic representation of pulsed ultrasound sine waveforms at different pulse repetition frequencies (100 Hz, 1 kHz, 10 kHz, and 100 kHz) with constant duty cycle (20%). The number of cycles per pulse is adjusted accordingly to maintain constant I_{SATA} at 30 mW/cm².

To investigate the effects of varying PRF while maintaining constant duty cycle (DC) (20%) and I_{SATA} (30mW/cm²), LIPUS was applied at four distinct PRFs: 100, 1000, 10000, and 100000 Hz. The carrier frequency (f_c) was held constant at 1 MHz across all conditions. The duty cycle was fixed by adjusting the number of cycles (N_{cycles}) per pulse, which yielded 2000, 200, 20, and 2 cycles per burst for PRFs of 100, 1000, 10000, and 100000 Hz, respectively, according to the following relationship:

$$DC = PRF \times \frac{N_{cycles}}{f_c} \quad \text{Equation 15}$$

A schematic representation of the resulting waveforms is shown on Figure 25.

4.3.2 Varying ultrasound pressure and duty cycle at a constant I_{SATA} of 60 mW/cm²

This experiment investigated how variations in ultrasound pressure and duty cycle influence cellular response when the spatial average temporal average intensity (I_{SATA}) was maintained constant at 60 mW/cm². The DC was increased to 20, 40, and 60% by proportionally reducing the peak-to-peak ultrasound pressure (p_{pk-pk}). This adjustment ensured that the total energy delivered remained consistent across all conditions.

All calculations were performed within the linear operating regime of the transducer, as confirmed by the voltage-pressure sweep presented in Chapter 2, Section 2.5.2. The condition of 80-160 kPa peak-to-peak pressure, and 20% DC was selected as the reference for the calculations, which was previously identified as optimal (Chapter 3). To maintain a constant I_{SATA} while increasing the duty cycle, the required pressure amplitude for each condition was calculated using the expression:

$$I_{SATA} = p_{pk-pk}^2 \times DC \quad \text{Equation 16}$$

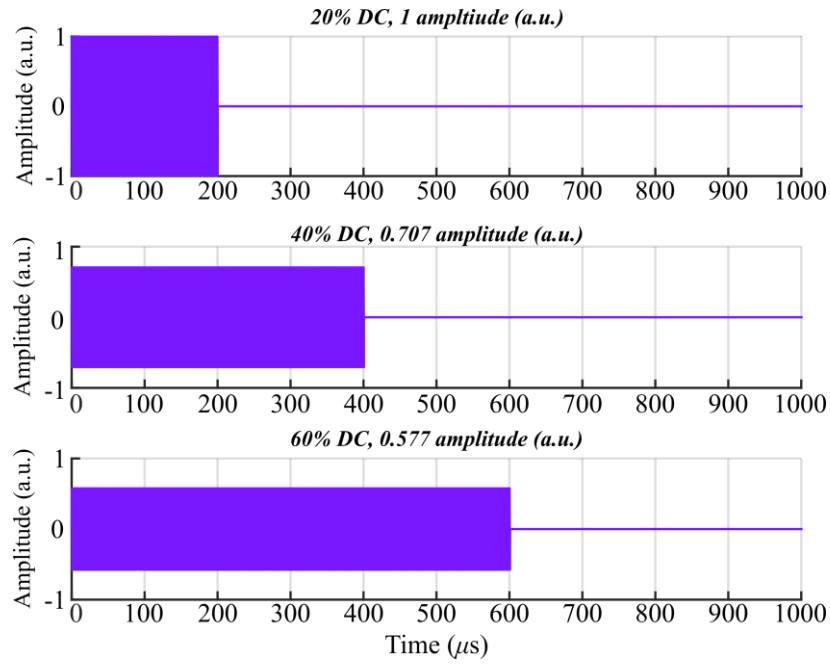


Figure 26. Schematic representation of the pulsed ultrasound sine waveforms used in the experimental conditions, all corresponding to I_{SATA} of 60 mW/cm². Equivalent I_{SATA} were achieved using different combinations of DC (20%, 40%, and 60%) with the corresponding scaling in ultrasound pressure.

Therefore,

$$p_{pk-pk} \propto \sqrt{\frac{1}{DC}} \quad \text{Equation 17}$$

This inverse square root relationship ensured that as the *DC* increased, the ultrasound pressure decreased accordingly. Pressure was modulated by adjusting the transducer input voltage and verified using a calibrated hydrophone. The waveform schematics are presented in Figure 26. This experimental design enabled the investigation of the temporal effects of ultrasound (via DC modulation). All other parameters, including carrier frequency (1 MHz) and PRF (1000 Hz) were held constant. A summary of the two investigations in this chapter is presented in Table 3.

The conversion between measured on-axis pressure and spatial-average intensity depends on the beam uniformity ratio $BUR = \frac{I_{SPTA}}{I_{SATA}}$, which accounts for the non-uniform spatial distribution of intensity across the beam. In this study, BUR corrections were not applied

Table 3. Summary of exposure parameters used across the experiments in this chapter.

Experiment Section	Peak-to-peak pressure (kPa)	PRF (Hz)	Burst length (ms)	DC (%)	I _{SATA} (mW/cm ²)	Exposure duration
4.4.1. PRF-DC	56-113	100, 1000, 10000, 100000	2000, 200, 20, 2, respectively	20	60	10 minutes
4.4.2. Ultrasound pressure – DC	80-160, 57-114, 46-92	1000	200, 400, 600	20, 40, 60	60	10 minutes

explicitly because all calculations were anchored to the hydrophone-measured pressures within the characterised -6 dB field region, thus implicitly incorporating the beam's spatial averaging within the characterised -6 dB field region.

4.4 Results

4.4.1 Cellular response decreases with increasing the modulation frequency at a constant I_{SATA}

This experiment explored the effects of pulse repetition frequency on cell response by varying across 100, 1000, 10000, and 100000 Hz corresponding to pulse widths of 2000, 200, 20, and 2 ms respectively while all other parameters including the carrier frequency (1 MHz), duty cycle (20%), I_{SATA} (30 mW/cm²) and ultrasound pressure (56-113 kPa) were held constant. This experiment employed an I_{SATA} of 30 mW/cm² and an exposure duration of 20 minutes per sample, reflecting parameters commonly used in commercial devices.

The nucleus shown in Figure 27A showed significant differences in area upon exposure to varying PRFs. A statistically significant (***) $p < 0.001$ increase was observed at 100 and 1000 Hz compared to the untreated control, followed by a progressive and significant

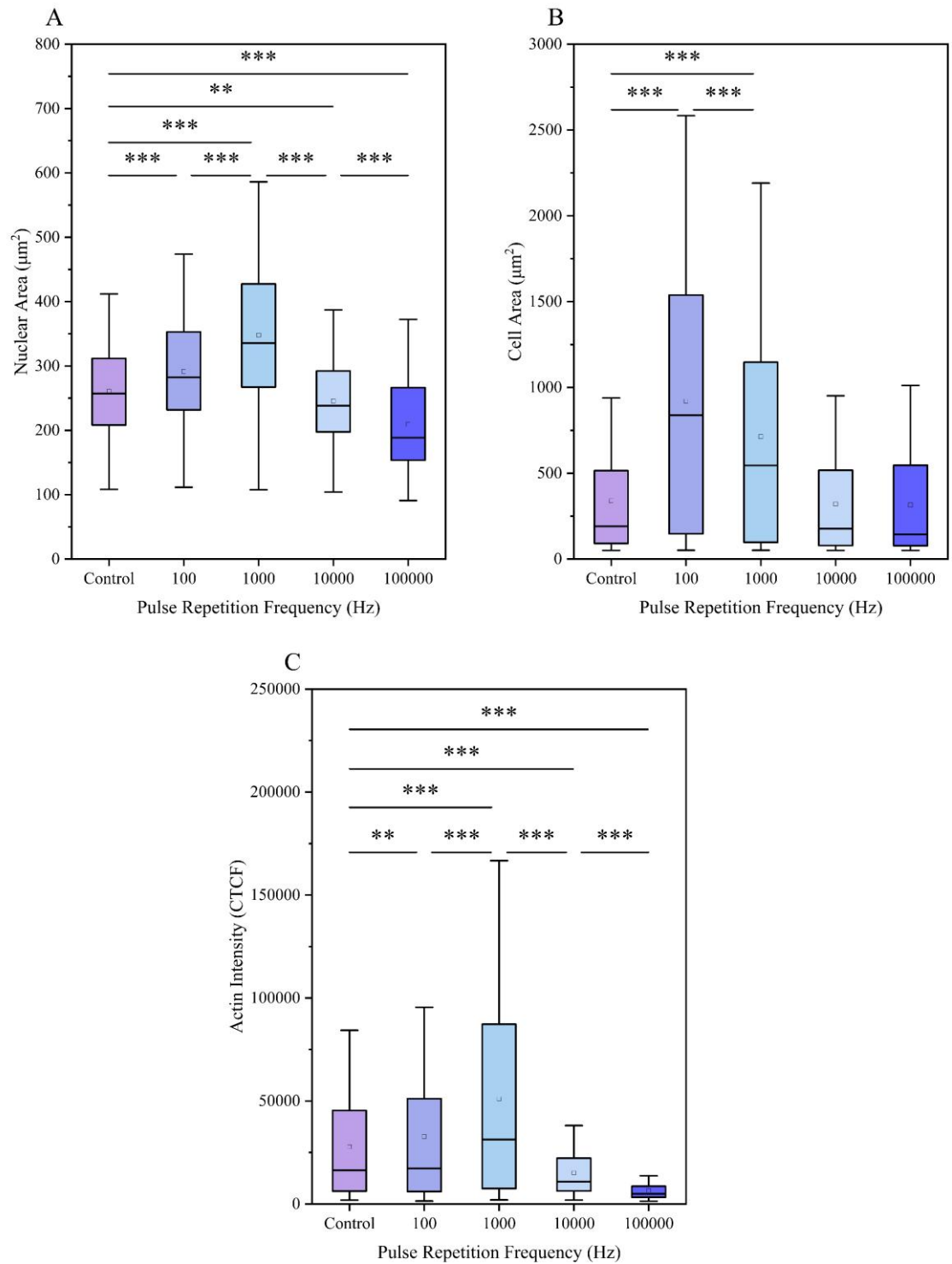


Figure 27. Effect of PRF on MG63s at a constant I_{SATA} . (A) Nuclear area, (B) Cell area, (C) Actin intensity (CTCF), following LIPUS exposure at 0 (control), 100, 1000, 10000, 100000 Hz pulse repetition frequency, 20% DC, and $I_{SATA} = 30 \text{ mW/cm}^2$. Boxplots show interquartile ranges, with medians (horizontal lines), means (squares), whiskers representing $1.5 \times \text{IQR}$, and individual points denoting outliers. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test (***p < 0.001, **p < 0.01). (E) Representative fluorescence images of MG63 cells showing nucleus (DAPI, blue), actin filaments (FITC-phalloidin, green), vinculin (TRITC, red) and merged channels at 40% DC.

decrease at 10000 and 100000 Hz. The highest mean increase in nuclear area was reported at PRF of 1000 Hz, despite all exposures being delivered at constant I_{SATA} .

A similar trend was observed in cell area (Figure 27B), with a statistically significant mean value at lower frequencies (100, 1000 Hz), compared to the control, while for all other frequencies it was similar to control levels. Interestingly, cell area was significantly higher at 100 Hz compared to 1000 Hz. Actin (Figure 27C) also showed a similar trend to cell and nuclear area with increased intensity at 100 and the greatest increase at 1000 Hz compared to the control. A significant decrease was observed at higher frequencies ($***p<0.001$).

Collectively, the results indicate that cells respond strongly to 100 and 1000 Hz PRF, as suggested by the increased nuclear and cell area as well as actin intensity. The higher actin intensity observed at 1000 Hz in combination with a smaller cell area compared to the 100 Hz condition suggests a denser and more tightly packed actin network. This likely reflects increased actin polymerisation, which is typically associated with focal adhesion maturation. However, additional analysis of focal adhesion-related proteins, such as vinculin expression and distribution, would be required to confirm this link.

These findings demonstrate that waveform frequency strongly modulates MG63 cell morphology and cytoskeletal organisation even under constant I_{SATA} . Cells exhibited the most favourable morphology and highest levels of actin CTCF and nucleus area at 1000 Hz, suggesting that this regime was most effective in promoting cytoskeletal and nuclear response. The responses indicate that cells are sensitive not only to total energy delivered but also to the temporal pattern within the stimulation waveform.

4.4.2 Cellular responses diminish with increasing duty cycle and decreasing ultrasound pressure at constant I_{SATA}

In this investigation, to keep I_{SATA} constant at 60 mW/cm² the duty cycle was systematically varied while ultrasound pressure was adjusted accordingly. The specific combinations used and their nomenclature in brackets, were 80-160 kPa pressure and 20% DC (160_20), 5-114 kPa at 40% (114_40) and 46-92 kPa at 60% (92_60). All other ultrasound parameters were held constant including carrier frequency (1 MHz), and 1000 Hz pulse repetition frequency for 10 minutes.

