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# Pulsed Amplitude Modulated Ultrasound Increase the Rate of Mineralization in Osteoblast Cells

A thesis presented  
by

Sardar Muhammad Zia uddin  
to

The Graduate School  
in Partial Fulfillment of the  
Requirements  
for the Degree of

Master of Science

in

Biomedical Engineering  
Stony Brook University

August 2009

State University of New York

The Graduate School

Sardar Muhammad Zia uddin

We, the thesis Committee for the above candidate for the Master of Science Degree hereby  
recommend acceptance of this thesis

Yi-Xian Qin, Ph.D

Thesis Advisor

Professor, Department of Biomedical Engineering

Michael Hadjiargyrou, Ph.D

Defense Chairperson

Associate Professor, Department of Biomedical Engineering

Wei Lin, Ph.D

Res. Assistant Professor, Department of Biomedical Engineering

This thesis is accepted by the Graduate School

Lawrence Martin

Dean of the Graduate School

Abstract of the thesis

**Pulsed amplitude modulated ultrasound increase the rate of mineralization in  
osteoblast cells**

by

Sardar Muhammad Zia Uddin

Master of Science

in

Biomedical Engineering

Stony Brook University

2009

Low-intensity pulse ultrasound has shown capability in promoting bony ingrowth and accelerating of fracture healing. Ultrasound has been known to increase calcium deposition in the MC3T3, a pre osteoblast cell line, in which different intensities ranging from 30 – 2000 mW/cm<sup>2</sup> show increase in the rate of mineralization. While changing of the loading frequency can alter the sensitivity of bone's adaptive response, it is proposed that the amplitude modulation of ultrasound can provide optimized mechanical stimulus to osteoblast and trigger cellular responses such as conformational changes in the transmembrane proteins to initiate downstream signaling pathways to increase the rate of mineralization. To make the ultrasound effect more enhanced and targeted at the focal point, this study uses a novel approach of applying pulsed Amplitude Modulated Ultrasound (pAMUS) to osteoblast cells. The objective was to analyze the effects of pAMUS on the rate of mineralization and enzymatic activity in

osteoblast cells. The study hypothesized that pAMUS will increase mineralization in osteoblast cells. The pAMUS signal of 45 kHz and 100 kHz was generated using two signal generators with different frequencies. Pulsed signal was amplified through a power amplifier and processed to two identical focused ultrasound probes, focusing at the same point in the culture dish. The effects of pAMUS were evaluated using an pAMUS signal of 45 KHz and 100 KHz with 20% duty cycle. The hydrophone scanned verified the formation of a focal point equal distance (16mm) from the surface of both transducers. Intensity profile using computer controlled 2D scanner showed circular focal point with a diameter of approximately 10mm.

The effects of the signal was studied using MC3T3 cells cultured in 10mM beta-glycerophosphate and 50µg/ml ascorbic acid at time points Day 7,12 and 18. The cells were analyzed for calcium mineralization using Alizarin Red staining and Calcium assay at OD<sub>650nm</sub> and for alkaline phosphates (ALP) activity using ALP assay at OD<sub>405nm</sub>.

The results from the study showed a significant increase in ALP activity at Day 12 and 18, when stimulated with pAMUS. At Day12, pAMUS stimulated samples , 45 and 100 KHz (28.89 ± 4.01 µM / 10<sup>4</sup> cells and 33.51 ± 3.20 µM / 10<sup>4</sup> cells respectively) samples showed a significant increase p< 0.0005 relative to samples stimulated with pulsed ultrasound (5.59 ± 2.70 µM / 10<sup>4</sup> cells) and no ultrasound( 5.60 ± 1.02 µM / 10<sup>4</sup> cells) . Day 18 control (pulsed ultrasound, 27.60 ± 7.74 µM / 10<sup>4</sup> cells) and experimental (pAMUS, 45 and 100 KHz , 30.48 ± 5.44 µM / 10<sup>4</sup> cells and 30.21 ± 9.63 µM / 10<sup>4</sup> cells respectively ) showed a significant increase from sham groups (7.06 ± 4.04 µM / 10<sup>4</sup> cells, p< 0.001) but there is not significant difference between control and experimental

groups at Day18. Significantly higher ( $p < 0.01$ ) level of matrix calcification was observed at Day 18 in cells samples stimulated with pAMUS at 45 and 100 KHz ( $0.37 \pm 0.01$  mM and  $0.43 \pm 0.03$  mM) and control samples ( $0.33 \pm 0.02$  mM,  $P < 0.05$ ). These results imply that pAMUS increases the rate of mineralization in osteoblast cells when compared with pulsed ultrasound and no ultrasound samples.

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## List of Symbols

$f_1$	Ultrasound frequency 1
$f_2$	Ultrasound frequency 2
$M$	Modulation index
$C$	Carrier Amplitude
$\Phi_c$	Carrier signal Phase
$M$	Modulated Amplitude
$\Phi_m$	Modulated signal Phase
$F_c$	Carrier Frequency
$F_m$	Modulated Frequency

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## List of Abbreviation

pAMUS	Pulsed amplitude modulated ultrasound
LIPUS	Low intensity pulsed ultrasound
pUS	Pulsed Ultrasound
ALP	Alkaline phosphatase
MMP-13	Matrix metalloprotease 13
Alpha MEM	Alpha Minimum Essential Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Solution
Pen-Strep	Penicillin-Streptomycin
ECM	Extracellular Matrix

## Acknowledgements

I will like to thank my mentor and advisor Dr. Yi-Xian Qin, the principle investigator of the lab for giving me this opportunity and courage, and helping me work towards my Master's Thesis. I learned new techniques and skills under his supervision. He always encouraged me to think critically and creatively in my experimental designs and take extensive care and caution in my data analysis.

I will render my thanks to Dr. Michael Hadjiargyrou, for his ever present guidance for the past 7 years. It is him who inspired me to follow research as a career choice, to look at things positively and critically, and to always keep my hopes high and be optimistic in life and career. He was always available to discuss results from this study and give his suggestion to improve experimental methods.

I am grateful to Dr. Wei Lin for serving in my thesis committee and providing me an in-depth understanding of ultrasound signals and biomechanical processes that can be involved in signal transduction. He, over the numerous meetings gave me input on experimental design and progress of the study.

I will also like to thank my colleagues, Dr. Jiqi Cheng, Fred Serra-Hsu , Suzanne Farreri, Minyi Hu, Murtaza Malbari, and others for providing a helping hand when needed. I will like to particularly mention Jiqi Cheng and Fred Serra-Hsu as they helped me great deal from the start to the experimental setup.

Finally I will like to thank Stony Brook University and the Department of Biomedical Engineering in particular for giving me the opportunity to learn and do research

## Hypothesis

*Pulsed amplitude modulated ultrasound (pAMUS) will accelerate mineralization in osteoblast cells in comparison to pulsed ultrasound exposure.*

## Specific Aims

**Sub-Hypothesis 1:** Focused pAMUS can be achieved by a pair of focused ultrasound transducers with burst pulse signals.

**Specific Aim 1:** Designing and verification of the Experimental setup to achieve pAMUS system at specific focal point using hydrophone.

**Sub-Hypothesis 2:** Acoustic energy at concentrated area has potential to promote mineralization in osteoblast cells.

**Specific Aim 2:** Quantifying Calcium deposition and ALP activity in MC3T3 cells after pAMUS and comparing with pulsed ultrasound.

## Background

The skeleton has always been an important organ for the survival of any organism through the selection process of evolution. It provides physical support and structure around which other organs of an animal grow. In the case of humans this frame work is provided by a complicated structure of bones which has evolved through millions of years to its present shape. Human bones are extremely efficient in providing support over the span of human life but with increasing age, bones tend to lose their strength. As the human body grows old, it tends to lose its bone mass, which can lead to osteopenia and in severe cases to osteoporosis. Beside the aging effect, the daily rigor of human life and day to day activity can lead to stress fracture or fractures, for instance, 5.6 million fractures occur annually in the United States [1]. Bone is really good in healing itself over the time but some fractures, about 5 – 10%, fail to heal in sufficient time so they are either characterized as delayed (6-9 months) or in severer cases non-unions (more then 9 months). These cases are common in aging people or people with systematic disorders like diabetes, obesity, osteopenia and osteoporosis [2]. Furthermore, today's space age also affects bone health as astronauts lose a significant amount of bone mass in their time in space [3-5] and same kind of effects has been seen in patients with long term bed rest [6]. To cure all these issues billions of dollars are spent in health care beside the mental and emotional fatigue and hardships caused [1]. Thus there has been an ongoing research to understand the healing process in bone and enhance the process of bone healing.

After decades [7-15] of active bone research, the process of bone healing is still not fully understood. There are two main factors in bone healing, rate of formation of mineralized matrix by osteoblast and resorption of matrix by osteoclast. The balance of these two events determines the rate of bone formation and regeneration in case of injury. Mechanical, optical, electrical and ultrasound stimulations have been applied in different variations to enhance the mineralization of bone by osteoblasts and osteocytes.

Mechanical stimuli have been known to increase cell proliferation and differentiation in bone cells [12, 13, 16, 17] and induce bone growth following Wolff's Law [18]. In 1892, German surgeon Wolff J presented a theory: "Every change in the form and function of a bone or of their function alone is followed by certain definite changes in their internal architecture, and equally definite alteration in their external conformation, in accordance with mathematical laws." According to this theory, bone grows in response to mechanical stress and strain it experiences and adapts to facilitate the mechanical demand on the tissue. Furthermore in accordance with his theory, Wolff also proposed a biological mechanism of bone regeneration by "interstitial bone formation"-creating new bone fragments within old bone tissue. In contrast, Roux W[10] proposed more dynamic model of bone growth by resorption of old bone and formation of new tissue to replace new bone. Recent advancements in understanding of bone formation show that Roux was right as bone tends to be a dynamic structure which sustains and adapts it self through resorption and formation of new tissue.

However, Wolff's law of bone remodeling is important since he associated bone adaption with mechanotransduction by either micro-damage in bone tissue or strain in the mineralized bone tissue. Wolff assumed that these changes in the tissue would be sensed by mechanical sensors spread all over the tissue and respond to any biomechanical signal by emitting chemical signals to other cells within the tissue. Even though first part of the Wolff's law turned out to be incorrect, the mechanotransduction concept is considered to be correct as bone cells response to mechanical stimulus and

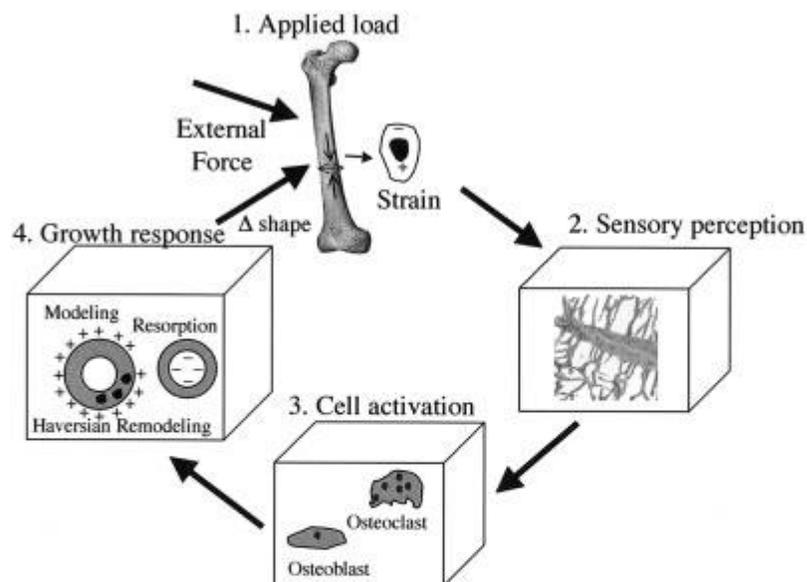


Fig 1: Localized response of bone tissue to mechanical stimulus (Strain). Strain is sensed by sensory receptors in the bone tissue which activates cell differentiation and proliferation and increase the rate of bone formation and reduce the rate of resorption. This process is also known as Haversian Remodeling. This leads to bone growth response and change in tissue mechanical characteristics.

[Figure adopted from Person et al. (2004)]

relay that signal to neighboring cells but more local than assumed by Wolff.

Figure 1[19] represent a more recent understanding of Wolff's law. Pearson et al (2004)(fig 1) discusses the big picture of bone response to mechanical stimuli (strain) as it gets sensed by mechanical receptor bone cells. These receptors initiate signaling cascades which activate a cell proliferation and differentiation in bone cells, locally. Due to increases in cell activation, the rate of bone formation increases and the rate of bone resorption decreases. This dynamic response is also known as Haversian Remodeling. Haversian remodeling enables bone tissue to grow and better adapt to changing mechanical demands. According to this model the basic functioning component of Wolff's law or Haversian Remodeling are bone cells, osteocytes, osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation and laying down bone matrix by extracting different proteins such as osteocalcin, osteopontin, osteonectin and collagen. These proteins form a dense matrix called osteoid. Osteocytes are formed when osteoblasts mature and form osteoid around themselves. Osteocytes are specialized cells that are linked to each other through gap junctions and processes passing through the cellular matrix forming channels known as canaliculi. Osteocytes are thought to function as sensory cells in bone tissue along with bone formation and ion exchange within the osteocytes network[20-22]. Osteoclasts are bone resorption cells and they breakdown old mineralized matrix, which allows osteoblast to form new

matrix. Synergy of all these components enables bone tissue to respond to mechanical stimulus.

