Chapter 5
Biomimetic Assemblies by Matrix-Assisted Pulsed Laser Evaporation

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Abstract The Matrix-Assisted Pulsed Laser Evaporation (MAPLE) technique emerged more than one decade ago as an alternative and complementary method to Pulsed Laser Deposition (PLD) in view of transferring organic materials onto solid substrates. In contrast to PLD, MAPLE proved to be a less harmful approach for transporting and depositing delicate, heat sensitive molecules. Since origin, MAPLE developed fast and was generally applied for organic biomaterials. It turned recently to inorganic compounds and has become a competitor to PLD. An important benefit of MAPLE is the capability of transferring films of nanoparticles with largely extended active areas. Such films can play an essential role in biology, pharmaceutics or sensing applications. This chapter reviews the mechanisms and recent progresses of MAPLE in thin film assembling for biomimetic applications in drug delivery systems, biosensors and advanced implant coatings.

5.1 Introduction

MAPLE was developed as a derivation of PLD and introduced [1, 2] for depositing thin films of organic and polymeric materials with a minimal thermal and chemical decomposition. In PLD, solid and compact inorganic materials are laser ablated and transferred via plasma onto a parallel substrate. Nevertheless, even for low laser energies, organic materials would be damaged by this approach. In MAPLE, organic biomolecules are dissolved in a laser wavelength absorbent solvent which is next frozen to form a solid target and exposed to laser irradiation. This way, the violent

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interaction of photons with the active material is diminished since the main fraction of energy is absorbed by the solvent. The energy is converted to thermal energy which helps to vaporize the solvent molecules entraining the organic molecules. The volatile solvent molecules are eliminated by vacuum system while organic molecules are reaching a facing substrate. There is a similarity of MAPLE with Matrix-Assisted Laser Desorption/Ionization (MALDI) [1, 3] but in the latter case the matrix is more complex from the chemical point of view and not appropriate for deposition.

With respect to solvent processing techniques, MAPLE preserves all the advantages of PLD and, in particular, it allows a better control of the film thickness and surface morphology, enhanced film/substrate adhesion, multi-layer deposition and patterning. Furthermore, being a non-contact procedure, eliminates a major source of contamination and can be integrated with other sterile processes. A more detailed discussion is found in Sect. 5.5.

Biomimetics emerged as a novel technological approach based on the biodiversity of the natural environment in order to reproduce the structure, physiology and function of biological entities. By mimicking the organization and the mechanisms in human body, the aim is to find solutions of replacing or repairing affected parts by the creation and design of new bioinspired materials. Human structural assembly is composed of complex configurations from nanometric to macroscopic scale. Although there were many attempts to develop it, the biomimetic nanotechnology is still in formative years, with no applications on the market. Nonetheless, exploring and imitating the biology at the nanometer scale is challenging and could bring new ideas and solutions in different domains such as tissue engineering, drug delivery systems or biosensors. During the last decade, one new approach of nanomaterials and of nanotechnologies focused on tissue engineering and regenerative medicine fields with the view of developing new tissue substitutes with superior biological properties [4].

Research in biomimetic tissue engineering and regenerative medicine takes advantage of biology inspired structures and mechanism and follows generally three directions: the development of bio-inspired functional surfaces for repair and regeneration of damaged tissues, the use of biomimetic drug delivery systems integrated into engineering materials in view of a controllable local release to prevent e.g. post-surgical infections in orthopedic implants, and to utilize engineered tissues with biologically implantable biosensor microdevices in order to monitor physiology and disease [5–8].

Development of Bio-Inspired Functional Surfaces For Repair and Regeneration of Damaged Tissue

Nowadays, metallic prostheses or bone grafts are used to repair bone defects. Titanium (Ti) and its alloys are the materials currently chosen in orthopedical and stomatological implants due to their high corrosion resistance in biological aggressive media, low mass density, high specific strength and biocompatibility [9]. However, in some cases implant failures due to encapsulation by a fibrous tissue or mismatch in elasticity modulus between bone and implant lead to revision surgeries [9]. To improve the regenerative capacity of bone tissue, bone associated materials are used

for two main reasons: mimic the environment and speed up the healing processes at interfaces [10–12]. Thin bioactive coatings were developed on implant surfaces to form solid chemical bonds between implant surfaces and bone tissue in respect with long-term stability [12, 13]. Inorganic materials such as calcium phosphates (CaP) and in particular, hydroxyapatite (HA), which represents the major mineral phase of native bone, bioglasses (BG) or CaP- and BG- based composite materials are the most widely studied in the literature [14, 15] due to their biological properties for bone bonding and protein adsorption. On the other hand, organic structures are interesting beside their biocompatibility, because of their biodegradability and mechanical properties. Collagen-based matrices with complex pore orientation, pore size and alignment anisotropy resembling the extracellular matrix (ECM) scaffolds [16], porous crosslinked chitosan hydrogels [17], silk fibroin scaffolds with good stability [18], fibronectin (FN) crosslinked within hyaluronic acid hydrogels [19] were suggested for damaged soft tissues regeneration and repair. In view of mimicking the mineral-organic composition of bone and interaction processes, inorganic-organic composite materials were focusing recently a great attention. HA-gelatin (HA-GEL) composites were extensively studied since gelatin is a protein obtained by hydrolysis of collagen, the main organic phase of bone [20–22]. CaP introduction into biopolymer matrices such as polylactic acid (PLA) or poly (lactic acid-co-glycolic acid) (PLGA) were found to improve mechanical performance as compared to CaP alone [23]. Poly(lactide-co-caprolactone) (PLCL) with good mechanical properties and a bone-mimicking gelatin-apatite system were combined into a functional composite membrane with improved biological functions for hard tissues regeneration [24]. In addition to composites, which are intended to interfacial tissue reconstruction between soft (e.g. cartilage) and hard (e.g. bone) tissues, gradient biomaterials of multilayer coatings were synthesized for repairing or regenerating the functions of damaged parts at the interface of different tissue types [25].