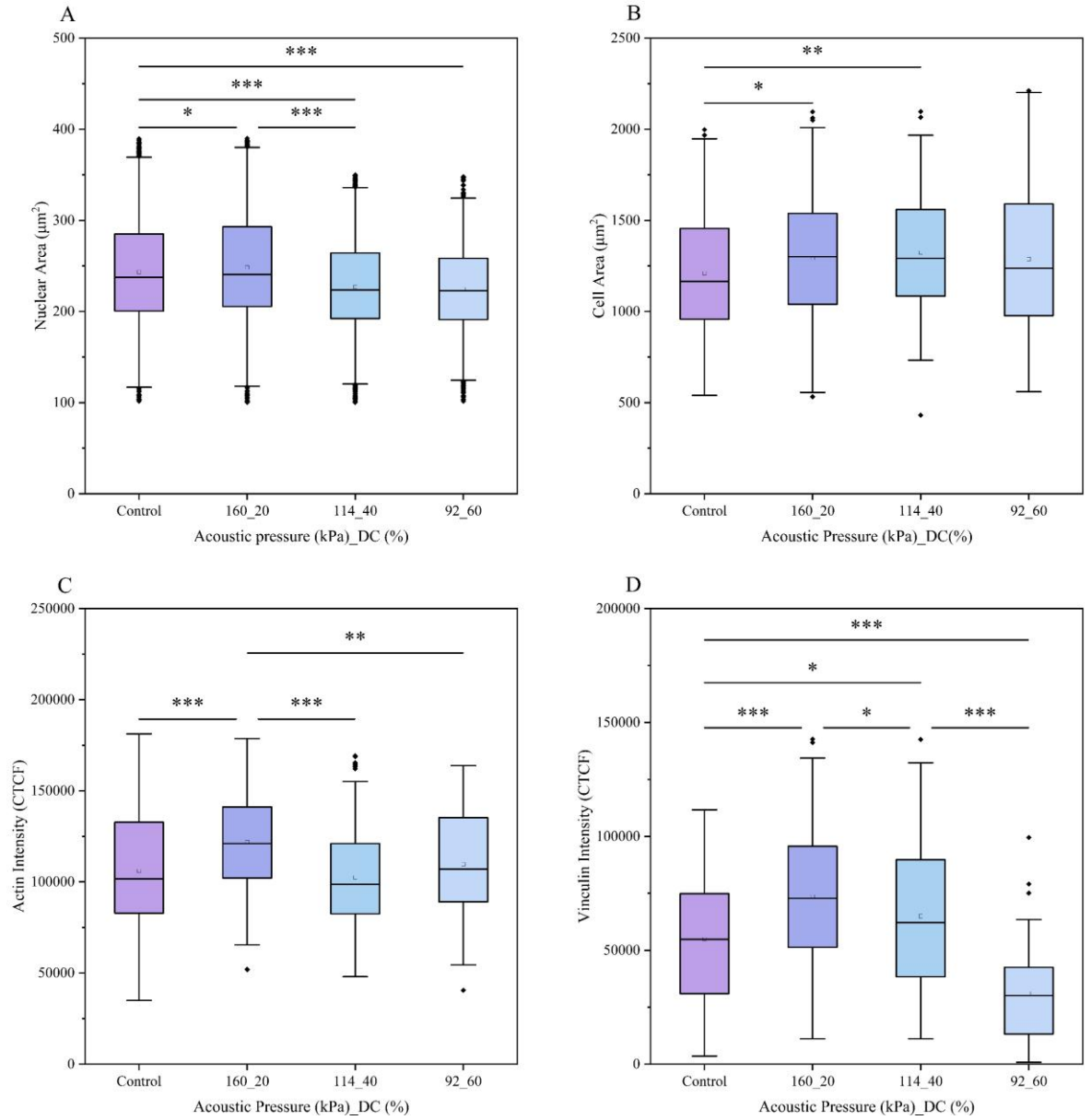


Figure 28. Effect of varying duty cycle and acoustic pressure under constant I_{SATA} on MG63s. (A) Nuclear area, (B) cell area, (C) Actin intensity (CTCF) and (D) Vinculin CTCF at constant I_{SATA} of 60 mW/cm² achieved by varying acoustic pressure and duty cycle: 160 kPa at 20%, 114 kPa at 40%, and 92 kPa at 60%, alongside an untreated control. Despite equivalent energy delivery, increasing DC combined with decreasing pressure resulted in progressively lower cellular responses. Boxplots show interquartile ranges, with medians (horizontal lines), means (squares), whiskers representing 1.5 x IQR, and individual points denoting outliers. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test (** $p < 0.001$, ** $p < 0.01$).

Nuclear area (Figure 28A) showed a statistically significant increase at LIPUS 160_20 compared to the untreated control samples, while all other conditions resulted in significant decrease in nuclear area compared to control. Cell area (Figure 28B) increased significantly ($*p < 0.05$) compared to control under the 160_20 condition, with the greatest increase observed under the 114_40 condition ($**p < 0.01$). In contrast, the 92_60 condition remained relatively unchanged compared to the untreated.

Actin intensity (Figure 28C) followed a more pronounced trend with statistically significant increase at the 160_20 condition compared to all other conditions. At 114_40 and 92_60 conditions, the intensity of actin did not change significantly compared to the control. Vinculin intensity (Figure 28D) however, increased significantly at 160_20 and 114_40, with both conditions showing strong increases ($*** < 0.001$). In contrast, intensity was significantly lower at 92_60 with a marked reduction relative to all other conditions.

Overall, the trends show that high ultrasound pressure at short duty cycles is more effective for ultrasound induced mechanosensing in MG63 cells. When comparing the 160_20 condition to the other stimulation regimes, a decline in actin and vinculin CTCF emerges, while cell and nuclear area either remain unchanged or decrease in area. This demonstrates that prolonging exposure duration by increasing the duty cycle, even at constant I_{SATA} , does not enhance the cellular responses. The differing response observed across the conditions exposed to ultrasound indicate that the averaged acoustic intensity alone is insufficient to predict cellular outcome, highlighting that individual ultrasound parameters are more influential than I_{SATA} in driving intracellular response.

4.5 Discussion

To evaluate if I_{SATA} is a reliable indicator of the mechanical stimulus experienced by cells, two sets of experiments were conducted in which ultrasound parameters were varied. In the first experiment, pulse repetition frequency was altered while keeping other parameters unchanged. In the second, duty cycle and pressure were adjusted together to compensate for one another and preserve a constant I_{SATA} , using the cellular response as a functional readout of the underlying mechanical stimulus to assess whether such fields are mechanically equivalent.

In the initial experiment presented in this chapter (Section 4.4.1), significant changes in cell area, nuclear area, and actin intensity were observed across conditions, demonstrating that PRF strongly influences the mechanically induced response of cells despite the total average energy delivery. At both 100 and 1000 Hz, cell area, nuclear area and actin intensity increased significantly compared to the untreated control. However, at 1000 Hz cell area and actin intensity increased significantly compared to 100 Hz. At frequencies beyond 1000 Hz, all measures of mechanosensing decreased significantly compared to the control. Similar PRF-dependent behaviour has been previously reported by Marvel *et al.*, who identified 1000 Hz as the most effective condition for promoting osteogenic responses in hASCs and hMSCs [85]. This agreement suggests the existence of an optimal PRF range beyond which effectiveness diminishes, further highlighting the importance of looking at LIPUS parameters as individual factors that affect cellular response and not describe them solely by using I_{SATA} .

From a mechanical perspective, altering PRF while holding the duty cycle constant alter the temporal distribution of the applied stimulus (or the number of cycles). Lower PRFs deliver fewer, longer pulses per second, whereas higher PRFs deliver shorter pulses in rapid succession. A possible explanation for the observed bioeffects is that a very high PRF limits the ability of mechanosensitive structures to recover and respond effectively to the repeated loading which would lead in accumulation of momentum. Whereas, at lower PRFs the recovery time and stimulation frequency may provide a more favourable environment for the cells, explaining the more favourable cellular responses at lower PRFs.

In the second experiment, duty cycle was increased while pressure was decreased accordingly to maintain constant I_{SATA} . Nuclear area exhibited a significant increase at 160_20, followed by a stepwise reduction at 114_40 and 92_60, suggesting progressively decreasing nuclear response. In contrast, cell area continued to rise from 160_20 to 114_40, before plateauing at 92_60, indicating that moderate changes in duty conducted different effects on cell spreading. Actin intensity increased significantly at 160_20, and no further enhancement at the other conditions. Similarly, vinculin expression peaked significantly at 160_20, was elevated at 114_40 and declined at 92_60, reinforcing the notion that higher pressures at lower duty cycles provide a more effective mechanical stimulus for focal adhesion related mechanosensitive responses.

This observation is particularly relevant given the established roles of actin and vinculin in cellular mechanotransduction as well as the role of the nucleus which indicates altered genetic information, essential for mechanotransduction to take place. Actin forms the primary structural component of the cytoskeleton, while vinculin links focal adhesions to the actin network, enabling transmission of mechanical signals between the extracellular and intracellular environment [48].

Despite matched I_{SATA} across all conditions, higher pressure and lower duty cycle combinations produced greater increases in actin intensity, vinculin intensity, cell spreading, and nuclear tension than conditions employing lower pressures and longer duty cycles. This suggests a threshold dependent mechanism for cellular mechanosensing. Mechanosensitive structures require a critical peak force to switch into active conformations. Low pressure regimes, regardless of the extended duration, fail to reach this activation threshold. Furthermore, high-pressure pulses induce a greater strain rate at the cell substrate interface, generating the rapid mechanical tension necessary to drive the observed responses. This rationale informed the investigation presented in Chapter 5, where the influence of strain rate is isolated by modifying the pulse envelope geometry while keeping the peak pressure constant to decouple the effects of strain rate from pressure.

These results align with previous literature highlighting that the biological effects of LIPUS are governed not only by the total energy dose, but also by the temporal distribution and peak magnitude of acoustic forces, emphasising the importance of waveform structure in modulating cellular responses [82], [158]. Temperature monitoring during exposure showed no measurable increase within the Biocell, and the calculated MIs remained well below the cavitation threshold (>0.7), further confirming that the observed effects are due to LIPUS exposure and not secondary thermal or cavitation mechanisms (Appendix B).

4.6 Conclusions

The work presented in this chapter advances the understanding of LIPUS bioeffects by demonstrating that response to LIPUS cannot be predicted by I_{SATA} alone. Variations in PRF, DC, and pressure amplitude produced significant differences in actin and vinculin

intensities as well as cell and nuclear area, emphasising the importance of waveform characteristics in modulating cell mechanosensitivity.

The parameter range investigated in this study was restricted to selected PRF values and three pressure-DC combinations and therefore does not capture the full spectrum of possible ultrasound settings. Within this range, the observed effects were consistent across biological replicates and reproducible between experiments, supporting the reliability of the trends reported. However, it remains unclear whether the observed effects are driven primarily by pressure magnitude, strain rate, or the resulting deformation induced at the cell-substrate interface. To ensure physical reliability of the findings, all ultrasound waveforms used throughout this thesis are characterised using LDV to measure displacement directly on the cell substrate, despite of the substrate material properties. This is particularly important as substrate properties can alter the mechanical input cells receive under otherwise identical ultrasound conditions.

Chapter 5: Influence of waveform rise time on cellular response and membrane displacement in LIPUS

5.1 Introduction

The previous chapters established that cells are sensitive to ultrasound pressure, duty cycle, pulse repetition frequency, and spatial-average temporal-average intensity. While these parameters are essential for characterising the ultrasound field and for safety, they describe the fluid environment rather than the mechanical stimulus experienced at the cell-substrate interface. Relying purely on these bulk metrics fails to capture the instantaneous, real-time substrate displacement or dynamic acoustic radiation force, which are more likely to describe cellular response [94].

At the cellular level, the effective mechanical stimulus is heavily mediated by local conditions at the cell-substrate interface, where substrate stiffness influences force transmission and mechanosensing through the molecular clutch [70], [159]. Therefore, the mechanical stimulus experienced by the cells may differ substantially from the externally applied input. Furthermore, mechanobiology studies have suggested that cellular responses depend not only on the magnitude of the applied force, but also on the rate at which it is applied (force loading rate or strain rate) [70], [160]. For instance, altering the signal windowing profile (e.g. from rectangular envelope to smoother Hanning envelope) modifies the onset phase of the applied stimulus and therefore the rate at which mechanical stimulation is imposed on the substrate. However, in ultrasound-mediated mechanotransduction, the influence of strain rate remains poorly understood.

Directly measuring the substrate displacement under different LIPUS regimes is therefore essential to identify the precise mechanical cues governing ultrasound bioeffects. In this chapter, LDV was utilised to directly measure the displacement amplitude generated at the cell substrate and investigate the effects of pulse envelope profiles. A strain rate experiment was also conducted to evaluate how altering the strain rate by changing the signal windowing profile impacts the subsequent cellular responses. These investigations aim to establish a direct link between ultrasound waveform design, substrate kinematics, and cellular response.

5.2 Literature review

In mechanobiology, cellular response has been shown to depend on the force magnitude as well as the rate at which it is applied, often referred to as the force loading rate [160]. In parallel, it has been proposed that cells respond to deformations of the ECM rather than to force magnitude alone [72], [161], [162]. This deformation is transmitted via the vinculin tail domain and propagates through the cytoskeleton, enabling initiation of mechanotransductive signalling cascades [48], [161]. Nanoscale substrate displacements have been shown to modulate cytoskeletal organisation, focal adhesion assembly, and lineage-specific gene expression [163], [164]. Reilly and Engler reported that both the intrinsic mechanical properties and the dynamic deformation of the substrate can influence cell fate [159].

A study using substrate stretching on a custom PDMS membrane device demonstrated that cellular mechanosensing is governed by the temporal dynamics of force application, with talin-dependent adhesion reinforcement and YAP nuclear localisation occurring within a specific loading-rate regime [160]. Notably, a 1 Hz progressive triangular waveform promoted structural reinforcement, whereas a 1 Hz square waveform which snaps quickly to maximum amplitude led to cytoskeletal fluidisation rather than reinforcement, a disruptive response comparable to that of the 2 Hz triangular waveform. Furthermore, increasing the stretch amplitude elevated the overall force loading rate, promoting talin reinforcement at intermediate levels, whereas, higher tested loading rates led to softening of the actin cytoskeleton. These results demonstrate that cells do not register isolated geometric parameters like frequency and amplitude alone. Rather, cells are sensitive to the force loading rate, determined by the product of matrix stiffness and the displacement velocity of the applied waveform, which is heavily dictated by the shape of the applied waveform.

More recently, a technique known as nanokicking was developed, in which MSCs are subjected to vertical nanoscale vibrations in the range of 10-30 nm. This technique demonstrates that very small amplitude mechanical signals are sufficient to activate mechanotransduction pathways and guide MSC lineage commitment and fate.

Research by Dalby and colleagues demonstrated that human MSCs exposed to 1 kHz, vertical nanoscale vibrations (16-30 nm) can undergo osteogenic differentiation, including

mineralisation, in 3D collagen gels in the absence of chemical osteoinductive cues [163], [164]. By varying vibration frequency while keeping biochemical conditions constant, the group showed that changes in vibration frequency led to changes in substrate displacement and corresponding differences in cellular outcomes, suggesting that the characteristics of substrate motion strongly influence cellular response. Notably, these studies reported little upregulation of osteogenic markers at frequencies beyond 1 kHz, despite investigating frequencies of up to 5 kHz, suggesting the existence of an optimal frequency range within this mode of stimulation. However, because changing the frequency also inherently alters the substrate displacement velocity and loading rate, these results likely reflect a cellular response to shifting loading dynamics rather than to the numerical frequency alone. In sinusoidal motion, these parameters are tightly linked, therefore their individual contributions could not be independently resolved in this setup.

It is worth noting that while 1 kHz nanokicking applies a continuous 1 kHz sinusoidal substrate displacement, LIPUS utilises 1 kHz strictly as a pulse repetition frequency to envelope a much higher 1 MHz acoustic carrier. Consequently, LIPUS subjects cells to 1 MHz acoustic particle velocities and transient radiation force pulses at 1 kHz intervals, rather than a single 1 kHz sinusoidal motion.

More recent work by Ambattu *et al.* extended this concept for continuous waves in the MHz regime and demonstrated that osteogenic differentiation can also be induced at MHz frequencies (10 MHz) using nanoscale displacements of up to 10 nm in magnitude [165]. Unlike the purely out-of-plane vibrations employed in nanokicking studies, stimulation was delivered through surface-reflected bulk waves, which generate a hybrid motion comprising both in-plane (longitudinal) and out-of-plane (transverse) components. This was achieved by tuning the substrate thickness and sound wave wavelength such that the surface wave leaks through the substrate thickness, reflects from the opposite surface, and couples with the original wave to form a hybrid surface and bulk wave [166].

Consequently, the cells are exposed to a combination of pressure and shear waves. Furthermore, because substrate acceleration scales with the square of excitation frequency, this MHz stimulation regime translates to extremely rapid displacement velocities and substantially larger instantaneous force loading rates. This mode of stimulation induced earlier osteogenic commitment occurring within days following short stimulation periods (10 min/day), in contrast to prolonged continuous excitation required in kHz studies.

Together, these findings emphasise that cellular responses to nanoscale mechanical stimulation are influenced by dynamic temporal factors rather than displacement amplitude alone. While nanokicking and surface-reflected bulk wave regimes operate within a similar nanometre displacement range, they differ in excitation frequency, force generation and directionality. These differences collectively highlight that the rate and velocity of energy delivery are primary drivers of biological response. Ultimately, these studies highlight the importance of considering the precise loading dynamics and kinetics when interpreting ultrasound-induced cellular responses.

