Imaging and modelling tissue structure to inform the development of musculoskeletal therapies

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Abstract

Biomanufacturing is moving rapidly towards recreating the complex, hierarchical structures of native tissues. With such advances comes a need to provide a detailed characterisation of the physical interaction between synthetic structures and the biological environment, and to provide increasingly detailed and/or specific targets for synthesis/manufacturing. The musculoskeletal system is a functionally challenging environment in which to apply these synthetic constructs, reflected in the difficulties faced by current treatment approaches. Limited information on the functional role of low-level structural features provides a further challenge. Here, we discuss imaging and modelling approaches for providing this characterisation, focusing on scanning probe microscopy, nonlinear optical methods and vibrational spectroscopy for probing structure, and numerical modeling to explore the potential roles of observed structural features.

1. Introduction

It is increasingly recognised that the extracellular matrix is a dynamic, nano- and micro-structured environment that provides mechanical, chemical and structural cues to cells [1-4]. By expanding our understanding of how cells interact with this environment, and particularly how such structures can modulate cell behaviour, it may be possible to improve current treatments or develop new ones. Attempts to recreate natural structure, or apply synthetic structures, to influence cell behaviour are widespread, and use a number of different approaches. These approaches can be broadly described as: top down – modifying the surface of a bulk material to produce a desired morphology, chemistry or surface energy (e.g. surface patterning, etching); bottom up – producing a bulk material made up of many nano/microscale structures (e.g. electrospinning, self-assembly); or a combination of the two.

As advances in biomanufacturing provide new opportunities to recreate the complex native environment of cells, or to subtly modify a material environment to elicit a certain biological response, improved characterisation techniques are required to guide and assess that development.[5] Here, we will explore some imaging and modelling approaches to this end, with a focus on sub-micron structure in the musculoskeletal system.

1.1. Surface engineering

Evidence is emerging that nanoscale surface features have interesting effects on cell behaviour [6-8], though our understanding of these cell-surface interactions is still in its infancy. Nanotechnology research has provided a means to create controlled surfaces that allow the study of these interactions, and to produce the fundamental knowledge to improve clinical outcomes [3]. In the laboratory, cell-surface interactions can be studied using lithography techniques to...
modify topology [9], combining this with surface coatings [10] or as part of a hierarchical patterning approach [11] to create highly controlled and repeatable surface features (Figure 4). A particular advantage of this approach is the ability to decouple parameters such as the size, depth, spacing, shape, and regularity of surface patterns from the complex physical and chemical environment of cell culture. A recent proof of concept study [12] has shown that patterns can be efficiently combined using new scanning probe techniques, providing libraries of up to 25 million features over the nano to microscales. This opens the possibility for large-scale screening studies that can inform the development of a new generation of biomaterials, utilising nanoscale patterns of stiffness, topology and chemistry to modulate biological response.

For in-vivo application such as in orthopaedic implants, large surface areas and irregular shapes make lithographic techniques impractical. Control over surface nanostructures can, however, be achieved through immersion in liquids using etching, oxidation or self-assembly to create the patterns. Oxidative nanopatterning have been shown to modify surface topology depending on the time of immersion, which can be used to encourage osteoblast and discourage fibroblast proliferation [13], aiding the integration of bone with the implant. Importantly, these techniques can be readily applied to implants and scaled for production [3]. Combining structural and biochemical stimulation, additional immersion treatments can be applied to induce collagen growth processes [14] or to add monolayers for binding bioactive compounds [15, 16] to further modulate biological response. However, such techniques are unsuitable for recreating the semi-ordered structure of the matrix, which is critical to cell response [9].

To create semi-ordered nanostructured surfaces that can be scaled to large, clinically relevant sizes, we are using a novel method of high-energy grazing ion bombardment. [17] The extremely local (~10 nm³) nature of the energy deposition in the high-energy regime leads to the creation of nanosized ‘hillocks’ (dots) on a surface with order determined by grazing angle (Figure 1). By varying irradiation parameters and therefore density and level of order of the hillock structures, human MSC differentiation can be directed towards bone or cartilage.

