CHAPTER 2

Enhancement of Mechanical Signals for Tissue Engineering Bone

Y. Yang* and A. El Haj

Summary

Mechanotranduction is known to play an essential role in bone tissue remodelling and repair. Membrane ion channels such as voltage operated calcium channels (VOCC) have been shown to be a critical component of the bone cell transduction pathway with agonists and inhibitors of this pathway having profound effects on the load signal. At physiological magnitudes, mechanical load results in an elevation of specific matrix protein mRNAs and thus, the amount of the matrix proteins. Tissue engineering has progressed into a most promising therapeutic direction for treatment of damaged tissues or organs over the past decade. Generation of matrix proteins with required quantity and quality via the tissue engineering methodology is paramount to form functional tissues. Here we propose to manipulation of mechanotransducer for tissue engineering bone by up-regulation/controlling matrix protein production. Our research focuses on the optimisation of mechanotransduction pathways; creation of 'mechano-active' scaffold. The effect of substrate modification on cell adhesion and biological conditioning of cell-scaffold constructs utilising bioreactors on matrix protein production are also investigated. Our results demonstrate that manipulating mechano-sensitive ion channels and attenuating the opening of calcium channels, which enhances the mechanical signals, may be an effective strategy for amplifying matrix production via mechanical stimulation. This may be applied to bone tissue engineering and potentially engineering of other load-bearing connective

*Correspondence to: Y. Yang, Institute of Science & Technology in Medicine, Keele University Medical School, Stoke-on-Trent, UK.
E-mail: bea00@keele.ac.uk

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Introduction

Over the past decades, bone tissue engineering has undergone considerable development and now demonstrates a great potential for improved treatment or replacement of damaged bone in comparison with conventional therapies. Bone is a dynamic organ with enormous capacity for growth, regeneration and remodelling. Among the most potent modulators of bone remodelling and regeneration are mechanical signals. Many studies have shown that mechanical forces stimulate the synthesis of bone extracellular matrix and may even enhance the mechanical properties of developing tissues. There is ample evidence for the importance of mechanical forces in facilitating bone remodelling. Mechanical loading of physiologically relevant magnitudes has been shown directly to initiate bone modelling in animal models (1). In contrast, lack of load has been shown to promote tissue atrophy and bone loss (2). How the bone cells sense the stress and convey the mechanical signals into biological events (reactions) is an area of increasing research activity. A number of mechanisms which describe the transfer of stress induced signals into the cell and the alteration of the cells' function or activity in response to the mechanical signal, i.e. mechanotransduction, have been proposed. Intensive investigations have focused on mechanotransducers via membrane ion channels and integrins when mechanical forces are exerted on cells via the extracellular matrix (3).

Bone tissue engineering involves mimicking and creating a complex biomechanical environment for cell-cell and cell-matrix interactions. Such an environment should promote maturation of the cell-scaffold construct *in vitro*. Eventually the construct should enable the modulation of key mechanotransduction pathways when implanted in a dynamic loading environment *in vivo* within the patient.

Our primary research in bone tissue has worked on identifying critical steps in the mechanotransduction pathways and in particular investigating the role of membrane

ion channels (4, 5). Based on the study of bone tissue, we have proposed the manipulation of the membrane ion channel to enhance mechanical signals for bone tissue engineering. In addition, to safeguard the efficient delivery of mechanical signals from substrate or scaffold to the cells, better adhesion, i.e. cell-matrix interaction, must be achieved. Our aim is to enhance mechanical signalling for improved production of engineered bone, ultimately resulting in matured and improved implant integration. In particular, we have applied two strategies to achieve the goal by which we can translate our understanding of cellular biomechanics in the field of bone tissue engineering.

1) Development of a 'mechano-active' scaffold to deliver agonists of mechanosensors in a controlled manner leading to enhancement of the mechanical signals received by the cells;
2) Exploiting improved surface coating agents for scaffolds to generate enhanced cell adhesion and ensure efficient delivery of external mechanical strain.

