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Chapter 4

Biopolymer Thin Films Synthesized by Advanced Pulsed Laser Techniques

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http://dx.doi.org/10.5772/61734

Abstract

This chapter provides an overview of recent advances in the field of laser-based synthesis of biopolymer thin films for biomedical applications. The introduction addresses the importance of biopolymer thin films with respect to several applications like tissue engineering, cell instructive environments, and drug delivery systems. The next section is devoted to applications of the fabrication of organic and hybrid organic–inorganic coatings. Matrix-assisted pulsed laser evaporation (MAPLE) and Combinatorial-MAPLE are introduced and compared with other conventional methods of thin films assembling on solid substrates. Advantages and limitations of the methods are pointed out by focusing on the delicate transfer of bio-macromolecules, preservation of properties and on the prospect of combinatorial libraries' synthesis in a single-step process. The following section provides a brief description of fundamental processes involved in the molecular transfer of delicate materials by MAPLE. Then, the chapter focuses on the laser synthesis of two polysaccharide thin films, namely Dextran doped with iron oxide nanoparticles and Levan, followed by an overview on the MAPLE synthesis of other biopolymers. The chapter ends with summary and perspectives of this fast-expanding research field, and a rich bibliographic database.

Keywords: Biopolymers, MAPLE and Combinatorial-MAPLE, thin films, biomedical applications

1. Introduction

During the last decades, thin film layers have proved to be in the forefront for continuous developments in nanosciences and nanotechnologies [1]. Several research fields are nowadays related to thin films with a broad range of potential applications. Thin (<1 μm) and very thin

films (<100 nm) have already proven as key prospectives for advances in semiconductor devices, wireless and telecommunications, integrated circuits, solar cells, light emitting diodes, liquid crystal displays, magneto-optic memories, audio and video systems, compact disks, electro-optic coatings, memories, multilayer capacitors, flat-panel displays, smart windows, computer chips, magneto-optic disks, lithography, micro-electro-mechanical systems, and multifunctional protective coatings, as well as other emerging cutting-edge technologies [1]. Among them, biopolymer thin films area hot topic for biomedical applications like tissue engineering and cell instructive environments, drug delivery systems, antimicrobial surfaces, or biosensors.

Both natural and synthetic biopolymer thin films are representatives for engineering of the cell/biomaterial interface in view of controlling cell behavior, with a high impact for the *design of instructive surfaces* for tissue repair or cell supports [2]. Several techniques for the synthesis of biopolymer thin films such as dip-coating, spin-coating, drop-casting, and Langmuir–Blodgett are currently used. Separately, each technique exhibits advantages and drawbacks, generally allowing the assembling of a limited class of compounds [3, 4].

Pulsed laser technologies have extensively confirmed to be versatile for the fabrication of high-quality biomaterial thin films as they ensure the stoichiometry preservation [5]. However, in case of biopolymers, Pulsed Laser Deposition (PLD) induces an irreversible damage of the organic materials' composition due to high laser intensities [6]. This limitation is avoided by the new technique called matrix-assisted pulsed laser evaporation (MAPLE), which allows transferring delicate, large molecular-mass organic compounds [7-9]. Indeed, after the first implementation in the late 1990s [10], MAPLE proved to attain its maturity with the synthesis of functional, high-quality organic thin films, as reported by Guo *et al*. [11]. Laser technologies have thus been shown to successfully apply to transfer polysaccharides [12-18], proteins [19-23], or even living organisms like bacteria [24, 25], fungi [26], or mammalian cells [27-29].

Among the three main classes of natural biopolymers (polynucleotides, polypeptides, and polysaccharides) [30], this chapter focuses on the advanced laser synthesis of two polysaccharide thin films, namely Dextran doped with iron oxide nanoparticles and Levan. A detailed literature survey overviews the MAPLE synthesis of other biopolymers. Moreover, the possibility to fabricate combinatorial libraries of biopolymers by advanced laser technique in a single-step process is mentioned.

The use of polysaccharides as biomaterials has evolved over the past several decades, covering biomimetic approaches. A simple classification divides the polysaccharides as derived from non-mammalian or mammalian sources. The first group includes Alginate, Chitin, and Dextran, among others, which possess similar saccharide structure although having different origins [31, 32]. The interest in these materials is due to their relatively easy extraction and purification of large quantities at low cost. Mammalian polysaccharides, such as the Glycosaminoglycans chondroitin sulfate, Hyaluronan, and Heparin, possess chemical similarities to the non-mammalian ones but their isolation is more complicated [33-35]. Their unique biological functionality, e.g., specific binding with multiple proteins, has raised increased interest for application in the field of biomaterials, as reviewed in Ref. [36].

Like other non-mammalian polysaccharides, Dextran is not present in human tissues, being expressed by bacteria such as either *Leuconostoc mesenteroides* or *Streptococcus mutans* [32]. It is a complex branched glucan (polysaccharide made of many glucose molecules) consisting of chains of different lengths (from 3 to 2000 kDa). Dextran is highly water-soluble and easily functionalized through its reactive hydroxyl groups [37]. As a biodegradable and biocompatible biopolymer, it was investigated as a blood plasma replacement in the early 1940s [38]. Dextran could be functionalized with nanoparticles [18, 39, 40] or conjugated with different polymers [41-44] for various biomedical applications as described in *Section 4.1*.

Among natural polysaccharides, Levan is a high molecular weight, water-soluble bacterial exopolysaccharide (β2,6-linked fructan) [45]. It is produced by microbial fermentation of *Halomonas smyrnensis* AAD6^T batch cultures grown on pretreated sugar beet molasses [46]. Due to its anticancer [47], anti-inflammatory [48], anticytotoxic [46], and antitumoral [49-51] properties, Levan is a promising biopolymer with a huge potential for biomedical applications. Some other application fields are in textiles, cosmetics, wastewater treatment, food, and pharmaceutical industries [46, 50, 52]. Sima *et al*. [12] reported for the first time on pure and oxidized Levan nanostructured thin films assembling by MAPLE (*Section 4.2*). The authors evidenced a high potential for cell proliferation for both coatings (with certain predominance for oxidized Levan) by *in vitro* colorimetric assays.

The latest progress achieved in the development of new materials or innovative properties is often based on combinatorial processes. Usually, the fabrication of a multicomponent organic coating is performed by film casting procedures [53, 54]. Compositional and/or thickness gradient thin films of polymers are produced by premixing different polymer solutions, followed by applying a linear temperature gradient [55]. Subsequently, stem cells were exposed to molecule combinations arrays, looking for synergistic effects that could direct cell fate [56]. Other studies reported binary combinations of an adhesive molecule and a growth factor in view of parallel testing of several environmental media to control the evolution of neural stem cells [57]. This approach is based on previously tested well-defined concentrations of biomolecules to obtain the desired combinations.

This chapter introduces an innovative solution for the synthesis of combinatorial libraries of biopolymer thin films by Combinatorial-MAPLE (C-MAPLE) technique (*Section 4.3*). The compositional gradient occurs in this case naturally along the substrate by the simultaneous laser irradiation of two cryogenic targets and thin-film co-deposition. A representative example is for Levan (L) and oxidized Levan (OL) biopolymers, *in vitro* cell culture assays illustrating characteristic responses of cells to specific surface regions [13]. The versatility of the method for the synthesis of hybrid materials and further possible developments are summarized.

2. Laser-based techniques for the synthesis of biopolymer thin films

2.1. Complementary techniques for organic thin films' fabrication

Nowadays, there is a large body of experimental studies focused on the deposition of thin, uniform, and adherent films of numerous types of polymers, organic materials, and

biomaterials (soft materials). The goal is the fabrication of controlled structures (or nanostructures) essential to be used, for instance, in diverse areas such as medical field, packaging, cosmetics, clothing fabrics, food additives, industrial plastics, water treatment chemicals absorbents, biosensors and detectors, or data storage elements [58]. For this purpose, one needs to choose an appropriate deposition method which should depend on the physical–chemical properties of the biomaterial, requirements for film quality, type of the substrate, and the production costs [25].