Use of Biomimetic Drug Delivery Systems

The second approach in biomimetic tissue engineering resorts to biomimetic principles that have been extended to drug delivery systems. Such systems are integrated into engineering materials in view of constructing cellular microenvironments for different biomedical applications. Delivery of a drug into a precise place via bioactive molecules is of a great significance for tumor or damaged tissues treatment. Based upon a biomimetic approach antibiotics with calcium phosphate coatings were obtained on titanium alloy (Ti6Al4V) substrates capable to release the drug with a pH-dependent rate [26]. This approach is considered helpful to prevent post-surgical infections in orthopedic implants. Calcium apatite has also been used for the retention and local delivery of osteogenic factors such as recombinant human bone morphogenetic protein-2 (rhBMP-2) to locally induce the osteogenic transdifferentiation, [27], which means that mature cells could differentiate into bone forming osteoblasts. Thermosensitive polymers connected with peptides act as a dual stimulus-sensitive polymer capable of both, forming a gel at body temperature and allowing degradation in the presence of peptides. The controlled biodegradation has potential application

in delivery systems, where the polymer gels can release the incorporated drug in a bioresponsive manner [28], sensitive to variations of physiological environment.

Engineered Tissues With Biologically Implantable Biosensor Microdevices

Bioreceptor entities such as proteins, peptides, enzymes or antibodies have been extensively studied for biosensing applications. In this respect, natural (biomimetic) recognition elements introduced in biomaterials extend the range of application of biosensors. Immobilization of biomaterials on artificial devices are usually attained by self-assembled monolayers (SAM), Langmuir-Blodgett (LB) films, or layer-by-layer (LBL) assembling [29]. Genetically manipulated proteins capable to fabricate 2D or 3D structures via bottom-up processes to control mineralization in biological systems were synthesized [30]. Engineered tissues containing biologically implantable biosensor microdevices which are able to observe tissue functions are expected to improve the feedback loop of implementation by the biosensor recordings [8]. Enzymes were stabilized via encapsulation in liposomes, polymers or gels to maintain their activity for longer time and be used as biosensors [31].

5.2 Biomimetic Design-Mimicking Aspects of a Natural Organism

Research studies demonstrated that all living systems are governed by molecular processes at nanometric scale. In particular, cellular organization and tissue characteristics depend on the extracellular matrix (ECM) composition. ECM is a non-cellular part present in tissues and organs and has significant roles in tissue morphogenesis, cellular differentiation or homeostasis [32, 33]. It is also involved in the physical arrangement of the cellular constituents. ECM is composed of water, proteins and polysaccharides acting as mediators between cellular components and the growing microenvironment [32]. It is a hierarchical complex structure with nanometer to the centimeter spatial order. Tissue-derived cells require strong attachment to a solid surface to ensure viability and growth. In contact with solid surfaces, the cells are adhering and communicating via integrins, trans-membrane receptors that mediate interactions between the cytoskeleton and the ECM. Studies confirmed the biological productive interaction of a material surface organized at nanometric scale [4]. In fact, human cells are in permanent contact with nanostructured surfaces [34]. In case of an implant, surface features (such as morphology, composition and structure) noticeably influence the adsorption of proteins which consequently mediate the cellular adhesion, proliferation and differentiation [35, 36].

Following a biomimetic approach, the future biomaterials will benefit from the understanding of the biological processes such as wound healing or inflammation and will develop reactions with high accuracy. The natural materials are generally composites based on polymers and minerals exhibiting a diversity of properties depending on their structure at various length scales [37]. To avoid an uncontrolled cellular

response to surfaces, both, nanoscale and microscale characteristics are therefore desired. In a recent review, porous structures were proposed for tissue replacement and regeneration that could mimic hierarchically structured porous natural materials [38]. It was stressed upon the different scales of porosity in natural materials (which ranges from nanometers to millimeters): pores with small dimensions (nanometers) are in charge with the bioactivity and protein interaction, micrometer sized pores are involved in cellular attachment, while larger ones are responsible for cellular growing, blood flux, mechanical resistance and implant functionality. In this view, the use of inorganic materials (such as ceramics) is not always convenient for the structural or functional restoration of a tissue, particularly in large bone defects. It exists therefore a growing interest to deliver and release in a controllable manner biologically active proteins, such as growth factors, adhesion proteins, or antibodies via inorganic layer dissolution and to stimulate the cellular response toward mineralization [39]. Deposition of composites, patterns or multilayer structures were found clinically significant and innovative designs were proposed [40].