1) Development of a 'mechano-active' scaffold

It is well known that calcium channels are present in excitable tissue capable of generating a large action potential (6). The presence and the importance of calcium channels in bone cells have been studied since the 1980s. It has been found that regulation of Ca^{2+} entry via activation of the diverse plasma membrane Ca^{2+} channel is an important signalling pathway in bone cells. Patch clamping measurements have revealed the presence of several types of calcium channels, both voltage-sensitive and voltage-independent. These calcium channels, often coupled with intracellular calcium release, can respond to hormonal, cytokine, and especially mechanical stimulation (7).

In our laboratory, we have focused on the early signalling pathways for mechano-transduction (4). We have demonstrated that membrane channels, in particular voltage

operated calcium channels (VOCC), have an important function in mechano-transduction in bone cells (5). Walker *et al.* (8) have shown that a dramatic flux of intracellular calcium appeared using fluo-3 AM techniques when applying a mechanical signal by optical tweezers or stretch to an underlying membrane of an individual human or rat osteoblast. This response is modulated by the VOCC blocker, nifedipine, and the VOCC enhancer, Bay K8634. In addition, we have shown that the production of matrix proteins, such as osteopontin and osteocalcin, are elevated in response to mechanical loading and that this response is strongly inhibited by the calcium channel blocker, nifedipine, and greatly enhanced by the calcium channel agonist, Bay K8644 (9).

Extending this modulation mechanism to tissue engineering, a mechano-active scaffold was proposed to be produced in our laboratory, with the capability of manipulating membrane ion channels to enhance mechanical signals. The core technique is to create a biodegradable scaffold containing slow release agonists which enhance the load signal and stimulate increased matrix synthesis. The advantage of this system is two-fold: the matrix is laid down in the region according to the mechanical signals, and the control of the augmentation is limited to a mechanical switch rather than continuous as in the case of bioactive growth factor-containing scaffolds. The calcium channel agonist used in this project is Bay K8644, an L-type Ca^{2+} channel agonist which prolongs single channel opening time without affecting the closing time (10).

Materials and methods

In this project, the polymer used for fabricating the scaffold was poly(l-lactide) (PLLA), a medical grade material, PURASORB™ supplied by Purac Biochem bv Gorinchem, Holland with a molecular weight of 360,000. The calcium channel agonist, Bay K8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluoromethyl)-phenyl]-3-pyridine-carboxylic acid methyl ester, was purchased from Calbiochem. Calf collagen type I solution in 0.1% acetic acid (Sigma) was used to coat the scaffolds without further modification.

The porous three-dimensional scaffolds were formed by a solvent-evaporating and salt-leaching technique with sodium chloride as the leachable component (11). Chloroform was used as the solvent. The pore size of the scaffold was controlled to range from 250 to 350 μm and porosity was estimated as 90% by the weight fraction of PLLA and salt in the scaffold. Cylindrical scaffolds with dimensions of ϕ 8 x 8 mm were produced.

The scaffolds were coated with collagen type I at a concentration of 7.5 $\mu g/cm^2$ by soaking in the collagen solution for two hours, denoted as PLLA/C, in order to enhance the adhesion of cells to the scaffolds. For processing calcium channel agonist incorporated scaffolds, the scaffolds were first soaked with Bay K8644 solution for two hours. After removing the solvent by evaporation, the treated scaffolds were then coated with collagen type I as described above, denoted as PLLA/C/Bay. The initial concentration of Bay in the scaffold was controlled at approximately 60 nM/cm^3.

A human osteoblast-like cell line MG63 was employed for this study; a low cell passage number of 20 was used. 1 X 10^6 cells in 100 μl medium were seeded in each of the cylindrical PLLA/C and PLLA/C/Bay scaffolds. The cell-scaffold constructs were cultured in αMEM medium supplemented with 10% fetal calf serum, 50 μg/ml of ascorbic acid, 10 mM of β-glycerophosphate at 37°C and 5% CO_2.