There exist techniques that can be used to deposit highly uniform biopolymers thin films and micro-patterns [59], like laser-induced forward transfer (LIFT) [60] or Matrix-assisted laser desorption/ionization (MALDI) [61]. Besides, sol-gel, layer by layer (LbL), aerosol spraying, dip coating, and spin coating are techniques that entail liquid solutions of the material in a volatile solvent [7, 62, 63]. A common method to obtain surfaces with a single biomolecular layer is Langmuir–Blodgett (LB) dip coating, using self-assembled monolayers [7, 64, 65].

In spite of the rich list reported in the literature of biomaterials deposited thin films, these techniques have their own merits but also disadvantages (e.g., manufacturing of a limited class of biomaterials). In order to obtain a better quality of thin films, these techniques should allow the control of several parameters during and after deposition. In short, in the case of LB method, key parameters have been identified to be crucial for obtaining high-quality thin films, e.g., deposition speed or transfer surface pressure [66]. These parameters directly affect the adhesion strength between film and substrate, causing delamination of the layer or generating discontinuities in the structure, which worsen the homogeneity of the films [67]. Also, this technique is limited to very thin layers [68, 69].

Sol-gel is a largely used method, successfully applied to obtain organic/inorganic hybrid coatings [70, 71] through precipitation by chemical reactions in liquid medium. In spite of its advantages, sol-gel technique will never reach its full potential due to some limitations, e.g., poor coating adhesion, low wear-resistance, involvement of liquid media that could impede the multilayer assembling (affect interfaces), high permeability, limit of the maximum coating thickness (~0.5 μm) [72], and difficulties in controlling porosity. On the other hand, a thick organic coating often results in failure during thermal process. Moreover, sol-gel is a substrate-dependent technique, and the thermal expansion mismatch hampers the wide application.

Other drawbacks that restrict the application of these methods are related to the choice of the solvent or liquid media issues during multilayer assembling, difficulties in obtaining large-area uniform thin coatings, or that the methods are time- and material-consuming [73-77].

It is widely accepted that the surface topography has a significant influence in a wide range of organic materials applications. In addition to surface topography and chemistry, thin film adhesion to the substrate also plays an important role. According to the literature, the current coating techniques [78, 79] provide inadequate coating adhesion to the substrate.

Nowadays, it is considered that laser-based technologies are among the main, most powerful tools for fabrication of micro- and nano-arrays of a wide range of different biomaterials with controlled thickness (with the precision of 1 Å), good adhesion to the substrate, and specific surface properties. Moreover, these methods permit the relatively uniform spreading of

http://dx.doi.org/10.5772/61734

material over rather large areas, control of substrate temperature, low material consumption, and stoichiometry conservation of the growing film.

Nevertheless, when using UV lasers such as excimers operating at 193- or 248-nm or frequency tripled Nd:YAG lasers at 355 nm (6.4–3.5 eV/photon) to obtain thin films of very complex delicate biomolecules, irreversible damage of the chemical bonds and consequent compositional modification are induced. Consequently, PLD technique is not a viable option for fabrication of complex biomolecules such as polymer thin films [6].

2.2. Matrix-Assisted Pulsed Laser Evaporation (MAPLE)

Discovered at the end of the 1990s at the Naval Research Laboratory, MAPLE has become nowadays an active area of research [9, 80]. Developed as a complementary method to PLD, MAPLE has introduced new advancements in laser methods deposition of thin films. In short, MAPLE provides gentler pulsed laser evaporation, a less damaging approach for transferring many different compounds including small or large molecular weight species, such as organic and polymeric molecules [81].

MAPLE technique has been successfully applied to obtain thin films of sensitive materials avoiding thermal decomposition and irreversible degradation under the action of electric or magnetic fields. Applications targeted development of biosensing, chemical sensing, and biochemical analysis, as well as drug delivery systems and the developing of a new generation of implants [19, 82-86]. In MAPLE, the laser-induced material ejection is generated backward from a solid cryogenic target. The expulsed substance is assembled on substrates, where it forms a thin film with a thickness from a few to several hundreds of *nm*. The incident laser pulse used for MAPLE initiates two photo-thermal processes in the matrix: evaporation of the frozen composite target and transfer of the material onto the substrate. The schematic of MAPLE setup is presented in Figure 1.

Typically, the target consists of base material (less than 10% wt.) dissolved/suspended into a laser wavelength absorbing solvent when in frozen state. The organic material molecules reach sufficient kinetic energy by collective collisions with the evaporating solvent molecules, ensuring a controlled transfer on the substrate, in gas phase. Since the receiving substrate is usually kept at room temperature and the sticking coefficient of the solvent is nearly zero, the evaporated solvent is efficiently pumped away by the vacuum system. The solvent and solute concentration should be selected so that: the solute can be dissolved to form a dilute, particulate-free solution; most of the laser energy has to be absorbed by the solvent molecules rather than the solute ones; and no photochemical reaction is produced between solvent and solute. By optimization of the MAPLE deposition parameters (laser wavelength, repetition rate, laser fluence, solvent type, solute concentration, substrate temperature, background gas and pressure), the process can proceed without significant material decomposition [6, 25, 81]. It was demonstrated that MAPLE could provide more crystalline layers as compared to PLD method from the same materials [87]. Recent comprehensive reviews on MAPLE deposition of organic, biological and nanoparticle thin films illustrated large potential (drug delivery, biosensors, etc.) applications of thin coatings obtained by this method [9, 88].

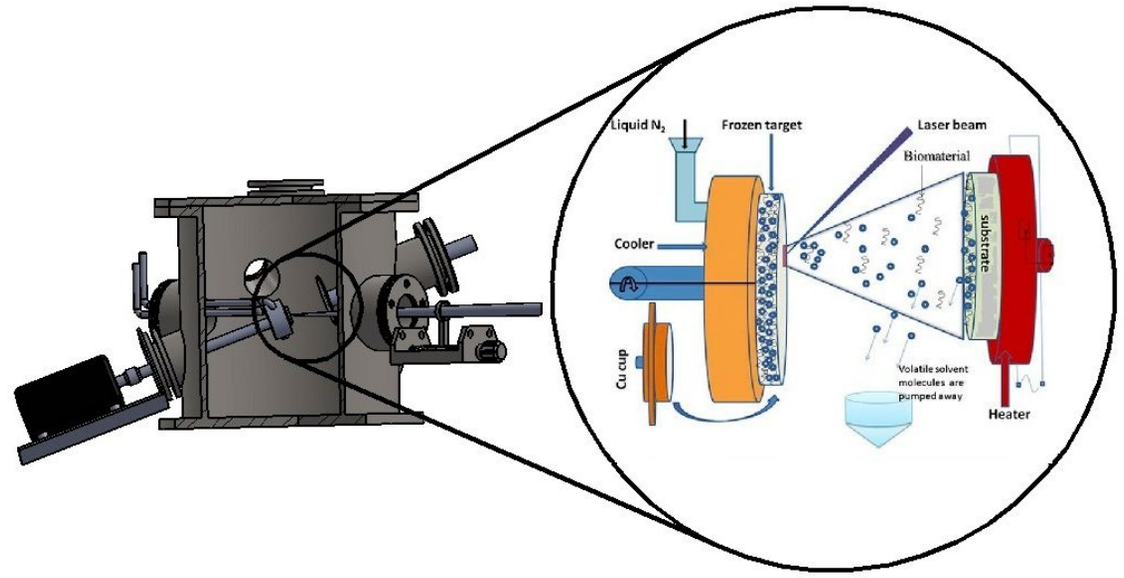

Figure 1. The experimental setup of MAPLE method.

2.3. Compositional library thin films fabrication by Combinatorial-MAPLE

Recently, improvements have been made on MAPLE technique based on developing a new concept for the synthesis of functionalized biomaterials surfaces. Combinatorial-MAPLE technique was introduced as a new approach for the fabrication of gradient organic/inorganic thin films, for the identification of best bioactive surfaces able to modulate and control cell behavior [13]. In C-MAPLE experiments, two targets are simultaneously evaporated by laser beams. Different lasers could be used, having different characteristics (pulse duration, wavelength, repetition rate), or the beam of one laser is divided into two beams (Figure 2) by an optical splitter.