1.2. Three-dimensional structures

Driven by the biomimetic ideal of recreating the three-dimensional structure of the natural extracellular matrix [1], a range of nanostructured bulk materials have been produced for studying and applying regenerative medicine strategies. The field of nanostructured biomaterials, whether nanoscale structures (e.g. fibrils, foam struts) or nanoscale features on microscale structures, is enormous (see refs [1, 4, 18] for an in-depth description). One of the most interesting areas in this field takes a bottom-up approach to creating nanostructured biomaterials through self-assembly [19, 20], following the strategy by which all biomolecules interact and self-organise to form the structures that govern functionality. This is particularly true of the peptide-based strategies [21] that reproduce the development of the natural matrix, and therefore have potential to recreate the complex architectures and interactions in extracellular matrices. Self-assembling peptide amphiphiles have been used to form nanofibres for cartilage regeneration by displaying a high density of binding epitopes to transforming growth factor β-1 [22], and for modulating osteoblast behaviour in bone scaffolds [23]. Articular cartilage-like matrices have also been produced by combined self-assembly of collagen, hyaluronic acid and aggrecan [24].

Like surface modification, much of the potential for these three-dimensional nanostructured materials to advance musculoskeletal research and/or clinical treatment is in combining nanostructure with chemical and mechanical stimulation. Here, the field is evolving from an emphasis on recreating matrix porosity and mechanical stability [25-27] (that is, a passive view) towards recreating the dynamic nature of the matrix [28, 29] (an active view). Cell behaviour can be predisposed through embedding bioactive compounds within fibres [30], or hierarchically scaling the three-dimensional fibril structure [31]. Combining such approaches, particularly targeting the release of multiple therapeutic agents at optimised ratios, physiological doses, and in specific spatiotemporal patterns has considerable potential for a range of therapies [32]. Although this potential is far from being realised in the musculoskeletal environment, the large body of work on scaffold production and maturing synthesis technologies have led to consistency in structural properties. This can be used to apply the emerging knowledge of cell-surface interactions [7, 9, 12, 33] in the more complicated three-dimensional setting of the musculoskeletal system.
2. Characterisation strategies for biological and biomaterials in the musculoskeletal system

With such advances in material design and the fundamental knowledge of cell-material interactions comes a need to describe this not just in terms of expression and synthesis, but rather in terms of the way matrix is laid down or structured in a biomaterial system. Before effective tissue engineering strategies are applied, it is further necessary to better understand the target, that is, the native environment, at the fundamental levels of tissue structure and cellular interaction. One of the most versatile and important tools for this is the atomic force microscope (AFM) [34], which is emerging as the primary tool for working at the nanoscale. Atomic force microscopes work by scanning a cantilever with a very sharp tip over a surface, with imaging modes working on the simple principle of beam deflection. Bringing a sharp tip near to a surface will cause it to deflect towards the surface due to Van der Waals forces, after which it will flex away from the surface will cause it to deflect towards the surface due to Van der Waals forces. The deflection, and using it as feedback, morphological [35-37], mechanical [38-40], electrical [41, 42] and chemical properties [43] can be resolved at scales below 10 nm.

![AFM images](image)

Figure 2: Multiple modes of AFM applied to tendon reveal organisation of matrix components, and measurement of their mechanical properties. (a) Kelvin Probe Force Microscopy; (b) Piezoresponse Force Microscopy; (c,d) AMFM-derived loss tangent and stiffness.

In the rheumatology and musculoskeletal fields, AFM can be used to image collagen organisation and polarity in the early stages of joint disease, interactions between matrix components in musculoskeletal tissues, toxic nanoparticles in cells, the synthesis of matrices in stem cell-based therapies, and many other applications. Modifications of these technologies to combine optics to the scanning probe allows super-resolution optical and spectral imaging using techniques such as near field scanning optical microscopy [44] and tip-enhanced Raman spectroscopy [45]. As such, the AFM can be used to observe and manipulate biological machinery in its native environment, with characterisation strategies ranging from simple topographical scanning to advanced lab-on-tip methods.