After 3 weeks' static culture, the scaffolds were subjected to one week's mechanical loading with a compression and perfusion system (Fig. 1) consisting of a medium reservoir, peristaltic pump and bioreactor chamber with a piston (12). The cell seeded constructs were located between the base of the bioreactor and the piston. The piston, with 7 symmetrically arranged medium outlets, acted as a medium distributor to deliver and guide the medium to be spread evenly on the cell-scaffold constructs from the centre to the end of the piston. The whole loading system was placed inside an incubator for the loading and perfusion period. The compression load was applied for 1 hour per day and continued for one week at 0.1% strain level and 1 Hz frequency mimicking physiological conditions. After 24 hours' post-culturing from the final loading, the total

RNA in the loaded and unloaded constructs was extracted using a modified guanidium thiocyanate method (13) as described by Gu *et al.* (14). The total RNA was quantified by spectrophotometric analysis of the absorbance at 260 nm.

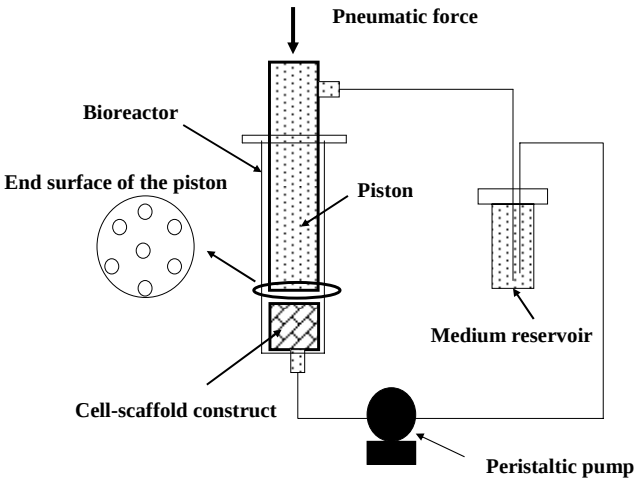


Fig. 1: Schematic set-up of the compression/perfusion bioreactor. The cyclic compression was applied by pneumatic force to the top of the piston.

Real-time quantitative PCR was performed to analyse mRNA expression of collagen type I and CBFA-1 using the Roche Molecular Biochemicals LightCycler system with SYBR Green 1 dsDNA fluorescence detection. The 20 µl reaction volume contained 100 ng cDNA, 0.5 µmol/l forward and reverse primers, and 1X LC master-mix containing reaction buffer, Taq DNA polymerase, dNTP mix, SYBR Green 1 dye, and 4 mmol/L $MgCl_2$. Collagen type I PCR cycling parameters consisted of an initial denaturation step at 95°C for 30 secs, followed by 40 cycles at 56°C for annealing (5 secs), 72°C for extension (7secs) and 95°C for denaturation (<1sec). CBFA-1 parameters were initial denaturation at 95°C for 30 secs, followed by 40 cycles at 62°C for annealing (3 secs),

72°C for extension (11.6 secs) and 95°C for denaturation (<1 sec). Gene expression was calculated using a serial dilution of amplified human genomic DNA stock standard, supplied by Roche Molecular Biochemicals, and reported as the number of copies of the gene expressed.

Results and discussion

In this study, we set out to investigate whether we can create a dynamic mechanical environment and enhance mechano-signalling in our mechano-active scaffolds. Two genes, collagen type I and CBFA-1, have been selected to evaluate the enhancement effects. As shown in Figure 2, the level of collagen I expressed as a number of copies per 100 ng cDNA was greatly elevated in response to load. This load-related elevation was further enhanced by incorporation of the Bay agonist within the scaffold. The same trend was found for the gene CBFA-1.