The two beams are independently focused onto the surface of each target, containing the frozen solutions to be irradiated. To grow uniform thin films, the targets are continuously rotated (from 1 to 80 rpm). This arrangement is preferred to avoid drilling and allow for the expulsion of materials in an accurate mode. To deposit high-quality films with controlled thickness, one should choose for each compound the appropriate fluence and number of laser pulses to reasonably balance the deposition rate along the length of the substrate. This particular combinatorial setup allows for the smooth and isotropic interpenetration and mixing of the two substance fluxes evaporated form the targets, resulting in the deposition of a continuous and uniform composition gradient. A gradient of composition from 100% A material to 100% B material is thus obtained on a substrate, as schematically depicted in Figure 3.

This method opens the possibility to both combine and immobilize two or more materials, dissolved in different solvents, using diverse wavelengths. Further, by investigating the obtained structures by physical, chemical, and biological methods, one could select the best compositions that can be synthesized from the two components. The advent of Combinatorial-MAPLE could open new research frontiers in identification of the best dosage between several organic and/or inorganic materials with great prospective for many applications.

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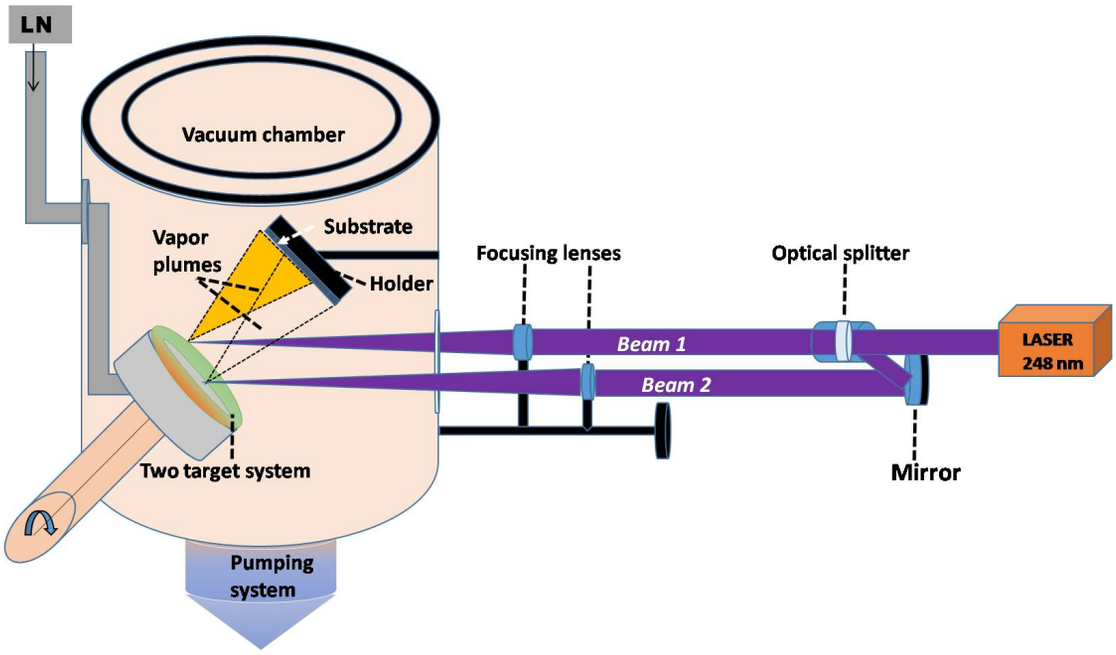

Figure 2. The experimental setup of C-MAPLE method.

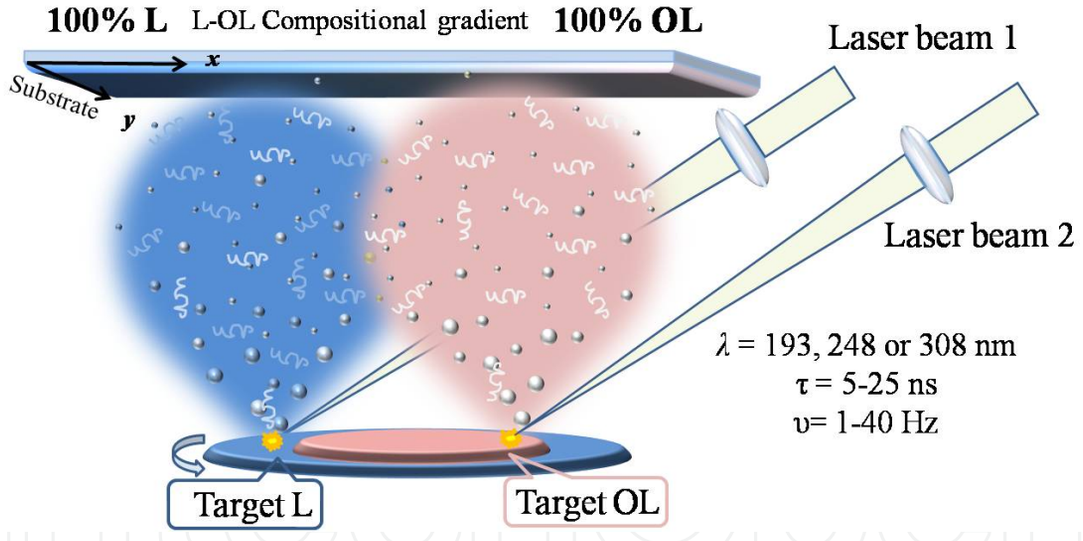

Figure 3. The design of the experimental setup.

3. Theoretical aspects of laser–matter interactions: Insights for MAPLE

The understanding of the fundamental processes responsible for the molecular transfer (physical mechanisms governing the ejection) of delicate materials when suspended in a volatile matrix in form of an icy target should be investigated in order to explain the experimental results. More concretely, the large surface roughness specific to MAPLE depositions could not be accounted for in terms of the first explanations advanced in Ref. [6], where it is hypothesized that the matrix absorbs laser energy and converts it into thermal energy which

will vaporize the solvent molecules. Then, the active material molecules are displaced onto the collector surface after collisions with solvent molecules. Moreover, the studies have proved that it is mandatory to make an appropriate choice of transfer parameters (laser wavelength, incident fluence and pulse duration, type of solvent, substrate nature and temperature, and nature and pressure of the background gas). When these conditions are met, MAPLE ensures the "soft" ejection and transfer of delicate material molecules preserving their chemical structure and very likely their functionality and biologic activity. Accordingly, the proper choice of solvent and deposition conditions is essential for getting the best possible compromise between films bioactivity and morphology.

Before 2007, no theoretical or computational works for a better understanding of MAPLE process have been conducted. Leveugle and Zhigilei [89] developed for the first time a computational model (a coarse-grained molecular dynamic (MD) model) to explain the basic mechanisms related to laser–material interaction and non-equilibrium processes and the resulting film characteristics, especially morphology. The authors demonstrated that even at low concentration (0.1–5% wt.) of active material in the matrix, the active molecules can influence the molecular ejection and subsequently the morphology of the films. The MD simulations were conducted for a laser wavelength of 337 nm with pulse duration of 50 ps and incident laser fluences in the range 3–9 mJ/cm^2. It was revealed that below the ablation threshold of the matrix (3.5 mJ/cm^2), only an evaporative process occurs. In this regime, the matrix molecules solely get vaporized and no active molecules are ejected. For laser fluences superior to this threshold, an explosive process takes place accompanied by ejection of clusters and liquid particles from the MAPLE target. This results in the deposition of thin coatings with a high roughness morphology. In addition to this, the simulations predicted that also the composition of the target surface can be modified by the ablation process induced by matrix evaporation. It is expected that an increase of active material concentration in the target is produced, especially in a multipulse irradiation regime.

The ejection of clusters composed of solute and matrix molecules and the rough surface of the MAPLE coatings seem inevitable. However, the quality of the growing films can be, at least partially, controlled by the temperature of the substrate and possible post-deposition treatments.

