4th 3B's Symposium on *Biomaterials and Stem Cells in Regenerative Medicine*

3B's Research Group Auditorium– AvePark, Caldas das Taipas, Guimarães, Portugal
Date: 08 June, 2015

Chairmen: João F. Mano and Rui L. Reis
3B's Research Group, University of Minho, Portugal

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Program

9:00-09:10	Welcome	*João F. Mano / Rui L. Reis*
	Natural-based materials for regenerative medicine (Discussion leader: Nuno Neves)	
9:10-9:20	Introduction	*Nuno Neves* (3B's-UM)
9:20-10:10	Multifunctional protein-based biomaterials (keynote lecture)	*Tony Weiss* (Univ. Sydney)
10:10-10:40	Marine origin biopolymers	*Tiago* Silva (3B's-UM)
10:40-11:10	Cross-linking strategies of natural-based polymers	*Catarina Custódio* (3B's-UM)
11:10-11:20	Break	
11:20-11:40	Supramolecular assembly of natural-based molecules and macromolecules	*Iva Pashkuleva* (3B's-UM)
11:40-12:10	Microfluidic-based methodologies to process biomacromolecules	*Luca Gasperini* (3B's-UM)
12:10-12:40	Green-based technologies to process green polymers.	*Ana Rita Duarte* (3B's-UM)
12:40-14:20	LUNCH BREAK	
	Cells and biomaterials in tissue engineering strategies (Discussion leader: Alexandra Marques)	
14:20-14:30	Introduction	*Alexandra Marques* (3B's-UM)
14:30-15:20	Combinatorial analysis of biomaterials (keynote lecture)	*Morgan Alexander* (Univ. Nottingham)
15:20-15:50	Platforms for high-throughput analysis of natural-based biomaterials	*Mariana Oliveira* (3B's-UM)
15:50-16:20	Multi-scale analysis of complex scaffolds for bone TE	*Miguel Oliveira* (3B's-UM)
16:20-16:30	Break	
16:30-17:00	Cell sources for bone TE: isolation and characterization	*Andreia Carvalho* (3B's-UM)
17:00-17:30	Complex cells-(natural-origin)hydrogel systems	*Dillip Bishi* (3B's-UM)
17:30-18:00	Vascularization strategies in bone TE	*Rogério Pirraco* (3B's-UM)

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Multifunctional elastic protein-based biomaterials

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Sir Samuel McCaughey Chair, Professor of Biochemistry & Molecular Biotechnology
1. Molecular Bioscience, University of Sydney
2. Charles Perkins Centre
3. Bosch Institute
4. Royal Prince Alfred Hospital
5. Scientific Founder, Elastagen P/L

Elastin provides structural integrity, biological cues and persistent elasticity to a range of important tissues, including the vasculature and skin. Its critical importance to normal physiology makes it a desirable component of biomaterials that seek to repair or replace these tissues. The recent availability of large quantities of the highly purified elastin monomer, tropoelastin, has allowed for a thorough characterization of the mechanical and biological mechanisms underpinning the benefits of mature elastin. While tropoelastin is a flexible molecule, a combination of optical and structural analyses has defined key regions of the molecule that directly contribute to the elastomeric properties and control the cell interactions of the protein. Insights into the structure and behavior of tropoelastin have translated into increasingly sophisticated elastin-like biomaterials, evolving from classically manufactured hydrogels and fibers to new forms, stabilized in the absence of incorporated cross-linkers. Tropoelastin can also be modified or blended with other natural or synthetic moieties, including proteins like silk fibroin, to augment existing capabilities or confer additional architectural and biofunctional features to compositionally pure materials for the manufacture of particles, fibers, gels, tubes, sheets and films. The resulting materials can be tailored to possess specific strength, elasticity, morphology, topography, porosity, wettability, surface charge, and bioactivity. This extraordinary tunability of elastin-based constructs enables their use in a range of biomedical and tissue engineering applications such as targeted drug delivery, cell encapsulation, vascular repair, nerve regeneration, wound healing, and dermal, cartilage and bone repair.