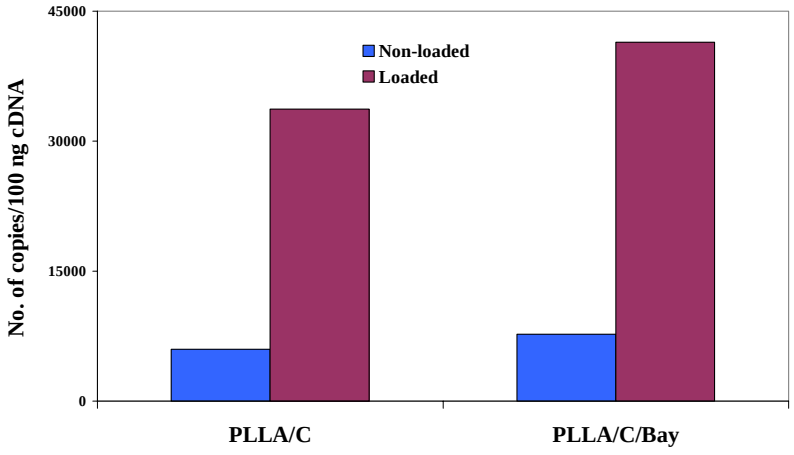


Figure 2a

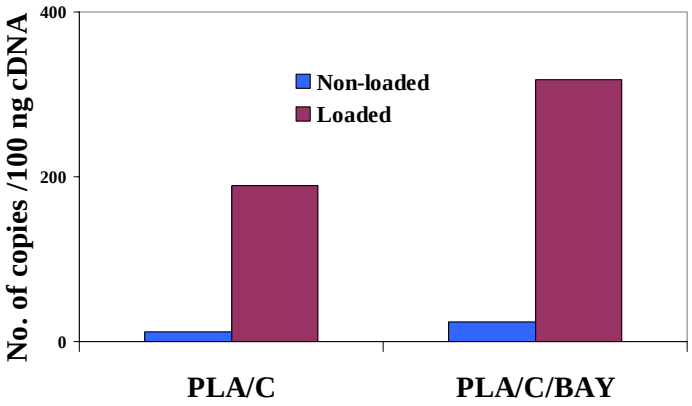


Figure 2b

Fig. 2: Real-time PCR data showing mRNA levels for total 4 weeks' culture. (a) Collagen type I; (b) CBFA-1. PLLA/C denotes the scaffolds coated with collagen type I only; PLLA/C/Bay denotes the scaffolds coated with collagen type I and incorporating the calcium channel agonist, Bay.

Collagen type I is the main element of extracellular matrix in bone. CBFA-1 is an osteoblastic differentiation marker. The fact that the extracellular matrix production increases in response to mechanical loading confirms the dynamic feature of bone modelling and remodelling. Application of a drug delivery technique to incorporate a calcium agonist into the scaffold has achieved the positive effect of amplifying mechanical signals.

A number of other chemical and bioactive agents such as heparin, trypsin inhibitor, insulin etc. have been proposed for delivery as therapeutics in slow release PLLA type scaffolds or substrates (15-17). Incorporation of Bay K8644 into PLLA type scaffolds follows these principles closely but the purpose of using this type of agent is novel. The

common feature of continuous release bioactive materials is that the agent is always present with time and there is no means of controlling the levels to which the cells are exposed. In the case of Bay, the agonist is continuously released but only acts when the calcium channels are open in response to the mechanical load. It is also possible that the mechanical forces on the scaffold will increase the release of Bay from the PLLA scaffold. Hence, the agent will act to amplify the load signal preferentially on the cells which are exposed to load rather than on those which are not. This suggests that the amplification will occur specifically to the load environment to which the implant is subjected.

2) Exploiting better surface coating agents for scaffolds or substrates

Since the first interaction between cells and a substrate (scaffold) is cell adhesion, the surface properties of the scaffold become a key factor in governing the success of engineered tissues. Without effective adhesion, the cascade of cellular events in a dynamic environment may not occur. The attachment of cells to their support matrix is important in determining cell shape, and ultimately proliferation, and in maintaining proper cell function and tissue integrity (18). Cell adhesion anchors cells to a substrate and provides positional signals that direct cellular traffic and differentiation (19).

A number of researchers have demonstrated that coating the scaffold surfaces with single extracellular matrix (ECM) molecules such as collagen, fibronectin, laminin, or even pre-soaking scaffolds in medium, improves cell seeding efficiency and spreading. However, the majority of the research work on cell adhesion behaviour is based on the evaluation of the cell-substrate adhesion in a static environment (20, 21). In bone tissue engineering, mechanical conditioning becomes a vital step to mature and enhance the tissue growth. Therefore effective cell adhesion is required in bone tissue engineering, allowing the cascade of cellular events in a dynamic environment to occur, especially with respect to mechanical stimulation. Without proper attachment of cells to the

substrate, the cells will be detached when a mechanical force is applied and a loss of cell population will occur. It is expected that the requirement for cell-substrate adhesion strength in such circumstances is higher. In this study, we take a step towards increasing cell-substrate adhesion by coating substrates with whole extracellular matrix extracted from autologous cells and testing the adhesion strength in a dynamic mechanical environment.