Based on this model, it is possible to avoid or minimize the deposition of molecular clusters in MAPLE and achieve a molecule-by-molecule deposition of ultrathin films without significant roughness by selecting the appropriate set of transfer parameters.

Water, "the universal solvent," is perhaps the most versatile matrix for biopolymers. A frozen aqueous solution is an attractive medium since such an icy matrix has turned out recently to yield promising results for biomolecule transfer from targets to selected substrates [21, 90, 91]. However, the laser light at 248 nm is not very efficiently absorbed by the ice matrix, but is on the other hand less harmful to the bonds in the polymer than light at shorter wavelengths [92].

When using a water matrix, the ablation process could be related to local overheating of absorbing areas constituted by biomolecules in the outmost surface layer, heating the solvent

in their vicinity [93]. In vacuum conditions, the water solvent starts boiling at room temperature, the vapors transporting the biomolecules toward the substrate surface. The material ejection is consequently produced at lower temperature than the degradation threshold.

Another mechanism based on *nonhomogeneous absorption* could be applied for low laser fluence, when the mass ejection is produced by surface evaporation, but also for higher fluences when the expulsion is governed by the hydrodynamic ablation [94]. A frozen MAPLE target contains not only the molecules of active materials and of the matrix but also different phases such as ice cracks, air bubbles, or other defects. These phases were suggested to be involved in light absorption or scattering processes during laser irradiation of the heterogeneous frozen target. Accordingly, the absorption was found to be higher in ice as compared to water. The laser absorption can be increased by the addition of other compounds in the solution, which introduce local modifications of material properties [9].

These MD simulation and models allow achieving two main objectives of MAPLE, which are as follows: (*i*) to avoid photo-chemical and photo-thermal molecular fragmentation (also called "bond scission") characteristic to PLD, and (*ii*) to achieve deposition of highly uniform thin films that cannot be obtained by solvent-based coating methods.

4. Biopolymer thin films for biomedical applications

4.1. Hybrid Dextran–iron oxide thin films

The development of hybrid biomaterials, in particular in the form of thin films, has received a growing interest in the last decades mainly due to their biomedical applications. It is generally accepted that both synthetic and natural biopolymers could be used in biomaterials research, because of their unique structures that allows for a specific functionalization for desired applications [36]. Moreover, embedding metal and/or metal oxide nanoparticles (NPs) into an organic and/or inorganic matrix could lead to the fabrication of a novel generation of *smart biomaterials*, with optimized properties [95].

As known, Dextran is a natural biopolymer that can be synthesized from fermentation of sucrose-containing media [31, 37]. Its structure consists of linear (1 → 6)-α-D-glucose, with branches extending mainly from (1 → 3) and occasionally from (1 → 4) or (1 → 2) positions accounting for a 5% degree of branching [36]. Due to its specific properties (neutral and water-soluble, easy to functionalize through its reactive hydroxyl groups, biodegradable, biocompatible, long-term stability), it is intensively used in several biomedical applications like an antithrombotic (antiplatelet) to reduce blood viscosity, and as a volume expander in hypovolemia [96]. Moreover, Dextran-based coatings were proven to develop well-defined surface modifications that could induce specific cell interactions and enhanced performances in long-term biomaterial implants [97].

In the last decades, biocompatible iron oxide NPs have attracted increased consideration due to promising properties for the biomedical field. Applications reported in the literature are related to: contrast agents in magnetic resonance imaging (MRI) [98, 99], *in vitro* cell separation

[100], *in vivo* diagnosis of cancer [101], targeted destruction of tumor tissues by hyperthermia [102, 103], and targeted drug delivery systems since allows activation by applying an external magnetic field [104, 105]. However, for such applications the NPs must combine several different properties like high magnetic saturation, biocompatibility, and interactive functions at the surface. Accordingly, in most of the cases, a further modification of their surfaces is mandatory by applying thin organic and/or inorganic coatings [106] in view of binding to drugs, proteins, enzymes, antibodies, or nucleotides [107]. The most studied iron oxide NPs are maghemite γ-Fe_2O_3, and magnetite Fe_3O_4 with single domains of about 5–20 nm in diameter. Details about their chemical synthesis, surface engineering, and effectiveness for biomedical applications were reviewed by Gupta and Gupta [107].

Although emerging applications envisioned, it was only recently reported that the nanosized feature of particles could be associated to cytotoxicity [108, 109], at least when large amounts of NPs have to be used. Only NP concentrations below 100 μg/ml are considered safe [110]. The growth of hybrid thin films consisting of Dextran and maghemite γ-Fe_2O_3 NPs using MAPLE technique was reported by Predoi and coworkers [18]. The authors investigated the biocompatibility, an essential requirement for the introduction of iron oxide into the human body, but also the influence of the NP concentration on the biomimetic properties of the synthesized coatings.

The chemical synthesis of iron oxide NPs was performed following a classical co-precipitation procedure, according to Bee *et al*. [111]. For investigations, the obtained particles were dispersed in deionized water, the pH being adjusted to 7 using aqueous ammonia. The total iron concentration of the suspensions determined by redox-titration was 0.38 mol L^{-1}. Well-crystallized NPs, having an average size of 8.3±0.3 nm were obtained, as visualized in high resolution Transmission Electron Microscopy (HRTEM) images, the corresponding Selected Area Electron Diffraction (SAED) pattern indicating the reflection of the cubic maghemite γ-Fe_2O_3 phase [18].

Dextran and Dextran–iron oxide composite thin films were deposited by MAPLE. Different solutions consisting of 25,000 Da molecular weight Dextran (10 % wt.), iron oxide NPs (0–5 % wt.), and distilled water as matrix solvent were used for target preparation. Before each deposition, 5 ml of the obtained solution was dropped in a copper holder of 3 cm diameter and 5 mm height and immersed in liquid nitrogen (77 K) to freeze a solid target. The pure and hybrid coatings were grown on SiO_2 glass substrates by applying 25×10^3 subsequent laser pulses. After optimization trials, the incident laser fluence on the target surface was set at 0.5 J/cm^2.

The structure of hybrid Dextran–iron oxide thin films obtained from the composite targets was first analyzed by X-Ray Diffraction (XRD). The diffraction patterns revealed the presence of the peaks assigned to the cubic maghemite γ-Fe_2O_3 structure. This observation is in good agreement with SAED analysis indicating that the NPs' crystalline phase was preserved during laser processing. Moreover, the average size $\langle d \rangle$ of the nanocrystalites computed using Scherrer's formula [112] evidenced values around 7.7 nm, in accordance with the average size of the NPs determined from HRTEM micrographs [18].

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Figure 4 illustrates the structural characteristics of the coatings inferred by Fourier transform infrared spectroscopy (FTIR). The graphs illustrate the spectra of iron oxide NPs (Figure 4.A) and Dextran used for the preparation of the MAPLE targets (Figure 4.B), as well as the spectra of the Dextran–iron oxide thin films synthesized by MAPLE from the composite targets containing 5% wt. (Figure 4.C) and 1% wt. (Figure 4.D) maghemite γ-Fe_2O_3, respectively. All spectra exhibit the bands assigned to OH stretching (ν OH) and HOH bending (δ OH) vibrational bands at 3480 cm^{-1} and 1700 cm^{-1} due to adsorbed water molecules [113]. The bands observed at 620 cm^{-1} and 580 cm^{-1} in the spectrum of the iron oxide NPs correspond to the Fe–O vibration modes of γ-Fe_2O_3 [114, 115]. In the FTIR spectrum of Dextran-NPs, the characteristic absorption bands of the polysaccharide can be observed [114, 116-118]. They are summarized in Table 1. One could notice that the spectra recorded in case of the hybrid coatings are very similar to the spectra of the starting materials. Furthermore, the intensity of the bands corresponding to the maghemite γ-Fe_2O_3 phase increases with increasing NPs concentration in the composite targets used in MAPLE experiments.