In the last five decades different kinds of mechanical stimuli have been applied to bone tissue to understand the mechanotransduction properties of bone cells and how mechanotransduction affects the rate of matrix formation. One of these stimuli is application of ultrasound to bone cells or tissue. Ultrasound is an acoustic radiation above human audible frequencies. Ultrasound travels through the medium (liquid or solid) as pressure waves and display properties of longitudinal waves. When these waves come across another medium (bone surface), they go through refraction, reflection and diffraction and create shear waves [23, 24] within the extracellular matrix. Extracellular matrix is a gel of polysaccharides (hyaluronic acid, heparin sulfate), and fibrous proteins (collagen and elastin), which are connected to cells through transmembrane proteins present in the cell membrane. Secondary waves formed due to ultrasound induce mechanical changes like stress and strain in extracellular matrix (ECM), which get sensed by cells as they are physically attached to ECM. Cells sense these stress and strains via mechanosensors present on membrane as proposed by Wolff's law. These biosensors transform mechanical signal into chemical signals by initiating conformation changes in the cell's cytoskeleton and other anchored proteins inside the cell. These changes can lead to upregulation of different transcription factors, genes and result in formation of functional enzymes (such as ALP, MMP-13) and structural proteins (collagen), which are required for mineralization of matrix. The

enhanced mineralization of matrix in response to mechanical stimuli can lead to formation of osteoid and maturation of osteoblasts to osteocytes [25-27].

Several studies have shown the effects of mechanical stimulation on differentiation and proliferation of osteoblasts [28-31]. Both sustained and dynamic mechanical stimulus has been studied relative to osteoblast differentiation and results show that cells response better to dynamic or cyclic stimulations [31-36]. Furthermore cyclic or dynamic loading is more representative of physiological conditions as bone tissue get stimulated by muscular contraction and relaxation. The dynamic or cyclic stimulation is imitated in ultrasound stimulation by applying Low intensity pulsed ultrasound (LIPUS). Low intensity is used to reduce the thermal effects during stimulation and maintain optimal temperature.

An ultrasound wave provides a pressure waves which induces biochemical events in bone cells [16, 37, 38]. The mechanism of ultrasound effects on cell proliferation and differentiation hasn't been fully understood completely. Thus different studies have applied different ultrasound parameters to optimize bone growth. The most studied parameter has been intensity ranging from 30 to 1000mW/cm<sup>2</sup> [39]. Most of the ultrasound applications use a pulsed ultrasound signal, which is on for 200µs and off for 800µs in one complete cycle. These settings are preferred keeping in mind that cells react best to cyclic stimulations and 20% on/off cycle respond best to muscular contraction in physiological conditions. Most studies ultrasound frequencies relative to bone healing or bone cell proliferation ranges between 1 – 3 MHz.

Effects of LIPUS have been studied relative to intercellular activity, cytokines release [40], gene expression notably aggrecan [41], calcium mineralization[42], Akt pathway[43], Potassium influx[44], angiogenesis [45], adenylyl cyclase activity and TGF- $\beta$  synthesis[46]. All these studies and many others suggest an increase in rate of bone healing in the presence of LIPUS stimulations but no study clarifies the mechanism through which LIPUS induces these changes or increases the rate of bone healing. Considering the encouraging results from both *in vitro* and *in vivo* experiments, the FDA approved use of first ultrasound stimulator in 1994.

The exact mechanism through which LIPUS enhances bone cells properties and increase the rate of bone healing is unknown but there is an interesting correlation between LIPUS stimulation and cellular responses due to flow induced shear stress and strain. Ultrasound is known to induce acoustic streaming and cavitations, both of which can induce fluid flow through extracellular matrix [47] and shear stresses and strains to osteoblasts, which are known to respond to them in a similar fashion as they do to LIPUS[13, 33, 34, 48-50]. To sense these shear stresses and strains, osteoblasts use integrins. Osteoblasts stimulated by LIPUS have shown higher accumulation of integrins in stimulated cells and an increase in activity of proteins down stream of integrin associated pathways [43, 49, 51-54].

Beside acoustic streaming ultrasound also has a thermal effect on the cells. Absorbing of ultrasound signal can result in an energy change into heat energy [55, 56] which can increase cellular temperature up to 1°C. This change may be small but some

enzymes such as MMP-1 or collagenase can be sensitive to such changes[57]. Further on the organ, level such small changes can stimulate blood flow to extremities which can have profound effect on the behavior of bone tissue[58].

LIPUS has been shown to enhance bone growth and increase the rate of mineralization, but the application of high energy ultrasound to tissues can lead to tissue necrosis due to the thermal effects of ultrasound depending on the intensity and duration of application [59, 60]. Furthermore repeated application of ultrasound can cause third degree burn marks and irritations at the site of application and patient compliance and understanding of usage can enhance these effects. Also, as these stimulators are designed for bone tissue, usually embedded in muscle and other kind of tissues, how high energy ultrasound will affect the functionality of surrounding tissue as ultrasound has to pass through it to reach to bone may cause concern. These surrounding tissues cause the loss of ultrasound energy; this can reduce the efficiency of treatment. Ultrasound pressure wave can induce cavitations in soft tissue surrounding the bone as they have higher concentration of microbubbles compare to bone tissue. Feril et al have discussed that formation of cavitations in cellular membrane can lead to cell death if cell is unable to repair disorientations in the cellular membrane. These cavitations depending on the ultrasound energy can also affect other organelles in side the cell. Furthermore prolonged ultrasound treatment can induce formation of free radicals inside the cells which can reduce cells response and induce cell necrosis [60].

Spatial resolution of ultrasound beam is an important factor considering the wave signal pattern in near field ( $D^2/4\lambda$ ,  $D$  – diameter of transducer element,  $\lambda$  is wave length of the signal) and far field. Ultrasound waves pattern close to the surface (near field) of the element are resultant of pressure waves produced by point sources on the transducer. The waves generated interact with each other and go through interference and form a resultant wave pattern. In the near field region beam edges are not sharp, and represent an average intensity at the axial distance. In far field, distance greater than  $D^2/4\lambda$ , the beam diverges and the wave pattern resembles the pattern produced by single point source at the center of transducer disc. For the pulsed field frequency spectrum is usually peak around a central frequency. Non uniform intensity in near field makes it hard to quantify the field intensity levels accurately in near field. Furthermore, near field has higher energy levels thus has enhanced thermal and non thermal effects. Far field is much more uniform energy and intensity spectrum but it diverges with distance thus energy dissipates significantly with small changes in distance. The reduction in energy level can reduce the thermal and mechanical effects of ultrasound significantly.

To enhance the effect of ultrasound on the target fracture side, a more focused, directed and low energy ultrasound signal is required which can focus on damage tissue and have its most effect on the site of lesion rather than affecting surrounding tissue in same manner as of target tissue. Furthermore signal should be modulated such that it has most strength or focal point at the point of fracture.

The objective of this study is to study and compare the effects of pAMUS and pulsed ultrasound on calcium deposition and ALP activity in osteoblast cells. pAMUS is pulsed amplitude modulated signal, which is a lower energy signal generated at focal point by combination of two low energy ultrasound signals. The resultant signal formed at focal point in amplitude modulated as amplitude of signal varies. This study used pulsed ultrasound to imitates the effects of low intensity pulsed ultrasound but with significantly lower energy to make it comparative to energy used by pAMUS. Thus enable us to study the effect of different ultrasound signal on osteoblast cells. The low energy pulsed ultrasound for this study was generated with same setup and confocal properties. Table 1 illustrates the differences and similarities between pAMUS (*experimental*), pulsed ultrasound (*control*). The objective of this study is to optimize the ultrasound signal for bone remodeling and healing. The experiment was design to analyze the effects of modulation rather than intensity or energy.

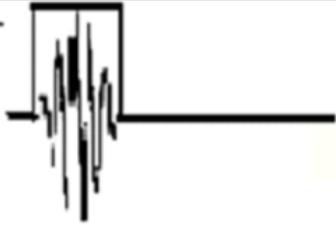
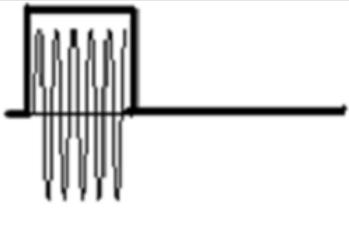
Ultrasound	pAMUS (experimental)	pulsed Ultrasound (control)
Intensity	$\sim 5 \text{ mW/cm}^2$	$\sim 5 \text{ mW/cm}^2$
Modulation	Amplitude modulation	no modulation
Duty cycle	20%	20%
Focused	Focused	Focused
Signal		

Table 1: Compares the parameter of pAMUS (Experimental) and pulsed ultrasound signal. Intensity and duty rate for both signals were kept constant to analyze the effects of modulation on the rate of mineralization.

## Methodology

Considering the effects of high energy ultrasound on muscle and skin tissue along with energy loss to soft tissue surrounding the bone, this study analyzes the application of focused and low energy pAMUS in osteoblast cells. To get maximum signal strength at focal point, two focused ultrasound signals with different frequencies are focused at the same focal region. In this setup the focal region of cells imitates the site of feature at organ level. When two signals of different frequencies are combined at focal they form modulated ultrasound. Amplitude modulated signal consist of a “carrier Signal” and a “modulate signal,” the modulating signal changes the amplitude of the carrier signal. The maximum amplitude reached either at positive or negative axis is known as peak amplitude and in full cycle amplitude from positive to negative give peak to peak amplitude.

The setup in this study combines two ultrasound frequencies at focal point. Both signals are sinusoidal waves given by:

Signal 1:  $\sin f_1 t$

Signal 2:  $\sin f_2 t$

At focal point these two signals combine to give:  $\sin f_1 t + \sin f_2 t$

Using sum to product identities  $\sin f_1 t + \sin f_2 t = 2 \sin\left(\frac{f_1 + f_2}{2}\right)t \cos\left(\frac{f_1 - f_2}{2}\right)t$

$$F_1 = \left(\frac{f_1 + f_2}{2}\right)$$

$$F_2 = \left(\frac{f_1 - f_2}{2}\right)$$

$$2 \sin\left(\frac{f_1 + f_2}{2}\right)t \cos\left(\frac{f_1 - f_2}{2}\right)t = 2 \sin F_1 t \cos F_2 t$$

This equation is comparable to amplitude modulated signal which is given by:

$$m(t) = \sin(F_c t + \phi_c) \cdot \sin(F_m t + \phi_m)$$

For simplification  $\phi_c$  and  $\phi_m$  can be considered equal to zero as they describe initial phase of modulated and carrier frequencies.

$$m(t) = \sin F_c t \cdot \sin F_m t$$

$$F_c = F_1 = \left(\frac{f_1 + f_2}{2}\right)$$

$$F_m = F_2 = \left(\frac{f_1 - f_2}{2}\right)$$

$f_1$  = ultrasound frequency 1

$f_2$  = ultrasound frequency 2

$F_m$  = Modulation frequency

$F_c$  = Carrier frequency

$\Phi_c$  = carrier signal Phase

$\Phi_m$  = Modulated signal Phase

Both ultrasound signals would be focused at the same focal point thus providing the maximum energy to the cells in the focal region. The element of transducer is 13 mm and applied frequency is around 1 – 1.2 MHz, applying, near field limit equation:

$$\text{Near field Limit} = D^2/4\lambda = D^2f/4V$$

$\lambda$  = wavelength of ultrasound (1.2-1.5 mm)

f = frequency of ultrasound ( 1- 1.2 MHz)

V= speed of ultrasound (1482 m/s in medium(water))

Applying the near field limit equation given applied parameters give us near field range of 28.5 – 34.2 mm for 1 – 1.2 MHz frequency. In the current setup the distance between transducer surface and cell layer is approximately 16mm. Thus this setup applied near field ultrasound.

This setup reduces the energy and signal loss to surrounding soft tissue in case of *in vivo* application and applies more targeted approach to the area of interest. Besides applying maximum energy to site of the fracture, this approach also reduces the exposure of pre and post fracture tissues to high acoustic energy as individual probes used are of much lower energy.