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MARINE ORIGIN BIOPOLYMERS: ISOLATION AND USE ON BIOMATERIALS

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On marine environment one can find an uncountable diversity of materials, bearing all kinds of biological and chemical features, some of which are unique, without equivalent in terrestrial organisms. This is being an inspiration of scientists, engineers and medical staff for the development of a new range of therapeutic proposals. For instance, the discovery of new bioactive compounds synthesized by marine organisms is extending the pharmacopeia for assessing different pathologies, in particular cancer and inflammation. Besides, biopolymers and ceramics found in marine environment are being proposed as building blocks for the development of innovative biomedical applications, such as tissue engineering scaffolds or drug delivery devices [1]. In this frame, some examples of the sea of opportunities that is opening wide in front of us will be addressed, particularly focusing on its potential for regenerative medicine approaches. The presentation will address from the isolation of biopolymers and their characterization up to their processing into the development of biomaterials.
In particular, the production and further use of chitosan from a poorly explored marine source, squid pens, will be discussed [2], with chitosan being probably the most explored marine biopolymer. Collagen, a key material in the biomedical field, given the fact of being the most abundant protein in mammals, can also be obtained from marine organisms, including from byproducts, and be further used on the production of hydrogels or other polymeric systems [3,4]. Moreover, marine biopolymers can be also used for the tune of properties of developed biomaterials, namely to improve their surface and/or morphological properties envisaging enhanced cell culture efficiency [5]. Finally, examples of peculiar marine organisms will be discussed as potential inspiration for new scientific breakthroughs.

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Cross-linking strategies of natural-based polymers

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Regenerative medicine has been defined as the replacement of diseased or injured tissues with a regenerated functional tissue. Biomaterials play a pivotal role in tissue regeneration. They not only serve as scaffolds providing mechanical support to the cells, but actively influence cellular responses including cell attachment and proliferation [1].

Typically, scaffolds are fully formed outside the body and then implanted surgically. There is however, growing interest in being able to use minimally invasive procedures in regenerative medicine. Hydrogels have been extensively explored due to their many desirable properties for tissue regeneration such as high tissue-like water content and ability to provide controlled release of entrapped therapeutic molecules [2]. Additionally they can be formed at specific sites using injectable polymeric solutions that crosslink *in situ*, making the application to a designated site minimally invasive. The crosslinking stabilizes the polymeric structure by changing the polymer solution into a cohesive gel by restricting the ability of movement. This may be accomplished by different mechanisms, using physical, chemical or biological crosslinking routes.

Physical networks have transient junctions that arise from either phase transition behaviour of certain polymers or physical interactions such as ionic interactions, hydrogen bonds, or hydrophobic interactions.

Chemically crosslinked networks have permanent junctions and provide enhanced mechanical properties and in vivo stability. Current chemical crosslinking routes often require a coupling agent, catalyst, or photoinitiator, recently Diels-Alder "click" chemistry as been also explored to form stable polymeric networks.

Biologically triggered gelation provides crosslinking reactions that occur in response to the presence of specific biomolecules, such as enzymes, antigens or nutrients. The type and degree of crosslinking influences many of the network properties, like swelling properties, elastic modulus and transport of molecules within the hydrogels. Therefore, the design of a particular hydrogel should focus on those features that give rise to the desired properties most suitable for the biomedical application, including transport properties, tissue interactions, and chemical stability.

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Sugars versus Proteins in the design of supramolecular assemblies

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Molecular self-assembly (MSA) is of central importance to life: a variety of biological structures, ranging from proteins and nucleic acids to viruses and cell membranes, possess a highly precise organisation on the nanometer scale which derives from specific interactions at molecular level[1,2]. Thus, a thorough understanding of MSA provides an opportunity to further our understanding of the living systems on one hand and to develop new tools to control self-assembly on the other hand, i.e. the development of therapeutic agents.
The talk will be focussed on two classes of natural biopolymers, namely proteins and carbohydrates, and their ability to trigger specific bioresponses by self-assembly. So far, proteins and their short analogues, i.e. peptides, are the most used building blocks in bioinspired self-assembly approaches for tissue engineering and regenerative medicine [1,3]. Thus, several case studies with such systems will be reviewed and the main mechanisms triggering the self-assembly process will be discussed[4]. Alternative approaches, involving carbohydrates, will be also introduced[5]. Finally, the advantages, drawbacks and different applications in the biomedical filed of these systems will be presented.