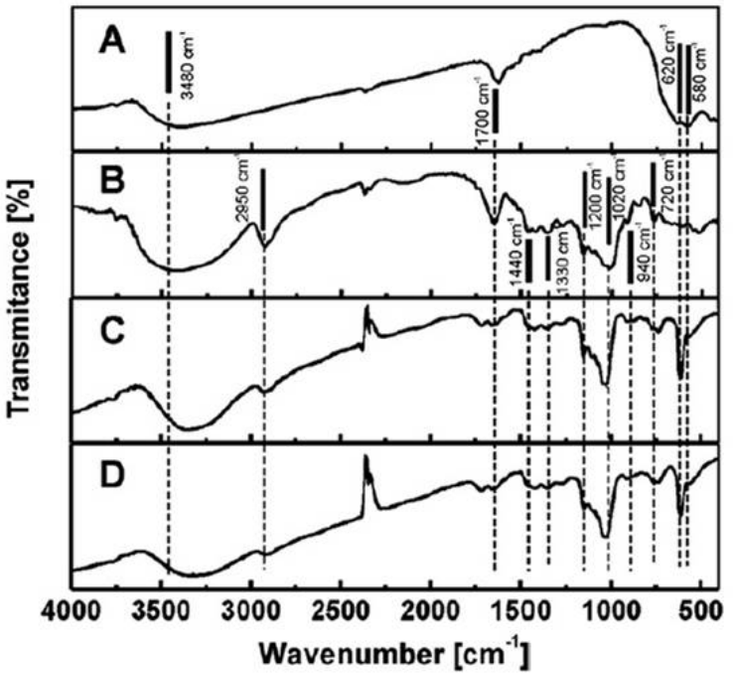

Figure 4. FTIR spectra of iron oxide NPs (A), pure Dextran (B), as well as Dextran–iron oxide thin films containing 10% wt. Dextran, 5% wt. (C) and 1% wt. (D) iron oxide NPs. (Reproduced with permission from [18])

The typical surface morphology of thin films deposited by MAPLE technique is characterized by an aggregated structure, consisting of micrometer-sized particles [17, 18, 22, 39, 119]. It is worth noting that a larger specific surface area was proven to induce an enhanced bioactivity, able to promote osteoblast differentiation, as reported in case of hybrid organic–inorganic thin films deposited by MAPLE [120, 121].

The biocompatibility of the Dextran–iron oxide thin films was demonstrated by 3-(4.5 dimethylthiazol-2yl)-2.5-diphenyltetrazolium bromide-based colorimetric assay, using human

liver hepatocellular carcinoma (Hep G2) cell line [18]. In Figure 5 are visible the inverted light microscope images of Hep G2 cells cultivated on pure Dextran (Figures 5.A, 5.B) and Dextran–iron oxide thin films obtained from composite targets (Figures 5.C, 5.D) after 24 (A, C) and 48 h (B, D) incubation time. The cultured Hep G2 cells form polygonal multicellular aggregates [122] as could be observed from figures, this morphology being preserved even after 48 h incubation time. At both iron oxide concentrations, the aggregates' size increased with the incubation time, but is still close to those grown on uncoated plastic slides.

ν (cm^{-1})	*Characteristic modes*
3480	OH stretching (ν OH)
1700	HOH bending (δ OH)
2950	ν (C\H) and δ (C\H)
1440	vibrational modes
1200	ν (C\O) vibrations
940	α Glucopyranose ring
720	deformation modes

Table 1. Characteristic absorption bands of Dextran biopolymers [18].

The results of the viability tests (MTT) of Hep G2 cells on pure Dextran drop-casted solution, Dextran and Dextran–iron oxide composite thin films obtained by MAPLE are presented in Figure 5.E, and compared to the cells cultivated on control samples (considered as having a viability of 100%). A small decrease of viability (~8%) was observed for Dextran–iron oxide thin films after 24 h incubation time. When increasing the incubation time, this drop increased but still remained below 12% for the Dextran thin film containing 5% wt. iron oxide, pointing to good biocompatibility [18]. Moreover, in a similar study, Ciobanu *et al.* [39] showed that Hep G2 cells adhered very well to thin films of Dextran-doped maghemite and exhibited a normal actin cytoskeleton, proving that these cells underwent normal cell cycle progression. As a result, the authors consider that hepatocytes adhered to hybrid thin films could be used as biosensors for different xenobiotics.

In summary, due to its properties, relatively low cost and availability, Dextran and its conjugates have increased utilization in the field of biomaterials. Non-laser-based techniques are intensively used to fabricate thin films and hydrogels as well [123-125]. The influence of Dextran and albumin-derived iron oxide nanoparticles on fibroblasts *in vitro* was studied by Berry *et al.* [126]. Magnetic composite thin films of Fe_xO_y nanoparticles and photo-cross-linked Dextran hydrogels are promising candidates for a broad field of applications from medicine to mechanical engineering [40]. Dextran-conjugated materials have been successfully investigated as controlled release delivery vehicles of indomethacin (a low molecular weight

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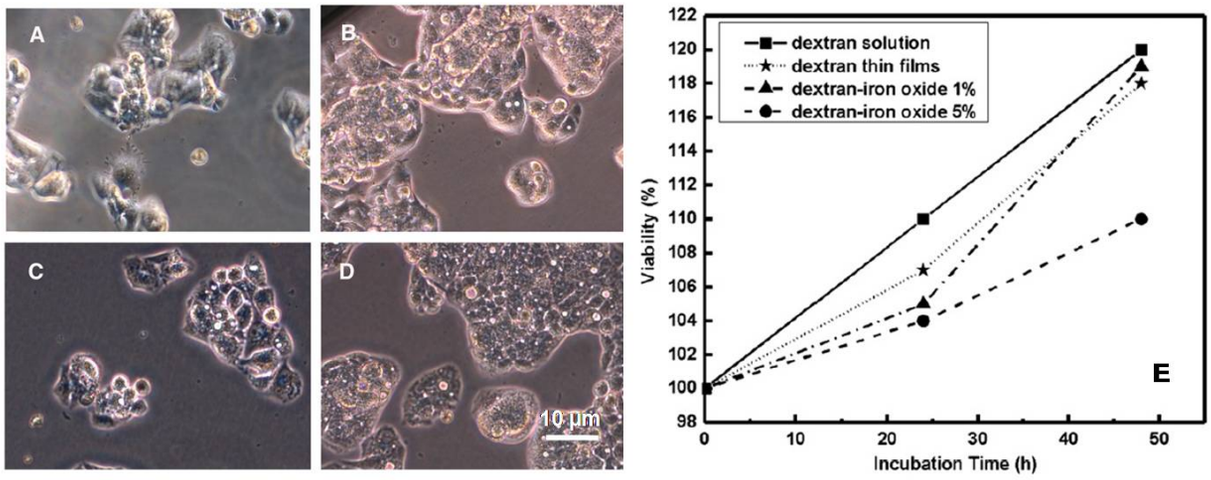

Figure 5. Inverted light microscopic images of Hep G2 cells grown on pure Dextran thin films (A, B), and Dextran–iron oxide thin films obtained from composite targets (C, D) after 24 (A, C) and 48 h (B, D) incubation time. Viability of Hep G2 cells grown on Dextran, and on composite thin films deposited by MAPLE technique (E). (Reproduced with permission from [18])

hydrophobic anti-inflammatory drug) [42], bovine serum albumin [43, 44], lysozyme, and immunoglobulin G [41, 44].