Pulsed ultrasound does not operate as a single-frequency stimulus; instead, it inherently combines low-frequency stimulation from the pulse repetition frequency with higher-frequency, rapid oscillatory motion of the medium in the order of MHz. As a result, cells exposed to LIPUS experience both nanometre-scale high-frequency mechanical oscillation and lower-frequency displacement arising from acoustic radiation force simultaneously. This distinction is critical as it indicates that pulsed ultrasound stimulation engages mechanosensitive processes through multiple mechanical cues acting across distinct cellular timescales, force amplitudes, and modes of substrate deformation, including both rapid oscillatory motion and sustained displacement during pulse application. Consequently, the effects of pulsed ultrasound cannot be directly compared to either purely kHz nanovibrational systems or continuous MHz excitation alone.

Chapter 4 demonstrated that different stimulation parameter combinations producing the same I_{SATA} elicited distinct cellular responses. One possible explanation is that these differences arise from variations in substrate displacement associated with changes in pressure amplitude. However, the mechanical stimulus experienced by cells is also influenced by local force transmission at the cell-substrate interface and the mechanical properties of the substrate. Consequently, accurate characterisation of substrate motion is essential for understanding how cells perceive and respond to ultrasound stimulation.

In practise, the ultrasound pulse onset and offset are often not explicitly reported, particularly in LIPUS systems. To our knowledge, there is no report in the literature examining whether pulse windowing of the applied acoustic radiation force, can influence cellular responses under controlled energy conditions. To investigate this, the standard 20% DC rectangular-windowed input signal used throughout this thesis was compared with two Hanning-windowed pulse configurations. Using a Hanning window, the amplitude of each

pulse gradually increases to its maximum value rather than rising instantaneously. The first Hanning condition was implemented with the same peak pressure as the rectangular waveform, allowing the effect of temporal loading profile to be examined independent of maximum pressure. However, because windowing inherently reduces the total energy delivered, a second compensated Hanning condition was implemented in which the peak pressure was increased to restore I_{SATA} to the same level as the rectangular waveform.

In the present system, cells adhere to a thin, tensioned mylar membrane constrained by the Biocell frame. While the previous chapters unravelled the effects of pulsed ultrasound parameters on MG63s, it was hypothesised that direct measurements of substrate displacement may provide additional insight into the mechanical stimulus experienced by the cells and aid the interpretation of the observed biological responses. Laser Doppler vibrometry was therefore employed to measure the amplitude and spatial distribution of membrane displacement under each stimulation condition. LDV is a non-contact method for measuring surface velocity and displacement (or surface deformation) with sub-nanometre resolution and has been widely employed to quantify vibration of large engineering structures to thin films and membranes under water [167], [168]. Together, the strain rate and LDV investigations aim to establish how waveform design influences substrate motion and the resulting mechanical cues experienced by adherent cells.

5.3 Chapter methodology

This chapter investigates how the rate of applied strain influences early mechanosensitive responses in MG63 cells cultured within the Biocell *in vitro*. Rectangular-windowed excitation was applied using previously optimised LIPUS conditions that produced the strongest cellular response and is compared with Hanning-windowed excitation under matched peak pressure and matched I_{SATA} conditions. In addition, LDV was used to characterise substrate displacement and frequency content across all pulsed ultrasound exposure conditions investigated throughout this thesis, to measure the resulting out-of-plane displacement of the cell substrate.

5.3.1 *In vitro* exposure

The *in vitro* experiments performed in this chapter followed the same experimental configuration, cell culture protocol, immunostaining, imaging, and statistical analysis procedures described in Chapter 3.

Signals generated in MATLAB were exported to a function generator and amplified to drive the ultrasound transducer using the same hardware previously described. Three distinct waveform conditions were tested during the experiments, displayed on Figure 29. The first condition employed a standard rectangular pulse at 20% DC serving as a control. In the second condition, the rectangular pulse was replaced with a Hanning windowed waveform, carefully scaled to preserve the same peak ultrasound pressure as the rectangular pulse. The third condition used a compensated Hanning-windowed waveform, in which the amplitude was increased to ensure the I_{SATA} matched that of the rectangular condition. All other parameters, including carrier frequency, PRF, and DC, were kept constant to allow for direct comparison across waveform types. For rectangular pulses, the reported pressure range (80-160 kPa) represents the spatial acoustic pressure distribution across the vessel floor during the active pulse phase. In contrast, for rumped pulses, the pressure dynamically scales over time from 0 to a peak spatial range within each pulse

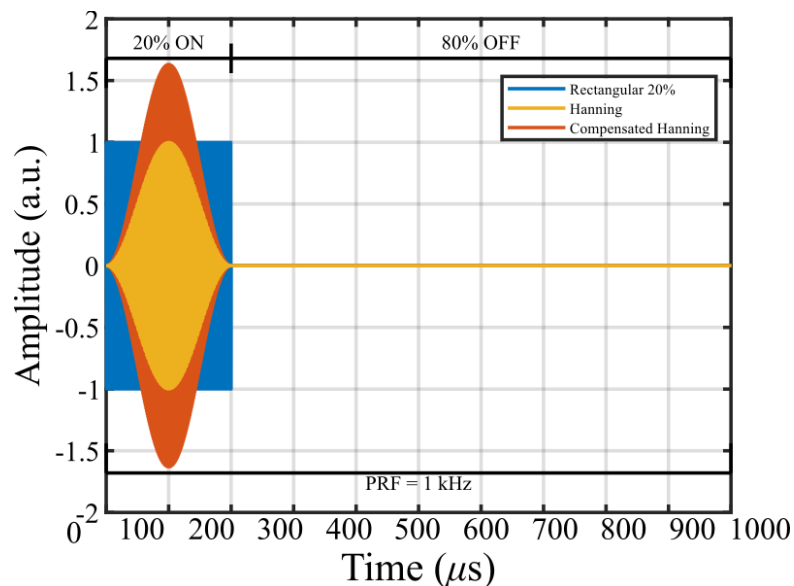


Figure 29. Time domain representation of a 1 MHz carrier burst at PRF of 1 kHz, and 20% DC (20% ON and 80% OFF) over a 1000 μ s interval. Three windowing conditions are shown of rectangular (blue), Hanning (yellow), and compensated Hanning (orange). The compensated Hanning-windowed signal restores I_{SATA} by increasing amplitude while maintaining a tapered envelope.

Table 4. Summary of stimulation conditions used to investigate the effect of pulse envelope shape on cell response and membrane displacement at a carrier frequency of 1 MHz, PRF at 1 kHz, and 20% DC for all conditions.

Condition	Waveform envelope	Peak-to-peak pressure (kPa)	I _{SATA} (mW/cm ²)
A	Rectangle window	80 – 160	60
B	Hanning window	0 – 160	22.5
C	I _{SATA} Compensated Hanning window	0 - 261	60

envelope. Despite matching either peak pressure or I_{SATA}, differences in substrate displacement were anticipated due to differences in waveform envelope and the resulting acoustic radiation force profile. The detailed waveform specifications are presented in Table 4.

5.3.2 Calculating I_{SATA}

I_{SATA} was determined following the approach described in Chapter 2 (Section 2.5.2) for the rectangular waveform. For the Hanning conditions, I_{SATA} was determined analytically based on the known energy characteristics of the Hanning envelope, as described below.

The Hanning window function ($\omega(t)$) applied to the carrier burst has the form of

$$\omega(t) = 0.5 \left(1 - \cos\left(\frac{2\pi t}{T}\right) \right) \quad \text{Equation 18}$$

Where T is the pulse duration and t is the instantaneous time variable.

Since intensity is proportional to the time-average of pressure squared, the relevant quantity is the mean of the squared window function:

$$\omega(t)^2 = \frac{1}{T} \int_0^T \omega(t)^2 dt \quad \text{Equation 19}$$

Expanding the window term gives:

$$\omega^2(t) = 0.25 \left(1 - 2 \cos\left(\frac{2\pi t}{T}\right) + \cos^2\left(\frac{2\pi t}{T}\right) \right) \quad \text{Equation 20}$$

Over one full cycle, the cosine term averages to zero, and the squared cosine term averages to 1/2.

$$\cos(\theta) = 0 \text{ and } \cos^2(\theta) = \frac{1}{2} \quad \text{Equation 21}$$

Substituting into Equation 20 yields,

$$\omega(t) = 0.25 \left(1 + \frac{1}{2} \right) = 0.375 \quad \text{Equation 22}$$

This shows that a Hanning windowed burst contains 37.5% of the energy of an equivalent rectangular burst with the same peak amplitude and duration. The corresponding reduction in I_{SATA} is therefore 62.5%.

Thus, the I_{SATA} of the unscaled Hanning condition was obtained by:

$$I_{SATA}(Hanning) = 0.375 \times I_{SATA}(Rectangular) \quad \text{Equation 23}$$

For the compensation Hanning condition, the input voltage was increased such that:

$$I_{SATA}(Compensation Hanning) = I_{SATA}(Rectangular) \quad \text{Equation 24}$$

Ensuring that only the shape of the amplitude envelope differed between conditions.

Because ultrasound pressure scales linearly with voltage, the intensity scales quadratically.

To restore the 62.5% energy deficit, the input voltage was adjusted such that:

$$V_{compensation} = \frac{V_{rectangular}}{\sqrt{0.375}} \quad \text{Equation 25}$$

5.3.3 Laser Doppler vibrometry setup

The LDV measurements presented in this chapter were performed using the same experimental configuration described in Section 2.6.7, with the same Biocell geometry, transducer-sample distance and tank arrangement (Figure 30). Briefly, the Biocell was mounted vertically and positioned 101 mm from the front face of the ultrasound transducer in a glass water tank. The transducer was positioned at the rear of the tank, increasing propagation distance to minimise reflections from the water-glass interface. The tank was filled with degassed DI water at 37 °C and acoustic absorbers were placed on the tank walls (excluding front wall) to further minimise reflections. A PSV-500 1D scanning laser Doppler vibrometer (Polytec, GmbH, Germany) fitted with the magnification lenses (close-up unit) was aligned normal to the mylar membrane and used to record the normal component of membrane velocity during exposure, as shown in Figure 30.

The LDV data were acquired in FFT mode. This approach was preferred over time-domain acquisition to optimise data storage and computational efficiency, given the high number of scan points. The LDV system was used to trigger the signal generator driving the 1 MHz transducer, ensuring each acquisition began at the same point in the excitation cycle. The

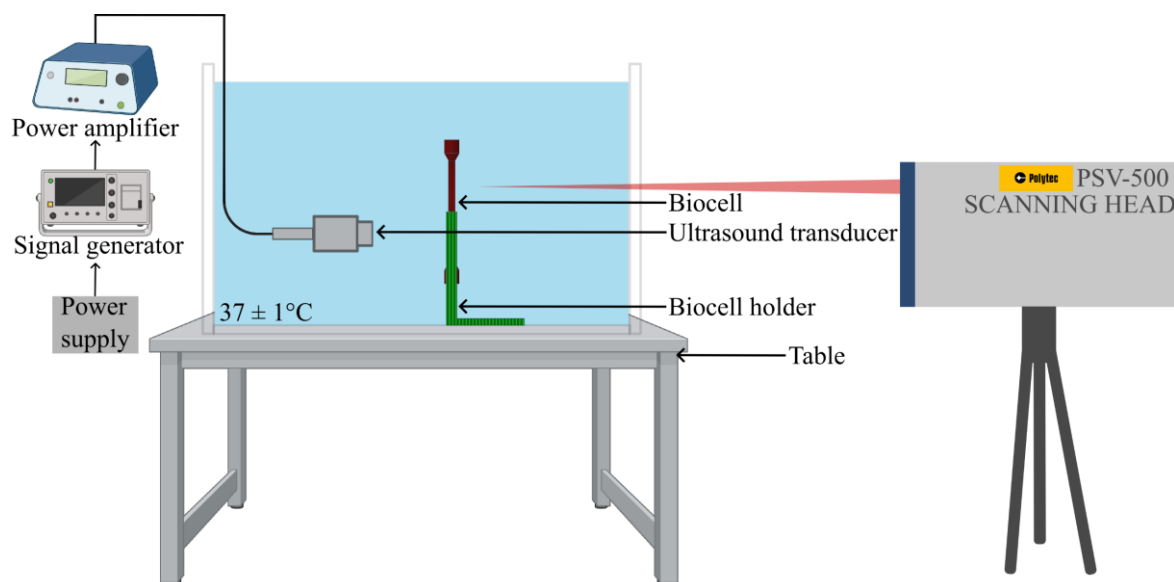


Figure 30. Schematic of the experimental setup employed for the non-contact laser Doppler vibrometry measurements of membrane displacement in submerged culture conditions. An ultrasound transducer, driven by a signal generator and power amplifier, delivers excitation through degassed deionised water maintained at $37 \pm 1^\circ\text{C}$ inside a glass tank. The acoustic wave hits the mylar membrane which is clamped and glued onto the Biocell frame and mounted on a Biocell holder. The PSV-500 scanning LDV head directs a laser beam through the transparent tank wall to measure the resulting membrane displacement without contact.

drive signal was also used as a reference to maintain phase matching between LDV measurements and the ultrasound output, allowing magnitude and phase measurements. Ultrasound stimulation was applied using the LIPUS conditions described in Chapters 3-5.

Velocity signals were sampled using the acquisition board (NI, PCI-6110) at a sampling frequency of 5.12 MHz, providing a frequency resolution of 19.53 Hz over a 2 MHz bandwidth, resulting in 102,400 FFT lines. The vibrometer was configured to record the normal vibration (+Z) velocity component, with a measurement range of 5 V (the lowest setting). A flattop window was applied to the vibrometer receive channel, and a reference was used. Speckle tracking was enabled to improve signal stability through the water path, and the tracking filter was set to fast. The tracking filter improves SNR, by effectively isolating the high frequencies from broadband background noise. This is achieved by phase-locking the receiver's internal oscillator to the carrier frequency returning from the moving mylar target. Measurements were averaged 3 times in complex averaging mode. The velocity decoder (DV-04) provided a 2 MHz bandwidth and a 200 mm/s range, ensuring adequate dynamic range for nanometre-scale displacement measurements. Magnitude and phase for each scan point were exported from the PSV software for further analysis.

The exported spectra were processed using a custom MATLAB script developed for this work (Appendix D). For each scan point, the Polytec export provided the velocity magnitude and phase, the frequency vector, and the X-Y location of the point in the scan. The script read all the files and converted magnitude-phase data into complex velocity spectra. Frequencies below 50 Hz were considered to be noise and therefore removed to improve SNR.

To identify the centre points of maximum membrane vibration, the RMS displacement was calculated within a fixed wide band of $1 \text{ MHz} \pm 200 \text{ kHz}$, and this value was used to rank all points. Ten points with the highest RMS displacement in this wide band were used to identify the locations of maximum vibration and were used for detailed analysis.

For these points, displacement was quantified separately in two fixed bands. A narrow band around the carrier frequency ($1 \text{ MHz} \pm 500 \text{ Hz}$) to extract the carrier-only displacement and a second band centred around the modulation frequency to extract PRF displacement. For each band, the complex velocity values were converted to displacement,

and the time domain signal was reconstructed. The peak-to-peak displacement was calculated from this reconstructed time-domain signal.