### **Specific Aim 1: Design and verification of focused pAMUS**

#### **Experimental Setup:**

To achieve the pAMUS signal, a setup was designed which can generate two different frequencies and those frequencies to be focused at one focal point. To generate the ultrasound signal, function generator (Model DS345 30 MHz Function /

Arbitrary Waveform Generator, Stanford Research Systems, Inc., *Sunnyvale, CA*) was used. The function of this function generator is to trigger the primary signal, which get fed into two secondary function generators, Function Generator 1 & 2 (AFG3021 Single Channel Arbitrary / Function Generator, Tektronix, Inc., Tequipment.net, Long Branch, NJ, USA). The Secondary function generator generates a pulsed ultrasound signal with 20% repetition rate. To get pAMUS both generators, must generate signals with different frequencies but same phase so there can be no destructive interference and 100% modulation can be achieved. Pulsed signal is then processed through a power amplifier (Model 2350 Dual Channel High Voltage Precision Power Amplifier, TEGAM,

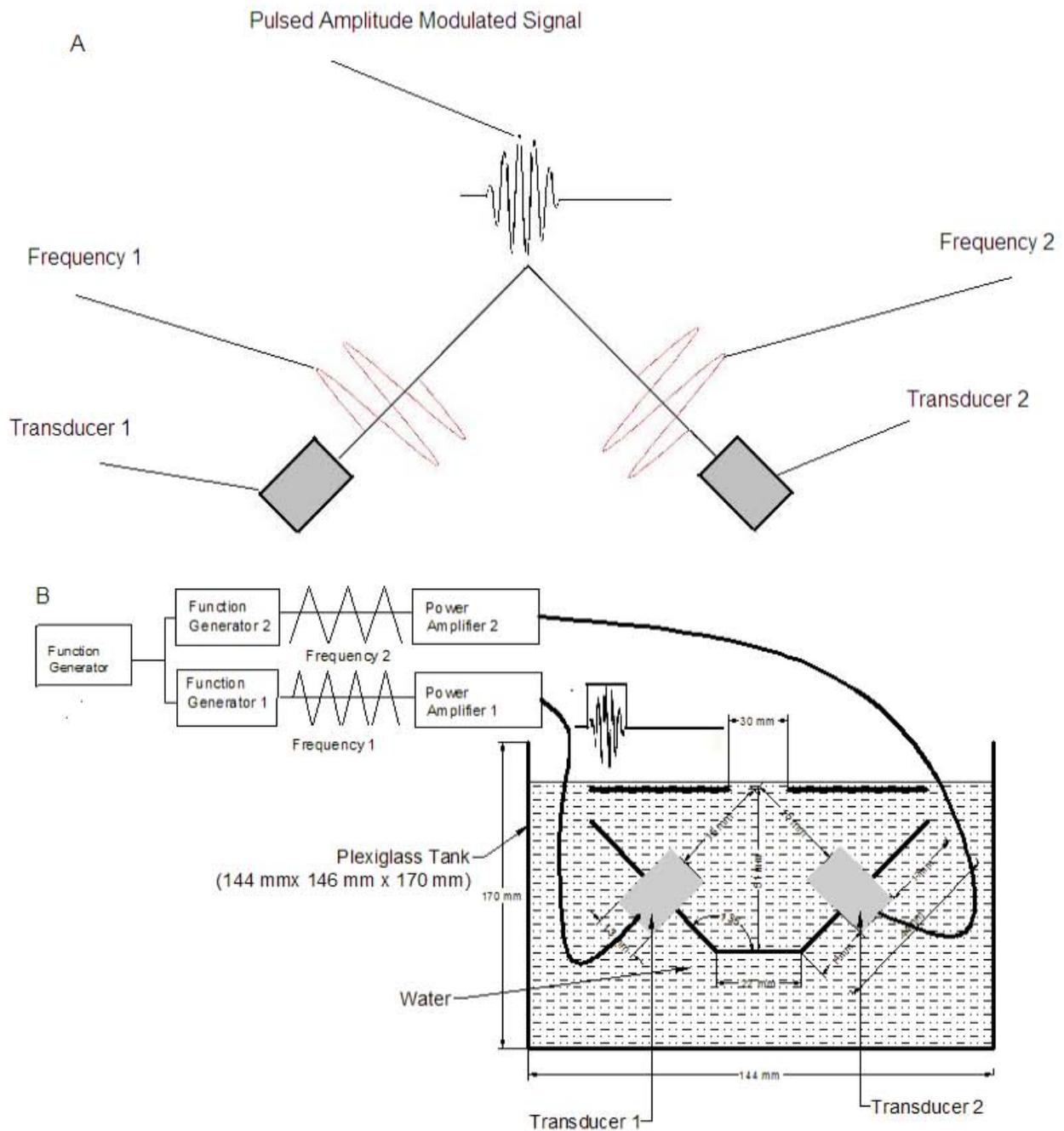


Fig 2: Formation of Amplitude Modulated signal and Experimental set up  
 A : Frequency 1 and Frequency 2 are focused at same focal point. Pulsed Amplitude Modulated signal is formed at focal point.  
 B: Experimental setup to generate two different frequencies to form amplitude modulated signal at focal point.

Inc., Geneva, OH, USA. Two focused transducers (Panametrics NDT, V303, 13 mm diameter, 16 mm focal point and 1MHz Olympus Corporation), are used to translate the signal into acoustic pressure waves. Figure 2 illustrates the experimental setup with detailed specifications and formation of amplitude modulated signal at focal point.

To get both transducers to focus at same point and as a result generate amplitude modulated signal, an aluminum holder was designed such that it can hold both transducers perpendicular to the holder and the angle between base and arms of stand were kept at  $135^{\circ}$  to horizontal. A plastic plate was design with a hole of 30 x 30 mm at the focal point of transducers. This plastic surface acted as a stage for the Lab-tek chamber slide and the hole provides the point of access for ultrasound to stimulate the cells. The set up was contained in a Plexi glass tank. The tank also held degassed water to fill the void between transducers and Lab-Tek Chambers as ultrasound can't travel through air. To verify the formation of amplitude modulated acoustic wave and to confirm the focal position, high performance hydrophone (HP Series High Performance Hydrophone Measurement System, Precision Acoustics, LTd. Dorchester,UK) was used. The hydrophone was attached to a computer controlled scanner which enabled the hydrophone to sweep through the surface in X and Y direction with resolution of 0.5cm. To verify the frequency both transducer were set to produce ultrasound at 1 MHz this frequency was chosen to test the system considering equations of Carrier frequency and Modulated frequency:

$$\text{Carrier frequency} = F_c = (f_1 + f_2)/2$$

$$\text{Modulation frequency} = F_m = (f_1 - f_2) / 2$$

there should be 0 MHz modulated frequency and 1 MHz Carrier Frequency. This provides a proper check for system and standard for the experimental frequencies. The wave signal was verified using oscilloscope (TDS 430A 400 MHz Two Channel Digital Real-Time Oscilloscope, Tektronix, Inc. Tequipment.net, Long Branch, NJ, USA) attached to the hydrophone. To confirm the formation of modulated ultrasound waves system was tested using different frequencies. The intensity of signal was measured using the results from hydrophone 2D scan data. Hydrophone measures the pressure changes at the focal point due to ultrasound signal. This data was plotted relative to XY axis using Matlab v7.1, the area under the graph represent the average intensity over the area of focal point which is approximately  $5\text{mW}/\text{cm}^2$ .

In this study two long wave amplitude modulated frequencies were studied, 45 KHz and 100 KHz. These values were selected after considering the literature search and recent studies which show that low frequency ultrasound, ranging from 20 – 140 KHz, can enhance enzymatic activity [61-65] in different cells. The activation frequency can be different for different enzymes[66]. The literature search didn't retrieve any particular low frequency for enzymes involved in bone mineralization.

Considering the modulation frequency equation, transducer 1 was set to 1 MHz and transducer 2 was set at 1.09 MHz and for 100 KHz transducer 1 was set at 1 MHz and transducer 2 was set at 1.20MHz. As explained in previous section modulated

frequency forms an envelope around carrier frequency thus cells can sense modulated frequency. To confirm the formation of desired frequencies focus region was scanned to confirm the wave pattern using oscilloscope.

**Specific Aim 2: Effect of pAMUS on Calcium Deposition and ALP activity**

**Cell Culture**

Murine Calvaria derived MC3t3, a pre osteoblast cell line (ATCC, LGC Promochem, France) was cultured in alpha-Modified Essential Medium ( $\alpha$ -MEM, Hyclone) supplemented with 10% Fetal Bovine Serum (FBS) and 100  $\mu$ l/ml Pen-strep in an incubator, set at 5% CO<sub>2</sub> and 37°C. Cells were initially cultured in 100 mm Petri dishes (Falcon™ Franklin Lakes, NJ). Cell took 4-5 days to reach to 90% confluency at which point they were either sub-cultured for experiments or preserved in liquid nitrogen. Cell count at confluency in 100 mm dish was approximately  $4 \times 10^6$ . All cellular work was done under sterile conditions under a Tissue culture hood.

Cryopreservation was done by rinsing cells with Phosphate-Buffered Saline (PBS) and detaching them with 3ml of 0.05% Trypsin in 0.53mM ethylenediaminetetraacetic acid (EDTA) for 5 min. Equal amount of Alpha-MEM was used to deactivate Trypsin. Cells suspension was centrifuged at 1000 RPM for 5 min. Supernatant was aspirated and cell pellet was resuspended in 0.75 ml of Alpha-MEM, 0.20 ml of FBS and 0.05 ml of dimethyl sulfoxide (DMSO) in a 1.5 ml cuvette and stored in an isopropanol container in -80°C over night. The isopropanol container was used to slow down the process of freezing and prevent formation of crystals as the cells freeze. After 24 hours cell were

transferred to liquid nitrogen.

To revive the cells, they were thawed in 37°C for 1min. Then, cells were suspended into 9 ml of pre-warmed Alpha-MEM and centrifuged at 1000rpm for 5 min supernatant was aspirated out and cell pellet was re-suspended in medium and plated into 100 mm Petri dish.

For the preliminary runs, cells were cultured in 35mm with seeding density of 50,000 cells. Considering the small focal region (diameter = 10mm) of the ultrasound signal, only 1% of the total area was stimulated with ultrasound. This problem was solved using more specialized Lab-Tek Chambers (Nunc™, Thermo Fisher Scientific, Rochester Site) with 4 wells. . Each well is 10mm in width and 18 mm in length, this setup enabled us to stimulate app 44% of the cells.

Using small chambers resolved the issue of stimulation area but due to small size of culturing area and application of acoustic wave over the span of experiment (18 day for 15 min each day), repeated medium changes and over confluency of cells, cell layer detachment was observed in at around day 7 along with medium color change (medium becomes pale yellow) around day 10. Cell layer detachment was overcome by starting ultrasound stimulations after 7 days of confluency to enable cells to make a stable extracellular matrix. To resolve medium color deterioration, medium was changed every day after day 10. Preliminary runs along with literature search were used to decide the time points, Day 7, Day 12 and Day 18. The early experiments was done using Day 7, 10 and 12 considering the previous studies [53, 67] which shows calcification of matrix

around day 9 in ultrasound stimulated samples. Later, Day 10 was eliminated after first run of experiments as there was no significant difference in ALP activity and matrix calcification. Initial experiments also showed higher level of ALP activity on Day 12 in pAMUS stimulated cells. Considering the function of ALP being a precursor to calcification experiment was extended to Day 18, as it should given enough time to observe significant matrix calcification.

For each experiment, cells were seeded at 10,000 cell per well. Cells were allowed to reach a confluent state and the experiment was started on Day 8 of post confluency. This allows cells to make their extracellular matrix and increase their adherence to the plate so that later application of acoustic pressure (ultrasound wave) wouldn't detach the cells from the surface. During the experiment, medium was changed on every 3<sup>rd</sup> day till Day 10 and every day onwards. To induce mineralization, 10 mM glycerophosphate and 50 µg/ml of ascorbic acid was added to cell culture medium at first day of experiment.

<b>Groups (n)</b>	<b>f<sub>1</sub> (MHZ)</b>	<b>f<sub>2</sub> (MHZ)</b>	<b>PAMUS (MHZ)</b>	<b>pUS (MHZ)</b>	<b>Duration (min)</b>
<b>Sham (8)</b>	0	0	0	0	0
<b>Control (8)</b>	1	1	0	1	15
<b>Experimental 1 (8)</b>	1	1.09	0.045	0	15
<b>Experimental 2 (8)</b>	1	1.20	0.1	0	15

Legend:

f<sub>1</sub> = Ultrasound Frequency 1

f<sub>2</sub> = Ultrasound Frequency 2

PAMUS = Pulsed amplitude modulated frequency

pUS = Pulsed Ultrasound frequency

Table 2: Group distribution and ultrasound parameters.

