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Chapter 9

Bio-Interfaces Engineering Using Laser-Based Methods for Controlled Regulation of Mesenchymal Stem Cell Response *In Vitro*

Valentina Dinca, Livia Elena Sima, Laurentiu Rusen, Anca Bonciu, Thomas Lippert, Maria Dinescu and Maria Farsari

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61516

Abstract

The controlled interfacial properties of materials and modulated behaviours of cells and biomolecules on their surface are the requirements in the development of a new generation of high-performance biomaterials for regenerative medicine applications. Roughness, chemistry and mechanics of biomaterials are all sensed by cells. Organization of the environment at the nano- and the microscale, as well as chemical signals, triggers specific responses with further impact on cell fate. Particularly, human mesenchymal stem cells (hMSCs) hold a great promise in both basic developmental biology studies and regenerative medicine, as progenitors of bone cells. Their fate can be affected by various key regulatory factors (e.g. soluble growth factors, intrinsic, extrinsic environmental factors) that can be delivered by a fabricated scaffold. For example, when cultured on engineered environments that reproduce the physical features of the bone, hMSCs express tissue-specific transcription factors and consequently undergo an osteogenic fate. Therefore, producing smart bio-interfaces with targeted functionalities represents the key point in effective use of hierarchically topographical and chemical bioplatforms. In this chapter, we review laser-based approaches (e.g. Matrix-Assisted Pulsed Laser Evaporation (MAPLE), Laser-Induced Forward Transfer (LIFT), laser texturing and laser direct writing) used for the design of bio-interfaces aimed at controlling stem cell behaviour *in vitro*.

Keywords: Bio-interfaces, laser processing, topography, protein-based coatings, stem cells

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1. Introduction

Currently, there is an increased interest within regenerative medicine applications in materials and methods for controlling the interface characteristics of materials in report with the modulated behaviour of cells and biomolecules on their surface. Tissue engineering approaches rely on guided tissue regeneration using materials that serve as templates for ingrowths of host cells and tissue, or on cells that have been implanted as part of an engineered device [1-3]. The factors considered within this context refer to understanding the extracellular and intracellular factors modulating cell functions, optimizing or replicating complex scaffold architecture and arrangement *in vitro* resembling to tissue, developing new materials and processing techniques compatible with bio-interfaces and enhanced strategies for low inflammatory response [4-14]. Although great progress has been made in materials engineering and fabrication of scaffolds and implant devices, there are still important issues to be clarified depending on the type of application, target tissue, materials and techniques available for biomaterial production [9-15].

Stem cells hold a great promise for a wide range of applications in regenerative medicine as well as key elements in model systems aimed at understanding fundamental processes in the field of developmental biology. Therefore, any progress in controlling their behaviour remains an important challenge [16-18]. Stem cells, from multipotent stem cells, pluripotent embryonic stem cells to induced pluripotent stem cells, have been the subject of numerous investigations related to identifying specific intrinsic and extrinsic regulatory cues, defining niches and characterizing their potential of self-renewal and differentiation [19, 20]. Consequently, a wide variety of key regulatory factors were discovered, from soluble growth factors, extracellular matrix (ECM) interactions and cell–cell interactions to micro- and nano-engineered environments or combinations thereof.

The domains of preventive and therapeutic healthcare and tissue engineering converge into an interdisciplinary field, where the interaction between materials interfaces and proteins or cells is dictated by materials bulk but more importantly by interface characteristics. Biomaterials are used widely for studying cell–substrate interactions, scaffolding or implant functionality [15-20]. The requirements for the use of natural or synthetic biomaterials are related to the degradation or stability (mechanical integrity) within fluids and fluid transport, the presence of cell-recognizable surface chemistries, the ability to deliver active biomolecules and to induce signal transduction.

The current trend is producing either 'smart' biomimicking synthetic or natural composites or enhancing bulk materials (e.g. metals, ceramics and polymers) by either chemical, physical and/or topographic surface modification or surface functionalization [15-17].

Natural biomaterials include collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), laminin, heparan sulphate proteoglycans, hydroxyapatite (HA) (being bioactive, biocompatible and of similar mechanical properties as native tissue), fibrinogen and fibrin cellulose, chitosan and silk fibroin. These were and are successfully used as 2D and 3D micro- and nano-engineered environments for stem cell culture. For example, Matrigel™, which contains a variety of ECM components, was used in tissue culture as such or in combination with various growth factors for improving neovasculature formation in an ischaemic mouse

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model [21], with a view to produce tube-like vascular structures [22]. When designing a new biomaterial, there are several aspects to take into account, such as micro- and nanoscale manipulation of scaffold composition, mechanical strength, control over porosity and 3D architecture, adequate surface area and adsorption kinetics and chemistry of bulk degradation of the scaffold, material-processing abilities, logistical issues of cost, compatibility with sterilization techniques and shelf life [14-16].

An enhanced biological response (surface–cell interactions, osseointegration, bone-to-implant interface strength, resistance for long-term functional loading) requires different surface treatments to be applied, from hydroxyapatite aggregation to Mg ion implantation, sandblasting or acid-etching. However, contamination of the surface with chemical compounds occurs often, which leads to modified biological response; so more *debris- or contaminant-free* techniques are necessary.

Therefore, besides material composition importance in addressing the current challenges in tissue engineering, the micro- and nano-fabrication methods provide the input for development of precise topographical architecture for 2D or 3D substrates. Various methods have been used, from self-assembly, lithography, photolithography, soft lithography, polymer demixing, phase separation and electrospinning [23-27] to surface roughness modification, with sandblasting, anodic oxidation and acid-etching [28, 29].

Laser-based method for surface modification is a promising alternative as it can be automated and is reproducible, does not generate contamination and, moreover, it can confer a variety of nano- and microstructures with increased roughness and stable characteristics for long-term bio-interaction assays. Laser deposition (i.e. Matrix-Assisted Pulsed Laser Evaporation) can create complex coatings onto 2D and 3D substrates, while direct writing and/or texturing of polymers represent two different approaches used to create 2D and 3D topographical features as physical guidance structures. This chapter will provide information about how bio-interfaces can influence cell fate, with a focus on their effect on stem cells and on bio-interfaces engineering using laser-based methods. In addition, it will cover the controlled regulation of stem cell response *in vitro* using biomaterials with defined features.

2. Surface topographical key factors for triggering stem cell response

Various factors are responsible for the behaviour of cells in response to their own niche. Considering that surface topography and chemistry represent major factors at cell–substrate interface dictating biological reactions, it becomes crucial to elucidate the complex interplay between cells, molecular signal pathways and the effect of external factors (e.g. an implant surface, functionalization and topographic features) within well-controlled cell culture systems. Nevertheless, since, in the last years, mesenchymal stem cells (MSCs) have been ideal targets in regenerative therapies, understanding of the pathways and the cues affecting stem cell differentiation to the needed lineage is critical. The ideal tissue substrate will therefore have to deliver the optimal combination of physical and biochemical signals for the spatio-temporal control of stem cell commitment and differentiation [30]. This section of the chapter focuses on describing current state of the art in topography controlling MSC fate.

2.1. Mechanical factors that influence stem cell differentiation

MSCs are multipotent cells able to differentiate to at least three lineages: osteoblast, chondrocyte and adipocyte. With a view to recreate specific niche microenvironments to direct MSC differentiation to a required phenotype, specific control elements have to be defined. Recent high-throughput approaches have aimed at defining specific chemical [31, 32] and/or biophysical [33, 34] factors controlling stem cell fate.

The formation, evolution and breakdown of the cellular components are highly influenced by the presence of topographical cues on the surface. The stress applied through focal adhesions *via* external forces and stiffness sensing induces the biological response of cells within their environment [35]. Matrix stiffness is the principal driver of stem cell specification [36], followed by soluble factors. Cultivating MSCs on extracellular matrix (ECM) derived from either osteoblasts or chondrocytes has revealed that cell-specific ECMs are capable of modulating the BMP-2-induced osteogenic versus chondrogenic differentiation [37]. A study regarding the matrix stiffness modulation of the effect of TGFβ on MSC fate concluded that stiffness is an important determinant for differentiation while requiring soluble input for unique lineage-specific outcome [38]. It has been shown that a pattern of mechanical stress is induced by the presence of textures in a nano- or micron-size range, which is directly correlated to cell spreading or arrangement in mESCs, but not in mESC-derived differentiated cells [39].

Stressing the cytoskeletal filaments networks has impact on alteration of nuclear protein assembly, gene transcription, DNA replication or RNA processing, due to nuclear shape changes, which generates modifications in chromatin organization [39], with further impact on gene expression programmes. Physical interactions between stem cells and ECM that govern cell fate have been reviewed in Ref. [40]. Several lessons have been more recently learned by studying polymer scaffolds that are important for understanding how the ECM proteins regulate MSC behaviour and for the future development of customized culture systems as already available for embryonic stem cells [41, 42]. Recent studies have revealed that it is the extracellular matrix tethering that regulates stem cell fate rather than substrate/ECM stiffness *per se* [43]. The authors showed that MSC differentiation is influenced by the stiffness of PAAm hydrogels but not of PDMS substrates. They concluded that differentiation on soft hydrogels is independent of substrate stiffness and is regulated by the ability of the cells to remodel the collagen fibres covalently linked onto the polymer structures. Controlled substrate geometries were designed using microcontact printing and fibronectin coating of patterned islands. Geometric features that increased actomyosin contractility promoted osteogenesis with an efficiency of 60–70% [44]. Using tunable stiffness, hydrogel researchers explored the lineage specification outcome when MSCs pre-cultured on soft or stiff substrates were transferred to gels of opposite stiffness [45]. They surprisingly found that rewiring of MSC lineage is possible by switching the biophysical microenvironment. Advanced tissue-mimetic matrices have been created using poly(octadecene-alt-maleic anhydride) (POMA) coated with fibronectin to tightly anchor MSC-derived ECM. [46]. This research paves the way for fabrication of native-like niches to control stem cell fate.