Materials and methods

PLLA film with a thickness of 10 ± 2 μm was produced by the solvent-casting technique. The film was then adhered to the loading area of a coverslip with a silicone adhesive and was of the dimensions of 24 x 30 mm. These are denoted as PLLA coverslips. The coverslips covered by the PLLA films in this way were still transparent. Primary human bone cells were used in the study; these were isolated from trabecular bone biopsies obtained from human tibial fractures. The cells were cultured with α-MEM supplemented with 10% fetal calf serum, 50 μg/ml of ascorbic acid, 10 mM of β-glycerophosphate. All subjects enrolled in this research have responded to an informed consent which has been approved by the University Hospital of North Staffordshire (NHS) Trust ethical committee.

Two methods were used to extract the whole ECM from the autologous cells for the coating. For the production of a homogenised cell coating, a cell pellet was ground in a mortar with PBS and the solution was collected and filtered with a 10 μm sieve to remove residual cell membranes. The PLLA coverslips were coated with the filtrate at room temperature for 2 hours at a concentration of 0.4 x 10^6 cells/per coverslip. For the lysed cell coating, 0.2x10^6 cells were seeded on a PLLA coverslip and cultured until confluent. The cells were lysed in water and exposed to 20 mM NH_4OH solution for 3 min., then rinsed in distilled water and PBS. In order to characterise these ECM coatings completely, other PLLA coverslips were coated with calf collagen type I at a concentration of 7.5 μg/cm^2. We compared 4 different coating systems: controls (non-

coating), lysed ECM, homogenised ECM and calf collagen type I coating. The same patient's cells were seeded on the coated coverslips at a cell seeding density of 100,000 cell/coverslip. An identical coating and seeding procedure was applied to glass coverslips for comparison. The cell morphology was observed under a Leica light microscope (Leica DM IRB) at two time points of 1 hour and 16 hours after seeding with or without hematoxylin and eosin staining.

To test the effect of the coatings on cell adhesion in response to mechanical loading, a four-point bending system was utilized to apply a force to the cells seeded on differently coated PLLA coverslips. This loading model can apply cyclic physiological levels of tensile and compression load to the PLLA coverslips. The schematic set-up of the loading model is shown in Figure 3. The bulk strain applied across the coverslips was measured to be approximately 1000 μstr strain by direct strain gauge measurements and formula-derived calculations. The mechanical tension was applied to the coverslips at 1 Hz frequency for 1 hour (22, 23). After 24 hours post-culture, the cells on the coverslips were fixed in 10% PBS buffered formalin and stained by hematoxylin and eosin (n = 3).

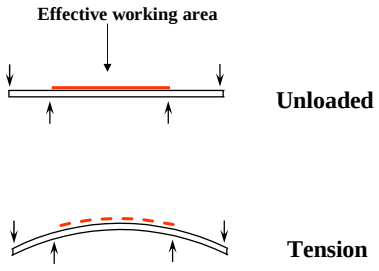
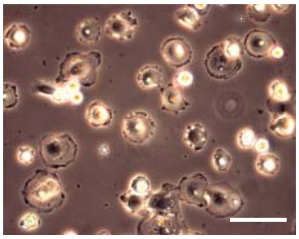


Fig. 3: Schematic set-up of the four-point bending system and effective working area on the coverslips. The cells were seeded and grown on the working area only.

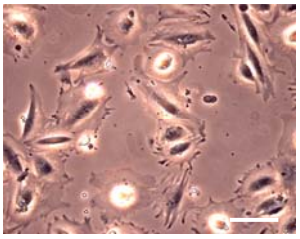
Results and discussion

Since the thin PLLA film on the coverslips remained transparent, the cell morphology on the PLLA substrates could be observed and recorded directly with a light microscope under transmission mode. The primary bone cells showed dramatically different attachment abilities to PLLA and plain coverslips with various coatings. This was judged by the shape of cells, round indicating lower adhesion and stretched or irregular shape indicating higher adhesion.