To study the effects of pAMUS, amplitude modulated frequencies of 45 KHz (0.045 MHz) and 100 KHz (0.100 MHz) were chosen. Considering the modulation frequency equation, generators were set at 1 MHz and 1.09 MHz to generate pAMUS waves of 45 KHz and for 100 KHz, frequency generators were set at 1.0 MHz and 1.2 MHz

Cells were distributed into four groups, Group 1 acted as Sham, cells were exposed to no ultrasound; Group 2 cells were exposed to pulsed ultrasound sound at 1 MHz with no amplitude modulation; Group 3 and 4 will be experimental groups with 100 KHz and 45 KHz pAMUS frequencies respectively. Control and experimental groups were exposed to ultrasound for 15 min each day. Table 2 shows the groups distribution

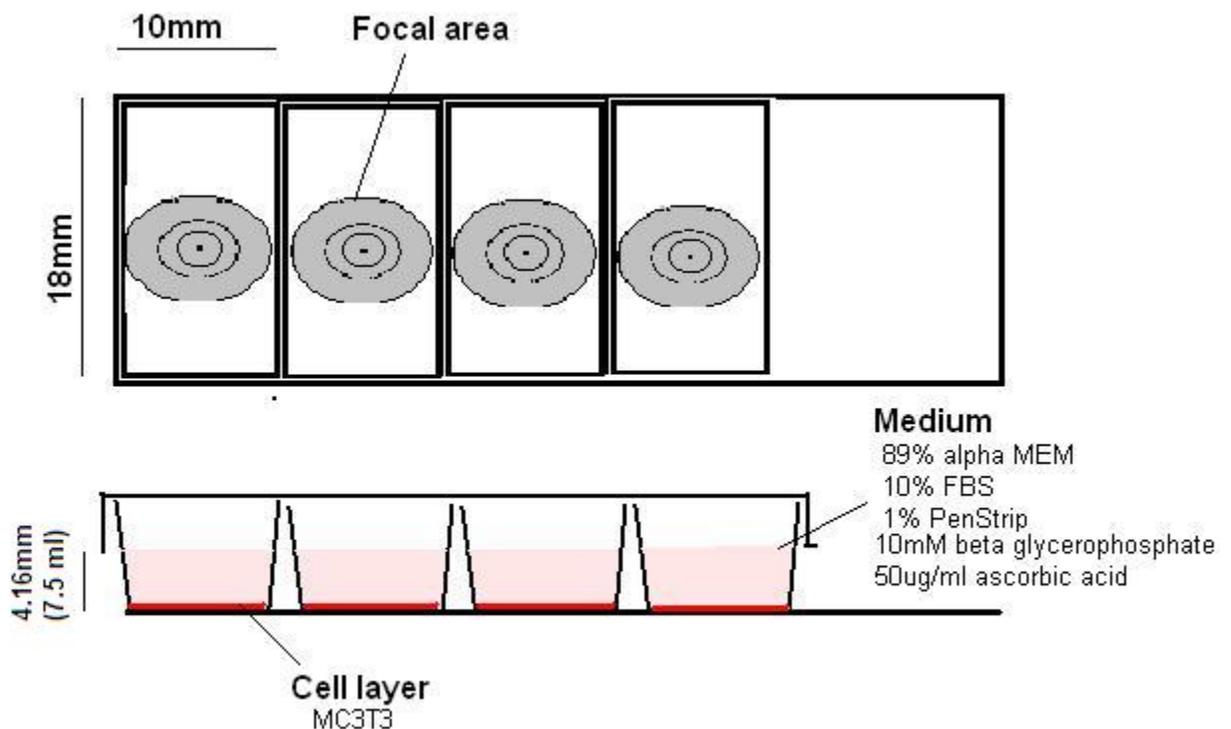


Fig 3: Lab-Tek chambers system, with Permanox base showing focal region (stimulated area)

and ultrasound specifications for each group. Groups were further distributed into three time points, Day 7, Day 12 and Day 18 and each group per day point has eight samples(n=8).

In order to place the cells at focal point, each well was placed on stage which had a hole of 30mm x 30mm. The gap between transducers and Lab-tek chambers was filled with degassed water. The whole set up was placed inside an incubator to provide the optimal conditions for cells to grow and proliferate. Fig 3, illustrates the Lab-tek chamber system and focal region. Each well was filled with 0.75 ml of medium. The volume of medium was chosen considering the formation of resultant standing waves. The frequency of ultrasound generated is 1 MHz with wavelength of 0.67mm. To avoid formation of standing waves the depth of the medium in the well shouldn't be an integer number of half-wavelengths of the ultrasound frequency. The depth of the medium in well comes out to be app 4.16mm.

**Alizarin Red Staining** (*Specific Aim 2*):

To analyze the calcium deposition and pattern in cellular matrix, alizarin red stain was applied to 3 samples from each group. At completion of ultrasound exposure for each group cells were washed with PBS and 1 ml of double filtered water was added to each sample after 24 hours of last stimulation. Cells were stored in -20°C till the completion of stimulations. Cells were thawed at room temperature and fixed with 70% ethanol for 1 hour at room temperature. After fixation, cells were rinsed with PBS and stained with 40mM Alizarin red Solution for 10 min, at pH 4.2 at room temperature, pH

was adjusted using 0.2N sodium hydroxide (NaOH). Cells were rinsed 5 times with water to reduce non-specific binding and left at room temperature for 1 hour to dry. Alizarin red (Chemicon International) staining was applied to cells at time points Day 7, 12 and 18 (n=3 per group per day point). The stain was analyzed using Axiovert 200M Inverted Microscope (Carl Zeiss, USA) at magnification of 2.5X and 10X. The amount of alizarin red stained bound to the mineral in each well was quantified by de-staining each sample in 10mM sodium phosphate containing 10% cetylpyridinium chloride at pH 7.0 for 15 min at room temperature, pH was adjusted using sodium Hydroxide and hydrochloric acid. Cells were destained for 15 min on a rotator at room temperature. 250  $\mu$ l of destained solution was added to 96 wells plate (Falcon<sup>TM</sup>, NJ) and the amount of alizarin red stain was measured by measuring optical density at 561 nm. 10% cetylpyridinium chloride solution attaches to alizarin red dye and form a purple precipitate. The amount of stain present in the sample determines the intensity of color which can then be quantified using spectrometer at 562 nm light wavelength. Every sample was measured three times and averaged to get more accurate reading. To calculate the relative density of stain in each sample was compared to a known concentration of alizarin red diluted with 10mM sodium phosphate containing 10% cetylpyridinium [68, 69].

**Calcium Assay** (*Specific Aim 3*):

Calcium combines with phosphate and forms hydroxyapatite. Hydroxyapatite gets deposited by osteoblast in extracellular matrix and causes mineralization of matrix, which gives bone its mechanical strength.

1N Acetic Acid (500ul) was added to the remainder of the samples(500ul), sham, control, 100 and 45 KHz at Day 7,12, and 18 (n=5, 5 replicated per group per day point), and was left at room temperature over night. Calcium reagent (Diagnostic Chemicals/Genzyme-Product 1420 Arsenazo III) (100 µl) was added to each sample (100µl). Absorbance was read at 650 nm. Overnight incubation in acetic acid allows the release of calcium from the cellular matrix. Arsenazo III reacts with calcium ion and form a complex, which forms a purple precipitate. The intensity of color indicates the amount of calcium in the matrix. The color intensity is measured at 650nm wavelength. All the readings were done in triplicates to get more accurate calcium mineralization readings. Different dilutions of calcium in water were used as standard and measured at 650nm to relative calcium concentration in the matrix.

**Alkaline Phosphatase Assay** (*Specific Aim 2*):

Alkaline phosphatase (ALP) is a membrane bound enzyme which releases phosphate from different molecules present in the cellular environment, thus providing phosphate for formation of calcium phosphate (hydroxyapatite). ALP turnover has been used as a marker for bone mineralization.

Cells samples were collected at Day 7, 12 and 18 (n=5 sample per group per day point). 1 ml water was added to each sample and preserved at -20°C. After the completion of experiment all the samples were thawed at room temperature and sonicated for 5 min to induce cell lysis. 500ul solution was extracted and was used for ALP activity assay. Remainder of the cell extract was stored for calcium assay.

*P-Nitrophenyl* Phosphate Liquid Substrate System (sigma)(100 µl) was added to the extracted samples from each group at D7, 12 and 18(100 µl)to each well of 96 well plate. Samples were then incubated for 60 min at 37°C in incubator, reaction was stopped using 100ul of 0.1N NaOH. Absorbance was read at 405nm. In the absorbance assay the cell lysate contains the ALP enzyme which acts on *P-Nitrophenyl* Phosphate substrate. The incubation time allows enzyme to break down the substrate, which changes the color intensity of the solution. The change in color intensity was detected by spectrometer at 405nm wavelength of light. The different in absorbance values show difference in enzymatic activity. All the samples were read three times to obtain an average and more accurate reading of enzymatic activity. The amount of ALP activity was determined using nitrophenol dilutions as standard.

## Statistical Analysis:

Sample number for the experiments was selected after doing an intensive literature research and looking at different studies which utilizes ultrasound application on cells. To analyze the significant changes between sham, control and experimental groups, statistical analysis were conducted using Student T-Test, One Way ANOVA and Newman-Keuls Test for Contrasts on Ordered Means.

*Student T-Test:* Considering the Normal distribution of the data and unpaired samples, the unpaired Student T-Test was used to analyze the significance difference in two groups. T-Test was conducted for control, 45 and 100 KHz samples relative to sham samples and then for experimental samples relative to control to study the level of significance of data experimental samples when compare to no ultrasound treatment and pulsed ultrasound treatment. p value of lower then 0.05 was considered significant. p –value is a statistical measure of the probability of obtaining a result which will support null hypothesis, no effect hypothesis.

*One Way ANOVA:* One way analysis of variance was used to test the hypothesis and see significant difference within the groups at each day point. p-value of lower then 0.05 was considered significant.

*Newman-Keuls Test:* Following ANOVA, Newman-Keuls test performed on data to find any particular patterns in subgroups of the samples

## Results:

### Field Verification (*Specific Aim 1*):

To confirm the focal point of the ultrasound field, high frequency ultrasound hydrophone was used to scan the field with resolution of 0.5cm. The results were recorded in the computer. Both transducers were set to 1 MHz frequency to provide a strong signal to confirm focal area. Fig 4 shows the image of 2-D intensity scan with circular focal area, with 10mm in diameter ( $78.54\text{mm}^2$ ); fig 4 confirms the formation of focused ultrasound field in region of interest. The image confirms the focal plane and area of the ultrasound. Fig 4a shows the ultrasound energy profile at the focal point measured relative to grayscale values using Matlab 7.1. The experimental setup was designed such that cells would lie in focal plane of the ultrasound to maximize the effects of amplitude modulated ultrasound (fig 4b). Lab-tek chambers with area of  $180\text{mm}^2$  were chosen to maximize the stimulated area, approximately 44% of cells.

The wave front pattern for pAMUS signal was detected using low frequency hydrophone (TC4013 Miniature Reference Hydrophone, 1 Hz-170 kHz, Reson A / S, DK) in the focal region. The wave form was captured by a digitized Oscilloscope attached to the hydrophone. To observe the formation of amplitude modulated waves two different sets of frequencies were used. In the first set we tested 1 and 1.1 MHz frequencies ( $\Delta f$ : 0.1 MHz,  $f_c$ : 1.05 MHz), the resultant modulated frequency of 0.047 KHz

was observed which lies in close approximation to theoretical value of 0.05KHz.In

second set frequencies of 1 and 1.045 MHz ( $\Delta f$ : 0.045 MHz,  $f_c$ : 1.0225 MHz) were tested,

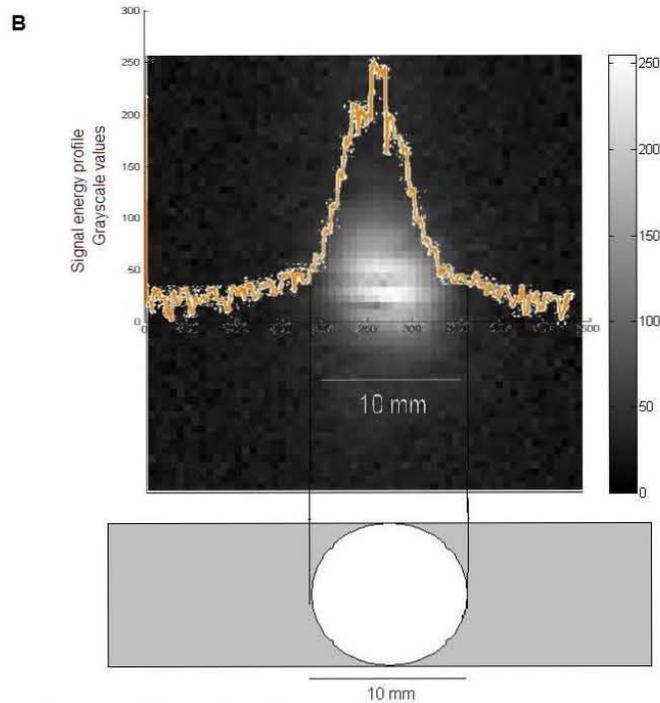
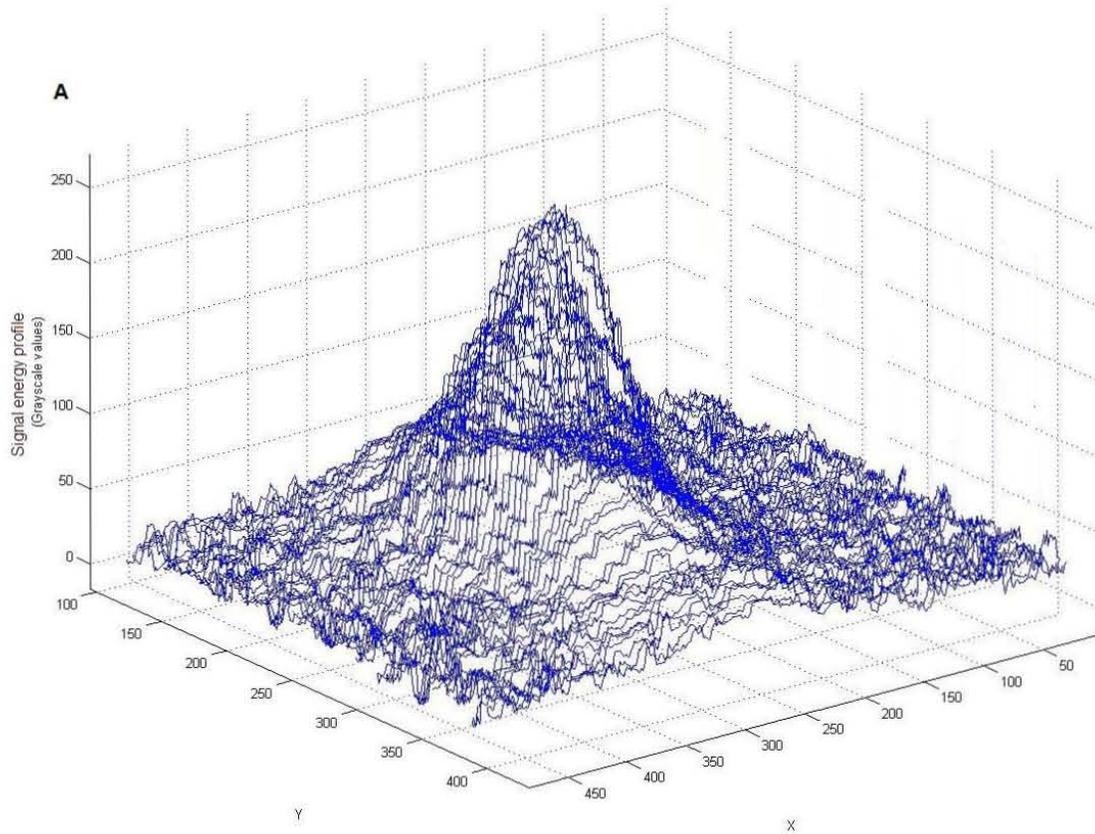