4.2. Nanostructured Levan thin films

High-purity biopolymers are now obtained by microbial fermentation. Levan is a natural polysaccharide produced from fructose by many microorganisms [127]. It is composed of d-fructofuranosyl monomers linked by β(2 → 6) units and β(2 → 1) branches. The carbohydrate structure of Levan synthesized by different microorganisms is rather similar, while small differences appear in degree of polymerization and branching unit [128]. Its specificity is related to furanose form of carbohydrate conformation with an important role in molecule dissolution [129]. Levan is less studied than other known polysaccharides such as Dextran or Pullulan, mainly due to the lack of information about its biocompatibility [130]. Because of unique combined properties like solubility in both oil and water, high molecular weight, and strong adhesion, Levan can be considered as a novel functional polymer with huge potential in industry, from foods, cosmetics, pharmaceutics to chemistry [131]. Indeed, its use as drug delivery matrix, antitumor agent, or protective coatings, stabilizers, or emulsifiers represent only few raised applications of Levans [132, 133]. The important issue is that for most applications or mass production, thin films and coatings are required. These structures compensate the cost by limiting the interaction of the environment with the product to the surface only. Films of polymers are currently produced by solvent casting or thermal processing [134, 135]. A concrete example is the case of a drug dose slow release, when the tablet coating consists of biopolymer with plasticizers for stabilization. It was demonstrated that by extrusion and molding, thick films of Levan can be produced by adding glycerol for cohesion [136]. There is however a high risk of poor adhesion, cracking, and peeling due to rather bulky aspect and consequently dissolution of the film. Thin films of dry and pure Levan are brittle, while

biopolymer nanocomposites exhibit improved mechanical properties in respect to the corresponding pure compound [137, 138].

MAPLE process was successfully applied to fabricate organized and nanostructured pure thin films of Levan (L) and oxidized Levan (OL) in vacuum. In order to produce functional aldehyde groups, the oxidation of Levan was carried out before laser transfer in dark oven at 50 °C for six days. MAPLE proved to be the only technique able to transfer nanostructured Levan thin coatings on solid substrates, which exhibited biocompatible properties *in vitro* [12]. The nanostructured aspect of the film that increases the specific surface area can consequently boost the material properties. Indeed, the behavior of Levan in aqueous solutions is difficult to predict and chemical methods fail in producing homogenous and uniform films. On the other hand, during MAPLE process the polymer molecules are transported by Dimethyl sulfoxide (DMSO) solvent molecules from which they separate during the transfer from the target to the solid substrate. Even though some solvent molecules accompany the polymer on the facing substrate, these proved beneficial in the film assembling. Most of the DMSO molecules are vaporized and removed from the reaction chamber by pumping system, whereas Levan molecules were transferred onto the substrates without degradation. In experiments, an excimer laser (KrF*, λ = 248 nm, τ = 25 ns) was used. Deposition process parameters such as fluence, pulse repetition rate, substrate temperature, or distance between the target and collector were optimized in order to grow biopolymer films on Si and glass substrates. DMSO was chosen as solvent as it does not chemically affect L or OL; it is highly volatile; and absorbs at 248 nm laser wavelength.

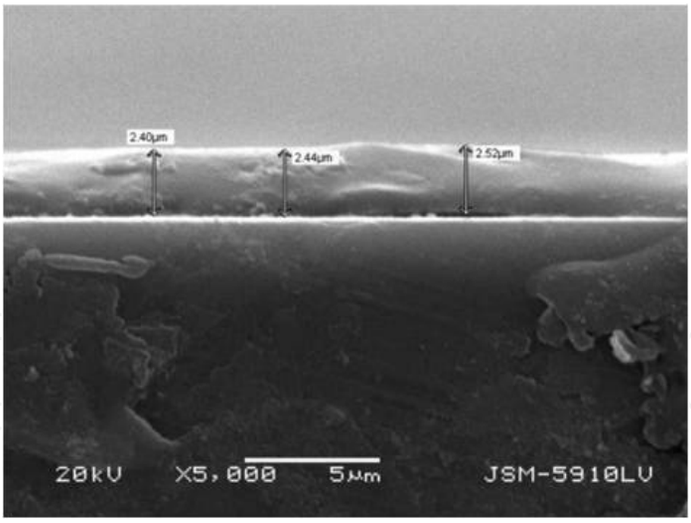

Figure 6. Typical XSEM of L thin films on glass obtained by MAPLE (Reproduced with permission from [12])

Levan films were compact in volume, exhibiting a good adhesion to substrate. As observed from cross-section SEM (XSEM) image in Figure 6, the film was rather compact, while the variation in height across films was low over a relatively large area, supporting the uniformity of the layer. The surface was smooth over large areas and homogenous. A growth film ratio of 0.012 nm/pulse was estimated.

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Uncommon two-dimensional ordered array was evidenced at film surface due the most probably to a controlled aggregation during the growing of the film (Figure 7). The nano-structured assembling appears when the solvent DMSO molecules evaporate from the heated substrate.

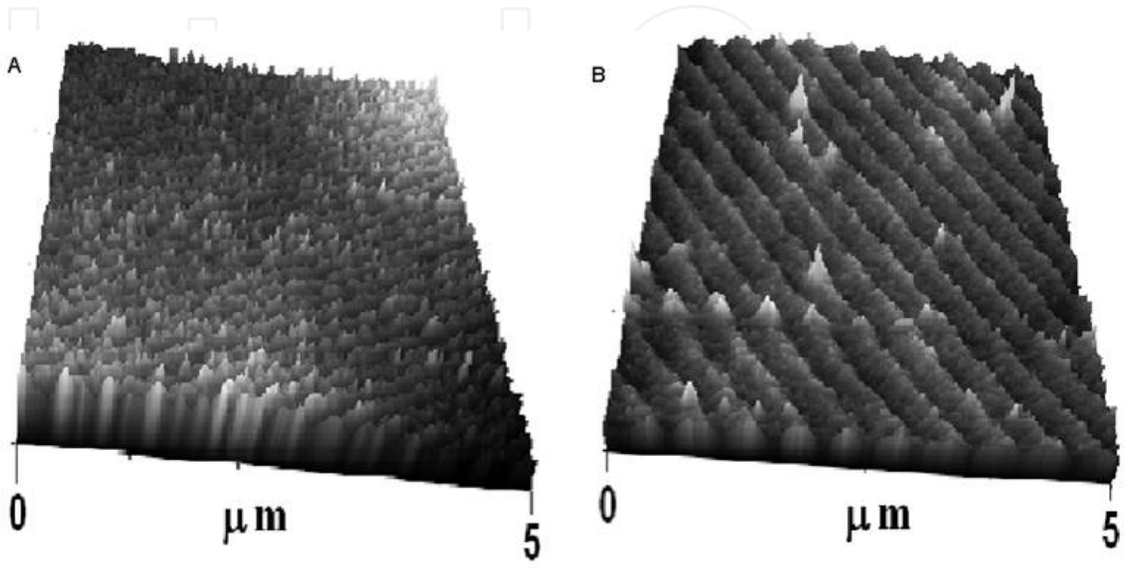

Figure 7. Typical AFM images of sample surfaces for (A) L and (B) OL coatings by MAPLE on Si (Reproduced with permission from [12])

The dynamics of polymers at surface is substantially altered especially when some solvent molecules induce rearrangements. Totally different to rigid ceramic or metal materials, the composition of the polymer varies also with the depth [139]. The morphology is quite similar for L and OL thin films obtained by MAPLE exhibiting a spatial orientation due to a collective influence of evaporation-induced assembly with the specific linkages of the linear structures of polysaccharides. These assembling morphologies were also found for nano-hydroxyapatite (nHA) – chitosan composites [140]. It is considered that the nanostructured assembling, which induces a larger specific surface area, boosts the surface properties of the biopolymer.

The effect of Levan films grown by MAPLE on cell viability and proliferation was investigated by interaction with bone cells. Their proliferation on Levan and control samples was found to be similar. The OL coatings induced an increased cell activity revealed by enhanced cell proliferation as compared with the simple L coatings. This is in accordance with the higher hydrophilicity of OL surfaces due to the acidic aldehyde–hydrogen bonds forming after oxidation [12].

4.3. Compositional libraries thin films by C-MAPLE

Combinatorial processes are required for the synthesis of new organic multicomponent thin coatings [54, 141]. In case of polysaccharides, co-electrodeposition is applied after materials

are dissolved or suspended in aqueous solution and integrated into thin films [142]. To produce thin film compositional libraries, premixing of biopolymer solutions followed by temperature gradient over the coating [55], casting processes [143], or flow-coating methods [144] have been used.