A material-inspired strategy of nano-assembling in biomedical applications for enamel repair was recently reported [41]. The combined action of a glutamic acid and nano apatite particles was capable to regenerate an enamel-like structure under physiological conditions.

A novel design based on graded materials was proposed. The structure consists of layers of biomineralized collagen, hyaluronic acid-charged collagen, and an intermediate layer of the same nature as the biomineralized collagen, but with a lower content of mineral. The aim was to develop composite osteochondral scaffolds organized in different integrated layers with biomimetic features for articular cartilage and subchondral bone [42].

5.3 Scaffold Fabrication and Deposition Methods

One novelty in the field of biomimetics is the hybrid deposition methods with the view of obtaining multilayer or nanocomposite structures for new applications and improved performances. Many attempts were mentioned to combine magnetron sputtering with cathodic arc deposition, plasma-enhanced chemical vapor and electron beam evaporation, or flash evaporation with physical vapor deposition to synthesize innovative inorganic films, which are reviewed comprehensively in [43]. In case of organic thin films it is mandatory to avoid thermal phenomena. Techniques such as sol-gel, dip-coating or spin-coating which are in present employed to obtain thin films of polymers or proteins are difficult to extend to multistructure generation because of solvent implication and combination problems.

In recent years, there was a major interest in thin coatings technology to fabricate multilayer coatings or high spatial resolution patterns on solid substrates with the view of developing appropriate deposition techniques for available biomaterials in suitable shape for specific applications [44, 45].

Table 5.1 summarizes widely used materials and deposition methods along with specific advantages, drawbacks and relevant examples.

Multi-nozzle Deposition Manufacturing (MDM) was introduced as a non-thermal process for manufacturing porous scaffolds for tissue engineering [14]. To obtain inorganic-organic composites, MDM was applied in a single-nozzle deposition process where the material was prepared by dissolving poly (L-lactic acid) into dioxane and mixing with tricalcium phosphate (TCP) particles. Thus, a macroporous scaffold structure was obtained by phase separation and solvent sublimation while consequently a growth factor such as BMP (bone morphogenetic protein) could be loaded into the scaffold by vacuum suction.

Organ-printing technology is a rapid prototyping computer-aided 3D method based on layer by layer deposition of different types of hydrogels and cells in order to fabricate 3D constructs for perfused, vascularized human tissues or structural and functional units of human organs [65].

Direct assembling of cells and extracellular matrices for the construction of functional 3D tissue/organ substitutes was achieved by an optimized cell-assembly printing technique [66].

However, rapid prototyping techniques working with hydrogels, laser-based, nozzle-based or printer-based systems, suffer from poor mechanical strength [67].

Another method to manufacture 3D hierarchical structures is the layer-by-layer microfluidics process which involves immobilization of a cell-matrix assembly, cell-matrix contraction, and pressure-driven microfluidic delivery to fabricate hybrid biopolymer structures for tissue engineering [68]. Electrospinning enables the fabrication of scaffolds with micro and nanoscale topography and high porosity [69]. A new generation of scaffolds comprising living cells was developed by electrospinning technology. Antibiotics, proteins as well as living cells were incorporated into the advanced scaffolds and electrospun [70].

Direct laser writing techniques such as Laser induced forward transfer (LIFT), allow for the deposition of biomolecule patterns without degradation and with high spatial resolution. Under the action of a laser pulse focused through a transparent layer on a thin metallic film, a small fraction of an organic material coating is transferred to a receptor substrate, which is placed closely and parallel. In this configuration, biomaterials such as polyethylene glycol and eukaryotic cells, were deposited with a spatial resolution of $\sim 10\,\mu m$ without damage of structures or genotype [40]. In other studies, microarrays of DNA have been spotted and were found capable to maintain gene discrimination capacity [60].

Biological laser printing (BioLP) has been proposed as an alternative to the above mentioned techniques for assembling and micropatterning biomaterials and cells. High-throughput laser printing of a biopolymer, hydroxyapatite and human endothelial cells was achieved demonstrating the capability of the method for three-dimensional tissue construction [59]. LIFT-derived cell seeding pattern was shown to modify the growth characteristics of cell co-cultures resulting in vessel formation and in an efficient regeneration of infarcted hearts after transplantation of a LIFT-tissue engineered cardiac patch [71].

Table 5.1 Common deposition methods of biomimetic thin films

Method	Advantage	Disadvantage	Materials
Plasma spray (PS)	Simple, not expensive	Poor coating adhesion to substrate/ weak ceramic-metal interface, limited to inorganic coatings	Hydroxyapatite (HA)/ Bioglass (BG) [46]
Radio frequency magnetron sputtering (RF-MS)	Uniform dense coating	Amorphous coatings, expensive, limited to inorganic coatings	Hydroxyapatite (HA)/ Bioglass (BG) [47, 48]
Pulsed laser deposition (PLD)	Film stoichiometry (especially for doped materials) and good adhesion	Small covering areas, limited to inorganic coatings	Hydroxyapatite (HA)/ Bioglass (BG) [13]
Sol-gel (SG)	Application to both organic and inorganic coatings	Poor coating adhesion; Involvement of liquid media limit multi-layer assembling	Hydroxyapatite (HA)/ Bioglass (BG) [49]
Electrophoretic deposition (EPD)	Simple processing equipment, application to both organic and inorganic coatings	Poor coating adhesion; Involvement of liquid media limit multi-layer assembling (affect interfaces)	Hydroxyapatite (HA)/ Bioglass (BG) [50]
Electrostatic deposition (ED)	Solvent-free coating process	Limited to single coating	Poly (lactic acid-co-glycolic acid) (PLGA) [51]
Sol-gel (SG)	Macroporous bioactive scaffold	Poor coating adhesion; Involvement of liquid media limit multi-layer assembling (affect interfaces)	Chitosan/BG composite [52]
Layer-by-layer (LBL)	Viscoelasticity/bioactivity	Involvement of liquid media limit multi-layer assembling (affect interfaces)	Chitosan/BG composite [53]
Adsorption on surface	Simple, rapid	Poor adhesion and uniformity, Involvement of liquid media limit multi-layer assembling (affect interfaces)	Fibronectin (FN), vitronectin (VN), collagen I [54]
Spin coating	Simple, uniform coatings	Solvent issue during multilayers, adherence	PLGA/polycaprolactone (PCL) composite [55]