Fig 4 Signal energy profile of focal point with grayscale values  
 A: Signal energy profile graph showing intensity values on gray scale.  
 B: Focal point measurement from hydrophone and stimulated area

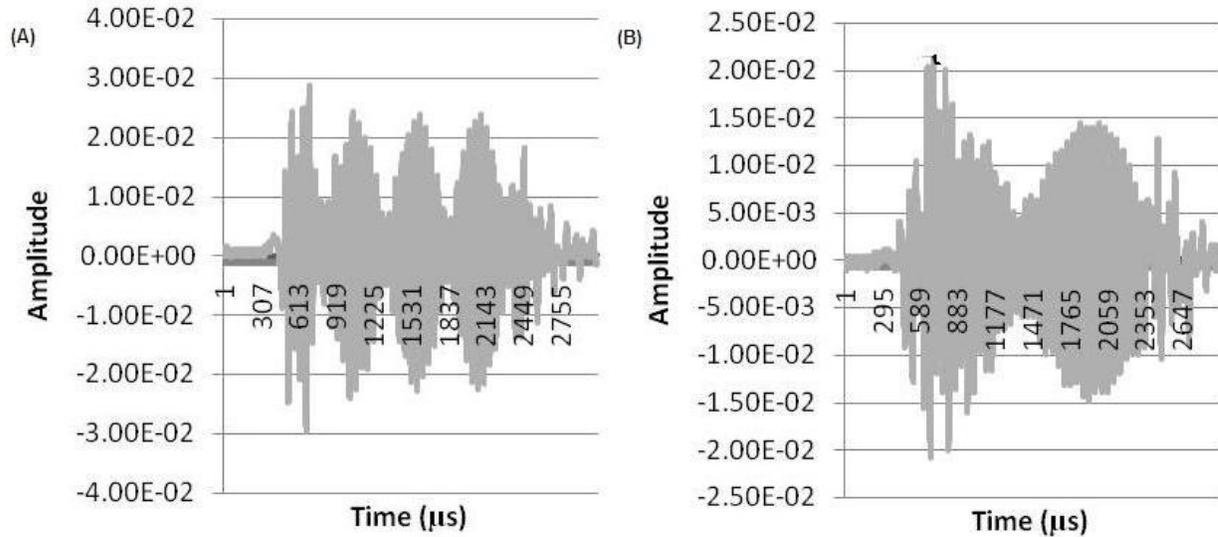


Fig 5a,b: Illustrates the wave form patten for amplitude modulated frequency Graphical representations of the beat waveforms detected at the focal point of the confocal ultrasound stimulation system using a low frequency hydrophone. In both graphs 'A' and 'B', the theoretical modulation frequencies (fm) closely matched the experimental modulation frequencies (fm) ('A': frequency 1: 1.0 MHz, frequency 2:1.1 MHz  $\Delta$ f: 0.1 MHz, fc: 1.05 MHz, Theoretical fm: 0.05 MHz, Experimental fm: 0.047 MHz) & ('B':frequency 1:1.0 MHz, frequency 2:1.045 MHz, Theoretical  $\Delta$ f: 0.045 MHz, fc: 1.0225 MHz, Theoretical fm: 0.0225 MHz, Experimental fm: 0.0229 MHz).

0.0229 KHz modulated frequency was observed in comparison to 0.0225 KHz. Fig 5a and b, shows the wave pattern for first and second set of frequencies respectively. Further figure 5 implies the formation of amplitude modulated frequency with carrier and modulated components.

### **Calcium Mineralization in MC3T3 cell (*Specific Aim 2*):**

Samples were collected at Day 7, 12 and 18 and stained with alizarin red to visualize calcium mineralization. Fig 6 and 7, shows the images the staining observed in sham, control and 100 KHz and 45 KHz at magnification of 2.5X and 10X for Day 12 and Day 18 respectively. At Day 12, Control, 45 KHz and 100 KHz samples show more staining than sham. Staining density between control, 100 KHz and 45 KHz doesn't show

any significant change. Furthermore, staining is distributed all over the sample rather than being concentrated at focal region of ultrasound stimulation. At Day 18, the

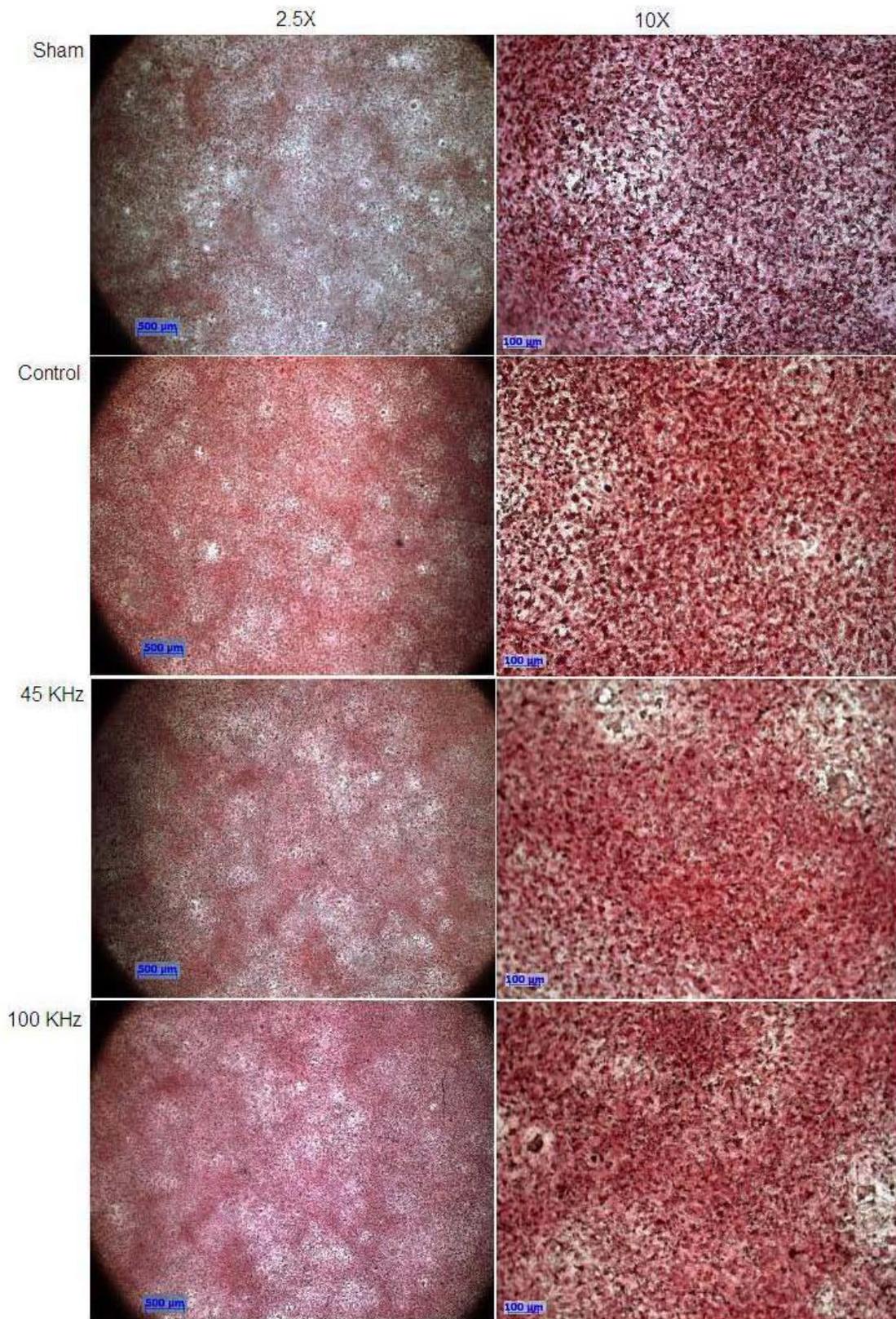


Fig 6: Alizarin Red staining at Day 12 in 2.5X and 10X magnification. Control, 45 KHz and 100 KHz higher level of staining compare to Sham samples. Control, 45 KHz and 100 KHz samples show no significant difference between them.

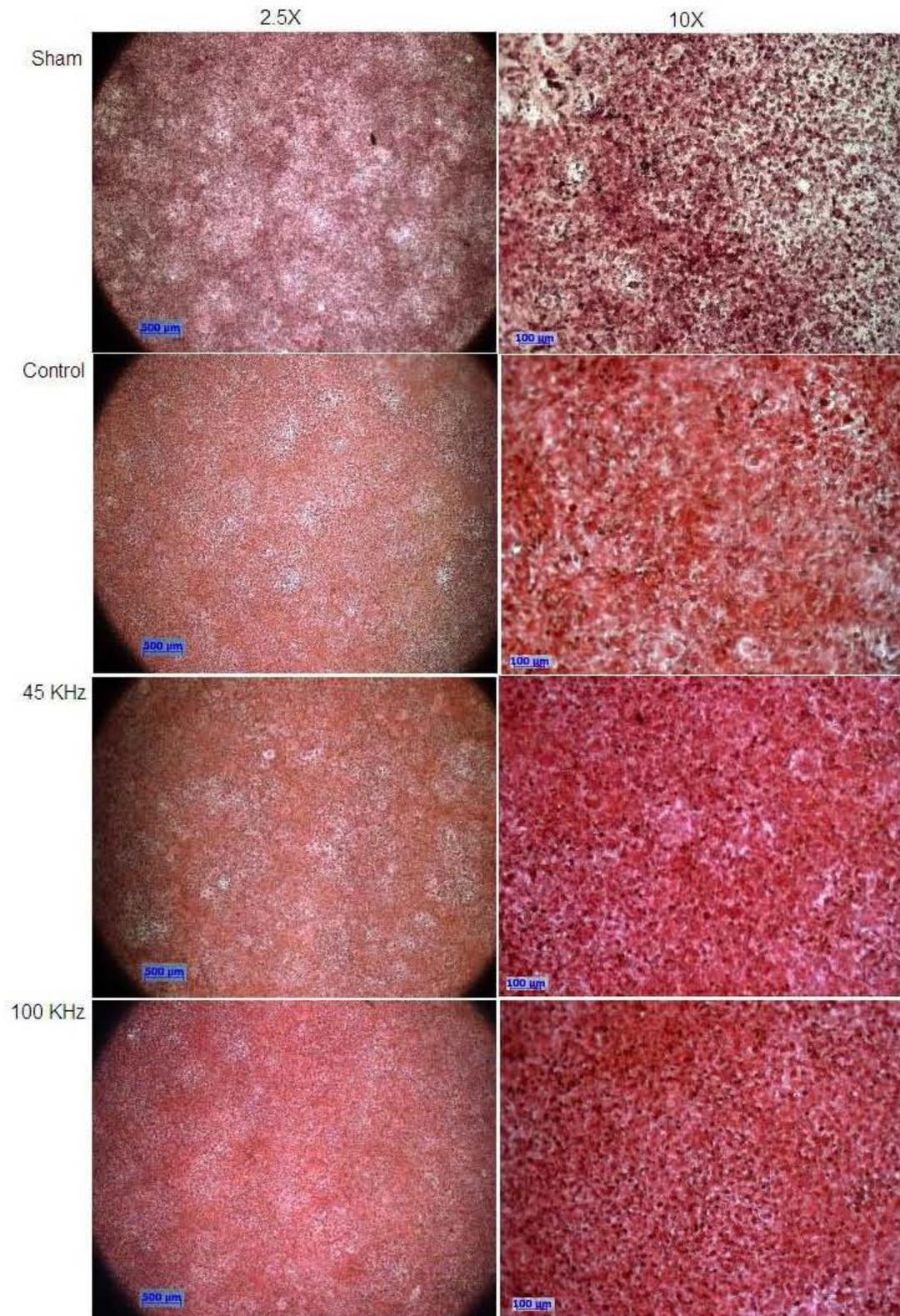


Fig 7: Alizarin Red Staining at Day 18 ,2.5X and 10 X Magnification  
 Ultrasound treated groups ( Control, 45 KHz and 100 KHz) show higher level of staining then shams. Control, 45 KHz and 100 KHz show strong staining evenly distributed in the cell layer. It is hard to make any conclusions based on images thus Cells were destined to quantify staining.

staining is dense in control and experimental samples, as soft spots (spots with no staining) are not present any more and stains cover almost all the cells. Stain is distributed over the surface of the sample, in control, 100 KHz and 45 KHz samples but all the cells don't show the same level of staining as some relatively dense regions of staining can be observed in each sample but these denser patches of stain do not form any certain pattern and are not localized to area of stimulation. Sham samples also show higher stain relative to Day 12 sham samples but there are still cells with no stain, showing formation of less or no calcium in that region of cell layer. Images show significant difference in staining density between sham and ultrasound stimulated samples but it is hard to draw conclusions between control, 45 KHz and 100 KHz samples just on the bases of images due to high density of staining in all samples. To obtain an objective data and analysis level of staining between ultrasound stimulated samples, alizarin red stain was destained with 10% cetylpyridinium in 10mM sodium phosphate for 15min at room temperature. Destained solution was analyzed at 561 nm

To evaluate the amount of calcium present in each sample, staining was de-stained using 10% cetylpyridinium in 10mM Sodium Phosphate. Fig 8a, shows the amount of alizarin red stain extracted from each sample and quantified at 561nm, show significant increase in control, 100 KHz and 45KHz,  $p < 0.05$  for control ( $0.76 \pm 0.08$  mM Alizarin Red),  $p < 0.005$  for 100 KHz ( $1.02 \pm 0.07$  mM Alizarin Red) and 45KHz ( $0.81 \pm 0.01$  mM Alizarin Red), relative to sham ( $0.46 \pm 0.03$  mM Alizarin Red) at Day 18.