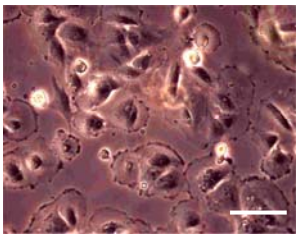
Figure 4 displays the morphology of primary bone cells on PLLA coverslips and plain coverslips with various coatings after one hour's culture. In general, cells attached better to plain coverslips than to PLLA coverslips. It is clearly shown that within one hour's culture, the cells barely attached to the non-coated PLLA surface, and a round shape morphology was generally present, which might be ascribed to the difference in hydrophility between the plain glass and the PLLA. PLLA is highly hydrophobic and the cell suspension was not even spread over the substrate. A stretched and irregularly shaped cell morphology dominated all of the coated PLLA substrates and plain coverslips. There was little difference in the cell attachment ability between the coated coverslips, i.e. the coating by collagen type I and the whole cell ECM extraction had similar effects on the initial cell attachment.



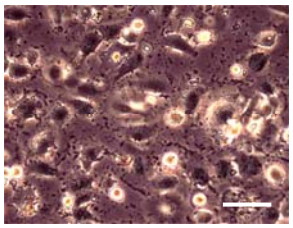
Non-coating



Collagen

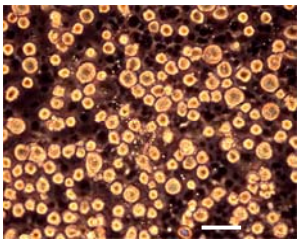


ECM (lysed)

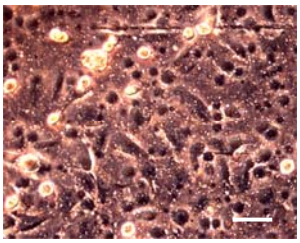


ECM (homogenised)

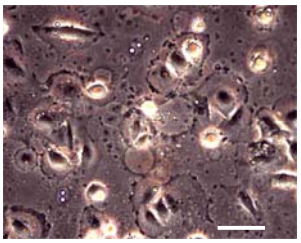
Figure 4a



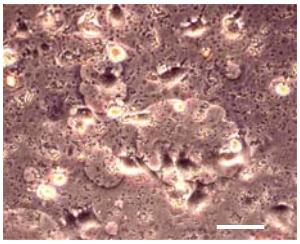
Non-coating



Collagen



ECM (lysed)

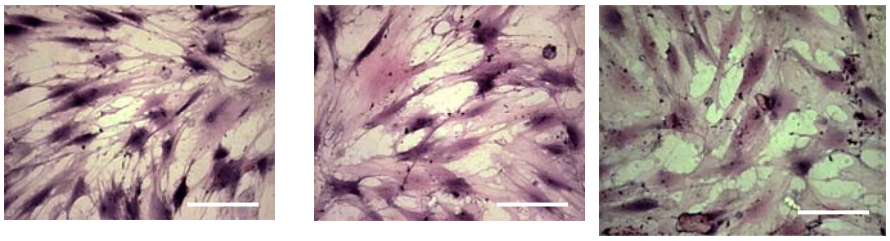


ECM (homogenised)

Figure 4b

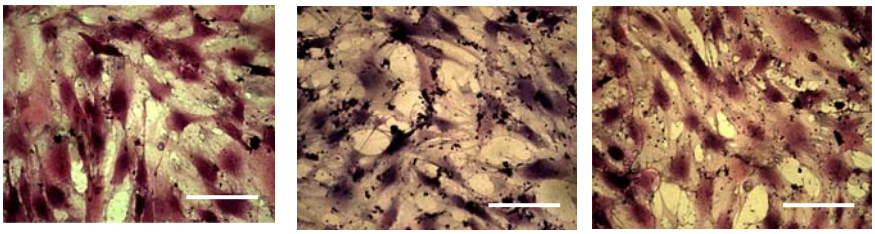
Fig. 4: Light microscopy images of primary bone cell morphologies on various coated coverslips after one hour's culture from seeding. (a) on plain coverslips; (b) on PLLA coverslips. Non-coating: control; collagen: calf collagen type I; ECM (lysed): EMC extraction from lysed autologous cell mixture; ECM (homogenised): EMC extraction from homogenised autologous cell mixture. Bar = 100 μm.