(continued)

Table 5.1 (continued)

Method	Advantage	Disadvantage	Materials
Langmuir-Blodgett dip coating	Monolayers	Limited to very thin films	Glucose [56]
Ink-jet printing	Thicker films	Possible nozzle blockage for composites	PCL/chitosan [57]
Multi-nozzle Deposition Manufacturing (MDM)	Porous structures	Possible nozzle blockage for composites	Poly (L-lactic acid) (PLA)/tricalcium phosphate (TCP) [14]
Laser-induced forward transfer (LIFT)	Patterns with high spatial resolution	Limited to patterns; difficulties for large area thin coatings	Hydroxyapatite (HA) [58, 59], proteins [40, 60, 61]
Matrix assisted pulsed laser evaporation (MAPLE)	Nanoparticulate films, application to both organic and inorganic coatings multilayers and multistructures	Small covering areas	Fibronectin (FN) [62], vitronectin (VN) [63], Octocalcium phosphate (OCP) [64]

2D structures of hybrid polymers were produced by Two-Photon-Polymerization (2PP). The scaffolds were tested in vitro for applications in tissue engineering with the aim of developing a dermal graft [72, 73].

One alternative single step fabrication method of complex constructions of multilayers and multistructures is MAPLE due to the potential of transferring and depositing both organic and inorganic materials. Because the transfer is supposed to be dry (the solvent molecules are eliminated during the transfer by vacuum pumping) the solvent implication is circumvented while the synthesis of multilayer structures keeps rather simple. The method is a non-contact technique which proved very versatile and challenging in respect with other laser based techniques [1, 74, 75]. One can produce by MAPLE coatings with adhesion better than by other methods whilst film uniformity and thickness on either rough or flat substrates can be well controlled. Moreover, using appropriate masks in MAPLE one can manufacture microsized samples (single or multilayered) for microarray chip applications [40].

5.4 Basics of MAPLE

Since its invention in late 1990s [2] as an alternative to spray coating of thin films for chemical vapor sensors [76], MAPLE was successfully applied to a large class of organic compounds for various applications [1, 77]. Thin films of pullulan [78] or triacetate-pullulan [79] polysaccharides for drug delivery, polyfluorene and polythiophene copolymers for metal-insulator-semiconductor and field-effect transistor (FET) structures [80], chemoselective polymers for microsensors [81] or proteins with applications in tissue engineering [62, 82] or biosensing [83, 84] were obtained. A recent review on MAPLE deposition of organic, biological and composite thin films summarized several potential applications of thin coatings obtained by this method [85]. MAPLE was recently used to deposit uniform, ultra stable and nanostructured glassy polymer films with superior thermal and kinetic stability [86].

5.4.1 Experimental Conditions and Mechanisms of MAPLE

According to the introductory remarks about the principle and prerequisites of MAPLE, a typical MAPLE experiment starts with the preparation of a homogenous solution consisting of a small amount of solute (0.5–5 % wt) dissolved in a solvent (matrix). Next, the solution is frozen with liquid nitrogen to obtain a solid target (Fig. 5.1), which is cooled and kept frozen during the laser irradiation and deposition process.

Adequate deposition substrates are chosen according to specific applications or analyses to be carried out. Before use, they are purged with acetone in an ultrasonic cleaner, ethanol and deionized water and dried with high purity N_2 gas. Subsequently, the substrates are placed parallel at an optimized distance in front of the

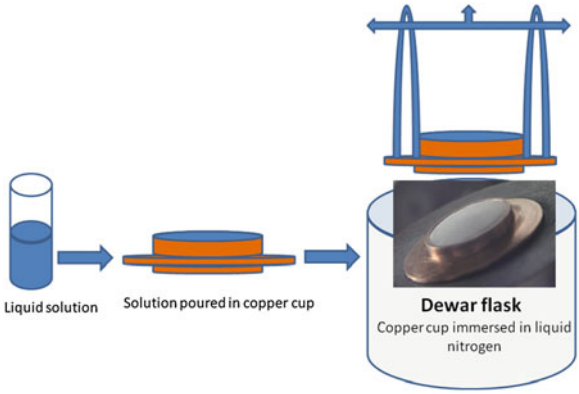

Fig. 5.1 Preparation stages of a solid frozen target starting from a liquid solution