5.4 Results

5.4.1 Effect of slow pressure rise via Hanning window on cell response and quantitative measurements of displacement on mylar membrane

In this experiment, the windowing function of the ultrasound signal was changed from a rectangular to a Hanning envelope, while maintaining a 20% duty cycle, 1 MHz carrier frequency, and 1 kHz modulation frequency. Three conditions were tested: Rectangular window ($I_{SATA} = 60 \text{ mW/cm}^2$, 80-160 kPa), Hanning window ($I_{SATA} = 22.5 \text{ mW/cm}^2$, 0-160 kPa), compensated Hanning window with increased pressure amplitude to restore I_{SATA} to 60 mW/cm^2 (0-261 kPa) while all other parameters remained unchanged.

Effect of Hanning window on membrane displacement

Peak-to-peak displacement was measured at 1 MHz and max displacement at 1 kHz, at 20% duty cycle, for the rectangle condition, Hanning-window ($I_{SATA} = 22.5 \text{ mW/cm}^2$) and I_{SATA} compensated Hanning windowed stimulation ($I_{SATA} = 60 \text{ mW/cm}^2$). Peak

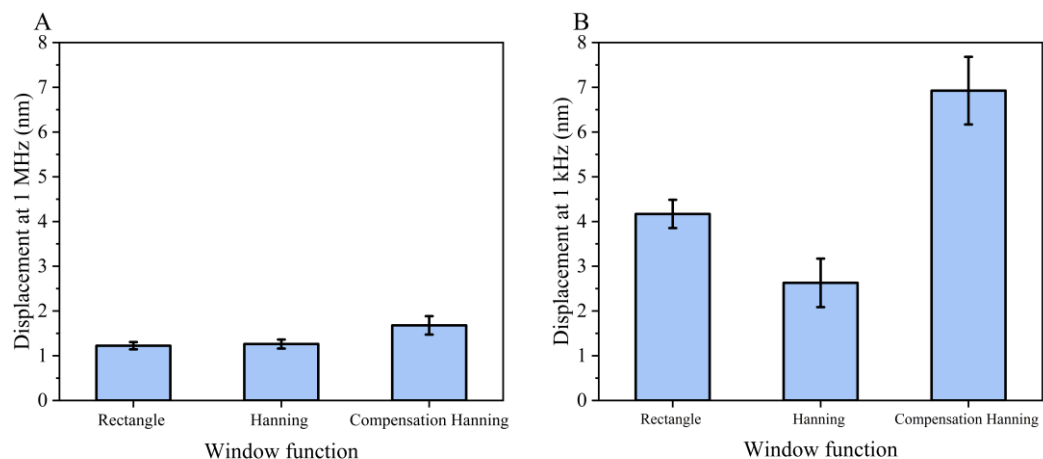


Figure 31. Effect of window function on membrane displacement. (A) Displacement measured at 1 MHz carrier frequency and (B) Displacement measured at 1 kHz modulation frequency. For each condition, 10 individual scan points were measured with three repeated measurements per point. Bars represent the standard error of the mean (SEM).

displacement at 1 MHz was 1.19 ± 0.08 nm for the rectangle condition, 1.26 ± 0.10 nm for the Hanning condition and 1.67 ± 0.20 nm for the compensated Hanning condition. For the modulation frequency (1 kHz) the max displacements were measured to be 4.17 ± 0.31 nm, 2.62 ± 0.54 nm and 6.92 ± 0.75 nm respectively (Figure 31).

Effect of Hanning window on cell and nuclear area, actin and vinculin intensity

Nuclear area (Figure 32A) showed a clear dependence on waveform window, with the rectangular condition producing significantly larger nuclei than both Hanning and compensated Hanning groups ($***p < 0.001$). The compensated Hanning condition resulted in much larger nuclei compared to the Hanning. Cell area followed a similar pattern (Figure 32B), with significantly greater spreading under the rectangular waveform compared to both Hanning conditions ($***p < 0.001$), which did not differ from one another. Actin intensity (Figure 32C) was also highest under the rectangular waveform, significantly exceeding both Hanning and compensated Hanning groups ($***p < 0.001$), again, with no significant difference between the two. Intriguingly, actin intensity showed the lowest mean signal under the compensated Hanning condition despite its higher peak pressure and measured displacement, consistent with high initial force loading rates causing transient cytoskeletal fluidisation rather than stress fibre assembly. Vinculin expression (Figure 32D) remained unchanged across all conditions.

Fluorescent imaging reveals no differences between the conditions on vinculin or actin localisation

Representative fluorescent images (Figure 33) reveal differences in cell morphology across the three conditions. The nucleus appears to be rounded with no obvious differences between the three conditions. Cells in the Hanning and compensated Hanning conditions appear smaller, and less spread compared with the Rectangle condition. Actin staining appears to be distributed across the cell area. The vinculin signal is visible in all conditions and a prominent vinculin signal at the periphery of the cell. These observations are consistent with the quantitative analysis of cell and nuclear area, and the CTCF values of actin and vinculin.

Collectively, the results demonstrate that modifying the pulse envelope significantly altered the cellular response, despite maintaining the same carrier frequency, modulation

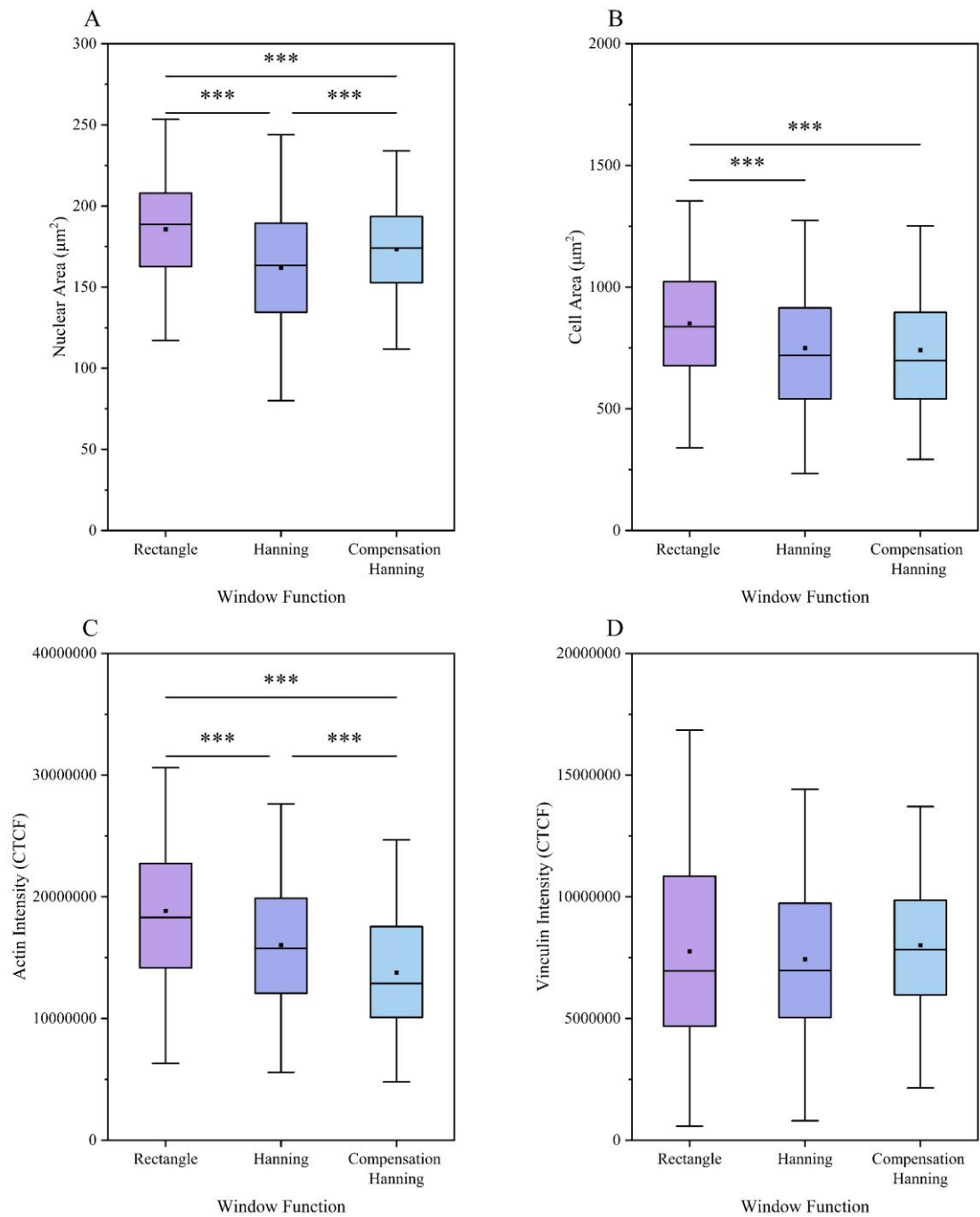


Figure 32. Effect of waveform windowing on MG63 cells. (A) Nuclear area, (B) Cell area, (C) Actin intensity (CTCF) and (D) Vinculin CTCF. The compensated Hanning window condition was adjusted in ultrasound pressure to match the I_{SATA} of the rectangular waveform ($I_{\text{SATA}} = 60 \text{ mW/cm}^2$). Boxplots show interquartile ranges, with medians (horizontal lines), means (squares), whiskers representing $1.5 \times \text{IQR}$, and individual points denoting outliers. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test (***) ($p < 0.001$).

frequency, and duty cycle across conditions. Hanning windowing changed the temporal profile of the applied stimulus by introducing a gradual rise and fall in amplitude, in contrast to the abrupt transitions of the rectangular waveform. This altered the loading dynamics experienced by the cells, including the displacement, and the rate at which force

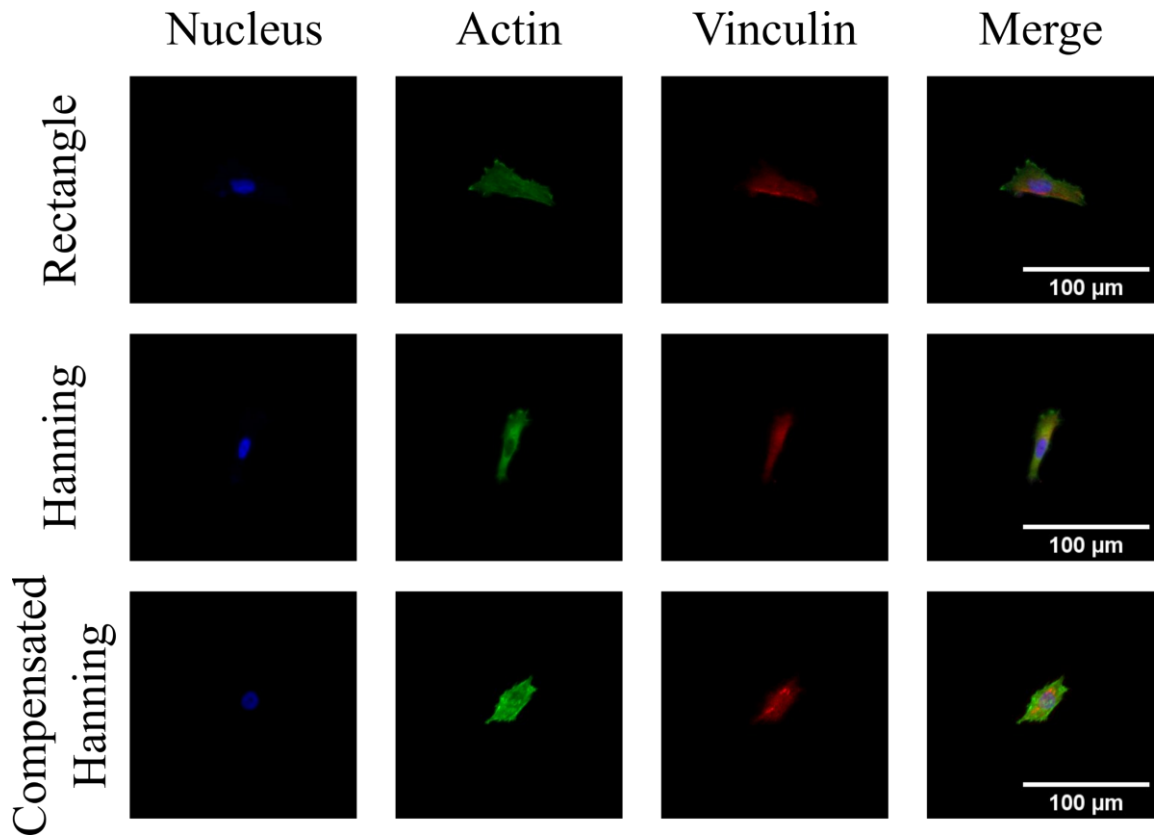


Figure 33. Immunofluorescence images of MG63 cells under different waveform windowing conditions (rectangular, Hanning, and compensated Hanning). Nucleus (blue), actin (green) and vinculin (red) are shown alongside merged images. Differences in cell morphology and cytoskeletal organisation are observed across conditions despite matched maximum ultrasound pressures between rectangle and Hanning and matched I_{SATA} between rectangle and compensated Hanning conditions.

was applied to the substrate. Notably, restoring I_{SATA} through pressure, did not recover the enhanced cell spreading, nuclear area, or actin intensity observed under rectangular stimulation, despite generated larger measured displacements. It is therefore evident that cellular mechanosensing under LIPUS is influenced not only by the conventional LIPUS parameters, but also by waveform-dependent temporal dynamics.

5.4.2 Membrane displacement under different ultrasound waveforms

LDV was used to quantify out-of-plane membrane displacement to better measure the mechanical stimulus on the cell substrate. Measurements were obtained under the same ultrasound exposure regimes applied in the cell experiments throughout this thesis, including pressure amplitude experiments, DC experiment, and PRF experiment from

Chapter 4, ISATA compensation experiments in Chapter 5, as well as the window function experiment described in this chapter.

Mylar membrane displacement varies linearly with pressure

Laser interferometry measurements were performed for the stimulation conditions presented in earlier in this thesis. In this experiment, membrane displacement was recorded in response to stimulation with a 1 kHz modulation frequency (Table 5) imposed on a 1 MHz carrier signal (Table 6) at 20% duty cycle for a rectangular window function at different pressures.

At 1 MHz (Figure 34A), the peak-to-peak membrane displacement increased progressively with higher input voltages. Peak to peak displacement ranged from 1.03 ± 0.05 nm at 65 kPa (10 mW/cm^2) to a maximum of 1.51 ± 0.09 nm at 388 kPa (200 mW/cm^2). The displacement recorded at 160 kPa (60 mW/cm^2), which was identified as the most biologically effective voltage from parallel cell experiments, was 1.19 ± 0.8 nm. A linear regression was applied to the displacement-pressure relationship ($R^2 = 0.98$) confirming that the displacement increased predictably across the tested pressure range. The measurements for displacement amplitude at 1 MHz are summarised in Table 5.