After 16 hours' culture, the cell morphology in most areas of the non-coated coverslips was similar to the coated ones, but the average cytoskeleton size in the coated coverslips was larger than in the non-coated one (Fig. 5a). However, there was no apparent dose response between the cell morphology and the cell number in the cell extraction solution. Increasing the cell number in the homogenised ECM solution did not result in an appreciable change in cell morphology, as shown in Figure 5b, indicating that using ECM extraction from small cell numbers can achieve a good cell adhesion effect.



Non-coating **Collagen** **ECM (lysed)**

Figure 5a



$0.2x10^6$ **$0.4x10^6$** **$0.7x10^6$**

Figure 5b

Fig. 5: (a) Light microscopy images of primary bone cells on various coated PLLA coverslips after 16 hours' culture; (b) the effect of cell number in ECM extraction. Non-coating: control; collagen: calf collagen type I; ECM (lysed): EMC extraction from lysed autologous cell mixture; ECM (homogenised): EMC extraction from homogenised autologous cell mixture. Bar = 100 μm.

The cell layer morphology after one hour's mechanical loading is shown in Figure 6. The difference between coated and non-coated PLLA coverslips, and between the different coating materials, was extraordinary. The cell layer on the non-coated PLLA coverslips was almost lost after one week's culture or one hour of the mechanical loading. The cell layer on the collagen coated PLLA coverslips remained integrated in the static culture, but lost the majority of the cell layer when subjected to mechanical loading. Strikingly, the cells on both of the whole cell ECM extraction coated PLLA coverslips remained intact before and after mechanical loading.