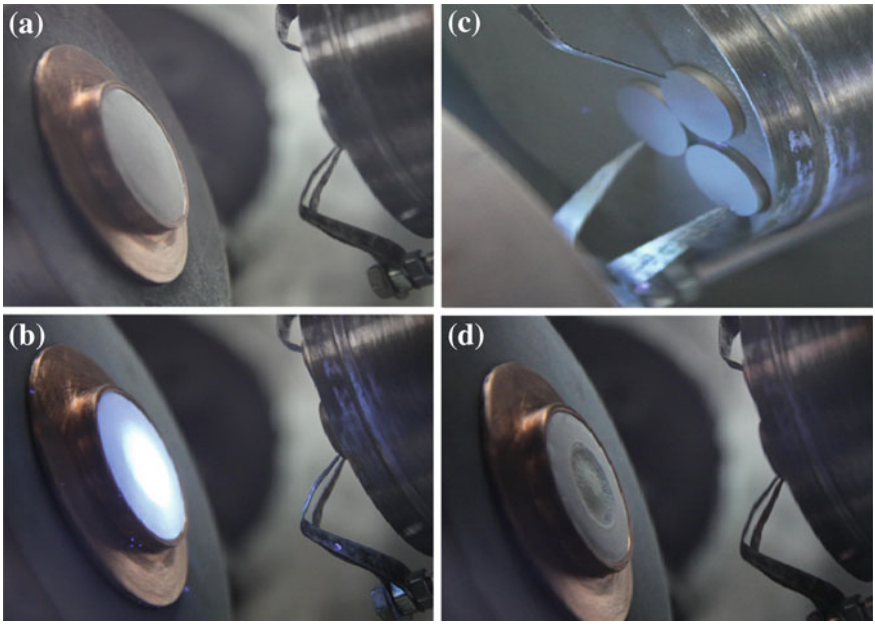

Fig. 5.2 Photos of a MAPLE configuration inside the reaction chamber: non-irradiated target mounted on cooler (**a**), cryogenic target illuminated during laser irradiation (**b**), deposition substrates dimly lit by irradiated target (**c**) and eroded target after multi-pulse laser irradiation (**d**)

rotating frozen target in the reaction chamber and gently heated below the degradation temperature of the solute (Fig. 5.2).

The solvent plays an important role in the MAPLE process, since it is the carrier of the solute molecules. Therefore it must be chemically inert and must not interact with the solute during laser irradiation. The frozen solvent must efficiently absorb

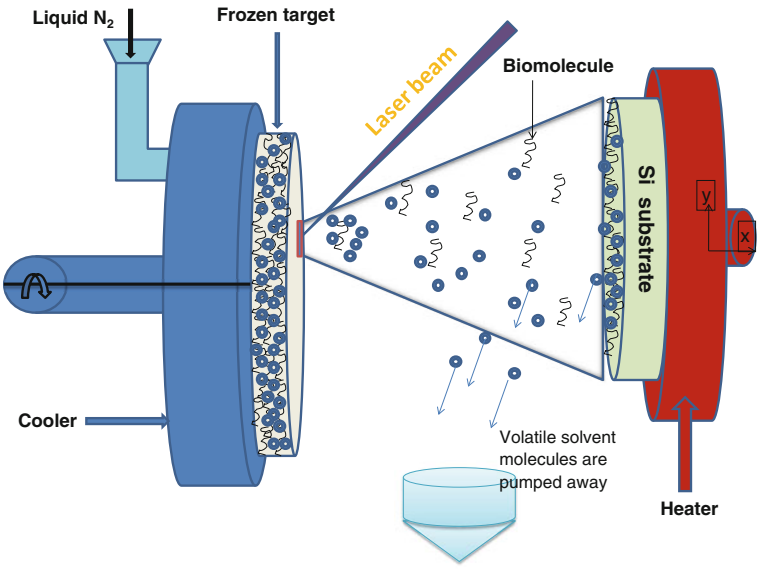

Fig. 5.3 MAPLE scheme. The solution is frozen and forms the target (*left*) which is cooled by a liquid nitrogen flow and maintained in constant rotation and translation. The laser beam (*center top*) irradiates the target, disrupting material from the surface which is deposited on the heated substrate (*right*). A warm substrate encourages solvent evaporation and adherence of film

the incident laser power and be easily evaporated. The volatile solvent molecules are vaporized and evacuated from the reaction chamber by the pumping system, guiding the solute molecules onto the substrates where they are deposited without degradation (Fig. 5.3). The most important MAPLE parameters are laser fluence, laser pulse repetition rate, substrate temperature, and target-collector separation distance. A dynamic pressure ($<10^{-1}$ mbar) is maintained inside the deposition chamber during the process. Typically, several hundred up to few thousand laser pulses are applied in order to achieve the desired thickness of the growing film.

The preferred laser systems in MAPLE experiments are pulsed UV lasers, such as excimer lasers (ArF* (wavelength: 193 nm), KrF* (wavelength: 248 nm), XeCl* (wavelength: 308 nm)), which generate pulses with a duration in the range of 20–30 ns or solid state Nd:YAG lasers (wavelength: 266 nm (fourth harmonic) or 355 nm (third harmonic)) with durations in the range of 5–10 ns. A typical applied laser fluence varies from 0.05 to 1 J/cm^2 [87]. In addition, a uniform intensity over the laser spot is generally desired (top hat profile) for a defined and stable material evaporation. At first sight, it seems difficult to apply MAPLE for the coating of large substrates due to the concentrated and localized (point-like) laser–material interaction. This drawback is circumvented by rotating and translating the substrates.