In comparison, maximum membrane displacement caused by acoustic radiation force at the PRF at 1 kHz (Figure 34B) were significantly higher across the same pressure range compared to MHz. The maximum displacement increased from 2.91 ± 0.18 nm at 64 kPa (10 mW/cm^2) to 10.94 ± 1.21 nm at 388 kPa (200 mW/cm^2). At 160 kPa (60 mW/cm^2) the max displacement measured at 4.17 ± 0.31 nm. This ultrasound pressure represents the optimal acoustic condition determined during the initial parameter optimisation experiments, confirming that a measurable mechanical displacement is delivered at the specific ultrasound pressure that was found to maximise the biological response. A linear regression including all points yielded $R^2 = 0.91$, confirming a linear relationship between pressure and displacement. Across the entire ultrasound pressure input range, displacement was consistently higher at 1 kHz than at 1 MHz. This was particularly evident at ultrasound pressure above 150 kPa.

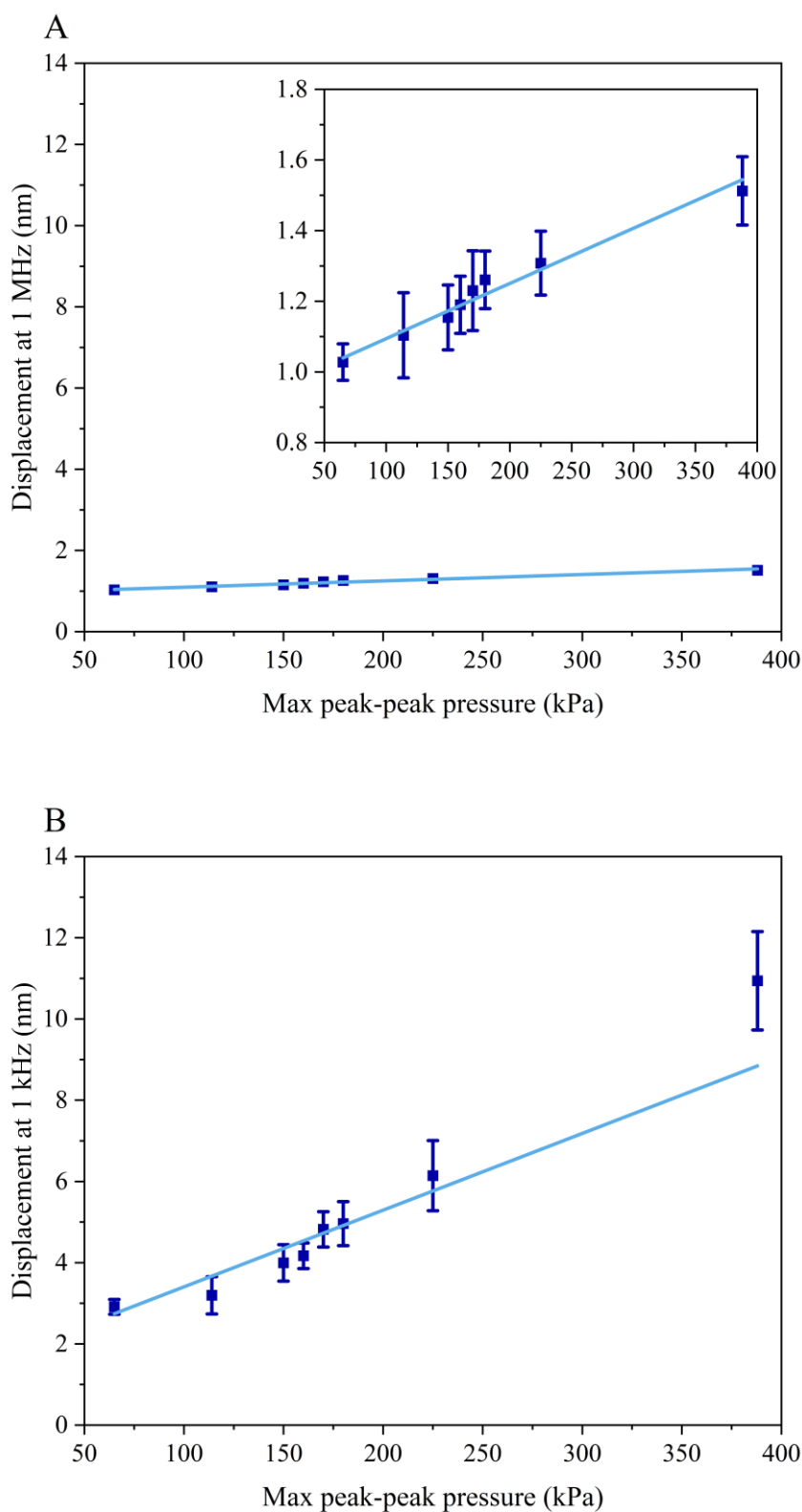


Figure 34. The relationship between mylar membrane displacement and output pressure as measured by LDV and hydrophone, respectively. (A) At 1 MHz carrier frequency, peak-to-peak displacement increased linearly with pressure. A linear regression was applied to all data points ($R^2 = 0.98$). (B) At 1 kHz modulation, the maximum displacement also increased with pressure. The lowest pressure point was excluded due to deviation from linearity, likely caused by low SNR. Linear regression of the remaining data yielded $R^2 = 0.98$. Measurements were obtained at 10 individual points, and three repeated measurements per point. Bars represent the standard error of the mean.

Table 5. Maximum measured substrate displacement amplitude at 1 kHz obtained using laser Doppler vibrometry, reported as mean displacement (nm), standard error of the mean (SEM) alongside the corresponding maximum peak-to-peak acoustic pressure for each condition.

Maximum displacement at 1 kHz (nm)	SEM	Max peak-peak pressure (kPa)
2.91	0.18	64
3.20	0.46	114
3.99	0.45	150
4.17	0.31	160
4.82	0.43	170
4.96	0.54	180
6.14	0.86	225
10.94	1.21	388

Table 6. Measured substrate displacement amplitude at 1 MHz obtained using laser Doppler vibrometry, reported as mean displacement (nm), standard error of the mean (SEM) alongside the corresponding maximum peak-to-peak acoustic pressure for each condition.

Peak to peak displacement (nm) at 1 MHz	SEM	Max peak-peak acoustic pressure (kPa)
1.03	0.05	65
1.10	0.12	114
1.15	0.09	150
1.19	0.08	160
1.23	0.11	170
1.26	0.08	180
1.31	0.10	225
1.51	0.10	388

Mylar membrane displacement is not affected by duty cycle

Figure 35 shows the peak-to-peak displacement amplitudes measured on the mylar membrane under pulsed 1 MHz ultrasound at 20% and 40% DC while keeping all other parameters identical at 1 kHz PRF and 160 kPa maximum pressure. When the carrier frequency alone was monitored using LDV, the peak-to-peak displacement increased slightly from 1.22 ± 0.08 nm (n=10) at 20% DC to 1.34 ± 0.0 nm (n=10) at 40% DC, based on a total of 20 spatial measurement points. A two-sample t-test comparing displacement amplitudes at 20% and 40% DC showed no statistically significant difference between the two conditions (p=0.33).

The modulation frequency induced a membrane motion that was substantially larger, consistent with acoustic radiation force driving slow substrate motion during bursting. In this case, displacement slightly decreased from 4.49 ± 0.16 nm (n=9) at 20% to 4.25 ± 0.21

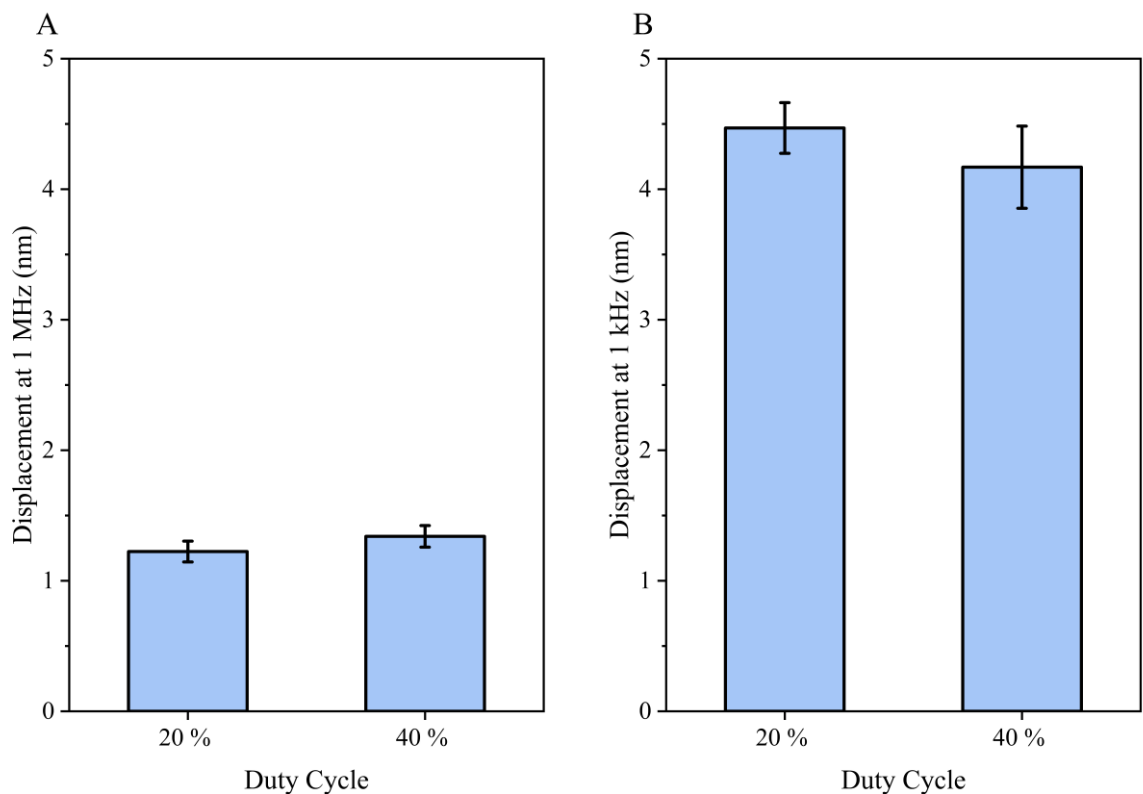


Figure 35. Measured displacement of the Biocell mylar membrane for the voltages used in the cell stimulation experiments, showing for the 1 MHz carrier frequency component (A) and the 1 kHz modulation frequency component (B) of otherwise identical ultrasound waveforms. Both carrier and modulation frequency displacement components follow a linear increase in displacement with voltage. In the case of the 1 kHz component, the lowest drive voltage (54 mVpp) was excluded from the linear fitting as this level is affected by low-amplitude spectral noise, making the modulation peak unreliable for fitting. For each condition, measurements were taken at 10 spatial locations and repeated three times. Bars represent the standard error of the mean.

at 40% (n=9). A two-sample t-test comparing the 1 kHz displacement amplitudes showed no significant differences between 20% and 40% DC (p=0.43).

The absence of a significant difference in displacement between 20% and 40% DC validates LDV measurements and confirms that membrane displacement amplitude is primarily affected by pressure rather than DC.

Mylar membrane displacement varies with PRF

Displacement was measured at the membrane surface for four different conditions (100, 1000, 10000, and 100000 Hz) at 1 MHz, and I_{SATA} of 60 mW/cm² and duty cycle at 20%, resulting in 2000, 200, 20, and 2 ms pulse width, respectively. At the carrier frequency (Figure 36A), the peak-to-peak displacement decreases steadily with increasing PRF, from 4.92 ± 0.38 nm (n=10) at 100 Hz to 0.14 ± 0.02 nm (n=10) at 100,000 Hz. Intermediate values were 1.22 ± 0.08 nm (n=10) at 1000 Hz and 0.26 ± 0.03 nm (n=10) at 10,000 Hz.

A similar trend was observed at the corresponding modulation frequency displacements shown in Figure 36B. Max displacement was the highest at 8.89 ± 1.65 nm (n=10) at 100 Hz, followed by 4.17 ± 0.30 nm (n=9) at 1 kHz and dropped to sub-nanometer levels at 10 kHz and 100 kHz, measuring 0.37 ± 0.06 nm (n=10) and 0.53 ± 0.05 nm (n=10) respectively.

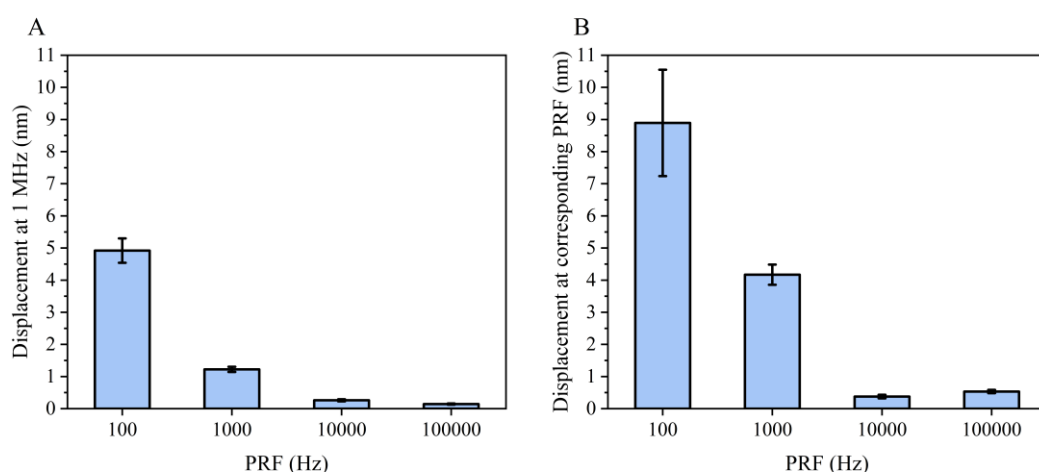


Figure 36. Effect of PRF on membrane displacement. (A) Displacement measured at 1 MHz carrier frequency and (B) Displacement measured at 1 kHz modulation frequency. Each point represents the mean of three repeated measurements at 10 individual points. Error bars show the standard error of the mean.

The apparent reduction in the extracted 1 MHz carrier frequency displacement with increasing PRF is likely influenced by the narrow, fixed digital bandpass filter (± 500 Hz) used in the MATLAB script to isolate the central carrier peak. A fixed band window was maintained across the conditions to ensure consistent processing and allow direct comparison between datasets. Although peak pressure and I_{SATA} were maintained constant, increasing PRF required progressively shorter pulse durations, reducing the number of carrier cycles per pulse and broadening the spectral distribution around 1 MHz. Consequently, a smaller proportion of the carrier-related spectral content was captured within the fixed processing window, leading to lower extracted displacement amplitudes at the carrier frequency.

Beyond signal processing considerations, physical displacement dynamics are also strongly dictated by the width of the pulse as PRF increases. At higher PRFs, maintaining a constant 20% DC requires progressively shorter pulse durations, from 2000 μ s at 100 Hz down to just 2 μ s at 100 k Hz, representing merely two full cycles of the 1 MHz carrier. For such ultra short excitation pulses, the onset and offset occur too rapidly for the substrate and surrounding medium to overcome mechanical inertia and reach steady-state displacement amplitude. The transient acoustic radiation force acts as a brief impulse rather than a sustained load, resulting in heavily damped, band-limited mechanical motion. Therefore, while peak acoustic pressure and I_{SATA} were held constant, the physical displacement decreases at high PRFs due to a combination of mechanical response time limitations during brief pulse windows and spectral energy broadening outside the fixed digital filter bandwidth.