These results show calcium increase at Day 18 in control, 100 KHz and 45 KHz relative to sham, highest being in 100 KHz sample, while control and 45 KHz samples showing

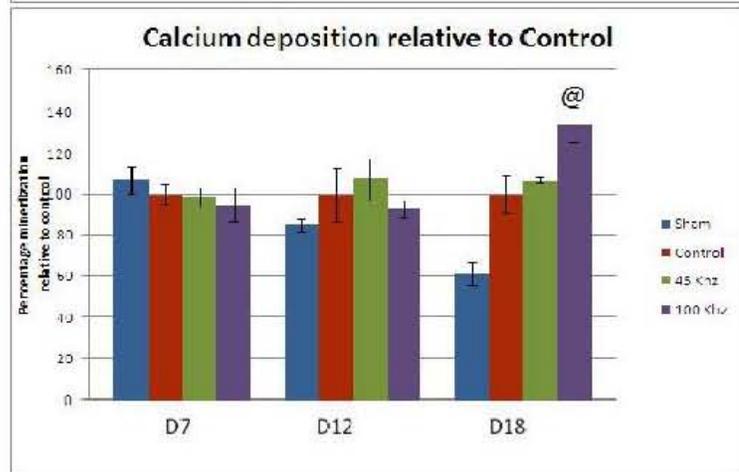
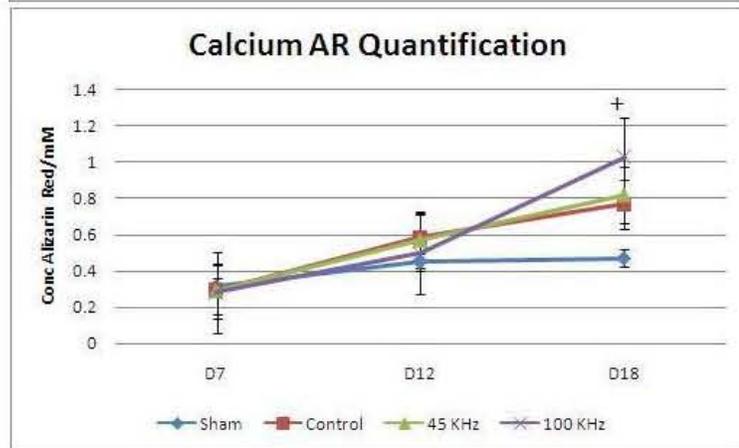
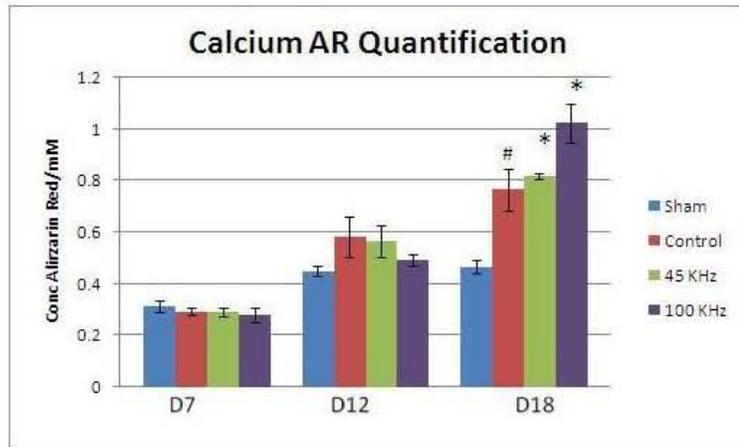


Fig 8: Calcium Mineralization in Alizarin Red Assay (561nm)  
 A: Calcium levels in Sham, Control, 45 and 100 KHz samples. Day 7 and 12 show no significant difference. Day 18 samples show significant increase in calcium deposition in Control, 45 and 100 KHz samples. pAMUS, 100 KHz sample show highest level of Calcium. (# =  $p > 0.05$ , \* =  $p > 0.005$ )  $\pm$  Standard Deviation.  
 B: Mineralization trend in Sham, Control, 45 and 100 KHz. Sham, control and 45 KHz show constant increase in calcium levels while a spike was observed in cells stimulated at 100 KHz (+ =  $p > 0.0001$  (ANOVA))  $\pm$  SE.  
 C: Calcium levels relative to Control. No significant difference at Day 7 and 12. At Day 18 45 KHz samples show 6.64% and 100 KHz samples show 33.74% increase relative to control. (@ =  $p > 0.05$ )  $\pm$  SD

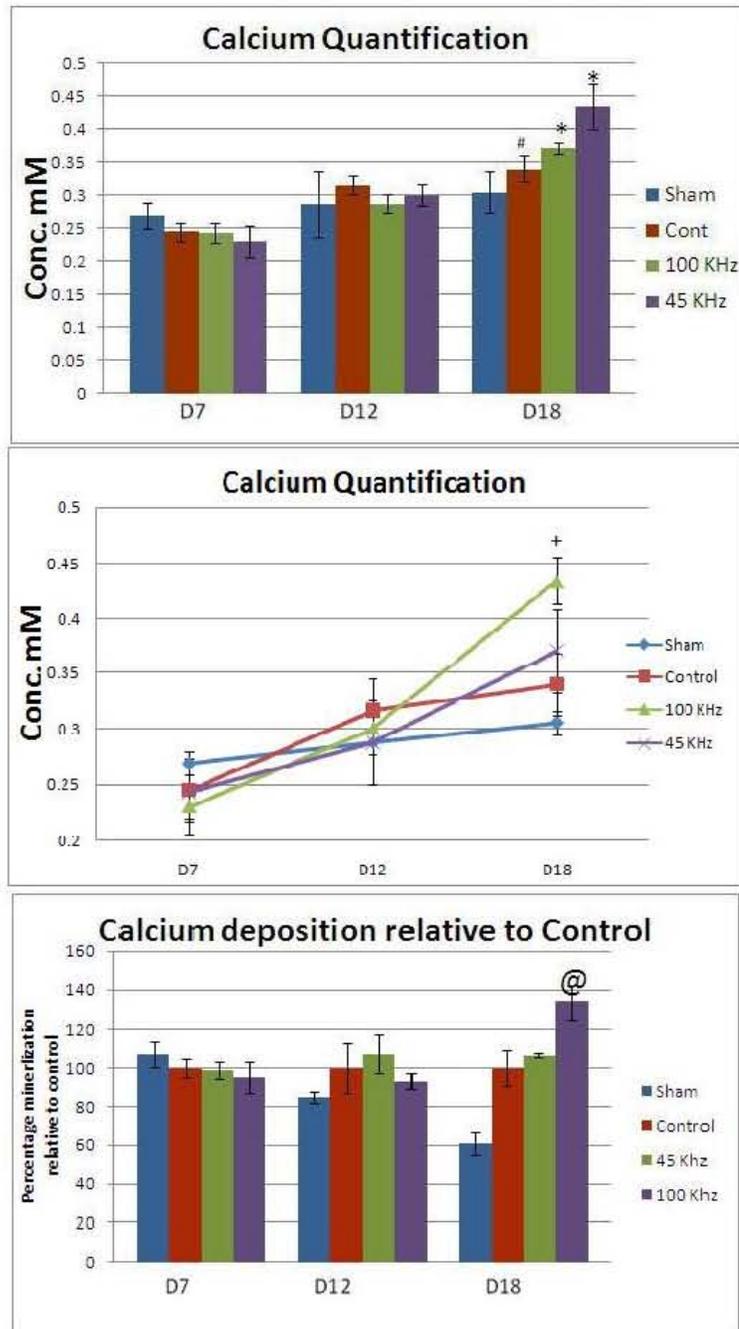


Fig 9: Quantification of Calcium present in Extracellular matrix using Arsenazo III measured at 650nm  
 A: Calcium levels show significant increase at Day 18 in Experimental (# =  $p > 0.05$ ) and Control Samples (\* =  $p > 0.01$ ). No significant change in calcium levels were observed at day 12. At day 7, Ultrasound stimulated samples show less calcium relative to Sham  $\pm$  SD  
 B: Mineralization trends: A constant increase of calcium levels are observed in Sham, Control and 45 KHz samples with relatively higher rate of increase in control and 45 KHz. 100 KHz samples sudden increase in rate of calcification after at Day 12.(+ =  $p > 0.0001$ , ANOVA)  $\pm$  SE  
 C: Calcium levels relative to control samples. Significant increase of 27.75 %, in matrix calcification in 100 KHz samples at Day 18 relative to Control. 45KHz sample show increase 9.14%, but not significant. (@ =  $p > 0.05$ )  $\pm$ SD

approximately similar levels of calcium mineralization. Fig 8b shows rate of calcium increase in sham, control, 100 KHz and 45 KHz. Cells stimulated at 100 KHz shows a rapid increase in calcium deposition after Day 12 while in case of control and 45 KHz samples show much more steady increase in mineralization. Alizarin red assay shows significant difference between 100 KHz and 45 KHz samples ( $p < 0.05$ ). Fig 8c shows, percentage increase in calcium deposition in 45 KHz and 100 KHz samples compare to calcium levels in control samples. Relative to control 100 KHz samples show  $33.74 \pm 8.37 \%$  and  $6.65 \pm 1.35 \%$  increase in calcium levels in 45 KHz samples. Mineralization increase significantly ( $p < 0.05$ ) in pAMUS samples at 100 KHz samples compare to samples stimulated with pulsed ultrasound.

Student t-test and ANOVA analysis followed by Newman-Keuls Test was performed on every day point to analysis significant difference at everyday point. Oneway-ANOVA and Newman-Keuls Test show significant increase in calcium level ( $p < 0.05$ ) in control, 45 KHz and 100 KHz relative to Sham at Day 18. The Newman-Keuls test show a significant calcium increase ( $p > 0.001$ ) in 100 KHz relative to 100 KHz Sample, 45KHz doesn't show significant ( $p < 0.5$ ) increase in calcium deposition at Day 18. pAMUS stimulated samples at 45 and 100 KHz show increase in calcification relative to pulsed ultrasound stimulated ( $0.76 \pm 0.08\text{mM}$ ) samples. Increase is significant enough in 100 KHz ( $p < 0.05$ ,  $1.02 \pm 0.07\text{mM}$ ) but not significant in 45 KHz samples ( $p < 0.05$ ,  $0.81 \pm 0.01\text{mM}$ )

To confirm the calcium mineralization, calcium assay was conducted using Calcium reagent, Arsenazo III and elevated at 650nm. The assay confirms the results from alizarin red staining with increase in calcium levels at Day 18(Fig 9a and b) for 100KHz ( $0.43\pm 0.03\text{mM}$ ,  $p<0.01$ ) and 45 KHz sample( $0.37\pm 0.01\text{mM}$ ,  $p<0.01$ ) and control samples( $0.33\pm 0.02\text{mM}$ ,  $p<0.05$ ) when compared to sham( $0.30\pm 0.03\text{mM}$ ). Furthermore, pAMUS samples at 100 KHz show significant increase from samples simulated with 45 KHz ( $p<0.01$ ) pAMUS. There were no significant differences in calcium levels at Day 12.

In both assays, Sham shows higher level of calcium than control, 100 KHz and 45 KHz at Day 7. Cells stimulated at 100 KHz pAMUS show a sudden change in calcium level after Day 12 but in the case of control and 45 KHz the rate of increase in calcium level is constant. Sham samples show a constant increase but at lower rate. Experimental samples (100 and 45 KHz) samples show  $27.75 \pm 10.13\%$  and  $9.11 \pm 2.50\%$  increase in calcium levels relative to control respectively (Fig 9c).