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Microfluidic-based methodologies to process biomacromolecules

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The goal of the project ComplexiTE is to screen the biological response of cells to different combinations of biomaterials as random 3D arrays cultured under defined conditions. Part of the project consists in the production of hydrogel microparticles containing combinations of biomaterials and cells. The encapsulation of mammalian cells within a semi-permeable hydrogel matrix is an attractive procedure for many other biomedical and biotechnological applications, such as xenotransplantation and maintenance of stem cell phenotype. The chosen strategy to encapsulate cells is through the adoption of a microfluidic approach Microfluidics is a technique dealing with the handling of fluids in microenvironments, such as microchannels where the flow of fluids is generally laminar (low Reynolds numbers). The laminar flow allows a fine control over the characteristics and composition of the microdrop but doesn't favor the mixing of the different biomaterials (1,2). Furthermore, to create microdroplets a microfluidic chip with a flow-focus or a t-junction is needed. The droplets of a hydrogel precursor are formed in a non-miscible continuous phase (oil and surfactant)(3) and then they can be crosslinked to form a hydrogel. In this talk some general aspect about microfluidics will be presented first, followed by a presentation of the materials, equipments, methods and some results related to the ComplexiTE project.

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The intersection between chemical and biomedical engineering: green technologies towards the development of enhanced biomaterials

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Despite the advances on biomaterials development and polymer processing technologies, this remains still one of the major scientific challenges that tissue engineering and regenerative medicine (TERM) faces to go from benchtop to bedside. Ideal scaffolds should be biocompatible, biodegradable and promote cellular interactions and tissue development and possess proper mechanical and physical properties. The preparation of 3D matrices must result, hereafter in structures with adequate porosity, interconnectivity, pore size distribution and compression properties which make then suitable for the tissue to be engineered. A wide range of biomaterials has been proposed for biomedical applications, from metals to ceramics and polymers. Due to their versatility, polymers are the straightforward choice. These must comply with different requirements such as hydrophilicity, biocompatibility, degradation rate, citotoxicity, among others. The use of natural based polymers in tissue engineering and regenerative medicine applications has long been proposed, precisely due to their chemical/biological versatility. Nonetheless, its processing using supercritical fluids only recently has started to received more attention from researchers. Supercritical fluids appear as an interesting alternative to the conventional methods for processing biopolymers as they do not require the use of large amounts of organic solvents and the processes can be conducted at mild temperatures. Different processing methods based on the use of supercritical carbon dioxide have been proposed for the creation of novel architectures able to fulfill the particular needs of each tissue to be regenerated and these will be unleashed in this presentation.

High Throughput Materials Discovery with Polymer Microarrays

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Polymer micro arrays have proven to be useful tools for the discovery of new synthetic materials which control cells.[1] This high throughput (HT) materials discovery approach is attractive because the paucity of understanding of the cell-material interface hinders the rational design of new materials.[2] The large number of polymer chemistries that can be investigated on a single polymer micro array act as a wide "net" in the search for materials that can achieve a certain cell response. Micro array hits are the starting point from which new materials may be developed.

Combinatorial acrylate libraries formed on standard glass slides were presented as a HT materials discovery platform by Anderson and Langer of MIT.[3] To complement materials screening, we developed the approach of HT surface characterisation employing a range of analytical techniques in collaboration with the MIT group.[4] This surface characterisation step is necessary to directly relate the effect of the material on attached cells to the actual surface on which they sit, and to enable effective scale up from micro array to culture ware dimensions. Application of chemometrics, to handle the large amounts of complex data, reveals the importance of certain surface moieties, guiding the process of materials discovery and increasing our understanding of the cell-material interface.

We have applied this approach to the identification of materials which resist bacterial attachment and biofilm formation with application in the reduction of medical device centred infection.[5,6] In the mammalian cell field, we have identified materials which show promise as synthetic substrates for pluripotent stem cell culture.[7,8] These materials require pre-treatment with expensive proteins such as vitronectin, a constraint which limits their commercialisation.[9]

Screening of arrays with greater chemical diversity than ever before, incorporating up to 140 monomers[10,11], is reported which leads to the identification of materials which support pluripotent stem cell expansion without pre-treatment of the substrate with protein. Materials which support maturation of cardiomyocytes with potential application in in vitro toxicology screening have been discovered.[12]

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Platforms for high-throughput analysis of natural-based biomaterials