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2.2. Engineering biosurface topographical cues at the micro- and nanoscale to control cell function

It has been shown that texturing of biomaterials increases cell adhesion in comparison to flat, smooth surfaces. Topography of a material can be characterized by different roughness parameters (Ra, Sa, Sm – measured in nanometres) determined by AFM, confocal microscopy, optical profilometry, SEM or tactile profilometry [47]. Knowing that the *in vivo* microenvironment is not flat, it is important to understand the function of cells grown on topographies that mimic natural conditions. Since the early definition of 'contact guidance' effect on cellular behaviour, a variety of different substrate parameters have been designed in micro and nano ranges, implying organization of the nanofeatures with different organization, chemistry and rigidity of the natural or synthetic biosurfaces, to meet the desired criteria and complexity of the native tissue architecture [48]. While *nanostructure* topography and roughness are important for cell adhesion, orientation, motility, antigen presentation, cytoskeleton polymerization, activation of tyrosine kinases and modulation of intracellular signalling pathways, *microstructure* texturing of biomaterials has impact on cell morphology, migration and tissue organization. Microfabrication technologies have been widely used in the development of substrates, scaffolds or biomaterials comprising precise topographical features.

A variety of nano- or microtopographies within hundreds of nanometres up to tens of microns range (e.g. nanoposts and nanogratings: 150, 400 and 600 nm in diameter and width; microposts and pits: 300 nm–10 μm in diameter, 3–50 μm in height) were obtained by various techniques (photolithography, electron beam lithography, reactive imprint lithography, etching, replications, hot embossing, injection moulding) [10-16] and used to stimulate hMSC-stimulated osteogenic differentiation. Although most of the studies present interesting and promising results, only a single or a small selection of parameters related to cell response were measured. Proposed platforms designed to study MSC fate regulation by both substrate mechanics and dynamic loading in 2D and 3D are reviewed in Ref. [49]. Noteworthy, few bio-interfaces obtained by polymer-processing approaches were able to initiate osseoinduction *per se*, without the need of differentiation factors. Seminal studies of Matthew Dalby *et al.* on electron beam lithography processed polycaprolactone (PCL) structures have proved that spatial organization of 120 nm pits in a square arrangement with centre-to-centre spacing of 300 nm and 50 nm offset in pit position had osteoinductive properties [50]. More recently, the same group produced substrates that are able to maintain MSC phenotype and multipotency by reducing the level of offset to almost zero [51].

2.3. Micro- and nanotopography of biosurfaces engineered by laser methods

Within the above-discussed context, by providing a control over the scale and patterning (both chemical and topographic), development in specific cell-regulating cues is implied, with the application ranging from basic cell biology to tissue engineering. Specific control over patterning involves microfabrication approaches: microcontact printing, abrasion, photolithography, hot embossing, electrospinning and laser ablation [52-57]. As most of the bioapplications require sterile conditions, laser-based techniques (i.e. laser direct irradiation or texturing, Laser-Induced Forward Transfer (LIFT), Matrix-Assisted Pulsed Laser Evaporation,

photopolymerization) are contact-free techniques and could be integrated with required sterile processes. Tailoring surface textures and their features on multiple scales can be controlled by directly and precisely processing by rapid scanning of focused laser beams (nanometre range up to the millimetre range on flat and curved surfaces). Moreover, by using ultrashort pulse lasers, the mechanical properties of the materials remain unchanged after the laser processing. In the cases where controlled biocoating is necessary, by using MAPLE, the thickness, porosity, architecture of mono- or multilayer can be easily tuned by controlling laser and target parameters. Among the main advantages, these are included: no limitations in the use of materials to be deposited or structured, no difficulty in controlling the thickness of the deposited layers, the ability to deposit multilayers without interlayer blending and compatibility in processing a wide range of nanoparticles, polymeric and biologic materials [52-57].

2.3.1. Engineering microstructured thin-film biosurfaces: gradients and porous surfaces by excimer laser direct texturing

The surface texturing in the context of specific surface roughness and architecture is also required for influencing cell directionality, proliferation and differentiation as the specific surface textures can be used to influence the functional properties. Using laser texturing, the surface topography of the material is altered, thus increasing the adhesion of the cells to the substrate and promoting the growth of these in a desired direction.

The most used approach was to modify surface roughness either by mechanical methods (high-pressure blasting with metal oxide particles, high-pressure liquid jet) or by chemical means (acid treatment), presenting the disadvantage of leaving the surface contaminated or undermining the structural integrity of implants. Similar to laser-based methods like direct laser metal sintering (DLMS), laser (i.e. CO_2) surface treatment modification has substantially broadened laser application for treating Ti alloys, allowing implants to be produced more economically than by traditional techniques. Laser irradiation using pulses with duration in the nanosecond (ns) to the femtosecond range can be used for direct surface texturing of a wide variety of materials: metals, ceramics and polymers.

In the recent works by Dinca *et al*. [58], texturing by excimer lasers (193 nm) and characterization of chitosan–collagen-based structures were performed with the goal of determining the optimal morpho-chemical characteristics of these structures for *in vitro* tailoring protein adsorption and cell and degradability behaviour. The chitosan–collagen surface processing by excimer lasers with 193 nm irradiation, combined with tilting of the sample, roughness gradients (from 5 nm to 213 nm) were obtained due to the change in fluence value over the exposed area. By monitoring four different types of mammalian cells (i.e. L 929 Fibroblasts, HEP G2 hepatocytes, OLN 93 oligodendrocytes and M63 osteoblasts) adhered onto structured chitosan–collagen surfaces, an indication on how cell growth is conditioned by the substrate topography was given. For example, if fibroblast cells had a preferential adhesion on the smooth regions (below 30 nm) of the structured films compared to the rough regions (up to 210 nm), osteoblast cells behaved completely opposite. Different behaviour was observed for the other two cell lines studied. The hepatocytes were round and separate on smooth surfaces, while spheroids were formed on rough surfaces. OLN exhibited bipolar

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elongated morphology to tri-polar branched cells within the areas with roughness in the range of 160–213 nm, while no cell integration could be seen on the areas characterized by the roughness below 120 nm.

Excimer laser processing can also provide other types of surface geometries (foams, bubbles) by varying wavelength, pulse number and sample positioning. Figure 1 presents such examples of chitosan–collagen surfaces generated by two different applied fluences using single pulse irradiation (248 nm KrF laser) onto horizontally placed samples. It can be observed that the laser irradiation of the polymer films caused different modifications of their morphological characteristics, and due to the local heat and pressure generated by the laser radiation, 2D and 3D polymer 'bubble' (Figure 1a–c), or 'sponge-like' (Figure 1d–f) structures on the surface were obtained.