Student t-test analysis shows a significant increase in calcium levels in pAMUS (45 and 100 KHz) samples ( $p<0.05$  and  $p<0.005$ , respectively) and control ( $p<0.05$ ). ANOVA does show significant difference within the groups at Day 18 and post hoc, Neuman-Keuls test show significant increase for control( $p<0.05$ ), 45 KHz( $p<0.005$ ) and 100 KHz( $p<0.001$ ). T –test also show significant increase calcification of matrix in 100 KHz samples ( $p< 0.05$ ) with compared to control samples

### **Alkaline Phosphatase Activity (Specific Aim 2):**

The enzymatic activity of alkaline phosphatase activity in cultures of MC3T3 was observed at 450nm using *P-Nitrophenyl Phosphate Liquid Substrate System* (sigma). Day 12 shows significantly elevated activity of alkaline phosphatase in 100 and 45 KHz approximately 5-folds ( $33.51 \pm 3.20 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.001$ ) and 4-fold ( $28.89 \pm 4.0120 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.001$ ) increase, in comparison to sham ( $5.61 \pm 1.02 \mu\text{m}/10^4 \text{cells}$ ) and control samples ( $5.59 \pm 2.7 \mu\text{m}/10^4 \text{cells}$ ) respectively (Fig 10a, b). Cells stimulated with pulsed ultrasound (Control,  $27.60 \pm 7.73 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.05$ ) show a significant increase of 4-fold ALP activity at Day 18 along with 100 ( $30.21 \pm 9.60 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.01$ ) and 45 KHz ( $30.48 \pm 5.44 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.001$ ) samples relative to sham ( $7.07 \pm 4.04 \mu\text{m}/10^4 \text{cells}$ ). Sham samples show a study increase in ALP activity but remain significantly less than control, 100 and 45 KHz samples. Although enzymatic activity in cell stimulated with 100 KHz pAMUS decrease on Day 18 but still remains significantly high compare to sham around 4 fold. Fig 10c shows the ALP activity relative to control samples. Day 12 experimental, 100 KHz ( $33.51 \pm 3.20 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.0005$ ) and 45 KHz ( $28.89 \pm 4.0120 \mu\text{m}/10^4 \text{cells}$ ,  $p < 0.0005$ ) samples show significant increase compare to control ( $5.59 \pm 2.7 \mu\text{m}/10^4 \text{cells}$ ). No significant difference in experimental samples (pAMUS, 45 and 100 KHz) was observed in Day 18 samples when compared to Control.

Student T-Test was used to analyzed the significant increase in pAMUS samples at 45 KHz and 100 KHz in ALP activity on Day 12 relative to sham and control ( $p < 0.0001$ ). ALP activity remains significantly elevated ( $p < 0.01$ ) in experimental groups and

increased significantly in control ( $p < 0.05$ ) compare to activity in sham samples on Day 18. Experimental groups (45 and 100 KHz) samples show significantly higher enzymatic activity ( $p < 0.0005$ ) on Day 12 relative to control samples while on Day 18 shows no significant difference between control and experimental samples.

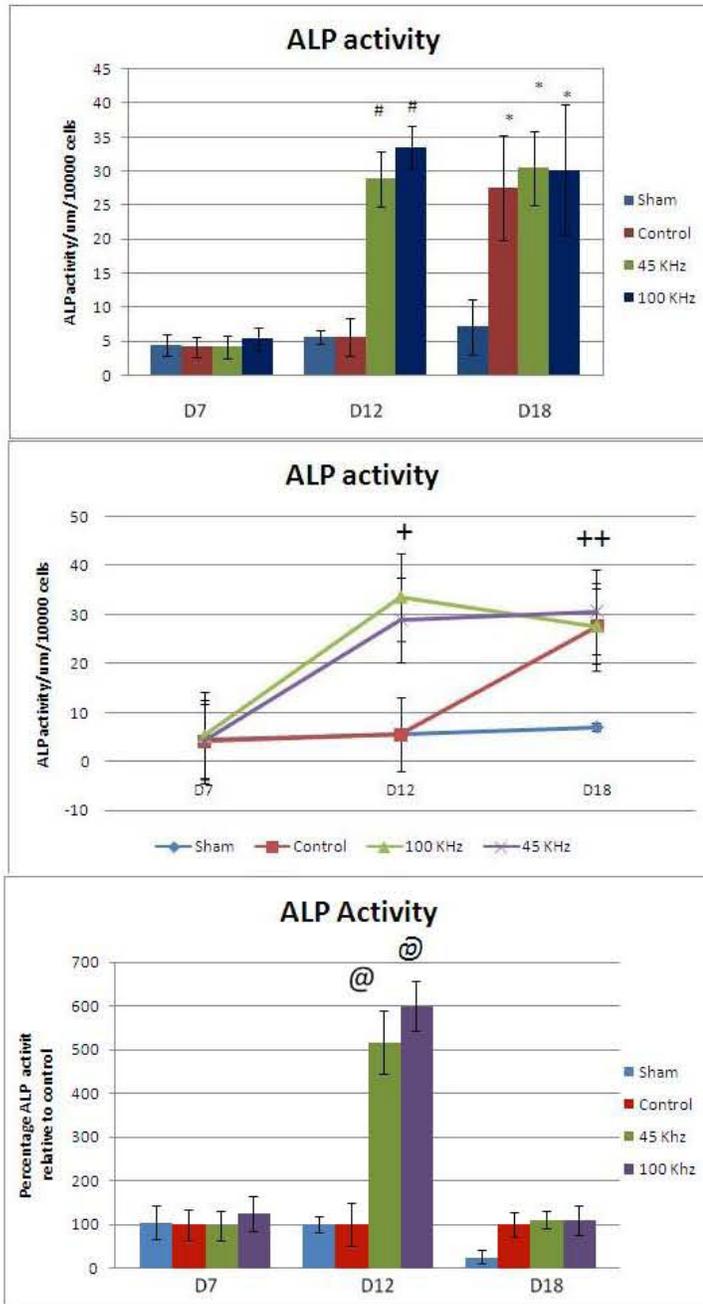


Fig 10: Alkaline Phosphate Activity, in Sham, Control, 45 and 100 KHZ samples measured at Day 7, 12 and 18  
 A: 5 fold increase in 100 KHz and 4 fold increase in KHz of alkaline phosphate activity was observed relative to Sham and Control at Day 12. Enzymatic activity also get significantly elevated in control samples at day 18. Experimental samples of 45 and 100 KHz continue to show 3-fold higher activity than sham. (n=5)(# = p > 0.0005, \* = p > 0.001) ±SD  
 B: Trends in ALP activity. Sudden increase in ALP activity of cells stimulated with pAMUS at 45 and 100 KHz at day Day12. Enzymatic activity is significantly elevated in control, 45 and 100 KHz samples at day 18. Sham displays a gradual increase in enzymatic activity (+ = p > 0.0001, ++ = p > 0.0005 ANOVA) ± SE  
 C: ALP activity relative to control. Significant increase was observed at Day 12. (5 fold in 100 KHz and 4 fold in 45 KHz). Day 7 and Day 18 doesn't show any significant change in ALP activity in experimental samples relative to Control. (@ = p > 0.0005) ± SD

## Discussion:

Our data has indicated that modulated ultrasound is capable to increase biomineralization in osteoblast-like cells. This study confirmed previous finding that low energy ultrasound is necessary to accelerate bone cell response. However, the data from pAMUS demonstrated stronger mineralization expression than pUS, which suggested that modulated ultrasound provide promising stimulation on cells for its turn over due it's enhanced acoustic properties. This study compared amplitude modulated signal to pulsed ultrasound signal at low intensity. The signal used for pUS was identical to signal used in LIPUS with respect to duty cycle and wave properties, while the only difference was in intensity levels. Considering pAMUS increased mineralization significantly relative to pUS, this potentially implies that pAMUS can further enhance bone mineralization if used at same intensity levels as of LIPUS in vitro, in vivo and even at the clinical application. The study used two transducers to generate focused pAMUS at focal point, which enabled this setup to generate higher energy at focal point relative to energy level of intial signals. On organ level this setup can provide more targeted approach to site of lesion along with enhanced energy and acoustic effects, focused pAMUS can generate. LIPUS treatment use a non focal ultrasound, which is susceptible to energy lose to pre and post bone tissue along with undesirable effects to soft tissue surrounding bone. This setup can potentially over this issue by using two low energy signals to produce a higher energy signal at site of interest thus reducing the energy lose and undesirable effects to surrounding soft tissue. The high levels of biomineralization

with pAMUS signal suggest that signal amplitude modulation plays an important role in mechanotransduction in osteoblast. More studies need to be conducted to know the exact mechanism through which enhanced mineralization is achieved but it can be speculated underlying mechanism of mechanotransduction are same as of LIPUS but pAMUS increases the mechanotransduction due to amplitude variability.

Low intensity pulsed ultrasound has been shown to increase bone mass in osteoporosis [70-72], accelerate bone fracture healing [25, 73-82], and treat non unions [83-87]. These studies describe increases in bone mass and mechanical strength. The enhancement of physical attributes in bone quality is achieved by promoting the rate of calcification of cellular matrix through elevated activity of different enzymes that are involved in process of matrix formation. Alkaline phosphatase and matrix metalloproteinase-13(MMP-13) are two critical enzymes that are involved in matrix formation in bone tissue. Thus activity of both these enzymes has been studied to determine the formation of bone tissue. Alkaline phosphatase is assumed to be involved in releasing for phosphate ions from other molecules in the cellular environment and further breaking down mineralization inhibitors like pyrophosphate. MMP-13 is thought to be involved in resorption and nucleation of mineral in the matrix. The coordinated functioning of these enzymes determines the rate of mineralization in cellular matrix. The activity of alkaline phosphatase[67] and MMP-13 [88] has been shown to increase after treatment with LIPUS (30 mW/cm<sup>2</sup>). These studies suggest that ultrasound accelerate matrix mineralization by elevating the activity of these enzymes.

Furthermore these studies provide one probable mechanisms through which ultrasound can enhance bone quality. Saito et al [89] show ultrasound affecting the linkages of collagen fibers in matrix which further increase the facilitate calcium deposition in to cellular matrix. These results from *in vitro* studies have also been transcribed into studies *in vivo*; in which application of ultrasound enhance bone mechanical and tensional strength [83, 90] significantly. Ultrasound has also been shown to promote differentiation in marrow derived cells[46, 91] , mesenchymal stem cells[92], osteoblasts [27, 93] and chondrocytes[94] isolated from rat cartilage.

The effects of ultrasound on cells have been studied over 5 decades now but still the mechanism hasn't been understood fully. Saito et al (2004)[89] discussed the cross-linking of the matrix can effect process of mineralization as ultrasound induced formation of more reducible and non-reducible collagen cross links ,which provide more proliferating environment for cells to differentiate. The mechanism of pAMUS induced bone cell response may closely relate to mechanotransductive factors, including acoustic pressure wave, generated mechanotransduction, acoustic cavitation, and acoustic streaming.

**Stimulation of acoustic pressure wave** over a period of time can increase the temperature of medium over time. This can affect the enzymatic activity within cells. Although temperature change in low intensity pulsed ultrasound and pAMUS are lower than 1°C but considering the enzyme kinetics and potential of exponential increase in enzymatic activity if the change in temperature lies in exponential range of enzyme

kinetics. Thus small change in temperature can induce larger change in enzymatic activity.

**Mechanotransduction** has been known to play an important role in promoting or inhibiting different biological processes within cells. Ultrasound wave is an acoustic pressure longitudinal wave. When this pressure wave comes across the cellular layer, it induces localized vibrations on the nanometer scale, in the direction of wave propagation, [95] in the cellular matrix. These localized vibrations along with some of refracted longitudinal pressure waves create shear stress waves in the intercellular space in the extracellular matrix. This along with cellular induction can explain calcium mineralization to be distributed rather than localized at region of stimulation. These vibrations and shear stress created by longitudinal waves is sensed by integrins. Integrins are a family of trans-membrane cell adhesion proteins, consisting of alpha and beta chains, which help cell to anchor to the extracellular matrix and sense any mechanical stimuli present in the extracellular matrix. Once integrin sense any mechanical stimulus, there is a conformation change in structure of intracellular chains of integrins which initiates the formation of focal adhesion complex and activates other signaling molecules. In the presence of mechanical stimulus integrin forms dense clusters. Yang et al [54] has shown formation of integrin clusters in osteoblast cells after stimulation with continuous ultrasound for period of 20min. LIPUS has shown to activate integrin intracellular signaling by formation of focal adhesion complex in fibroblast [96] and osteoblasts[53]. Studies done by Tang et al and Zohu et al suggest that ultrasound induced mineralization are controlled by multiple down stream

pathways such as MEK-1/ERK  $\frac{1}{2}$  pathway[96] and Akt/NF- $\kappa$ B pathway[53]. Blocking of any pathway can decrease the level of ultrasound induced calcification but wouldn't inhibit it completely. McCormick et al[97] show an increase of nitric oxide due to shear stress in LIPUS stimulated cells cause actin fibers to re-organize and form stiffer cytoskeleton which can induce transcriptional changes. Considering actin fibers are part of focal adhesion complex and formation of focal adhesion complex due to integrin activation will induce reorganization of actin fibers. Fig 10[25, 43] displays four potential pathways that can be triggered by ultrasound stimulation through integrin. Many studies [53, 98-101] link ultrasound stimulation and activation of integrin to increase level of cyclooxygenase 2, which is a critical enzyme in the production of prostaglandin E2, regulates calcium mineralization but this still doesn't explain the higher level of numerous other genes, protein and growth factor by LIPUS/ultrasound stimulation. In recent study done by Takeuchi et al [43] show higher level of  $\beta$ -Catenin in LIPUS stimulated cell. They associate  $\beta$ -Catenin high levels to downstream signaling to AKT/P13K pathway. It is possible these  $\beta$ -Catenin high levels are due to integrin/ focal

adhesion kinase (FAK) activation or potentially activation of Wnt/Frizzled pathway.