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One of the main challenges in tissue engineering (TE) is to obtain optimized products, combining biomaterials, cells and soluble factors able to stimulate tissue regeneration. Multiple combinations may be considered by changing the conditions among these three factors. However, the unpredictable response of each combination requires time-consuming tests. High-throughput methodologies have been proposed to master such complexity in TE [1-3], implementing the perspective of a rapid, efficient, and industry-paced discovery of adequate biomaterials. Moreover, these strategies allow a higher efficiency on the discovery of more truthful biomimetic cell niches [4].
The use of the first biomaterial miniaturized array– with the size of a microscopy slide - allowed discovering relevant and unexpected effects of 2D materials on embryonic stem cells proliferation and differentiation [5]. Since then, the design of microarray platforms has seen progress aiming to meet the criteria of complex TE biological environments. Particular aspects as compatibility with versatile biomaterials processing (namely configurations allowing for cells-3D biomaterials interactions [6,7]) and more effective data acquisition/treatment have raised the interest of researchers working in this field. Moreover, apart from *in vitro* array-based strategies, there is also an emerging trend consisting of *in vivo* high-throughput approaches that take in consideration important regeneration phenomena [8], often overlooked in *in vitro* tests.
Superhydrophobic surfaces patterned with wettable regions are a particular type of indirect writing platforms used for biomaterials studies [9]. It is generally accepted that superhydrophobic surfaces show water contact angles higher than 150º and low surface energy, effectively repelling water adhesion [10]. In such surfaces, biomaterials remain restricted to the wettable spots due to the wettability contrast between them and the superhydrophobic surrounds [6]. This approach allows patterning of water-based biomaterials with distinct shapes and heights, depending on the shape and area of the wettable spot, as well as on the volume dispensed. The technology is also compatible with distinct biomaterials processing methodologies, giving rise to hydrogels or porous scaffolds.
Following the optimization of wettability contrast-based chips for short-term *in vitro* cellular studies and *in vivo* implantation, ongoing work is now focused of the application of such platforms to study the osteogenic differentiation of adipose-derived stem cells (ASCs) in biomaterials based on natural occurring polysaccharides and proteins using distinct cell culture medium. This approach allowed hit-spotting biomaterial formulations leading to ASCs osteogenic differentiation in the absence of

typical osteogenic cues (e.g. dexamethasone, which has to be supplied cyclically to the cells). The hit-spotted polysaccharide/protein formulation is currently being studied to design an injectable biomaterial with osteogenic factors-free differentiating properties.

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Multi-scale analysis of complex scaffolds for bone TE

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Keywords: Biomaterials; Multiscale; Bone; Nanoparticles; tissue Engineering

The control of stem cell differentiation to become osteoblasts *in vivo* is still regarded as a challenge in stem cell-based and bone tissue-engineering strategies. Biodegradable nanoparticles systems have been proposed as intracellular drug delivery carriers of bioactive molecules. Our group has been proposing the combination of nanotechnology approaches, stem cell engineering and tissue engineering. The development of polymeric systems at nano- and micro-scales can allow controlling stem cells fate *in vivo*, resulting on a superior new bone tissue formation, thus opening new possibilities in the production of biocompetent and mature complex tissues for regenerative medicine applications.

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Cell sources for bone TE: isolation and characterization

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The possibility to regenerate or repair damaged/injured or malfunctioning tissues/organs has gained a new perspective with increasing progress in tissue engineering (1). The possible application to clinical scenarios of stem cell-based therapies, in combination with degradable biomaterials gave a tremendous impulse to the field of bone tissue engineering and regeneration.
Adult stem cells, and in particular mesenchymal stem cells, are powerful candidates for regenerative medicine applications, given their unique abilities to self-renew, to home toward injury sites, and to secrete angiogenic and anti-apoptotic paracrine factors, attracting other cell types to promote healing (2). Moreover they possess the capacity to differentiate into different lineages, such as osteogenic, chondrogenic or endothelial. First isolated from bone marrow, they can now be isolated from diverse sources. In recent years, adipose tissue as gained interest as a powerful easily accessible and abundant alternative source of adult stem cells, the so-called adipose derived stromal/stem cells (ASCs) (3).
Bone is a highly vascularized tissue, and therefore cells that participate in vasculogenesis and osteogenesis play central roles in bone formation. When designing a successful bone TE approach, major factors to be taken into consideration: the sources of cell players; the strategies used for differentiation of adult stem cells into osteoprogenitor and endothelial cellular components; and the thorough characterization of their bona-fide acquired differentiated phenotype. The adipose stromal vascular fraction (SVF) and ASCs have been successfully used as sources of both progenitor cells for osteogenesis and endothelial cells required for vascularization events (4-5).
The combination of stem cell biology strategies with biodegradable biomaterials hold the possibility to achieve a functional osteogenic/endothelial system that can be used in tissue engineering approaches for bone regeneration in clinically relevant scenarios.