5.5 Discussion

This chapter examined whether changing the rate of applied strain, through rectangular and Hanning-windowed pulsed ultrasound excitation, alters early mechanosensitive responses in MG63 cells despite comparable nominal ultrasound pressure or I_{SATA} . In parallel, LDV was used to quantify the resulting substrate motion and relate the observed cellular responses to the underlying mechanical stimulus. The findings demonstrate that the rectangular waveform consistently produces stronger cellular responses than the Hanning conditions and show that the rate at which force is applied, represents an important parameter that should be considered alongside conventional acoustic exposure parameters when optimising LIPUS protocols.

It is evident that both Hanning conditions led to reduced actin expression and cell area compared to the rectangular waveform, whereas differences in vinculin intensity were not statistically significant across conditions. The rectangular waveform applies the mechanical stimulus at a higher strain rate $\left(\frac{dF}{dt}\right)$ compared with the more gradually applied Hanning envelope, which may affect talin unfolding. In integrin-mediated mechanotransduction, the rate of force loading determines whether mechanosensitive talin unfolds before integrin-ECM bonds disengage. When talin unfolding occurs while integrin remains engaged, downstream signalling pathways are initiated, facilitating focal adhesion assembly [46], [68]. Here, peak acoustic pressure was matched across conditions to ensure that mechanical threshold required to induce focal adhesion signalling was reached. However, LDV displacement measurements revealed that matching nominal acoustic pressure does not guarantee matched physical substrate displacement, demonstrating that envelope dynamics significantly alter the actual physical stimulus delivered to the cells.

To explore whether peak pressure could override these envelope effects, the compensated Hanning regime increased peak pressure to match ISATA, testing whether higher peak force could offset the lower strain rate and promote greater focal adhesion formation or vinculin recruitment. Intriguingly, actin intensity was reduced further under the compensated Hanning condition despite its higher peak pressure and larger measured displacement. To match I_{SATA} , the compensated Hanning pulse requires a substantially higher peak amplitude, which steepens the transient force loading rate $\left(\frac{dF}{dt}\right)$ during the envelope ramp. When subjected to this combination of elevated peak displacement and rapid transient loading, the actin cytoskeleton may undergo strain-induced fluidisation or stress fibre disassembly rather than adaptive reinforcement.

To further characterise the mechanical input experienced by the cells, substrate displacement measurements were performed using LDV. These measurements revealed information about the frequency content of the ultrasound waveform that is not readily captured by conventional ultrasound parameters, providing further insight into the mechanical stimulus experienced at the cell-substrate interface. In addition to the carrier frequency component (1 MHz), a distinct low-frequency displacement corresponding to the pulse repetition frequency (kHz) was observed in the displacement spectra. This low-frequency motion arises from acoustic radiation force and reflects the modulation

generated by the waveform envelope rather than high-frequency oscillations of the pressure wave itself. Notably, the displacement magnitude at the modulation frequency was substantially greater than that at the carrier frequency, indicating that cells are exposed to a larger physical motion at the PRF.

However, whether the observed biological responses arise primarily from the 1kHz displacement, the 1 MHz oscillatory motion, or a combination of both acting in synergy is unclear. Previous studies have demonstrated that both kHz and MHz nanoscale vibrations are capable of inducing osteogenic responses [163], [164], [165]. Pounder *et al.*, measured nanoscale displacement of 0.15-0.55 nm on bone fragments at the 1 kHz pulse frequency of LIPUS using laser interferometry, although no carrier frequency displacement was reported [169]. While the displacements reported here are higher, as expected given the substantially different mechanical properties of bone compared with the mylar membrane used in the present work as well as the lower I_{SATA} (30 mW/cm²) employed by the authors, both studies confirm the presence of a distinct low-frequency displacement component during pulsed ultrasound. While the kHz component dominates substrate deformation, the nanometre-scale motion at the carrier frequency may still contribute via direct membrane perturbation, ion channel activation or localised acoustic microstreaming.

Methodological validation confirmed that displacement scaled linearly with input voltage and applied acoustic pressure at both 1 MHz and 1 kHz. Furthermore, the effect of duty cycle (20% vs. 40%) on displacement was negligible (two-sample t-test, $p > 0.05$), confirming measurement stability. In contrast, displacement at both carrier and modulation frequencies decreased steadily with increasing PRF. This reduction is driven by progressively shorter pulse durations required to maintain constant duty cycle at higher PRFs. In the frequency domain, compressing a signal in time inherently broadens its spectral distribution, causing energy leakage away from central peak. Because a narrow, fixed digital bandpass filter (± 500 Hz) was maintained across processing conditions to ensure standardised analysis, a smaller fraction of spectral content was captured within the fixed window at higher PRFs, resulting in smaller extracted displacement amplitudes.

To evaluate this, alternative filtering approaches were tested during post-processing in MATLAB. However, widening or altering the filter bandwidth admitted extra noise into the signal, degrading the signal-to-noise ratio and making displacement extraction unreliable. Therefore, the fixed filter bandwidth was retained as the most consistent

method for direct comparison across conditions. Beyond these signal processing considerations, a physical reduction in displacement is also expected at higher PRFs. For very short pulse widths, such as 2 μ s at 100 kHz, the onset and offset occur too rapidly for the membrane to overcome inertia and reach its full steady-state displacement amplitude within each pulse.

Finally, it is noteworthy that an unstimulated control was not included in this specific experiment. Therefore, direct comparison with baseline cellular activity is not possible. Nevertheless, the Hanning-windowed conditions retained visible actin and vinculin staining and retained morphologies consistent with adherent, spread cells, suggesting that the altered responses more likely reflect a diminished stimulatory effect relative to the rectangular waveform rather than the overall loss of viability or adhesion. Therefore, interpretations should be made relative to the optimised rectangular regimes established in Chapters 3 and 4. Together, these findings indicate that the cellular responses are governed not only by the magnitude of the applied acoustic pressure, but by temporal waveform characteristics, particularly the rate at which the load is applied at the cell substrate interface.

5.6 Conclusion

In conclusion, this chapter demonstrates that the temporal characteristics of the ultrasound waveform play a critical role in governing cellular response. Despite being delivered at equivalent I_{SATA} or pressure, rectangular and Hanning windowed waveforms produced distinct cytoskeletal responses, with the rectangular waveform consistently eliciting a stronger effect. These differences were not explained by acoustic intensity or peak pressure alone but were reflected in the resulting substrate displacements measured using LDV supporting the notion that direct measurement of mechanical vibration may provide a more representative descriptor of the stimulus.

The present findings support the hypothesis that cells are sensitive to the rate at which mechanical force is applied. It should however be noted that the stimulation parameters used here were originally optimised for rectangular pulse rather than Hanning windowed excitation therefore, the effectiveness of slower rise ramp waveforms cannot be excluded based on these findings alone. Overall, LDV measurements revealed that conditions

matched in conventional acoustic parameters produced distinct displacements, confirming that these parameters do not uniquely define the mechanical stimulus experienced at the cell substrate interface. Given the central role of substrate motion in mechanotransduction these results highlight the need to move beyond conventional ultrasound dosimetry parameters when interpreting ultrasound-cell interactions.

Chapter 6: Discussion

6.1 The influence of LIPUS waveform structure on MG63 mechanosensing

The work presented in this thesis has firmly demonstrated that I_{SATA} of 60 mW/cm², delivered at a pressure of 80-160 kPa, 20% DC, 1000 Hz PRF, with a rectangular window applied to the pulse, enhances the expression of markers indicative of cell mechanosensing in MG63s. These findings were obtained under acoustically characterised exposure conditions, where pressure, intensity, temperature, and substrate displacement were quantified. Establishing controlled and precisely measured in vitro exposure setups is essential when designing ultrasound experiments. Here, LDV serves as a precise tool to define the physical dose delivered to cells and confirm the involvement of integrin-mediated mechanotransduction. The highest temperature increase recorded across all investigations conducted was 0.2 °C, and the highest MI was 0.08 (Appendix B), confirming that the observed responses were unlikely to result from thermal effects or cavitation. Other effects such as microstreaming due to the presence of gas in the culture medium cannot be excluded. At the optimised conditions, the displacements recorded using LDV at the 1 MHz carrier frequency and the 1 kHz pulse modulation frequency were 1.19 ± 0.08 nm and 4.17 ± 0.31 nm, respectively.

Previous studies have reported a range of cellular responses to LIPUS, though many were conducted under uncontrolled experimental conditions using standard tissue culture plastic. Gupta *et al.* investigated the response of Saos-2 cells in multiwell plates positioned above the transducer in a water-filled anti-reflective chamber and observed reduced vinculin staining, disrupted actin morphology, and a loss of metabolic activity at I_{SATA} as low as 25 mW/cm², raising concerns about the potential for non-specific or damaging effects when ultrasound is applied without controlling the acoustic environment [124]. In contrast, Atherton *et al.*, stimulated MC3T3-E1 cells using the Exogen LIPUS device (30 mW/cm²) with the transducer immersed directly above the culture medium above the cells and demonstrated that LIPUS is sensed through integrin-mediated adhesions and that vinculin plays a central role in coupling the applied mechanical stimulus to downstream actin remodelling and Rac1 activation [77]. The present findings support this observation, as changes in vinculin expression were consistently accompanied by corresponding

alterations in actin organisation. However, the contrasting findings reported across studies highlight that differences in exposure setup, coupled with reliance on nominal device parameters alone, make meaningful comparison across studies challenging. Without a standardised exposure configuration and direct characterisation of the ultrasound exposure conditions experienced by the cells, it is difficult to arrive at a consensus regarding optimal ultrasound conditions for enhanced vinculin expression and consequently mechanosensing.

The results in Chapter 3 reveal that the cellular response to LIPUS is not proportional to acoustic intensity. Instead, actin and vinculin expression were enhanced within a relatively narrow range of exposure conditions and diminished at both lower and higher intensities. Similarly, increasing the duty cycle, did not improve cellular response despite increasing the total energy delivered. It is therefore established that cell responses to LIPUS are highly dependent on the individual acoustic parameters used to deliver the stimulus rather than on acoustic energy alone and also suggest the existence of an optimal window, where both insufficient or excessive stimulation produce weaker biological responses.

A key observation from Chapter 4 was that conditions delivering the same I_{SATA} produced markedly different cellular responses depending on the combination of pressure and duty cycle used to achieve that intensity. The greatest increases in mechanosensitive markers were observed at high pressure and lower duty cycle, indicating that cellular response is influenced by more than I_{SATA} alone. One possible explanation for these findings is that higher pressure amplitudes produced larger substrate displacements, thereby applying a stronger mechanical stimulus to the cells. This interpretation is supported by the LDV measurements, which demonstrated a linear increase in displacement with pressure amplitude. Similar observations have been reported by Hsu *et al.*, who also identified that increasing the duty cycle at otherwise identical conditions produces less favourable outcomes in human chondrocyte monolayers [154]. It can therefore be concluded that I_{SATA} is insufficient to describe the effective LIPUS stimulus experienced by the cells and that increasing the duty cycle is not an effective strategy for enhancing cellular response.

The same conclusion can be drawn about the effectiveness of I_{SATA} to describe dose from the investigation in Section 4.4.1. where, I_{SATA} was kept constant by altering PRF and the number of cycles within each burst. At lower PRFs actin expression and cellular morphology increased whereas the opposing trend was reported with increasing PRF. The LDV measurements revealed that increasing PRF reduced the displacement amplitude and

generated a weaker mechanical stimulus at the cell substrate, with displacement falling to sub-nanometer levels at 10 and 100 kHz, despite comparable I_{SATA} . However, this trend should be interpreted with caution as a portion of this displacement reduction is attributed to the post-processing constraints associated with spectral broadening at higher PRFs. Consistent with the present findings, PRF has previously been identified as an important determinant of cell response to LIPUS, with results showing that 1000 Hz PRF may promote the osteogenic commitment of hASCs [85].

The waveform envelope experiment in Chapter 5 (Section 5.4.1) revealed that the rate at which mechanical forces are applied also contributes to the effectiveness of mechanical stimulation. While the rectangular waveform delivers pressure abruptly, the Hanning waveform introduces a gradual rise and fall in pressure amplitude, altering the manner in which mechanical forces are applied to the cells. In terms of force mechanics, smoothing the waveform envelope alters the transient momentum transfer profile, which would be expected to produce a lower peak radiation force ramp during pulse onset compared to an abrupt rectangular transition. LDV measurements confirmed that altering the waveform envelope changed the mechanical stimulus delivered to the substrate. This supports the hypothesis that the rate at which mechanical forces are applied influences cellular response. One possible mechanism involves loading-rate-dependent processes such as talin unfolding and subsequent focal adhesion reinforcement, although this was not investigated in the present study.

Finally, while using LDV to measure 2D substrate displacement provides a highly controlled way to quantify mechanical stimulus at the cell substrate, translating these findings to 3D culture models or *in vivo* tissues introduces additional physical considerations. In 3D extracellular matrix or native tissue, cells experience complex viscoelastic matrix compliance, interstitial fluid movement, and multi axial strain distributions rather than planar surface displacement alone. Therefore, while 2D LDV measurements establish fundamental relationships between ultrasound waveform shapes and resulting substrate motion, future work translating these findings to 3D models will need to consider how the surrounding matrix alters force delivery to the cells.

6.2 Biological readouts for assessing LIPUS response

Cell spreading area, nuclear area, actin cytoskeleton intensity, and vinculin intensity serve as critical indicators of cellular mechanotransduction. The engagement of mechanosensitive vinculin at focal adhesions and the subsequent structural reorganisation of the actin cytoskeleton generate internal contractile forces required to alter cell morphology. As cell spreading area and actomyosin contractility increase, the tension is transmitted to the nucleus, causing it to spread and flatten, which ultimately drives transcriptional changes in cells [170]. Tracking the changes and repeatability of these four interconnected markers provides a robust readout of how cells respond to LIPUS.

Cell area emerged as a consistent and reliable readout across the different LIPUS conditions tested for MG63 cells. This aligns with the expected cell mechanics, as cell spreading is supported by cytoskeletal organisation and cell-matrix adhesions, meaning that changes in cell area would be anticipated to occur alongside alterations in actin and vinculin expression. Therefore, cell area serves as a practical morphological marker to assess the efficacy of ultrasound-induced bioeffects.

In contrast, the nuclear area displayed a non-consistent response across the exposure conditions investigated. Although significant changes were observed in some experiments, these findings were not reproduced across experiments and did not consistently align with alterations in actin or vinculin expression. It is possible that the effects on nuclear architecture may require long-term stimulation to become detectable. While cytoskeletal remodelling can occur within minutes of mechanical loading, downstream adaptations in nuclear morphology may require hours to days [171]. In this study, MG63 cells were only exposed to a single LIPUS treatment before analysis, which may not have been sufficient to elicit measurable nuclear adaptation. Clinically, LIPUS is typically applied for 20 minutes per day over several weeks, suggesting that cumulative or repeated stimulation may be necessary to induce measurable structural adaptations at the nuclear level.

Actin staining intensity proved to be a reliable and repeatable indicator of cellular response across the experimental conditions investigated. Similar trends were observed across independent experimental repeats, and the overall response remained consistent as the number of biological replicates increased. Furthermore, actin intensity was sensitive to changes in pressure, duty cycle, PRF, and waveform envelope, allowing favourable and

unfavourable exposure conditions to be distinguished. This makes it a suitable readout for assessing early mechanosensitive changes in MG63s upon ultrasound exposure.