Parallel to the experimental efforts related to MAPLE, theoretical considerations aiming at the explanation of the underlying processes were made. In this context, a model based on *complete evaporation* process was proposed for the interaction of the

laser beam with the frozen target [88]. Namely, it is supposed that the laser energy is absorbed by the matrix, converted into thermal energy necessary for the solvent molecules to be vaporized. Next, the solute molecules are transported by collisions with solvent molecules and deposited on facing collector.

Later on, molecular dynamic simulations demonstrated that a consequence of the interaction between a laser beam and the cryogenic target is the formation of solvent-solute clusters due to overheating [89, 90]. In support of the proposed *explosive-boiling* model, it was shown that the ejection of material can be the result of explosive evaporation or spallation also described as "cold laser ablation", a phenomenon which becomes more evident when increasing the fluence. This results in the deposition of thin droplet coatings with high roughness morphology. Another mechanism based on *local overheating* of absorbing outmost surface layer of biomolecules was proposed [91]. More concretely, the solute molecules are also absorbing laser energy and their temperature is increasing. The heating is transferred to the solvent which, under vacuum, starts boiling just above melting point. The material ejection is consequently produced at lower temperature than the degradation threshold.

A *nonhomogeneous absorption* mechanism was proposed as well. This mechanism could account for the two cases: for low laser fluence the mass ejection is produced by surface evaporation while at higher fluencies hydrodynamic ablation mechanisms is responsible for the expulsion [92]. A frozen target composed of solute dissolved in a solvent also includes 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 [92]. 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. An example is visible from Fig. 5.4 where the absorbance level at e.g. 248 nm is indicated for distilled water (d.w.) and solutions containing organic (TRIS–tris(hydroxymethyl)aminomethane), inorganic salts (NaCl) and proteins (bovine serum albumin–BSA) before and after freezing. These salts act in two ways: as protein stabilizers and absorption centers during laser-target interaction.

Based upon the above mentioned theoretical considerations, which confirm the important role of the chosen solvent regarding the properties of the ejected particles, films with quite small roughness and improved surface morphology were recently obtained [93–95]. In this view, properly selected solvent and a reduced solute concentration in the target allowed for an optimum absorption regime. Perfectly matching experimental conditions such as a proper selection of the solute-solvent mixture, suitable laser source (laser wavelength with respect to spectral absorption of the solute-solvent mixture), dynamic pressure, substrate temperature and target–substrate separation distance are important prerequisites for an optimized MAPLE transfer and deposition of the solute on the solid substrate.

Hence, a major issue within a MAPLE deposition process is related to the laser-target interaction, which essentially depends on the used solvent or solute. Moreover, the process becomes more complicated when introducing supplementary compounds in the solution. A MAPLE protocol, which defines appropriate experimental condi-

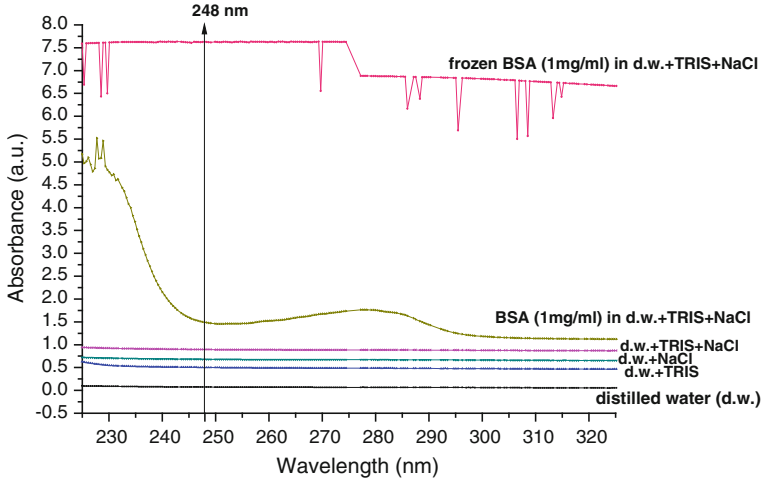

Fig. 5.4 Optical absorption spectra of pure distilled water, distilled water + TRIS (50 mM), distilled water + NaCl (150 mM), distilled water + TRIS (50 mM) + NaCl (150 mM), distilled water + TRIS (50 mM) + NaCl (150 mM) + BSA (1 mg/ml) and frozen solution of distilled water + TRIS (50 mM) + NaCl (150 mM) + BSA (1 mg/ml)

tions, should therefore be elaborated for each investigated solvent-solute mixture and laser wavelength combination.