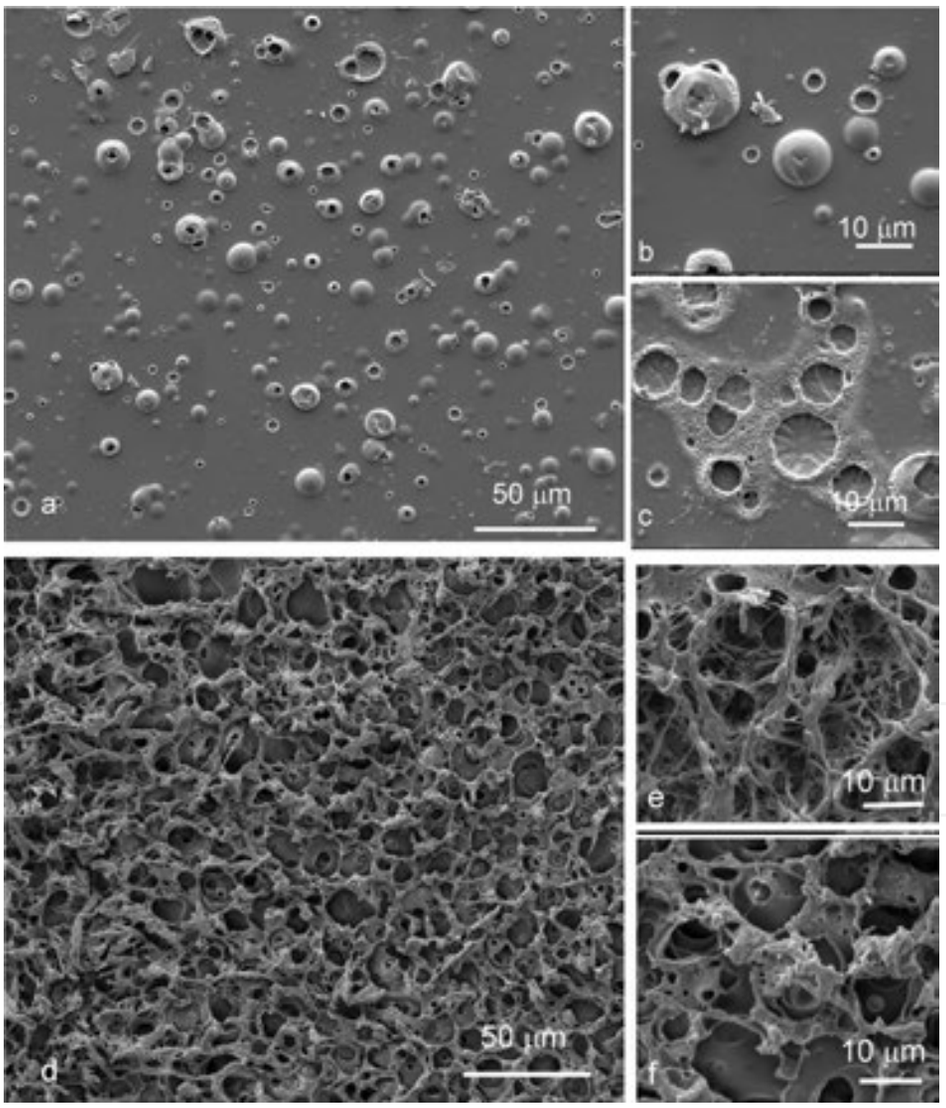


Figure 1. SEM images of various microbubble structures induced by KrF excimer single laser pulse (0.35 J/cm²) (a–c), and microfoam (d–f) (0.7 J/cm²).

In vitro studies showed an increased proliferation of mouse NIH/3T3 fibroblasts on chitosan foams obtained by excimer laser (KrF laser) irradiation as compared with untreated surfaces [59].

Obtaining microstructured thin-film surfaces and porous surfaces from naturally derived polymers (such as chitosan, collagen) could be used as artificial membranes for skin engineering and/or in cell directional growth and tissue regeneration.

2.3.2. Periodic nanostructured surfaces induced by femtosecond laser texturing

Nevertheless, machining and surface patterning of polymers and biopolymers using ultrashort pulse lasers take place with reduced mechanical and thermal deformation as compared with processing performed with longer ns laser pulses, and particular structures such as self-organizing textures can be fabricated on different materials (e.g. on metals, ceramics, semiconductors and glass). Self-organizing effects are caused by the laser material interaction and can be used to create patterns with dimensions independent of the focused laser spot size. Recent work by Rusen *et al.* [60] combined the advantages of natural biopolymer characteristics (chitosan) with the flexibility in surface texturing by ultrafast laser for creating functional microstructured surfaces for cell–substrate *in vitro* studies. A Ti: Sapphire femtosecond laser irradiation (λ = 775 nm and 387 nm) was used for tailoring surface morphological characteristics of chitosan-based films (i.e. polymer 'bubbles', 'fingertips' and 'sponge-like' structures). In the case of 'bubble' or 'fingertips' types of structures, the heights of the irradiated area were from several hundred nanometres up to few micrometres, and along with increasing fluences (beginning from the fluence of 1500 mJ/cm^2), the characteristic 'sponge-like' and even folded filaments at the irradiated area edges were observed. One example of 'fingertips' type of structure is shown in Figure 2, where regular ripple structures with a typical period of a few hundred nanometres were observed inside the created textures.

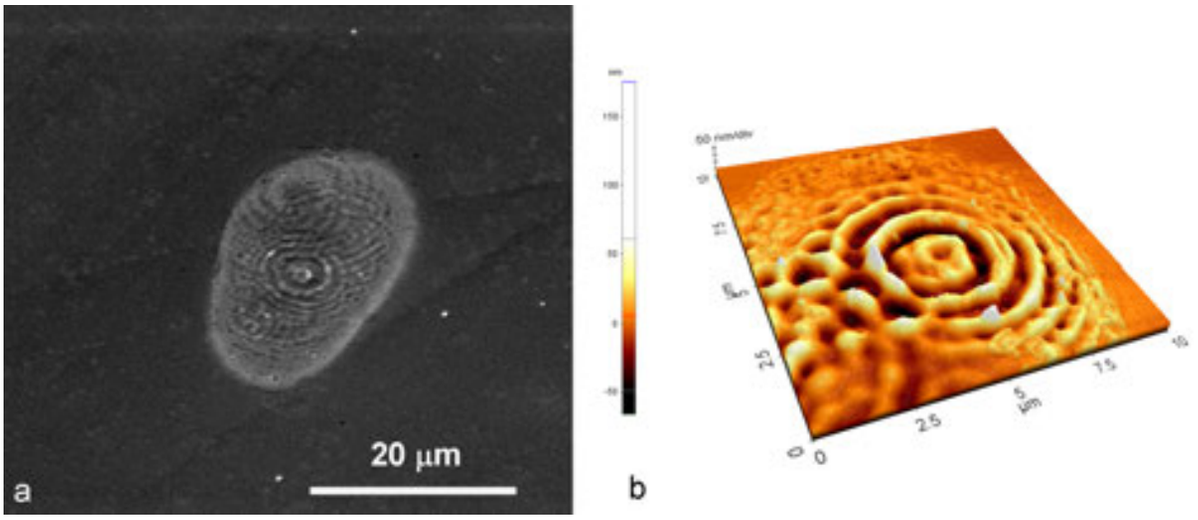


Figure 2. SEM image (a) and AFM image (b) of fingerprint-like structures obtained by Ti: Sapphire femtosecond laser irradiation (λ = 387 nm) in chitosan films.

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OLN cells cultured on the patterned surface showed that early cell growth was conditioned by specific microtopography and indicate possible uses for the structures as cellular platform applications.

A similar effect was observed in the case of ceramics. Zirconia sheet cut with the dimension of 10 × 10 × 5 mm^3 from Zirkonzahn (Zirkon Translucent-ZRAB0490, Lot ZB 0070A) was used as received before the laser irradiation (1.9 Jcm^{-2} fluence), while the traverse speed was set at 0.1 mm/sec. Droplet-shaped microcavity (DμC) arrays were obtained by setting 20 pulses per cavity and translating the samples in X, respectively Y directions with a fixed step of 35 μm (Figure 3). Control planar Zirconia substrates were used as a comparison throughout our study. It was shown that the increase in surface area induced increased spreading of cell on all directions, which triggered cell morphology modification towards polygonal shape (Figure 4a) and consequently increased circularity of stem cell nuclei (Figure 4b).

Cells grown onto DμC pattern (Figure 4, DμC, left, 2000× magnification) spread efficiently over the microcavities. Cytoskeleton filaments extended closely over their tubule-granular topography (Figure 4, DμC, right, 10000× magnification, arrows).

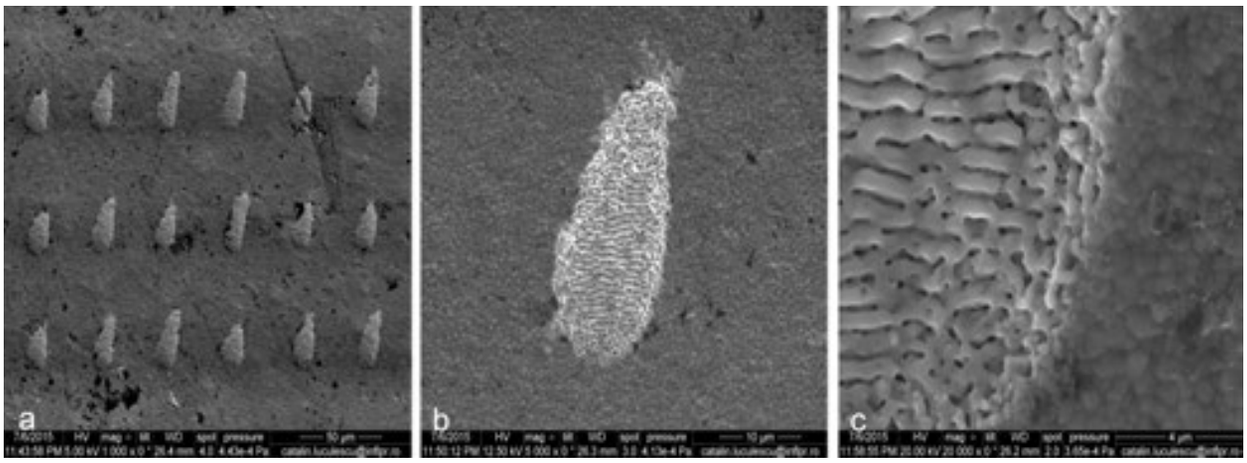


Figure 3. SEM images of droplet-shaped microcavity (DμC) arrays obtained in zirconia (a) and the corresponding close-up images of one DμC (b) and ripple-like structures inside each DμC (c).

2.3.3. Laser engineering contact guidance structures

'Contact guidance' mechanism was proposed by Harrison in 1911 [61], and since then, micro- and nanoridges were used to control not only cellular morphology and orientation but direct cell migration as well. Intriguingly, researchers proved by time-lapse microscopy that opposite to cells on the flat surface, mouse MSCs extended forward the long, thin process and left the wider edge trailing while migrating in both directions along the tracks, when seeded onto a groove pattern (10-μm pitch and 1600-nm step height) [62]. Consequently, cells occupy the bottom of the grooves while projecting long, thin extensions along the silicon gratings.