Vinculin intensity generally followed actin and cell area trends, consistent with its role as a marker of focal-adhesion associated cellular responses to LIPUS. Conditions that promoted greater cell spreading and actin organisation, particularly at 60 mW/cm² rectangular waveform, also produced more pronounced vinculin expression, often localised at the cell periphery. This supports the idea that focal adhesion-associated responses accompany cytoskeletal remodelling in response to mechanical stimulation. However, this relationship was not observed under all exposure conditions, as changes in actin expression and cell area were not always accompanied by corresponding changes in vinculin intensity. These results highlight the value of vinculin as a marker of cellular engagement with the substrate but also underscore the complexity of focal adhesion responses to mechanical stimulation.

Fluorescence microscopy and imaging was an effective method to visualise the localisation and intensities of actin and vinculin, however, raw intensity measurements rely on fixed endpoints rather than live cell imaging. For example, vinculin functions by clustering at focal adhesion along the cell periphery and a static snapshot fails to capture the rapid assembly, disassembly, and kinetic turnover during LIPUS stimulation. Similarly, while a brighter actin signal successfully captures an overall increase in filament mass, total intensity alone cannot distinguish whether this reflects filament lengthening, thickening or network reorganisation. Therefore, while imaging qualitatively confirms that LIPUS stimulates mechanosensing, fluorescence intensity remains a less specific and repeatable marker.

6.3 *In-situ* measurement of displacement as a metric for LIPUS-induced mechanical response

LDV was employed to measure the displacement of the mylar membrane at the cell-seeded interface, to better characterise the substrate motion generated during ultrasound exposure. Unlike hydrophone measurements, which cannot be performed at the cell substrate, this approach enabled direct measurement of the spatial distribution of displacement across the mylar membrane, allowing comparison with the pressure field while capturing the mechanical response at the point of cell attachment. By directly measuring substrate

displacement, LDV provided additional insight into the mechanical cues delivered to the cells that may underlie the study's observations. The measurements revealed a distinct displacement component at the modulation frequency (1 kHz) that would not be evident from the pressure magnitude alone without frequency-resolved analysis.

The results revealed that mylar membrane displacement varied systematically with ultrasound parameters, including input voltage, PRF, and waveform shape. While some trends, such as the linear relationship between voltage and displacement and the insignificant effect of DC on displacement, were expected, others revealed important discrepancies. In particular, the apparent reduction in measured displacement as PRF increased was unexpected. Importantly, the results demonstrated that equivalent LIPUS exposure conditions did not necessarily produce equivalent substrate displacements, highlighting an important distinction between nominal LIPUS parameters and the mechanical stimulus experienced at the cell substrate, providing an explanation for the distinct biological responses.

While acoustic pressure provides a more informative parameter, it does not inherently capture the frequency-dependent nature of the resulting motion. This is evident in the matched pressure condition achieved with Hanning windowing, in which cellular responses differed from those with a rectangular waveform, albeit not significantly in magnitude. Despite identical peak pressure, LDV measurements revealed substantially lower displacement at 1 kHz for the Hanning condition (2.62 nm) compared to the rectangular waveform (4.17 nm), demonstrating that equivalent pressure conditions do not necessarily produce equivalent substrate motion, providing a potential explanation for the differing cellular responses.

This has two key implications. First, it may explain some of the variability in cellular responses observed under otherwise matched ultrasound conditions. Second, it offers a potential pathway for improving the translation of *in vitro* data to *in vivo* applications. By quantifying the displacement at the cell surface, treatments could be tailored to achieve specific mechanical outputs at the target tissue. For example, a given displacement range could serve as a reference when optimising ultrasound parameters to stimulate a soft, early-stage hematoma versus a more mature callus. Furthermore, such measurements provide a quantitative framework for comparing mechanical inputs across studies and relating them

to cellular responses, enabling investigation of how parameters such as membrane tension and strain correlate with biological outcomes.

6.4 Experimental considerations

This thesis systematically examined how the structure of ultrasound waveforms influences mechanosensing in MG63. The work is framed around controlled manipulation of waveform parameters and direct quantification of the resulting mechanical stimulus. The experimental setup developed in this thesis enables the controlled stimulation of cells to ultrasound *in vitro* while remaining compatible with both fluorescence imaging and laser vibrometry measurements. In contrast to conventional rigid culture ware, where reflections at solid boundaries give rise to standing wave fields, herein, cells were cultured on a thin, acoustically and optically transparent membrane, reducing the likelihood of local minima and maxima, that may otherwise confound interpretation of biological outcomes.

The study design enables the systematic interrogation of waveform structure while accounting for their interdependence in MG63 cells. Rather than varying a single nominal parameter in isolation, pressure, duty cycle, pulse repetition frequency, and rise time were examined, including conditions in which I_{SATA} or ultrasound pressure was constant. This approach avoids overreliance on I_{SATA} as descriptors of sole description of dose and addresses a key limitation in the LIPUS literature, namely, the lack of direct quantification of the mechanical stimulus experienced at the cell interface.

Most LIPUS studies investigating osteogenic response employ MC3T3-E1, which is a murine pre-osteoblast cell line. In this work, the human osteosarcoma-derived MG63 cell line was selected and has been widely used in studies applying mechanical stimulation, primarily cyclic stretch, where increased osteogenic marker expression has been reported [172], [173]. While MG63s are highly responsive to mechanical stimuli making them a good model for examining focal adhesion behaviour in response to mechanical stimuli, their osteosarcoma origin may not fully represent the behaviour of healthy primary osteoblasts [146].

Furthermore, the biological measurements employed primarily capture early morphological and cytoskeletal responses and do not directly probe downstream signalling pathways or longer-term functional outcomes. As such, the observed changes should be

interpreted as indicators of altered mechanosensing rather than definitive evidence of specific mechanotransduction mechanisms. In addition, substrate displacement was quantified in the absence of cells. While this provides a controlled and reproducible reference for the applied mechanical stimulus, the presence of adherent cells may locally modify membrane mechanics and damping, potentially altering the precise displacement experienced at the cell-substrate interface.

Finally, all experiments were conducted using a two-dimensional *in vitro* model with cells adhered to a thin, flexible membrane. Although this configuration enables precise control of ultrasound delivery and mechanical characterisation, it does not replicate the structural or mechanical complexity of native bone tissue or three-dimensional extracellular matrices. Consequently, the ultrasound conditions identified here should not be assumed to translate directly *in vivo*. Rather, the findings emphasise the importance of considering waveform structure, boundary conditions, and substrate mechanics when interpreting and comparing ultrasound-induced cellular responses across experimental systems.

6.5 Conclusions and future work

The findings of this thesis demonstrate that cellular responses to LIPUS are strongly influenced by waveform design and cannot be described by conventional acoustic descriptors alone. By engineering a custom, highly controlled *in vitro* exposure system and pairing it with precise substrate displacement measurements, this work systematically isolated the biological impact of individual ultrasound parameters. Crucially, these findings invalidate the reliance on I_{SATA} as a primary dosing metric, while providing a foundational proof that bone cells directly perceive and actively respond to the LIPUS waveforms *in vitro* when the mechanical stimulus is properly controlled. While this work establishes a rigorous framework for evaluating early mechanosensitive pathways *in vitro*, fully evaluating the downstream intracellular cascades as well as translating these parameter interactions to the complex *in vivo* bone remain critical next steps.

Future work should extend the current 2D framework to three-dimensional culture systems to better represent the mechanically heterogeneous environments encountered *in vivo*. Because ultrasound induced mechanical displacement propagates differently through a 3D matrix compared to a cell monolayer, systematic characterisation across 3D environments

of varying stiffness and composition is essential for optimisation of LIPUS treatment. Furthermore, as the mechanical environment changes dynamically during bone healing, investigating these variations will reveal how tissue stiffness alters the displacement experienced by the cells. Because tissue stiffness increases dramatically during the progression from hematoma to soft cartilage callus and eventually to hard mineralised bone, maintaining a consistent mechanical stimulus at the cell level may require adjusting acoustic exposure parameters as healing progresses. Rather than relying on fixed pressure or intensity, future protocols may need to optimise for target substrate or matrix displacements, adapting the applied pressure to compensate for changing tissue mechanics at different repair stages.

To model and optimise these parameters across different healing phases, future studies could utilise 3D tissue constructs that mimic distinct mechanical properties and cell populations (e.g., MSCs, chondrocytes, and mature osteoblasts) of soft versus hard callus. Pairing these models with finite-element computational modelling would allow researchers to predict matrix displacement under ultrasound exposure and map how ultrasound energy attenuates as tissue stiffens. Computational modelling could also be used to address specific experimental limitations from this work. For example, simulating the temporal pulse spectrum and applying digital bandpass filtering in MATLAB could clarify the signal processing constraints observed at high PRFs, helping to separate digital filtering artifacts from actual physical drops in displacement.

Beyond early mechanosensitive focal adhesion related proteins investigated herein, future work is required to investigate downstream mechanotransduction. While this thesis focused on early morphological and cytoskeletal responses, future studies should examine how these initial mechanical cues are transduced into long-term signalling and transcriptional changes. Particular attention should be given to additional mechanosensory elements, including adhesion-associated proteins, cytoskeletal components, ion channels and nuclear components that may respond to LIPUS. This would help establish how ultrasound-induced displacement evolves into sustained biological effects.

Further insight into the mechanism of action of LIPUS could be gained by separating the effects of low frequency (1 kHz) mechanically induced displacement from high frequency (1 MHz) acoustic exposure. Comparing cellular responses under conditions that preferentially emphasise modulation frequency-driven displacement versus carrier

frequency effects may help identify which components of the waveform initiate mechanotransduction, and if more than one mechanism is responsible for LIPUS-induced bone healing.

Appendices

Appendix A: *In vitro* LIPUS exposure apparatus acoustics

When sound waves travel from one medium to another, part of the energy is reflected at the interface. The proportion of reflected energy is determined by the reflection coefficient, which depends on the acoustic impedance of each material, which is defined as

$$Z = \rho c$$

Where ρ is the material density (kg/m^3), and c is the speed of sound (m/s) in the medium. For a plane wave incident normally on a boundary between two media with thicknesses much greater than the wavelength, the reflection coefficient (R) is given by

$$R = \left(\frac{Z_2 - Z_1}{Z_2 + Z_1} \right)^2$$

Where Z_1 and Z_2 are the acoustic impedances of the first and second materials, respectively. Using values in the literature [141], boundaries such as growth media to air or polystyrene to air [174] exhibit near total reflection ($R = 0.99$), while interfaces like growth media to polystyrene show much lower reflection ($R = 0.054$) [134]. At the boundary between the media and piezoelectric element (e.g. PZT4) the reflection coefficient is considerably higher ($R = 0.842$), meaning that approximately 84% of the incident energy is reflected back toward the cell monolayer. Although many transducers include a matching layer with an impedance closer to that of water to reduce internal reflections, a significant portion of the energy is still reflected into the dish. These differences highlight the importance of considering boundary conditions when designing *in vitro* LIPUS experiments, as they can significantly affect the acoustic field distribution and the potential for multiple reflections that will interfere with the field and lead to standing wave formation.

Table A1. Estimated reflection coefficients at key material boundaries relevant to in vitro LIPUS setups. Acoustic impedance values are sourced from literature [141] assuming the growth medium has the same impedance as water. PZT4 = Lead Zirconate Titanate Type 4. Adapted from [138].

Boundary	Z_1 (kg/m ² /s)	Z_2 (kg/m ² /s)	R
Media / Polystyrene	1.5×10^6 [1]	2.4×10^6 [2]	0.054
Polystyrene / Air	2.4×10^6	415 [1]	0.999
Media / Air	1.5×10^6	415	0.999
Media / PZT4	1.5×10^6	35×10^6 [3]	0.842

Appendix B: Temperature monitoring and MI during exposure of cells to LIPUS

Mechanical Index (MI) was calculated using Equation B1 below where, f is the frequency in MHz

$$MI = \frac{\text{peak negative pressure}}{\sqrt{f}} \quad \text{Equation B1}$$

Table 7. Temperature monitoring and MI inside the Biocell during LIPUS exposure. Measurements were recorded prior to stimulation and at 5-minute intervals. Across all acoustic conditions from Chapter 3, temperature variations remained below 0.5 °C and the highest MI recorded was well below the cavitation threshold.

ISATA (mW/cm²)	Duty cycle (%)	PRF (Hz)	MI	Temp. at 0 minutes (°C)	Temp. at 5 minutes (°C)	Temp. at 10 minutes (°C)	Temp. at 15 minutes (°C)	Temp. at 20 minutes (°C)
10	20	1000	0.02	37.1	37.1	37.1	37.1	37.0
30	20	1000	0.03	37.1	37.1	37.0	37.0	37.0
50	20	1000	0.03	37.1	37.1	37.0		
60	20	1000	0.04	37.0	37.0	37.0		
70	20	1000	0.05	37.1	37.1	37.0		
80	20	1000	0.05	37.1	37.0	36.9		
120	20	1000	0.05	37.0	37.1	37.2	37.2	37.0
120	40	1000	0.05	37.0	37.0	37.0		
200	20	1000	0.07	37.0	37.3	37.3	37.3	37.2

Table 8. Temperature monitoring and MI inside the Biocell during LIPUS exposure. Measurements were recorded prior to stimulation and at 5-minute intervals. Across all acoustic conditions from Chapter 4, temperature variations remained below 0.1 °C and the highest MI recorded was well below the cavitation threshold.

ISATA (mW/cm²)	Duty cycle (%)	PRF (Hz)	MI	Temp. at 0 minutes (°C)	Temp. at 5 minutes (°C)	Temp. at 10 minutes (°C)	Temp. at 15 minutes (°C)	Temp. at 20 minutes (°C)
30	20	100	0.03	37.1	37.0	37.0	37.0	36.9
30	20	10000	0.03	37.2	37.1	37.1	37.1	37.0
30	20	100000	0.03	37.2	37.1	37.1	37.1	37.0
60	40	1000	0.04	37.0	37.0	37.0		
60	60	1000	0.04	37.0	36.9	36.9		

Table 9. Temperature monitoring and MI inside the Biocell during LIPUS exposure. Measurements were recorded prior to stimulation and at 5-minute intervals. Across all acoustic conditions from Chapter 5, temperature variations remained below 0.1 °C and the highest MI recorded was well below the cavitation threshold.