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Compartmentalization of combined cell populations in complex microcapsules of natural-origin hydrogels

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The emerging concepts of biodegradable biomaterials and biomimetics have opened up a new era in the field of tissue engineering to develop innovative tools for microfabricating bioartificial tissue constructs. The general strategy is usually to encapsulate cells within a scaffold, a structural device that defines the geometry of the replacement tissue and provides environmental cues that promote tissue regeneration. Co-cultures of distinct combined cell populations have been used to make more biomimetic microenvironments [1], whereas biodegradable biomaterials of natural origin are the blueprints of various successful tissue engineering strategies [2]. Hydrogels are three- dimensional (3D), hydrophilic networks composed of cross-linked hydrophilic polymer chains resembling native ECM architecture and functionality and can be modulated for micro-scale engineering. A cell-compatible hydrogel has the ability to control specific molecular interactions at the cell-material interface, such as receptor-ligand complexes that mediate cell adhesion and migration, bound or soluble molecule interactions facilitating proteolytic biodegradation or transcriptional events regulating cell phenotype, as well as focal adhesion interactions with substrates to transmit mechanical stimuli to cells [3]. The complex combination of different natural-based polymers and distinct cell populations, processed in the shape of miniaturised hydrogels structures could be used to engineer artificial stem cell niches and act as models to predict positive and synergistic actions over stem cell behaviour. In fact, cells react to the biomaterials surface by means of producing their own ECM proteins and by secreting growth factors and cytokines that act via paracrine, autocrine and juxtacrine mechanisms. Miniaturised hydrogel-based tissue constructs have been generated using emerging bottom-up approach such as microfluidics and more importantly such methodologies offers hydrodynamic cellular patterning to co-culture different cell types for manipulation of cell–cell interaction dynamics [4]. From tissue engineering standpoint, microfluidics-based 3D culture systems are optimal due to small culture volumes with minimal reagent consumption, lesser cell number requirement to test multiple experimental conditions and can easily be automated for high-throughput screening applications. In a proof-of-concept style, Tumarkin E et al. have developed a microfluidic platform for the high-throughput generation of hydrogel microbeads for mixed-type co-culture [5]. Microfluidics-based production of Janus droplets (containing two distinct compartments) offers a novel approach for fabricating unique 3D-culture

system to understand how the *ex vivo* expanded cell populations acclimatize to the complex microcapsule of various natural-origin hydrogels. Such complex tissue engineered micro-constructs could be used as suitable templates for screening adequate stem cells-biomaterials interactions in a high-throughput manner.

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Vascularization strategies in bone Tissue Engineering

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A comprehensive network of blood vessels is responsible for the critical role of transporting gases, nutrients and other molecules to and from most organs and tissues in the human body. Without such a system, tissue thickness would be restricted to 100 - 200 μm, the diffusion limit of oxygen[1]. Blood vessel formation can occur by two processes, *de novo* (vasculogenesis) and from preexisting vessels (angiogenesis). During embryonic development, the first blood vessels form from progenitor cells through vasculogenesis while angiogenesis is the main process responsible for post-natal vessel formation[2]. Any defect or problem affecting the blood supply to a given organ (ischaemia) can result in cellular death and, ultimately, loss of function. It is therefore of the utmost importance to develop adequate Regenerative Medicine strategies to address such issues, both in the context of tissue regeneration in general and in the context of tissue engineering. Recent approaches for the regeneration of ischemic tissues are based on boosting endogenous angiogenesis by growth factor or cell delivery. However, the lack of an adequate cell source [3] and the complexity of growth factor response [4] pose great challenges necessary to be overcome. In the case of bone tissue engineering applications, inadequate vascularization of the engineered constructs lead to cell death at the bulk of the scaffolds, implant failure and, ultimately, rejection[5]. The strategies researchers have used to deal with these issues often involve the use of angiogenic growth factors and scaffold design to promote vessel ingrowth[6], endothelial cells to prevascularize the constructs[7] and combinations of both [8]. The pitfalls of such strategies are deeply related with inadequate cell type used or limited control of growth factor delivery strategies[9]. Therefore, novel strategies encompassing an adequate cell source for neo-vascularization as well as deeper knowledge regarding the controlled release of angiogenic factors will be required for successful Bone Tissue Engineering strategies.

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