In order to screen for conditions to control MSC orientation and morphology, we have produced different microtopographies using excimer (Exitech KrF excimer laser, PPM-601E

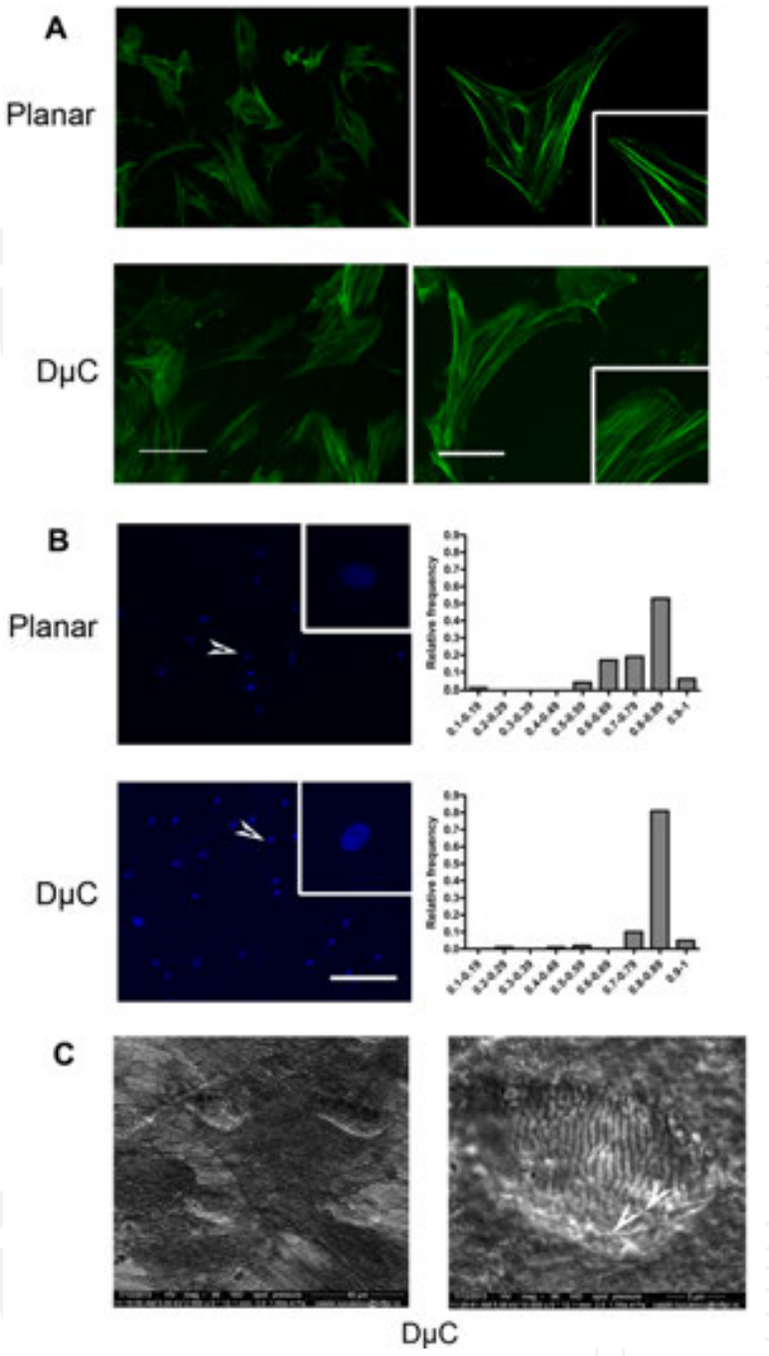


Figure 4. Modifications in cell and nuclei morphology upon growth on DμC arrays. (a) Fluorescence microscopy of hMSCs labelled for actin filaments at 8 days after seeding on femtosecond laser ablated droplet-like cavities as compared with planar zirconia. *Left panel* (10×) shows adhesion of osteoprogenitor cells onto irradiated micropatterns (scale bar = 200 μm); *right panel* (20×) evidences stress fibre organization onto the irradiated areas (sce bar = 100 μm) with emphasis on cell membrane protrusions (insets). (b) Nuclei orientation and shape. *Laleft panel* – fluorescence microscopy of DAPI-stained hMSCs nuclei 8 days after seeding onto DμC topographies or planar control. Insets provide high magnification details of a characteristic nucleus (pointed with white arrowheads onto the enlarged images). *Right panel* – frequency distribution of nuclei with specific circularity index. Note that the nuclei develop more rounded shapes on DμC arrays. Scale bar = 200 μm. (c) SEM analysis of hMSCs–zirconia interface emphasizing cytoskeleton organization (see white arrowheads on high magnification image).

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Gen 6 Tool), laser micromachining by mask projection with half-tone masks. By scanning the mask under the beam and the polycarbonate (PC) sample, micropatterns of different shapes (dots, cones, pyramids, pits, funnels, inverted pyramids) with a depth/height of 2.5 to 10 μm and a width of 5 to 25 μm were generated on a polycarbonate (PC) substrate.

Cells grown on microlens-like topography (Figure 5a) lay their body over the upper part of the structures and project long dendrites along these tracks (Figure 5b,c). When grown onto reversed U-shaped lines separated by deep grooves (2 μm) (Figure 5d), cells elongate and align to surface geometry. When grooves are less deep (Figure 5e) and more frequent (Figure 5f), cells are able to diverge from their aligned profile and respectively spread over a number of lines recuperating their fibroblast-like shape, while maintaining a controlled direction. Steep edges of the V-shaped lines' topography did not have the same restrictive effect (Figure 5g), as cells are able to protrude laterally *via* long filopodia that contact the peaks. When deep grooves are ablated into PC surface, cells tend to bridge the highest areas of the substrate, with membrane domains remaining suspended over the gaps. It is the case of V-shaped lines (Figure 5g,h), pyramids (reversed or standing) (Figure 5j,k) and reversed microlenses (Figure 5m) with increased heights, from 5 to 10 μm. On milder topographies of the same structures (Figure 5i,l,n,o), cells are able to access even the deeper parts of the substrate.

The surface morphological characteristics of chitosan films were tailored (i.e. ridges, grooves structures) by using multiple pulses from Ti: Sapphire femtosecond laser irradiation (λ = 775 nm and 387 nm) and varying the scanning speed (Figure 6),. *In vitro* tests on the patterned surface showed that early growth for both OLN and fibroblast cells (Figure 6 b,d). was conditioned by the microtopography and indicate possible uses of the structures in biomedical applications.

Substrates bearing anisotropic microscale [63] or sub-micron [64] geometries have been shown to control hMSC alignment and elongation and consequently cell differentiation [65]. MSC elongation was correlated with osteogenic commitment [66] while stem cell circularity inclines the differentiation balance towards adipogenesis [67]. Combinations of sub-microscale texture and microgroove patterns, as well as distinct direction of the texture in relation to that of the microgrooves, have been also subject to primary cell adhesion assays [68, 69].

Studies *in vivo* have confirmed that aligned topographies induce differentiation of progenitor adult stem cells, with remarkable impact for tissue engineering [70, 71]. Aligned topographies enhance osseointegration and orient the local microstructure of attached tissue. Moreover, textured interfaces prevent the colonization by fibroblastic and macrophage cells that arrive early during wound healing and initiate the encapsulation of smooth substrates [72].

2.4. 2D and 3D pattern formation by Laser-Induced Forward Transfer for steering cell adhesion

LIFT is a laser-assisted direct-write process in which the materials to be transferred are in the form of a rheological fluid, polymer-based composite or fine powder, placed on a transparent support and transferred by a single pulse onto a receiver. The main key feature that separates LIFT from other direct-write techniques is that it offers the advantages of controlled and