ISATA (mW/cm²)	Duty cycle (%)	PRF (Hz)	MI	Temp. at 0 minutes (°C)	Temp. at 5 minutes (°C)	Temp. at 10 minutes (°C)	Temp. at 15 minutes (°C)	Temp. at 20 minutes (°C)
22.5 (Hann)	20	1000	0.02	37.0	37.0	37.0		
60 (Hann_{Comp})	20	1000	0.08	37.0	36.9	36.9		

Appendix C: Nuclei area analysis MATLAB script

```

clearvars; close all; clc

% Settings
parent_folder = pwd;
pixel_area = (1/1.5567)^2; %  $\mu\text{m}^2$  per pixel
min_um2 = 200;
max_um2 = 600;
min_circ = 0.2;
min_seed_dist = 5;

% Output folder
out_dir = fullfile(parent_folder, 'nuclei_summaries');
if ~exist(out_dir, 'dir')
    mkdir(out_dir);
end

% Look for the condition folders
D = dir(parent_folder);
D = D([D.isdir] & ~startsWith({D.name}, '.'));

conditions = {'0 minutes', '10 minutes', '20 minutes'};
parsed = struct('name', {}, 'condition', {});

for k = 1:numel(D)
    folder_name = D(k).name;
    idx = find(strcmp(folder_name, conditions), 1);
    if isempty(idx)
        continue
    end

    parsed(end+1).name = folder_name;
    parsed(end).condition = conditions{idx};
end

conditions = unique({parsed.condition}, 'stable');
allAreas = cell(size(conditions));
debugCount = 0;

% Process images
for p = 1:numel(conditions)
    grp = parsed(strcmp({parsed.condition}, conditions{p}));

    for gi = 1:numel(grp)
        files = dir(fullfile(parent_folder, grp(gi).name, '*.tif'));

        for f = 1:numel(files)
            file_path = fullfile(parent_folder, grp(gi).name, files(f).name);
            I = imread(file_path);
            I8 = uint8(mat2gray(I(:,:,1)) * 255);

            % Threshold and clean up
            bw = imbinarize(I8, graythresh(I8));
            bw = imfill(bw, 'holes');
            bw = bwareaopen(bw, 30);

            % Watershed

```

```

Ddist = -bwdist(~bw);
Ddist(~bw) = -Inf;

minima = imextendedmin(Ddist, min_seed_dist);
Dmod = imimposemin(Ddist, minima);
L = watershed(Dmod);

bw(L == 0) = 0;
bw = imdilate(bw, strel('disk', 1));
bw = imfill(bw, 'holes');

% Show a few images to confirm
if debugCount < 2
    debugCount = debugCount + 1;

    bw0 = imbinarize(I8, graythresh(I8));
    bw1 = imfill(bw0, 'holes');
    bw1 = bwareaopen(bw1, 30);

    Ddbg = -bwdist(~bw1);
    Ddbg(~bw1) = -Inf;

    mini_dbg = imextendedmin(Ddbg, min_seed_dist);
    L_dbg = watershed(imimposemin(Ddbg, mini_dbg));

    figure('Name', sprintf('Debug %d', debugCount), 'Color', 'w');
    tiledlayout(2, 3, 'Padding', 'compact', 'TileSpacing',
'compact');

    nexttile; imshow(I8); title('Raw DAPI');
    nexttile; imshow(bw0); title('Binary');
    nexttile; imshow(bw1); title('Cleaned');
    nexttile; imshow(Ddbg, []); title('Distance map');
    nexttile; imshow(mini_dbg); title('Minima');
    nexttile; imshow(label2rgb(L_dbg)); title('Watershed');

    drawnow;
end

% Measure and filter nuclei
stats = regionprops(bw, 'Area', 'Perimeter');
A = [stats.Area] * pixel_area;
P = [stats.Perimeter];
C = 4 * pi * A ./ (P.^2);

keep = A >= min_um2 & A <= max_um2 & C >= min_circ;
allAreas{p} = [allAreas{p}; A(keep)'];
end
end
end

% Combine data
data = [];
labels = {};

for p = 1:numel(conditions)
    n = numel(allAreas{p});
    if n > 0
        data = [data; allAreas{p}];

```

```

        labels = [labels; repmat(conditions(p), n, 1)];
    end
end

% Export
maxLen = 0;
if ~isempty(allAreas)
    maxLen = max(cellfun(@numel, allAreas));
end

if maxLen > 0
    areaMat = nan(maxLen, numel(conditions));

    for p = 1:numel(conditions)
        A = allAreas{p};
        areaMat(1:numel(A), p) = A(:);
    end

    areaCell = num2cell(areaMat);
    areaCell(isnan(areaMat)) = {[]};

    outCell = [conditions; areaCell];
    txt_wide = fullfile(out_dir, 'nuclear_area_columns_by_condition.txt');
    writecell(outCell, txt_wide, 'Delimiter', '\t');

end

```

Appendix D: MATLAB script for LDV analysis of Biocell membrane displacement

```

clc;
close all;

% Settings from the LDV
df = 19.53125; % Hz
T_recon = 1 / df;
Nt = 131072;
NmaxBins = 3000;

% Number of points
n_individual = 3;
n_selected = 10;

% Reconstruction bands
fc_center = 1e6;
fc_halfwidth = 500; % Hz

prf_center = 1000;
prf_halfwidth = 200; % Hz

% ranges for FFT plots
fft_carrier_xlim = [0.95e6, 1.05e6];
fft_prf_xlim = [800, 1200];

% Noise settings
fmin = 100;
mad_thresh = 8;

% load the cache
root = pwd;
cacheFile = fullfile(root, 'magphase_fullspec_cache.mat');
force_rebuild = false;
is_mag_peak = true;

% Output folder
timestamp = datestr(now, 'yyyymmdd_HHMMSS');
outdir = fullfile(root, ['LDV_Output_' timestamp]);
if ~exist(outdir, 'dir')
    mkdir(outdir);
end

% Load cache if it exists or build it
X = [];
Y = [];
Fcell = {};
Vcell = {};

if isfile(cacheFile) && ~force_rebuild
    S = load(cacheFile);

    if all(isfield(S, {'X','Y','Fcell','Vcell','is_mag_peak'})) &&
        S.is_mag_peak == is_mag_peak
        X = S.X;
        Y = S.Y;
        Fcell = S.Fcell;
        Vcell = S.Vcell;
        fprintf('Loaded cached full spectra (%d points).\n', numel(X));
    end
end

```

```

end

if isempty(X)
    files = dir(fullfile(root, '*.txt'));
    if isempty(files)
        error('No .txt files found in %s', root);
    end

    nFiles = numel(files);
    X = nan(nFiles, 1);
    Y = nan(nFiles, 1);
    Fcell = cell(nFiles, 1);
    Vcell = cell(nFiles, 1);

    fprintf('Reading %d files and building cache...\n', nFiles);

    for k = 1:nFiles
        fpath = fullfile(files(k).folder, files(k).name);

        try
            [x, y, f, mag, ph_deg] = read_polytec_magphase_point(fpath);

            X(k) = x;
            Y(k) = y;

            if ~is_mag_peak
                mag = mag * sqrt(2); % convert RMS to peak if needed
            end

            Vcell{k} = mag(:) .* exp(1j * (ph_deg(:) * pi / 180));
            Fcell{k} = f(:);

            if mod(k, 50) == 0 || k == 1 || k == nFiles
                fprintf(' %4d / %4d %s\n', k, nFiles, files(k).name);
            end
        end

        good = isfinite(X) & isfinite(Y) & ~cellfun(@isempty, Fcell) &
~cellfun(@isempty, Vcell);

        X = X(good);
        Y = Y(good);
        Fcell = Fcell(good);
        Vcell = Vcell(good);

        save(cacheFile, 'X', 'Y', 'Fcell', 'Vcell', 'is_mag_peak');
        fprintf('Saved cache to %s\n', cacheFile);
    end

    % Select top points based on RMS displacement in a wide carrier band
    selection_band = [fc_center - 2e5, fc_center + 2e5];
    M_rms = band_x_rms(Fcell, Vcell, selection_band, fmin, mad_thresh);
    [idxTop_all, ~] = topN_indices(M_rms, n_selected);

    fprintf('Selected top %d points.\n', numel(idxTop_all));

    % Storage

```

```

carrier_t = cell(numel(idxTop_all), 1);
carrier_x = cell(numel(idxTop_all), 1);
carrier_f_raw = cell(numel(idxTop_all), 1);
carrier_V_raw = cell(numel(idxTop_all), 1);

prf_t = cell(numel(idxTop_all), 1);
prf_x = cell(numel(idxTop_all), 1);
prf_f_raw = cell(numel(idxTop_all), 1);
prf_V_raw = cell(numel(idxTop_all), 1);

% Main loop
for r = 1:numel(idxTop_all)
    i = idxTop_all(r);

    f_all = Fcell{i}(:);
    V_all = Vcell{i}(:);

    [fG, VG] = guard_full(f_all, V_all, fmin, mad_thresh);

    carrier_f_raw{r} = fG;
    carrier_V_raw{r} = VG;
    prf_f_raw{r} = fG;
    prf_V_raw{r} = VG;

    % Carrier frequency
    maskC = fG >= (fc_center - fc_halfwidth) & fG <= (fc_center +
fc_halfwidth);
    fC = fG(maskC);
    VC = VG(maskC);

    if ~isempty(fC)
        [~, tC, xC] = recon_pp_vec_T1df(fC, VC, Nt, NmaxBins, T_recon);
        carrier_t{r} = tC;
        carrier_x{r} = xC;
    end

    % PRF
    maskP = fG >= (prf_center - prf_halfwidth) & fG <= (prf_center +
prf_halfwidth);
    fP = fG(maskP);
    VP = VG(maskP);

    if ~isempty(fP)
        [~, tP, xP] = recon_pp_vec_T1df(fP, VP, Nt, NmaxBins, T_recon);
        prf_t{r} = tP;
        prf_x{r} = xP - mean(xP);
    end
end

% Summary
fileC = fullfile(outdir, 'carrier_1MHz_results.txt');
fileP = fullfile(outdir, 'prf_1kHz_results.txt');

fc_low = fc_center - fc_halfwidth;
fc_high = fc_center + fc_halfwidth;

prf_low = prf_center - prf_halfwidth;
prf_high = prf_center + prf_halfwidth;

```

```

FID_C = fopen(fileC, 'w');
fprintf(FID_C, ...

'Rank\tIndex\tX(m)\tY(m)\tpp_nm\tRMS_nm\tSNR_dB\tnBins\tdf_Hz\tBandLow\tBandHigh\tT_recon_s\n');

FID_P = fopen(fileP, 'w');
fprintf(FID_P, ...

'Rank\tIndex\tX(m)\tY(m)\tpp_nm\tRMS_nm\tSNR_dB\tnBins\tdf_Hz\tBandLow\tBandHigh\tT_recon_s\n');

for r = 1:numel(idxTop_all)
    idx = idxTop_all(r);

    % Carrier
    tC = carrier_t{r};
    xC = carrier_x{r};
    if ~isempty(tC)
        magC = abs(carrier_V_raw{r});
        [SNR_C, nBins_C] = snr_calc(magC);

        pp_nm = (max(xC) - min(xC)) * 1e9;
        rms_nm = rms(xC) * 1e9;

        fprintf(FID_C, ...

'%d\t%d\t%.6f\t%.6f\t%.3f\t%.3f\t%.2f\t%d\t%.5f\t%.0f\t%.0f\t%.6f\n', ...
    r, idx, X(idx), Y(idx), pp_nm, rms_nm, SNR_C, nBins_C, df, fc_low,
fc_high, T_recon);
    end

    % PRF
    tP = prf_t{r};
    xP = prf_x{r};
    if ~isempty(tP)
        magP = abs(prf_V_raw{r});
        [SNR_P, nBins_P] = snr_calc(magP);

        pp_nm = (max(xP) - min(xP)) * 1e9;
        rms_nm = rms(xP) * 1e9;

        fprintf(FID_P, ...

'%d\t%d\t%.6f\t%.6f\t%.3f\t%.3f\t%.2f\t%d\t%.5f\t%.0f\t%.0f\t%.6f\n', ...
    r, idx, X(idx), Y(idx), pp_nm, rms_nm, SNR_P, nBins_P, df, prf_low,
prf_high, T_recon);
    end
end

fclose(FID_C);
fclose(FID_P);

% Individual plots
n_to_plot = min(n_individual, numel(idxTop_all));

% Averaged signals time and displacement vectors for the Fc AND prf
[avgC_t, avgC_x, avgC_sd] = average_signals(carrier_t, carrier_x);
[avgP_t, avgP_x, avgP_sd] = average_signals(prf_t, prf_x);

```

```

% filter out freq above fmin before further manipulating the FFT

function [f2, V2] = guard_full(f, V, fmin, mad_sig)
good = isfinite(f) & isfinite(V) & f >= fmin;
f = f(good);
V = V(good);

if isempty(f)
    f2 = [];
    V2 = [];
    return;
end

% identify suspicious spikes in FFT using median absolut deviation and remove

mag = abs(V);
med_mag = median(mag);
mad_mag = mad(mag, 0);

spike = (mag > med_mag + mad_sig * mad_mag) & (mag > 10 * med_mag);
f(spike) = [];
V(spike) = [];

f2 = f;
V2 = V;
end

% calculate displacement within the freq band for Fc and PRF

function M = band_x_rms(Fcell, Vcell, edges, fmin, mad_sig)
N = numel(Fcell);
M = nan(N, 1);

for i = 1:N
    [f, V] = guard_full(Fcell{i}, Vcell{i}, fmin, mad_sig);
    if isempty(f)
        continue;
    end

    mask = f >= edges(1) & f <= edges(2);
    f_band = f(mask);
    V_band = V(mask);

    if isempty(f_band)
        continue;
    end

    v_mag = abs(V_band);
    x_rms = (v_mag ./ sqrt(2)) ./ (2 * pi * f_band);
    M(i) = sqrt(sum(x_rms.^ 2));
end
end

% find ttop points (max displacement)

function [idxTop, vals] = topN_indices(M, N)
[vals, idxTop] = maxk(M, min(N, sum(isfinite(M))));
end

```

```

% reconstruct time domain displacement from FFT

function [pp, t, x] = recon_pp_vec_T1df(f, Vpeak, Nt, NmaxBins, T)
if numel(f) > NmaxBins
    idx = round(linspace(1, numel(f), NmaxBins));
    f = f(idx);
    Vpeak = Vpeak(idx);
end

% convert velocity spectrum to displacement and calculate peak-peak

t = linspace(0, T, Nt).';
Xf = Vpeak ./ (1j * 2 * pi * f);
x = real(exp(1j * 2 * pi * t * f.') * Xf);
pp = max(x) - min(x);
end

% go back to FFT domain

function [f_fft, X_fft] = local_fft(t, x)
dt = mean(diff(t));
N = numel(t);

Xfull = fft(x);
f = (0:N-1)' / (N * dt);

halfN = floor(N / 2);
f_fft = f(1:halfN);
X_fft = Xfull(1:halfN);
end

% average multiple signals to get mean +/- SD

function [avg_t, avg_x, sd] = average_signals(tcell, xcell)
valid = ~cellfun(@isempty, tcell);

if ~any(valid)
    avg_t = [];
    avg_x = [];
    sd = [];
    return;
end

ref_idx = find(valid, 1);
tref = tcell{ref_idx};
Nref = numel(tref);

Xmat = nan(Nref, sum(valid));
k = 1;

for r = find(valid)'
    t = tcell{r};
    x = xcell{r};

    if numel(t) == Nref
        Xmat(:, k) = x;
    else
        Xmat(:, k) = interp1(t, x, tref, 'linear', 'extrap');
    end
end

```

```

        k = k + 1;
    end

    avg_t = tref;
    avg_x = mean(Xmat, 2, 'omitnan');
    sd = std(Xmat, 0, 2, 'omitnan');
    end

function shaded_mean_sd(t, mean_nm, sd_nm)
    upper = mean_nm + sd_nm;
    lower = mean_nm - sd_nm;

    hold on;
    fill([t; flipud(t)], [upper; flipud(lower)], [0.8 0.8 1], ...
        'LineStyle', 'none', 'FaceAlpha', 0.3);
    plot(t, mean_nm, 'b', 'LineWidth', 1.5);
    grid on;
    hold off;
end

```

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