AFM can be used to measure interactions within and between biomolecules, and determine the energy landscape of molecular recognition events (Figure 6) [43]. The distribution of charge over a surface can be mapped using Kelvin probe techniques, which measure the deflection caused by attractive or repulsive charges between the sample and a charged tip. Tips have been produced that are capable of measuring biologically relevant charges in a biological buffer solution, though problems with contamination limit some application [46]. In a similar way, tips can be modified by coating with molecules to determine their interactions with cell membranes, and the nanoscale arrangement of receptors in live cultures [47, 48]. This can further be combined with dynamic force spectroscopy to map energetic parameters. By detecting switching of an antibody on a tip, the kinetics of antibody–antigen association and dissociation can be mapped on the single-molecule level [49]. AFM techniques are constantly evolving, and with progress in control technology, molecular recognition methods and nanophotonics, we are only just beginning to unlock its capabilities.

2.1. Nonlinear optical methods

In the musculoskeletal system, collagen organisation is of principal interest for assessing matrix integrity. Second harmonic generation (SHG) has emerged as a label-free and non-destructive method for imaging this architecture. To date, however, it has generally been used qualitatively and on the scale of approximately one micron. By combining polarised imaging with a vectorial Green’s function model of SHG from collagen, the tens to hundreds of micron images can be quantitatively related to the underlying tens of nanometre scale structure within the laser focal volume [50]. Specifically, filling fraction, fibril diameter and fibre bundling can be determined. Through this model, the early structural changes in osteoarthritis and tendonopathy can therefore be probed, as well as healing processes and matrix organization on and around implants. Further, the development of interferometric SHG allows the orientation of γ(2) / piezoelectric domains to be determined [51], providing insights into cell signalling and mechanical behaviour.

Vibrational spectroscopy provides a means to study the concentration and arrangement of the major components of musculoskeletal tissues. Near infrared spectroscopy, due to its high penetration, ease of application and short acquisition times is particularly promising for clinical imaging applications [52, 53], and can characterise tissue damage in bulk samples with sensitivity surpassing that of currently available clinical tools [54]. Raman spectroscopy is being applied to characterise the extracellular matrix in laboratory-based studies of stem cell differentiation and to increase our understanding of early, bone-driven osteoarthritic processes in excised tissue (Figure 4).
3. Mechanical testing and modelling of ultrastructure

Through a combination of imaging, direct mechanical testing and modelling we are investigating the mechanical properties of tissues and the role of mechanics in disease and repair. Our philosophy is to use structure as the basis for understanding the mechanics of tissues and other biological materials, and to provide a link between the mechanical environment and biological feedbacks.

Using information from scanning probe and optical microscopy, we take a “bottom-up” approach to set the arrangement, orientation and properties of each constituent part, and assemble them into a (currently collagen-focussed) tissue structure that is simulated on a CPU. Solids are treated as nonlinear elastic based on experimental testing (e.g. single fibril tension experiments [55]) with damage feedbacks applied at critical levels of local strain. Fluid is simulated by solving Navier-Stokes equations on a GPU, with fluid-solid interactions calculated at each time step. Using structural and biological feedbacks, degradation and repair processes can be simulated with structural changes referenced against experiment.

Finally, we are using finite element analysis to explore the mechanical and signalling roles of structural features observed in experiments. Using spider silk as a biomimetic ‘ideal’ we are investigating the structural features and mechanisms underpinning its high mechanical performance. Although spider silk and silk-based materials have important limitations for biomaterial application, features such as rough microfibril structures may have potential for translation. In the musculoskeletal system, we are particularly interested in toughening mechanisms resulting from piezoelectric domains and proteoglycan distributions, and possible roles of piezoelectricity in cell signalling.
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