A combinatorial technology based on MAPLE for the blending of novel organic compounds was introduced. The new processing method called Combinatorial-MAPLE (C-MAPLE) was proposed to biopolymer compounds [13, 14, 22]. The composition gradient between two materials is achieved by laser co-evaporation of two distinct cryogenic targets and thin-film co-deposition process on solid substrate as described in *Section 3.2*. Two similar compounds such as L and OL but with different physical–chemical and biological properties were chosen in order to generate a compositional discrete library of the two organic compounds.

In experiments, an excimer laser source (KrF*, λ = 248 nm, τ = 25 ns) was used for target evaporation. The Si substrates or glass slides were placed at 4 cm far and parallel to the targets and slightly heated during laser deposition. In a configuration with a distance between the plasmas' centers of 2 cm (see Figure 2), one can obtain a 4 cm long deposition with edges consisting of only L and OL, respectively, and in-between discrete areas of L–OL blended compositions. The soft mixing of the two compounds evaporated from the two distinct targets results in the deposition of a continuous and uniform film with compositional gradient. A gradient of composition from 100% L at left corner to 100% OL at right corner (Figure 8.B) was thus obtained.

The compositional gradient of the film was followed by fluorescence microscopy, as a change in fluorescence emission between L and OL occurs. Levan contains fructose, which is highly fluorescent under green excitation (488 nm). OL loses the fluorescence because fructose is oxidized to aldehyde groups [145]. In Figure 8.A are presented optical and fluorescent pair images in which one can observe the increasing of fluorescence intensity from OL to L along the deposited sample (Figure 8.A and 8.C). This confirms the compositional gradient in the structure [13].

Cellular adhesion, spreading, and proliferation are processes dependent on surface composition and roughness. An optimal cell response to the surface characteristics is of great significance for tissue engineering and nanomedicine. The biocompatibility and cellular behavior to gradient films on glass and silicon substrates was thus evaluated. Initial cell–substrate interaction is shown by cell attachment, followed by adhesion and proliferation. The cell attachment efficiency and morphology is indicative of material biocompatibility. To clearly discriminate between the cell responses, the samples were cut in four equivalent pieces (OL, OL-L, L-OL, and L, respectively). L regions should consist of Levan only, OL of oxidized Levan only, while in OL–L intermediary areas one can expect to contain more OL than L and in L–OL areas more L than OL. The density and actin morphology of cells were evaluated on the four regions (Figure 9.A) at 40 min after cell seeding, as the proof of primary attachment. Similarities on all four zones and standard microscopy cover slips were indicative of biocompatibility and of the dynamic interaction of L/OL gradient coatings with bone cells. The quantification (Figure 9.B) showed that the cells preferred OL as compared to L areas. The

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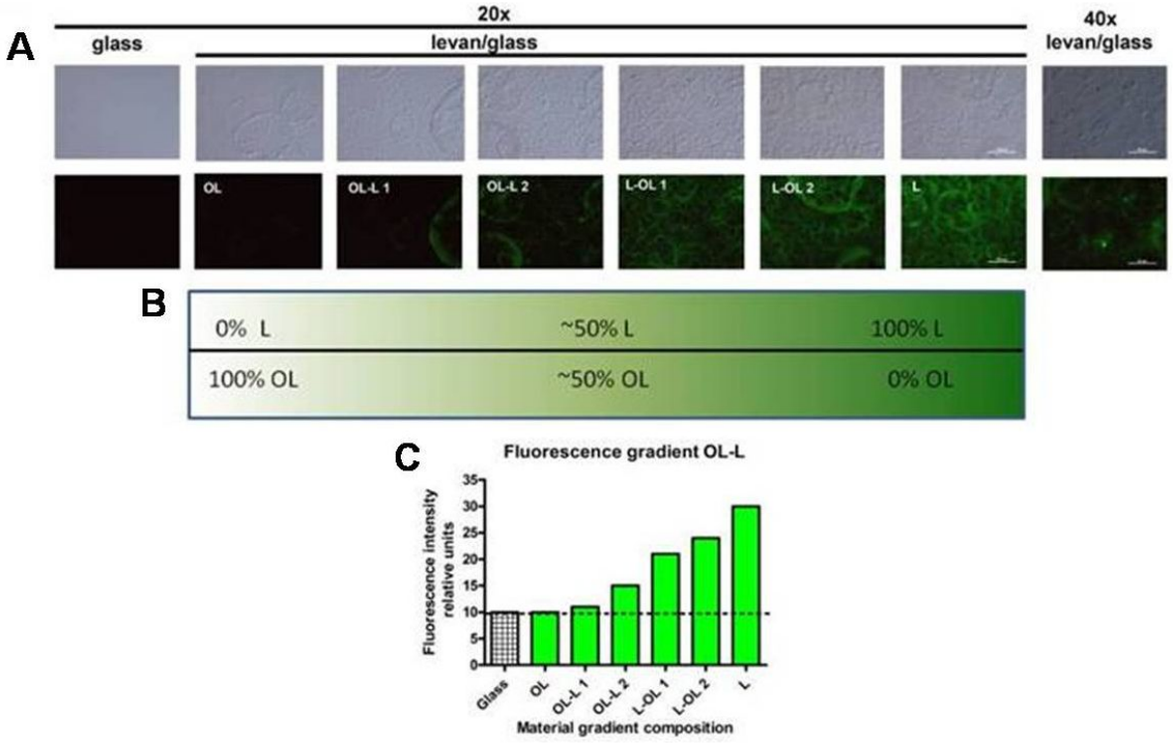

Figure 8. (A) Differential interference contrast and fluorescence microscopy images of OL (left) – L (right) gradient film obtained by C-MAPLE along the glass slide. Positive (Levan/glass) and negative (glass) controls are presented. Bar = 100 μm (20x) and 50 μm (40x). (B) Diagram of expected composition gradient obtained by C-MAPLE from OL and L targets. (C) Quantification of gradient regions fluorescence emission intensity using ImageJ histogram function. Glass background is set as threshold and depicted as dotted line (Reproduced with permission from [14]).

main result was the evident increased cell accumulation on L–OL film blends. This effect was explained by surface wettability associated with the presence of the appropriate amount of OL within L zones. Indeed, the degree of oxidation combined with surface hydrophilicity and roughness stay at the origin of improved bone cell proliferation on L–OL and OL zones. Interestingly, the cell density on OL areas was superior to standard cover slips. It was suggested that such compositional gradients could be used to screen specific nanostructured surface cues for tailoring cell proliferation or to modulate intracellular signaling pathways for specific biomedical applications [13, 14].

The new combinatorial laser technology opens the prospect to simultaneously combine and immobilize *in situ* and in well-defined manner two or more organic materials on a solid substrate by laser evaporation.

4.4. Overview of recent contributions in the field of thin films synthesis by MAPLE

An exhaustive list of other thin films grown by advanced laser techniques, as well as their physical- and biochemical characterizations for biomedical applications could be found in the literature [6, 81]. Several book chapters and review articles available to readers, spanning a broad coverage of both fundamental and applicative aspects, published in the last five years, are summarized in Table 2.