Thin coating processes are "wet" when the method involves solvent flow coating and air drying and "dry" when the liquid phase is avoided. A still open question is if the MAPLE process could be considered wet or dry. The concern is related to the possible transfer of solvent molecules along with the solute on the substrate. In this case, MAPLE could be considered wet. For example, in case of not very volatile solvents such as DMSO, some molecules are reaching the substrate and remain immobilized along with the solute. It is not dramatic in this case since DMSO is considered biocompatible. However, some further interaction with the biopolymer molecules or other film layer could affect morphology or even composition. MAPLE can be considered dry when using very volatile solvents such as toluene. This solvent is definitely undesirable, in particular for further biological investigations, but it is entirely pumped out by vacuum system and is not reaching the substrate. In case of proteins, water is completely eliminated during transfer but part of the buffer (inorganic and organic salts) is expected to reach the substrate and further act as stabilizers. We mention that nowadays, wet processes are employed in pharmaceutical production to coat drug tablets with a thin film which allows for the controlled release of the active substance. Dry processes are advantageous for multi-component coatings and are environmentally safe by avoiding the waste of organic gas or aqueous stream. The choice of a good solvent is therefore one important key to the successful transfer by MAPLE.

5.4.2 Reliability

Since polymers and proteins easily denaturate, precipitate or aggregate, special attention should be paid to the preparation of a homogenous starting solution. It is emphasized that this is a prerequisite for reproducible sample fabrication, especially in the field of biomimetics involving cell technology. Each material demands individual treatment and exposure conditions and is therefore discussed separately:

Deposited Polymer Films

Reproducible samples require well controlled film composition and morphology. The cluster formation after target irradiation by using a low solute concentration and low laser fluence should be avoided. This regime results on one hand in a reduced deposition rate and hence a slow film growth, but on the other hand yields a smoother film surface and composition conservation. Some morphological features specific to MAPLE deposited films were assumed to origin from evaporation of solvent remains in the coating, which accompanied the solute molecules to the substrate in case of explosive evaporation or spallation of target. This happens e.g. in case of not very volatile solvents like DMSO, for which the solvent molecules are reaching the substrate along with the solute. There, the solvent is slowly evaporated since the temperature of the substrate is moderately heated [96]. Heating carefully the substrate just below the degradation temperature of the solute one could improve the film assembling and morphology by better controlling the adhesion of the film and avoiding the formation of non-homogenous zones. Besides composition and morphology, the film thickness should be carefully monitored since MAPLE is not always an additive process. Thus, in some cases, when increasing the amount of solute deposited on the substrate it tends to become more compact then growing in thickness. This is not generally applicable and it happens for low quantities of transferred material. A combined effect of evaporation induced assembly after transfer of solute-solvent clusters with the specific linkage between the linear structures of polymers was found to influence film growth [93].

Deposited Protein Films

Proteins are sensitive biological macromolecules, consisting of one or more amino acids, which are held together by peptide bonds. While they are stable under normal ambient biological conditions (especially temperature), they are easily disrupted and denaturated at elevated temperatures [97]. Consequently, intense laser irradiation and the associated heating can cause irreversible structural changes of proteins, which affect drastically the protein or cell activity. Thus, a compromise must be sought between the protein stability in the solution, its freezing and the safe laser transfer and immobilization on the substrate. For this purpose, colloidal solutions containing organic and inorganic salts, which are beneficial for protein stability and increase the laser absorption of the matrix while protecting the protein, are used (Fig. 5.4). The aqueous solution is softly homogenized while its freezing is induced rapidly drop by drop by pouring in an already cooled copper container at liquid nitrogen temperature

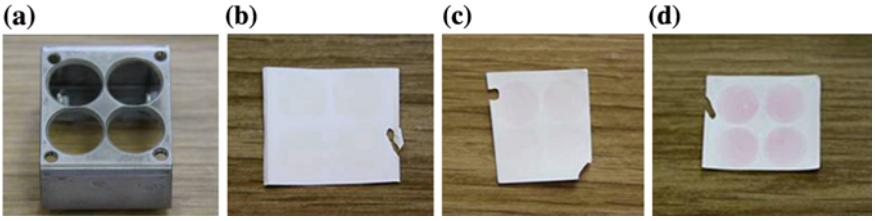

Fig. 5.5 Ponceau Staining on nitrocellulose paper after MAPLE protein transfer. Staining of bovine serum albumin (BSA) on nitrocellulose through a mask with four circles of 12 mm diameters: the used mask (**a**), staining of BSA after the MAPLE transfer from solutions of 0.1 mg/ml (**b**), 0.25 mg/ml (**c**) and 1 mg/ml (**d**)

(to ensure target homogeneity in volume). During freezing the protein remains folded to preserve its structure. After laser transfer, proteins are usually adsorbed onto solid surfaces via electrostatic and hydrophobic forces. The protein recovering from frozen state on surface is preferred in order to get the desired bio-effect. To this aim, various functionalized substrates should be tested. To control the material spreading from target and the deposition distribution on substrate in order to uniformly collect the solute, experiments on nitrocellulose paper (known to exhibit a good protein affinity) can be carried out at different target-substrate separation distances which stand for an important issue in MAPLE transfer. The proteins are collected through masks on paper surfaces of identical areas with actual deposition substrates (Fig. 5.5).

The deposition area is visualized by staining with Ponceau S Solution [62, 98]. By monitoring the gradient of color intensity on the nitrocellulose paper, the optimum separation distance between the target and the collector is selected as the best compromise between deposition efficiency and distribution uniformity [21]. To quantify the amount of protein deposited by MAPLE, a bicinchoninic acid (BCA) assay can be performed using bovine serum albumin (BSA) dilutions as standard [99]. The spectrophotometry data indicate the amount of deposited protein on area of interest [63].