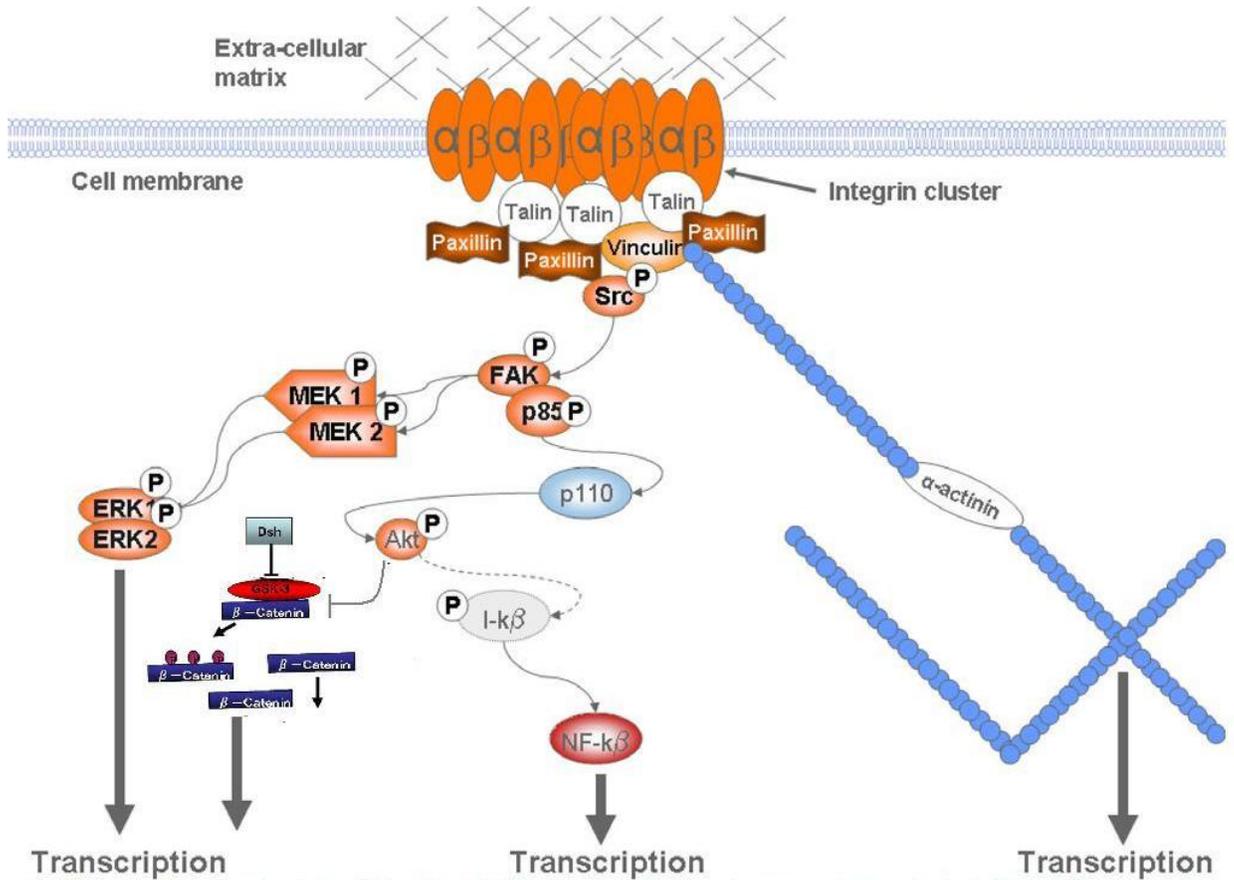


Fig 11: Adopted from Pounder et al. and Takeuchi et al. Different pathways that have been suggested to get activated through integrin signaling after low-intensity pulsed ultrasound stimulation.

Acoustic pressure wave have thermal and non thermal effects, depending upon different parameters of ultrasound like frequency, pressure amplitude, pulse duration, pulse repetition frequency and beam and scanning configuration along with tissue properties (e.g. absorption coefficient, density, perfusion, presence of free bubbles etc). Ultrasound applied in calcium mineralization is usually of low intensity thus thermal effects are minimal but considering enzymatic kinetics can still be important (discussed above). Non thermal effects of ultrasound include acoustic cavitations, radiation force, radiation torque and acoustic streaming[102].

**Acoustic cavitation** is defined as physical forces generated due to pressure waves affecting the micro-environmental gasses present within fluid. The characteristic compression and rarefaction causes microbubbles to expand and contract as pressure wave propagates through the medium[103]. Acoustic streaming is defined as physical forces by pressure waves providing a driving force for displacing ions and small molecules[104].

Acoustic cavitations are dependent on pressure amplitude, beam focal properties and presence of free bubbles with in the cellular matrix or cell membrane. On cellular level cavitations can be formed inside cell membrane due to the presence of gas bubbles. These cavitations can be formed due to several processes [105, 106]:

*a localized acoustic streaming within cell membrane:* when gas bubbles start to oscillate in response to ultrasound pressure wave and disturb the lipid bilayer.

*Sonochemistry:* gas bubbles can suddenly collapse and induce a momentary localized high temperature.

*Shock waves:* they are formed when gas bubbles start to oscillate and then burst with sudden impact and induce shock waves in region around them.

*Liquid microjets:* acoustic wave can induce non-uniformities into the bubbles which can form high velocity microjets. These microjets can penetrate tissue and generate secondary stress waves.

All these mechanisms basically disturb the lipid bilayer and increase permeability of the cell to nutrients or inductive factors present in extracellular environment.

Acoustic cavitations hasn't been considered as a mechanistic option in calcium mineralization due to highly dense and calcified matrix in bone tissue, which reduced formation of free gas bubbles in the matrix and low intensity ultrasound signals are not strong enough to disturb the cell membrane. Further calcification of matrix forms a hard tissue which can affect the attenuation properties of ultrasound.

**Acoustic streaming** is the formation of a steady and direct current flow in the acoustic field (extracellular matrix), in response to ultrasound pressure waves. These streams can be sensed by integrins which can initiate different pathways (discussed above). Further more the unidirectional movement of pressure wave due to acoustic streaming in medium can induce conformation changes which can activate or deactivate some functional proteins[107]

This study looks at the focused pAMUS signal which is different from LIPUS or continuous ultrasound signal that is generally used in matrix mineralization studies. Focused pAMUS provides targeted approach to a certain area in the matrix. Application of pAMUS on osteoblast cells shows elevated enzymatic activity and higher calcification of cellular matrix relative to cells that were either stimulated with pulsed ultrasound or no ultrasound. Results from the experiments prove the hypothesis that pAMUS accelerates calcification of cellular matrix and enhances enzymatic activity. The experiments in this study were designed to study of the effects of pAMUS on osteoblast but it can be speculated that the increased rate of mineralization and enzymatic activity would be due to enhanced acoustic properties such as focused ultrasound application, formation of higher amplitudes at focal point, which get

dispersed in the matrix and induce shear stress into cellular matrix. Formation of shear stress in cellular matrix can potentially activate integrin pathway or other mechanotransduction pathways and induce mineralization. Further experiments are required to understand the biomechanical function of pAMUS in osteoblast cells.

## Limitations

This study only looked at ALP activity and matrix calcification as markers for pAMUS effects on osteoblast cells, and more analysis would be required to determine the effects of pAMUS on transcription and gene levels. It would have also been beneficial to analyze the spatial distribution of the calcium mineralization in samples and see if there are any differences, which can't be accessed easily. This study doesn't look at the effects of carrier signal and modulated signal separately, thus can't establish if the effects on mineralization were due to modulated signal or if they were due to a combination of two signals.

Current study proved the acceleration of mineralization in osteoblast cells when exposed to pAMUS but considering the experimental setup, application of two transducers at certain angles and particular distances from focal points can be an issue. This setup requires a high level of precision as small changes in angle and distance can disturb the focal region. Furthermore, it is important to consider if such a setup can be used or adopted into *in vivo* application or not.

This study only looks at two experimental frequencies with stimulation times of 15 min

per day thus it can't address the question of application time and its effects on mineralization.

## **Further Studies:**

This study analyzes the enzymatic activity and calcification changes due to application of pAMUS and finds that pAMUS increases the rate of calcification in osteoblast matrix and significantly elevates ALP activity. It will be interesting to see the effects of pAMUS on transcription level and gene expression and activation. This can provide us with a more in-depth analysis of pAMUS effects on osteoblast functioning. It would be interesting to further integrate the pAMUS signal and see if the effect caused on mineralization is due to modulated signal or carrier signal or combination of both.

pAMUS application accelerates the calcification of osteoblast extracellular matrix but this study doesn't provide any lead into the mechanism through which it accelerates the process of mineralization. Therefore studies can be designed to study the shear stress, function of integrin and other mechanoreceptors when stimulated with pulsed amplitude modulated ultrasound.

This study just looks at two pAMUS frequencies thus it will be interesting to look at different frequencies and optimize the parameters for pAMUS with respect to frequency, stimulation time and intensity.

One of the limitations of this study was the experimental setup as it requires two transducers to generate pAMUS. It is possible to generate focused pAMUS signal using

one transducer so it will be interesting to see if one transducer setup can generate same results or not. If it does then that can make amplitude modulated signal much more adaptable for future *in vivo* studies and clinical applications.

## **Conclusion:**

This study looked at the effects of pAMUS (100 KHz, 45 KHz) on enzymatic activity of alkaline phosphatase and resultant mineralization of matrix in MC3T3 cells relative to cells which were stimulated by pulsed ultrasound signal and cells with no treatment. Considering the previous studies done on effects of ultrasound on matrix mineralization and enhanced properties of amplitude modulated ultrasound, we hypothesized that pAMUS will further accelerate calcified matrix formation.

Alkaline phosphatase activity is significantly increased in cells stimulated with 100 KHz and 45 KHz pAMUS at Day 12 compare to cells stimulated with pulsed ultrasound and cells with no treatment. ALP activity in control samples was elevated on Day 18. 100 KHz samples show decrease in ALP activity at Day 18 but still significantly higher than sham cells. 45 KHz samples show rather constant ALP activity between Day 12 and 18. Elevation of ALP activity in control samples was expected considering these samples were also stimulated with ultrasound. ALP activity suggests that pAMUS accelerate matrix formation even more than pulsed ultrasound samples. Calcium mineralization was highest in 100 KHz samples, followed by 45 KHz, control and sham samples respectively. 100 KHz pAMUS sample show 27-33% higher and 45 KHz sample show 6-

9% higher mineralization than samples stimulated by pulsed ultrasound signal.

Furthermore, alizarin red staining shows that higher calcium levels are not local to focal region as they were expected, considering only cells in the focal region were being induced by ultrasound (both pAMUS and pulsed ultrasound). This suggests that ultrasound induces cells locally but due to process of induction (cell signaling) within cells and acoustic streaming, the effect gets distributed in larger area than originally stimulated.

The ALP activity starts to show significant increase at Day 12 and Day 18 relative to sham samples but in the case of calcium deposition the only significant increase was observed at Day 18. These results make sense considering that ALP is a precursor to calcium deposition and ALP activity is required to provide phosphate ions for calcification process. Furthermore, cells stimulated with pAMUS at 45 KHz show a significant increase in ALP activity in Day 12 relative to control samples but calcium assays don't show a significant increase in the 45 KHz samples either at Day 12 or 18. Considering the complexity of mineralization process and activation of different enzymes it is possible that 45 KHz pAMUS ultrasound is not sufficient to increase the activity of other enzymes which play important role in mineralization process.

The data obtained from this study shows the formation of focused pAMUS in the region of interest (focal region). The cellular studies show higher levels of ALP activity and matrix calcification in pAMUS samples when compared to pulsed ultrasound and no ultrasound samples. The data supports that application of pAMUS further accelerates

the mineralization process in comparison to pulsed ultrasound. Osteoblast cells show higher sensitivity to pAMUS after 7 days of stimulation as after first 7 days there was no significant difference in pAMUS, pulsed ultrasound and no ultrasound samples. These results suggest that pAMUS signal can enhance mineralization more than low intensity pulsed ultrasound.

The enhanced mineralization in pAMUS relative to pulsed ultrasound suggests that amplitude modulation can further enhance the mineralization process in osteoblast cells and make changes in amplitude an important factor for future studies. Considering the similarities of pUS signal to LIPUS, the study suggests that pAMUS can potentially accelerates bone healing process relative to LIPUS treatment.

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