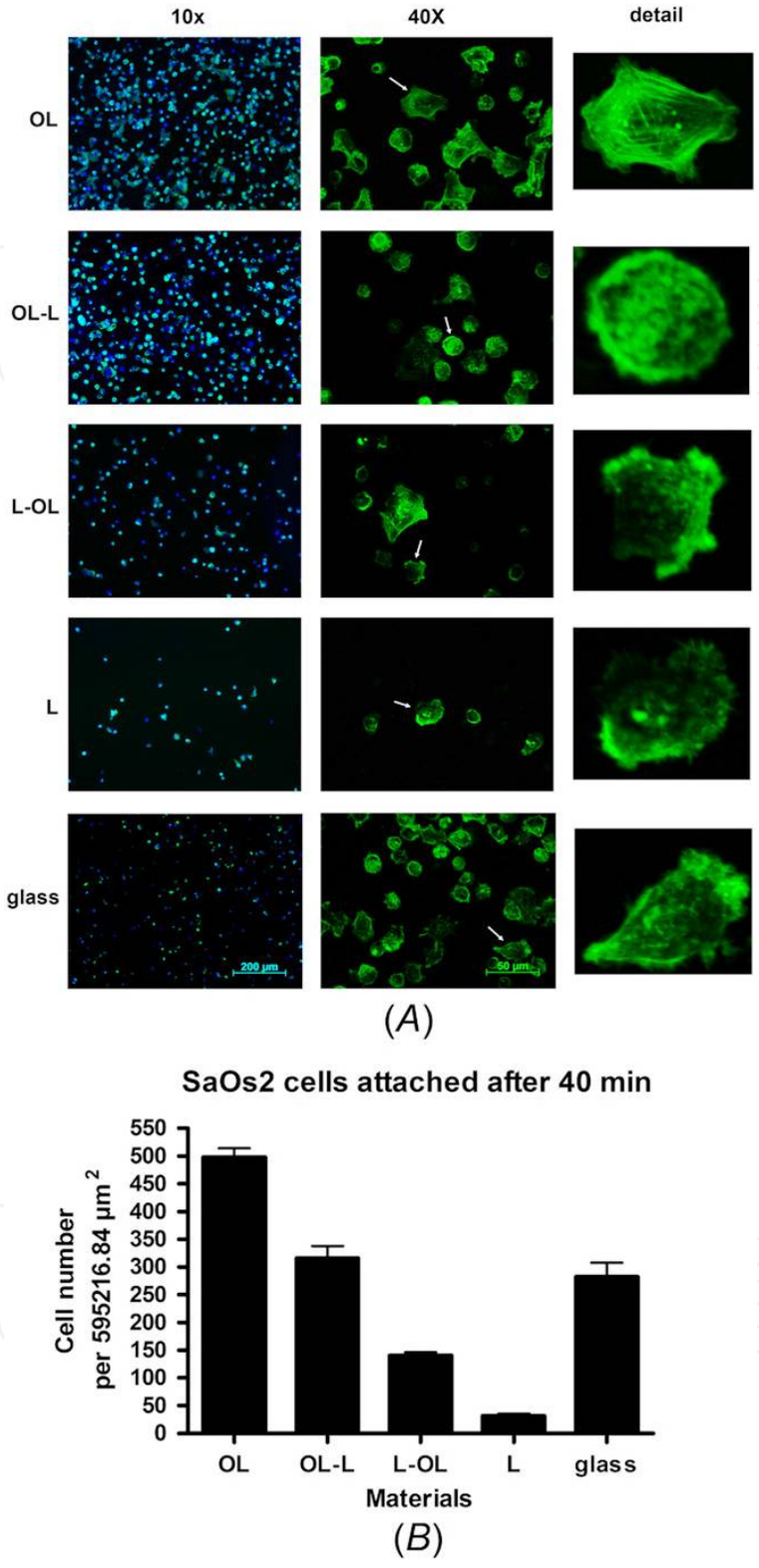

Figure 9. (A) Fluorescence microscopy of bone cells on combinatorial and control materials after 40 mins seeding. Different magnifications (10X and 40X) of cells labeled with Alexa Fluor 488-conjugated phalloidin (actin – green) and DAPI (nuclei – blue) are presented with details on cell morphology. (B) Quantification of cells by ImageJ nuclei counting function. Mean ± SEM is depicted on graph.

Materials	Applications	Title	Authors/Reference
Living cells enzymes, proteins and bioceramics.	Tissue engineering, stem cell and cancer research.	Topical Review: *"Laser-based direct-write techniques for cell printing"*	N.R. Schiele et al. [146]
Polymers (SXFA, POOPT (poly [3-(4-octyloxyphenyl) thiophene]), poly(9,9-dioctylfluorene) (PF8), Ge-corrole derivative (Ge(TPC)OCH3)), Proteins (horseradish peroxidase (HRP), insulin, bovine serum albumin (BSA)), Nanoparticles (TiO_2, SnO_2).	Biomaterials, gas sensing.	Chapter 9: "*Fundamentals and Applications of MAPLE*"	A. Luches and A. P. Caricato [7]
Hybrid organic–inorganic bionanocomposites [HA–sodium maleate (HA–NaM) copolymer, alendronate–HA].	Advanced biomimetic Implants.	Chapter 10: "*Advanced Biomimetic Implants Based on Nanostructured Coatings Synthesized by Pulsed Laser Technologies*"	I. N. Mihailescu et al. [8]
Polymers and biological molecules, biomaterials, nanoparticle films.	Drug delivery, tissue engineering, for gas and vapor detection, for light emitting devices, etc.	Review: "*Applications of the matrix-assisted pulsed laser evaporation method for the deposition of organic, biological and nanoparticle thin films: a review*"	A.P. Caricato, A. Luches [80]
Living mammalian cells and pluripotent stem cells (e.g., human dermal fibroblasts, rat neural stem cells, mouse embryonic stem cells).	In vitro cellular microenvironment, tissue engineering, regenerative medicine.	Review: "*Matrix-assisted pulsed laser methods for biofabrication*"	B.C. Riggs et al. [147]
Polymer and other soft matter thin films (Horseradish peroxidase, Ribonuclease A, Poly(ethylene glycol), Poly(3-hexyl thiophene), MEH-PPV).	Organic electronics, medical implants, drug delivery systems, and sensors.	Trends in Polymer Science: *MAPLE Deposition of Macromolecules*	Shepard, K. B. and Priestley, R. D. [148]
Biocompatible and biodegradable polymers ((PEG), (PLGA), mixtures	Biomimetic applications in drug delivery systems,	Chapter 5: "*Biomimetic Assemblies by Matrix-Assisted Pulsed*	F. Sima and I.N. Mihailescu [9]

Materials	Applications	Title	Authors/Reference
PEG-PLGA, poly(D,L-lactide), Levan); Extracellular matrix proteins (fibronectin, vitronectin); organic – inorganic composites.	biosensors and advanced implant coatings.	*Laser Evaporation*"	
Polysaccarides (Levan), Composite alendronate-HA, Enzyme ribonuclease A.	Biomimetic coating of medical implants, drug delivery systems, biosensing.	Chapter 11: "*Biomaterial Thin Films by Soft Pulsed Laser Technologies for Biomedical Applications"*	I. N. Mihailescu et al. [88]
Enzyme immobilization (Laccase).	Bio-Sensors.	Chapter 9: "*Deposition and Characterization of Laccase Thin Films Obtained by Matrix Assisted Pulsed Laser Evaporation*"	N. Cicco et al. [149]

Table 2. Overview of recent reviews and book chapters published in the field of thin films synthesis by MAPLE

5. Summary and perspectives

MAPLE synthesis of biopolymer thin film was applied to fabricate organized and nanostructured pure and hybrid polysaccharide layers. It was demonstrated that laser-based techniques allow for transferring complex, large molecular-mass organic compounds, avoiding their photo-thermal decomposition and/or irreversible damage. The functionality preservation was secured for Dextran and Levan coatings and derivatives, as revealed by cells' viability and proliferation *in vitro* tests. Combinatorial-MAPLE evidenced the possibility to generate compositional gradient thin films of two organic and/or inorganic compounds in a single-step process. Engineering the cell/biomaterial interface to control cell behavior has implications for the fabrication of instructive environments for tissue repair or cell supports.

The flexibility of the C-MAPLE method allows for the synthesis of new hybrid materials by correlating laser irradiation settings with the thermo-physical and chemical properties of the raw materials. This approach opens a great potential to the discovery of new drugs for the pharmaceutical industry and for drug release applications from biodegradable polymeric coatings. Intelligent materials synthesized on discrete areas exhibiting desired properties such as controlled rate of coating dissolution stands for a future challenge. An expansion of combinatorial organic domain can be stimulated by laser technologies. The strong advantages are the control of the preferred density of functional groups at the surface, among which we mention chemical composition and/or physical properties on nanometric areas and the fabrication of multicomponent gradient layers.

Acknowledgements

The authors acknowledge the support of UEFISCDI under the contracts 19_RO-FR/2014 and PNII-RU-TE-2014-4-1790, -1273 and -0396.

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