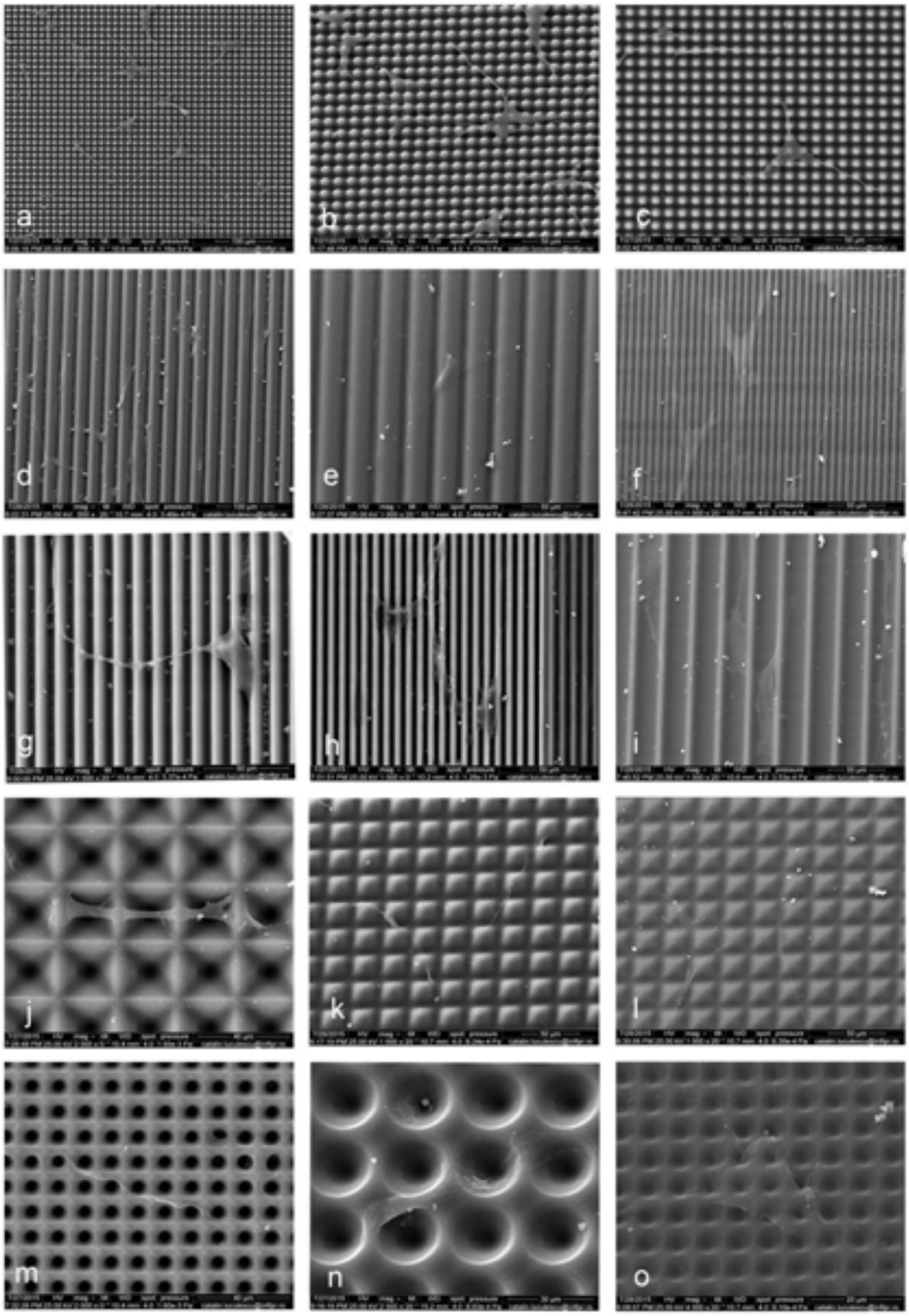


Figure 5. Interaction of hMSCs with polycarbonate microtopographies obtained by KrF excimer laser irradiation. SEM analyses were performed 72 hours post-seeding of cells onto structured microlenses (a–c), reversed U-shaped lines (d–f), V-shaped lines (g), U-shaped grooves (h,i), reversed pyramids (j), pyramids (k,l) and reversed microlenses (m–o).

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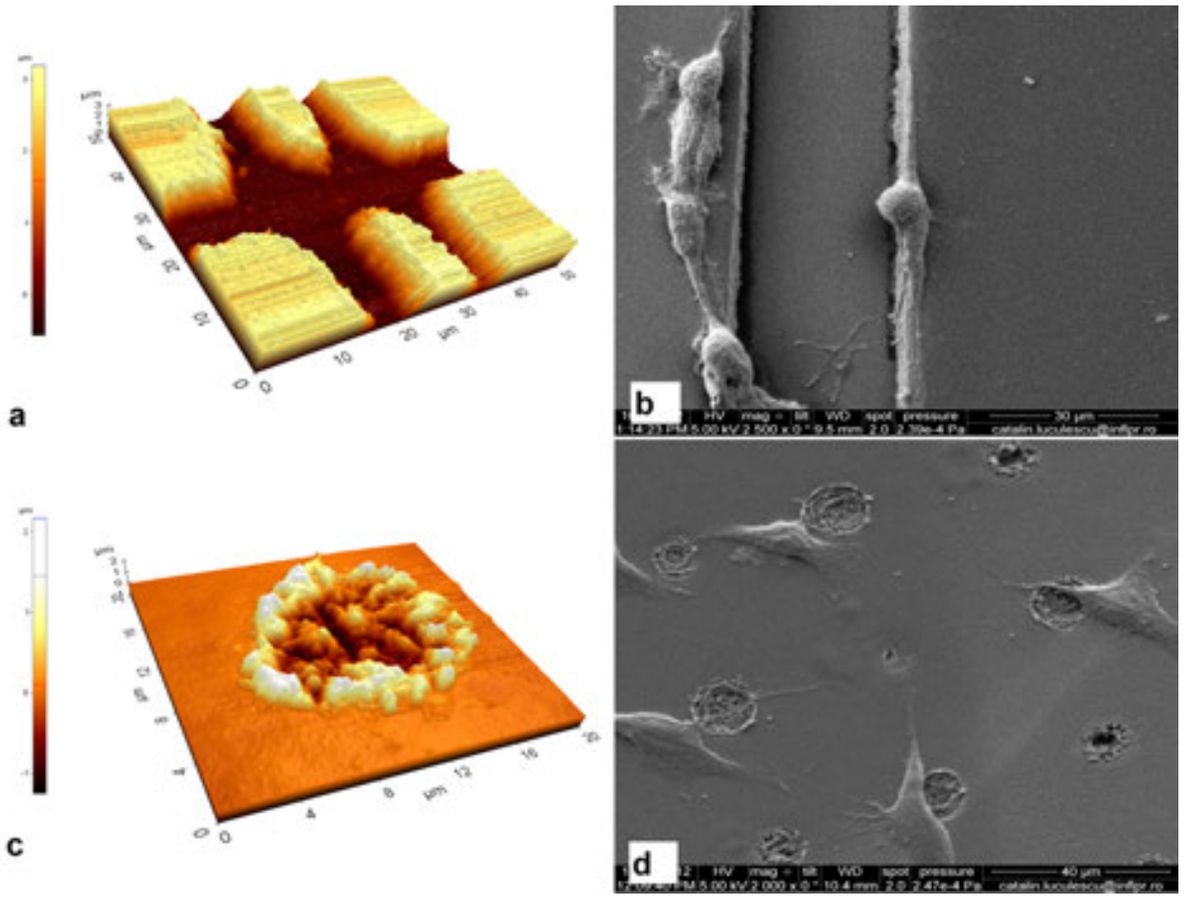


Figure 6. Architecture of chitosan ridges (a) and pits (c) scaffold made by fs multiple pulses irradiation. 3D AFM images (a,c) and SEM images (b,d) of the corresponding OLN and fibroblast cells response and organization onto the different surface textures.

localized micrometre-size pixels/clusters, similar to inkjet printing, but faster (up to 200.000 pixels/sec) and without the limitation in the nature of the material to be transferred [73-81]. The technique does not depend on the donor material properties, which therefore allows the use of non-soluble organic compounds, and the realization of complex 2D and 3D structures onto any type of surface *via* a single laser shot. Although LIFT was successfully used for transfer of biological compounds (peptides, enzymes, proteins, cells), its ability to form patterns to be used as a biological platform for studying cells behaviour is still under progress. Two-dimensional spatially controlled polyethyleneimine (PEI) micropatterns were obtained by Dinca *et al*. [82] using Dynamic Release Layer-assisted LIFT (DRL-LIFT) onto PEG-coated glass substrate to study the adhesion and cellular behaviour of SH-SY5Y human neuroblastoma cell confinement and behaviour within the created patterns. We demonstrated that the separation distances between the polymer pixels and on the surface chemistry influence the positioning of neuron aggregates inside the polymer pattern and the formation of interconnecting neurite fascicles. In this context, new directions were related to combining the advantages of DRL-LIFT with a soft substrate (i.e. Thermanox) for obtaining surface functionalization concomitantly with micro- and nano-'porous' polymeric structures. The structures obtained with different topographical properties were used as LIFT-printed platforms for fibroblast and

oligodendrocyte cellular behaviour study [83]. In all cases, the transfer was achieved using a single pulse from a XeCl excimer laser (Compex, Lambda Physik, 308 nm, 30 ns). Not only 2D polymer pattern can be obtained by DRL-LIFT, but also 3D patterns [84]. For example, polystyrene microbeads arrays were printed by LIFT for selective attachment of OLN cell adhesion and the study of changes in cell shape and spreading induced by the beads arrangement (Figure 7).