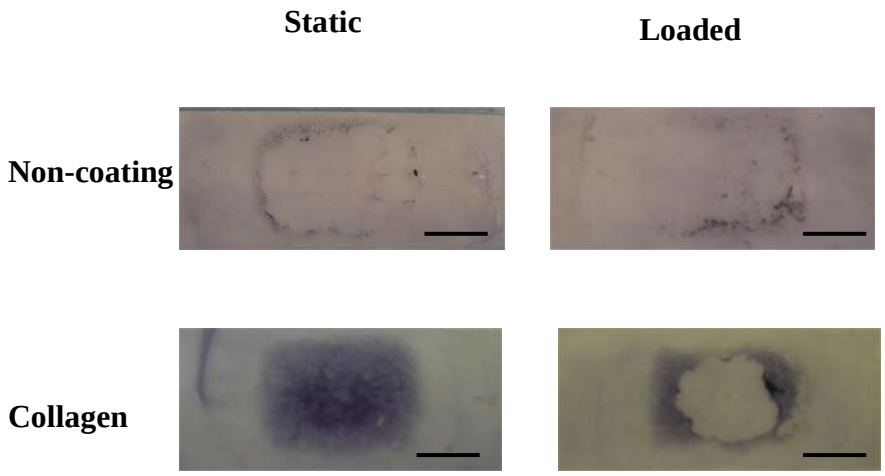


Figure 6-1

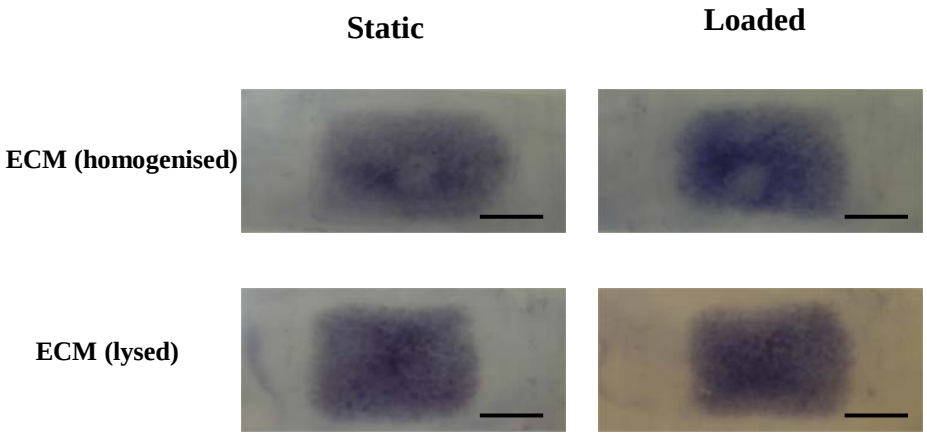


Figure 6-2

Fig. 6: Cell layer morphologies on various coated PLLA coverslips after the cells were subjected to static culture and mechanical loading. The cells were stained by H & E. Bar = 10 μm.

The cell-matrix/substrate interaction plays a pivotal role in bone cells sensing mechanical signals within their environment. Tissue culture studies indicate that many cells adhere to solid surfaces in three ways, namely focal adhesions, close contacts and extracellular matrix contacts (24). It is via these adhesions that applied mechanical signals are transmitted to cells through a substrate, resulting in the activation of intracellular mechanotransduction pathways believed to perform an essential role in the upregulation of matrix protein synthesis required during bone tissue production. The widely accepted process of cell adhesion to substrates is via RGD sequences which are present in many ECM molecules. Collagen, glysin and fibronectin are the commonly selected ECM molecules used as coatings to enhance cell adhesion.

PLLA coverslips coated with collagen, one of the main components of ECM containing RGD (Arg-Gly-Asp) sequence which is specific to the fixation of cell membrane receptors such as integrin (25, 26), showed a great improvement in cell adhesion compared to the non-coated PLLA coverslips in the static and short period culture, which confirms the role of RGD in cell attachment. However, the adhesion strength was not great enough to withstand a mechanical strain at physiological level. In comparison, the coating with whole cell ECM extraction exhibited a more robust attachment in response to mechanical force.

The lysed cell solution or the homogenised cell mixture contains various glycoproteins, such as fibronectin, laminin, collagen and other ECM molecules produced by the autologous cells, and also includes some growth factors, which will promote cell adhesion and growth in the first attachment and proliferation stages. Having such a wide range of adhesive molecules within the coating agent for substrates leads to tougher adhesion strength, which is a prerequisite for mechanical stimulation of the cells. As a result, the cells were not detached in response to mechanical loading; indeed the mechanical strain might stimulate further proliferation. Therefore, an intact cell layer remained after loading.

Using autologous ECM extraction as a coating material offers extra merits. It avoids any potential risk of immunological reactions or disease transmission. For instance, the common collagen solution used as a coating agent for scaffolds is of bovine derivation. Since the serious outbreaks of transmissible bovine spongiform encephalopathies (BSE), the control of the use of animal derivatives in medical devices has become stricter. With regard to bone tissue engineering, it is thus important to seek alternative, safer coating agents both to improve cell-substrate adhesion and to enable mechanical signal transduction in mechanically conditioned cell-scaffold constructs. Autologous ECM extraction has the potential to be a prime candidate for this purpose.

Conclusions

The creation and application of mechano-active scaffolds for bone tissue engineering in our work demonstrate that manipulating ion channels and attenuating the opening of calcium channels may be an effective technique to enhance mechanical signals, which lead to amplified matrix production. Furthermore, mechanical loading can act as a switch to control the amplification in such scaffolds.

Autologous ECM extraction provides a better coating material for bone cells to adhere to a PLLA substrate. The coating produced enhanced cell adhesion and enabled the withstanding of mechanical stimulation at a physiological level, thus safeguarding and enhancing the mechanical signals to the cells.

The application of the thin film on coverslips allows us to establish a convenient and reliable method to study cell morphology on opaque polymers in response to different surface treatments and mechanical stimulation. These studies increase our understanding of mechanical conditioning for bone tissue engineering.

Acknowledgements

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