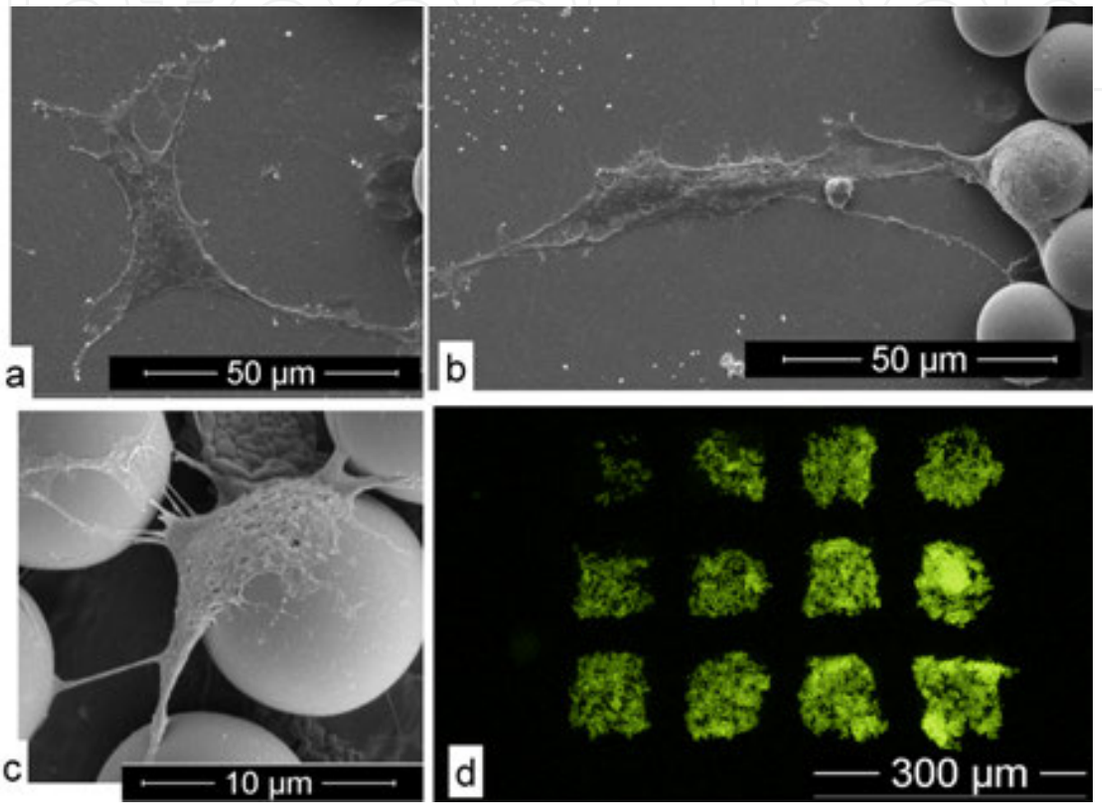


Figure 7. Interaction of OLN cells with polystyrene microbead microtopographies obtained by LIFT. SEM analyses were performed 24 hours post-seeding of cells onto control surfaces (a) and transferred microbeads (b–c). Fluorescence micrographs (d) of OLN cells cultured for 24 hours on PS microbead array.

Cells grown on microbead arrays (Figure 7a) spread onto flat surfaces and start projecting long dendrites along the bead-patterned array area (Figure 7b), their body adhering over the upper part of the structures (Figure 7c). Interestingly, when washed gently to check the adhesion strength, the remaining cells were grouped strictly over the microbead pattern, as seen in Figure 7d.

2.5. Biofunctional coatings onto 2D and 3D surfaces obtained by laser evaporation: Matrix-Assisted Pulsed Laser Evaporation

Although Pulsed Laser Deposition (PLD) was successfully used for deposition of few polymers (polyepichlorhydrin [85], poly(methyl methacrylate) [86], polyhydroxybutyrate [87] polyisobutylene (PIB)), proteins (silk protein, bovine serum albumin protein [88]) and crystalline HA

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on Ti implants [89] onto various substrates, when biological molecules are implied, another technique, namely MAPLE, has been used for deposition of various composites or hybrid biofunctional materials [52-57, 90, 91]. Both methods are performed under vacuum, comprising a rotating target and substrate holder, with the difference in target preparation. If for PLD the solid target is made of alloys, metals, ceramics, etc., in the case of MAPLE, the material (1–5% in weight) to be deposited is suspended into a solvent and frozen. The target is irradiated and the resultant laser plasma plume transports the molecules on the substrate placed in parallel and at a short distance (3–5 cm) [53, 90].

The coating characteristics are related to wavelength, pulse duration, repetition rate and, especially in the case of MAPLE, of solvent absorption and target composition and percentage (preferable under 5–10% in mass). For example, using the same laser parameters (Nd: YAG, 266 nm, 150 kpulses), HA coating with different aspects, from highly porous (Figure 8, left) to compact (Figure 8, right) agglomeration of HA nanoparticles, was obtained when target composition was changed by different solvents.

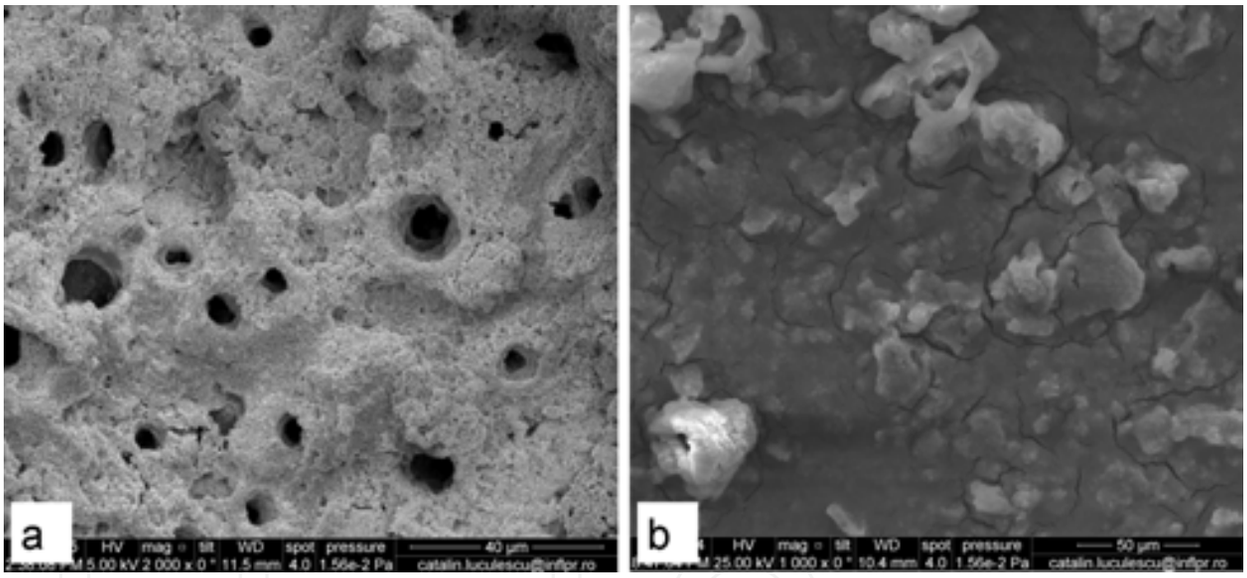


Figure 8. SEM images of HA coatings deposited on glass by MAPLE (266 nm, 150 kpulses, 500 mJ/cm2, and 2% HA in chloroform and DMSO target composition) when different solvents led to the formation of different topographies. Porous structures were obtained when chloroform was used (a) while the DMSO led to more compact arrangement of the film (b).

It is known that adherent cells may sense and respond to micro/nanoscale ECM topographical cues through cell–ECM adhesive interactions. To note, the characteristic size dimension of topographical features at the cell–ECM interface as fibrillary collagens or fibronectin fibrils in the ECM of connective tissues is in the broad size range between about 10 nm and 10 μm. The MAPLE process was shown to produce protein and protein–copolymer coatings with a wide range of morphologies and without changing the functionalities of the proteins embedded in the polymeric matrix [90, 92]. MAPLE-deposited fibronectin layers were shown to induce superior osteoprogenitor cell attachment *via* dense and extended stress

fibres [93]. Levan and oxidized levan nanostructured thin films were similarly synthetized and shown to support osteoblast cell proliferation [94]. Moreover, biopolymer thin-film gradients were successfully obtained by combinatorial MAPLE (C-MAPLE) in a single step using simultaneous irradiation of two targets containing levan and oxidized levan [95]. Distinct areas of the gradient were shown to impact differently the MAPK cell signalling pathway, which indicates a potential application of these assemblies for cell fate regulation [96]. Control can be exerted over the morphology and thickness of MAPLE coatings by varying four process parameters: solution concentration, applied fluence, deposition distance and deposition time. Combinations of these parameters can generate thin films with complex morphologies. For example, low concentration protein (0.5–1%) and high fluence (500–700 mJ/cm^2) would create fibrillary structures, similar to the ECM structure and topography in the case of collagen (Figure 9) and chitosan (Figure 10). AFM images of chitosan and collagen reveal fibrillary, porous or conglomerate types of structures. Conglomerates can be seen especially at chitosan at low and medium fluences, while higher fluence can influence the morphology in periodic ways, creating a variety of new shapes on the surface.

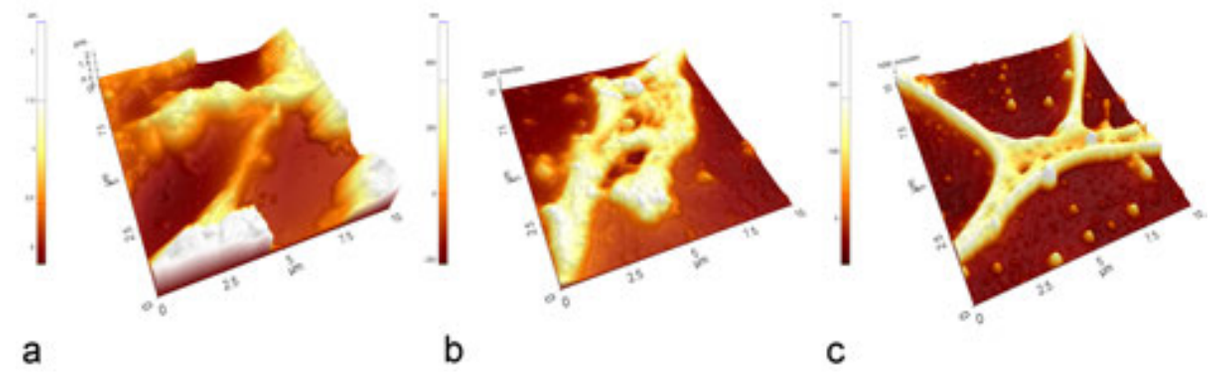


Figure 9. Examples of AFM images (10 × 10 μm^2) of fibrous collagen structures obtained by MAPLE (Nd:YAG laser, 266 nm), using different fluences: (a) 700 mJ/cm^2, (b) 600 mJ/cm^2 and (c) 500 mJ/cm^2)

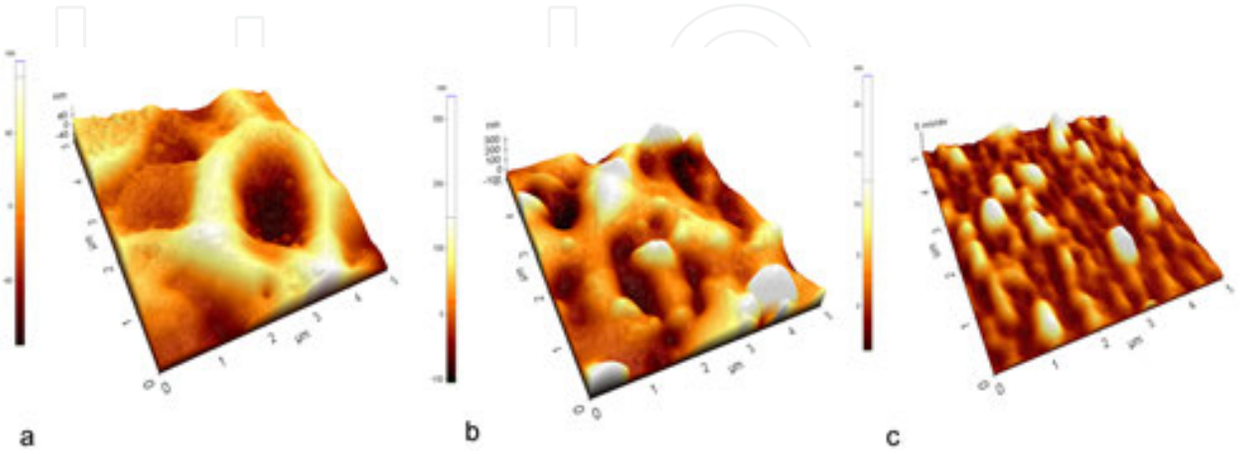


Figure 10. Examples of AFM images (5 × 5 μm^2) of porous and fibrous chitosan structures obtained by MAPLE (Nd:YAG laser, 266 nm), using different fluences: (a) 700 mJ/cm^2, (b) 600 mJ/cm^2 and (c) 500 mJ/cm^2)

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AFM images of chitosan and collagen reveal fibrillary, porous or conglomerate types of structures. Conglomerates can be seen especially at chitosan at low and medium fluences, while higher fluence can influence the morphology in periodic ways, creating a variety of new shapes on the surface. More importantly, MAPLE can be easily used to coat 2D and 3D structures [92]. For example, PMMA structured substrates obtained by photolithography (Figure 11a) were coated with laminin (Figure 11b) and the effect on OLN cells was studied (Figure 11c–e). SEM images of OLN-93 cell after 2-day culture are shown in Figure 11. The cells cultured onto laminin-coated structured PMMA substrates span from bipolar elongated morphology, with preferential alignment along the line arrays with line dimensions below 10 μm.

We also used laminin (Figure 11g–j) to coat structures that were previously generated by laser irradiation of polycarbonate (PC) (Figure 11f) and studied the combined effect of topography and chemistry on MSCs. We observed that cells aligned to the 1.5-μm separated lines both on PC (Figure 11f) and on laminin-coated structure (Figure 11g–j) as compared to the random orientation they have on the laminin-coated non-irradiated PC areas (Figure 11h). A more detailed analysis showed that cells that extended partially to smooth areas between the lines (delimited by white dots in Figures 11i1 and j1) developed small protrusions from their lamellipodia (Figure 11i1,j1 – white arrows), regardless of cells that encountered the flat surface laterally (Figure 11 i–i1) or along the lines (Figure 11 j–j1). However, when cells adhered with the whole body onto the laser-processed microtopography, they 'scanned' the environment with long, thin filopodia (Figure 11i,j – red arrows).

2.5.1. From 2D to complex 3D biosurfaces: Multi-Photon Lithography

Multi-Photon Lithography (MPL) is a 3D printing technology that allows the construction of readily assembled structures with sub-100-nm resolution [97, 98]. It is based on the phenomenon of non-linear photon absorption and polymerization; the beam of a sub-picosecond-length pulse laser is focused inside the volume of a transparent photosensitive material, causing it to absorb two or more photons and polymerize locally. Moving the beam according to a CAD model, one can fabricate a realistic micromodel of this design.

Despite it being fairly new technology, MPL very quickly found application in tissue engineering and cell growth investigations, and, to date, several biomaterials have been employed to this purpose [99]. These are mostly negative photoresists, such as hydrogels [100-104], acrylate materials [105, 106], the epoxy-based photoresist SU-8 [107] and organic–inorganic hybrid materials (Figure 12)[108-113]. Special mention should be made of the 3D structuring of natural polymers and proteins, pioneered by Campagnola *et al*., who worked on cross-linked proteins, such as bovine serum albumin (BSA), fibrogen and fibronectin, and collagen [114-120]. Seidlits *et al*. have crosslinked protein inside a hydrogel for neural cell guidance [121]. BSA and also avidin using flavin mononucleotide as a photoinitiator were crosslinked by Turunen *et al.* [122], while Torgesen *et al*. photopolymerized BSA encapsulating a live *C. elegans* worm [123]. The same team mixed BSA with a modified gelatin, to make stable and well-defined MPL-made scaffolds [124].

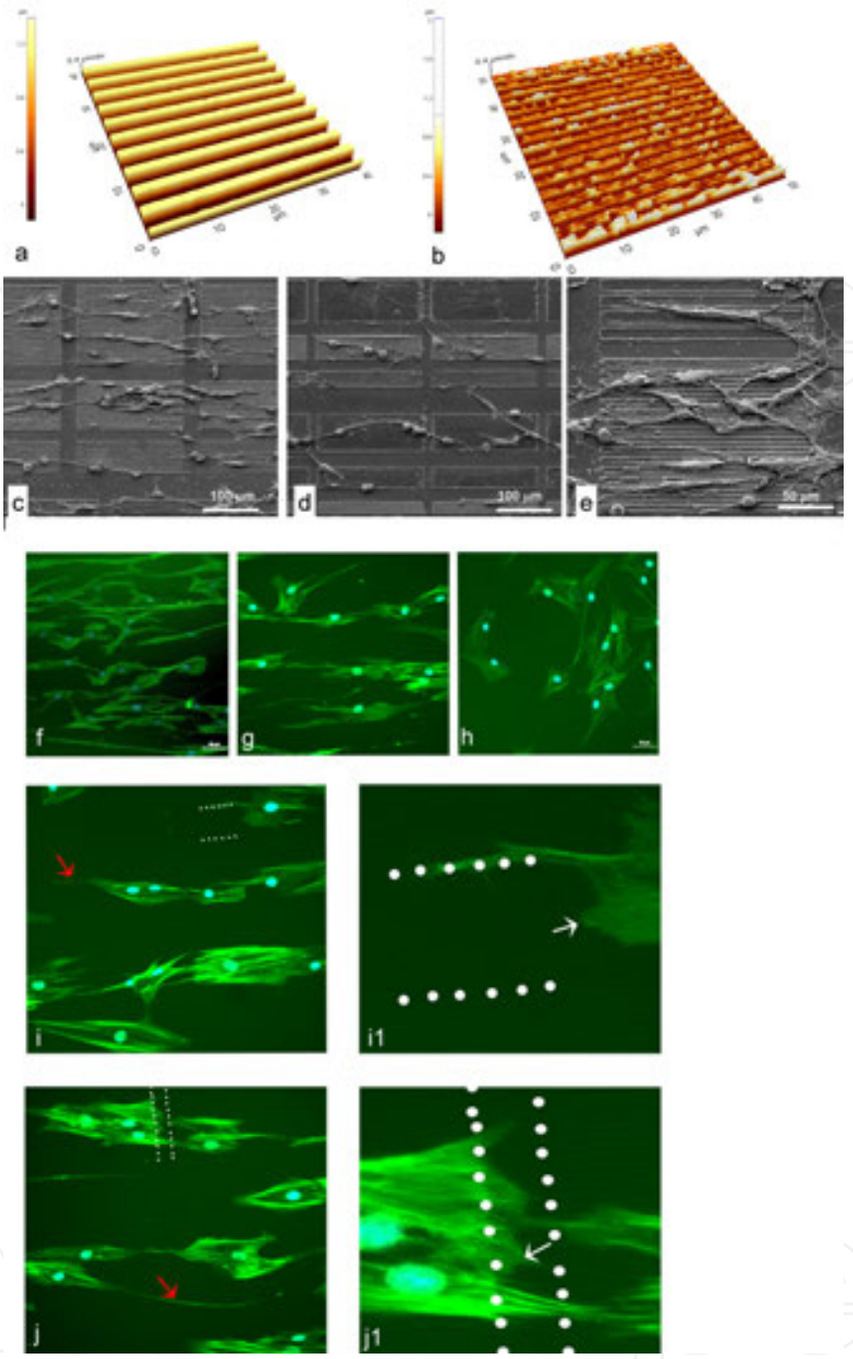


Figure 11. Cell distribution onto laminin-coated line structured PMMA and PC substrates. AFM analysis of PMMA microgroove profile before (a) and after (b) laminin coating. SEM images of OLN-93 cell alignment after 2-day culture on laminin-coated PMMA topographies (c–e). Fluorescence images of MSCs cells aligned to the 1.5 μm separated lines on PC (f) and laminin coated structure (g-j). Random orientation of MSCs on the laminin-coated non-irradiated PC areas (h). Close up images of the small protrusions from MSCs lamellipodia (i1,j1 – white arrows) onto treated areas.

There is also a lot of work on modifying natural polymers for MPL, such as polycaprolactone (Figure 13) [125], polylactide (Figure 14) [126, 127], gelatin[128] and hyaluronic acid [129]. This avenue of research has provided the most promising results, as far as biodegradable materials are concerned.

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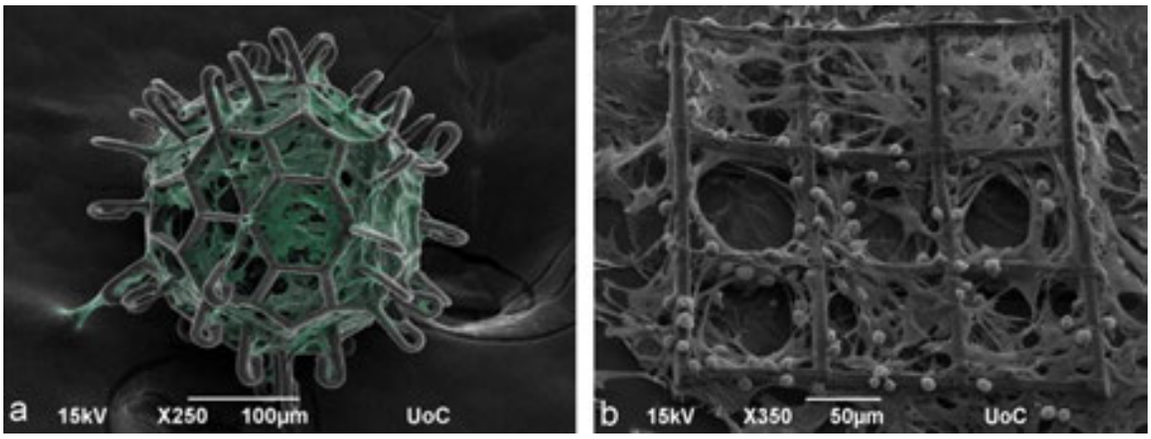


Figure 12. A 3D free-standing scaffold made of an organic–inorganic hybrid material loaded with pre-osteoblastic cells, coloured green for easy-viewing [113] (a) and a scaffold decorated with mineralized amyloid peptides and loaded with pre-osteoblastic cells [110] (b).

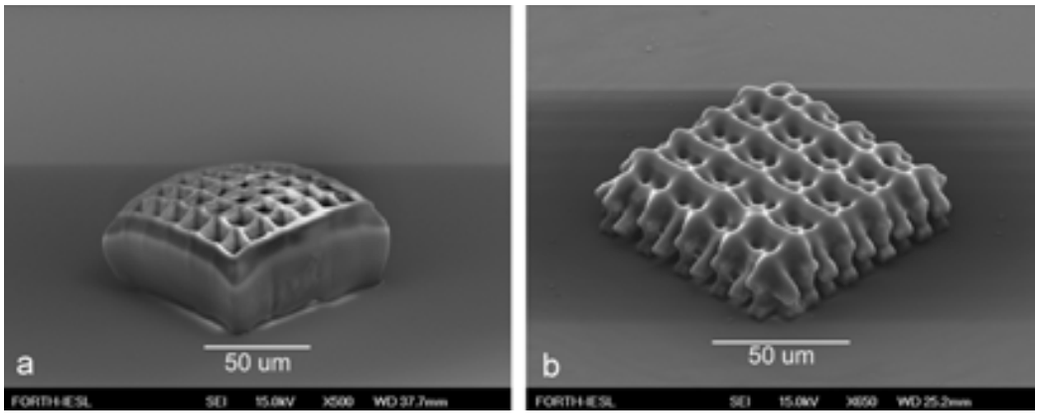


Figure 13. scaffolds made using chemically modified poly-e-caprolactone [125].

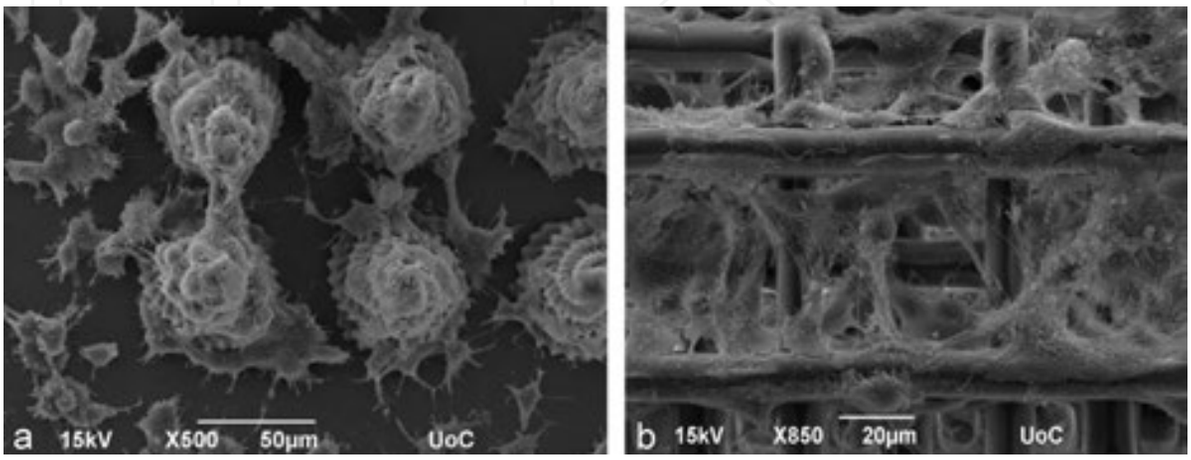


Figure 14. Cells growing on polylactide structures [126].

3. Conclusions and future prospects

Combining topographical features for mimicking the natural architecture of the ECM environment with chemical factors, such as biologically active molecules for controlling interfacial interaction between biological entities and materials, provides a challenging approach for identifying important factors that determine stem cell fate, such as extrinsic signals from the niche that impact gene expression.

This chapter briefly reported the recent progress in our research and others over topographical and chemical cues of biosurfaces on cells with accent on the hMSCs.

Although significant progress has been achieved in this field, the materials discussed above still have obvious limitations in practical applications that need to be overcome in the future. Our results indicate that substrate micropattern features play a key role in hMSCs' spreading response and more importantly that even smoother patterns than grooves and ridges are able to change cell shape. Whether the substrate conformations presented are able to induce stem cell commitment to a specific differentiation pathway is a topic of future investigation.

Although the majority of the previous works in this field have used simple topographical features such as grooves, future attempts should be more focused on realistic substrates with a higher degree of biomimetic relevance to impose multidirectional cues within the cellular microenvironment. Such novel substrates will enable addressing questions on how cells globally integrate biophysical signals from their surrounding microenvironment. Furthermore, topographical features could be integrated with chemical stimuli, such as soluble factors, to enhance cellular process, such as stem cell differentiation. The continuous advancements in the field of cell–substrate topography interactions will not only benefit fundamental biological studies but also have significant implications in the field of tissue engineering through fabrication of engineering synthetic and implantable substrates with controlled features. Therefore, the design of topographical and chemical features for engineering smart bio-interfaces with multiple and synergetic functionalities represents the key point in effective use of hierarchically topographical and chemical bioplatforms targeting controlled regulation of stem cell fate.

Conflict of interest

The authors declare no competing interests.

Acknowledgements

The research leading to these results has received funding from the Romanian Ministry of National Education PN-II-PT-PCCA 239/2014. V.D. acknowledges the financial support from Sciex Grant project no.12.313 and from a grant of the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PN-II-RU-TE-2011-3-0289. L.E.S. is grateful for the financial support from the European Social Fund POSDRU 2007–2013 through the contract POSDRU/89/1.5/S/60746 (postdoctoral fellowship). Partial funding was obtained

from the Romanian Ministry of Research and Education, through the Romanian Academy Project 1/2011 of the Institute of Biochemistry.

Author details

Valentina Dinca[1*], Livia Elena Sima[2], Laurentiu Rusen[1], Anca Bonciu[1], Thomas Lippert[3], Maria Dinescu[1] and Maria Farsari[4]

*Address all correspondence to: valentina.dinca@inflpr.ro

1 National Institute for Lasers, Plasma and Radiation Physics, Magurele RO, Bucharest, Romania

2 Institute of Biochemistry of the Romanian Academy, Bucharest, Romania

3 Paul Scherrer Institute, General Energy Research Department, Villigen PSI, Switzerland

4 Institute of Electronic Structure and Laser (IESL), Foundation for Research and Technology Hellas (FORTH), Heraklion, Greece

Valentina Dinca and Livia Elena Sima contributed equally to this work

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