5.5 MAPLE: From the Origin to Biomimetics

5.5.1 Application to Organics

MAPLE provides a more gentle mechanism than PLD for transferring different compounds, including large molecular weight molecules. It is generally acknowledged that PLD is limited in case of organics due to the high laser intensity which can cause irreversible damage to polymer or protein chains. Nevertheless, there were a few attempts to apply PLD to polymers at very low laser fluence [100–103]. The technique failed, in particular, in case of more sensitive biopolymers [78, 104], enzymes or proteins [91, 92].

MAPLE was applied from the very beginning to organic biomaterials in order to develop structures, which mimic parts of organism [105]. The huge number of materials with potential interest for biomedical applications allowed for a fast expansion of the method which rapidly improved and developed in search for an appropriate niche. The following survey illustrates the development and application scenarios of MAPLE:

Polymers for Drug Delivery Systems

Biodegradable polymer coatings are applied to implants for controlled and local drug delivery. A poly(DL-lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) composite in a multilayer configuration was deposited by spin-coating technique and dipyridamole was loaded as a drug into the surface nanopores [55]. This configuration was found effective as drug-delivery platform over 70 days for drug-eluting implants, in particular for cardiovascular stent applications. A PLGA coating with sirolimus (rapamycin—an immunosuppressant drug used to prevent rejection of an implant) was deposited by a dry-powder electrostatic process. During the 90 days of coating absorption, the drug was released and prevented inflammation [51].

Thin coatings of biocompatible and biodegradable polymers with potential as carrier vectors for drug delivery such as polyethylene glycol (PEG) [40], (PLGA) [96], mixtures PEG-PLGA [106, 107], poly(D,L-lactide) [108] or triacetate-pullulan [78] among others were obtained by MAPLE without noticeable chemical degradation. Among biomimetic "smart" solutions, some fascinating materials (e.g. polysaccharides such as chitin which is found in fungal and yeast cell walls or mollusk shells [109] or other biopolymers from microbial sources [110]) with exceptional properties are developed by biological organisms. In particular, Levan is an exopolysaccharide which can be used as food or feed additive and is distinguished from other polysaccharides by its low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid and alkali media, high holding capacity for water and chemicals, and good biocompatibility [111–113]. MAPLE application to obtain nanostructured thin films of Levan was reported for the first time in [93]. Thin coatings of desired thickness could be attractive to control the rate of dissolution for drug release and delivery applications. The nanostructure feature has the potential to enhance the biopolymer specific surface area for applications as carriers in drug delivery systems. An unusual ordered array was observed by AFM (Fig. 5.6), the most probably forming by the solvent (DMSO) evaporation induced nano-assembling combined with the specific linkages between the linear structures of polysaccharides. MAPLE samples exhibited a compact structure, with good adhesion to substrate and a homogenous nanostructured surface, fully compatible with potential use in biology or medicine [93].

Proteins and Enzymes for Biosensors

Biomimetic materials with sophisticated three-dimensional design, well-defined pattern and tunable properties used for drug carriers and tissue engineering can be also used to monitor biological microelectromechanical systems and diagnostics. They can respond to in vivo environmental changes and secure controlled parameters for

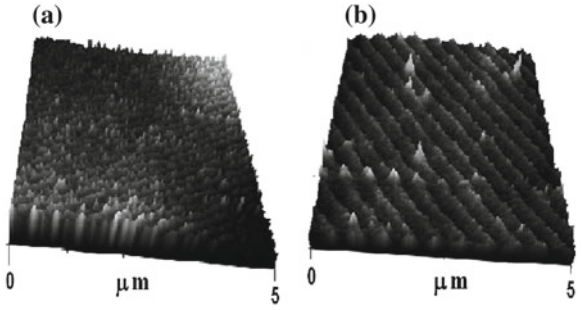

Fig. 5.6 Typical AFM images of polymer surfaces for (**a**) Levan and (**b**) Oxidized Levan coatings by MAPLE on Si. Reprinted with permission from Biomacromolecules [93]

drug release, cell interaction, mechanical properties, or permeability [114]. The need to elucidate fundamental mechanisms of growth and the structure of biological systems in response to new biomaterials is one challenge for developing miniaturized protein or enzyme based biosensors [109]. MAPLE could provide in this respect an alternative to "wet" methods in view of obtaining patterns [105] or multi-structures since the solvent issue is avoided [94]. The accurate control of the expulsed material and layer thickness or the uniform and homogenous distributions of the material on the substrate are the real advantages of the method.

Insulin and horseradish peroxidase (HRP) were the first proteins deposited by MAPLE [115] as active biomolecules that could be used in biosensors with the goal of fabricating a functional microfluidic device. In the same study efforts have been paid to develop a polymer (poly(ethylene glycol -PEG)–protein(HRP) composite film with increased adhesion to surface. The structure and activity of the proteins were found unaffected and the method was proposed for depositing active biomolecules for sensor or microarray applications.

MAPLE was applied to immobilize urease (an enzyme which catalyses the hydrolysis of urea in biological systems, monitoring the nitrogen concentration of the human serum in the form of urea–a measure of the kidney function) on solid collectors with the aim to develop a sensor based on a biomimetic principle, a strategy that mimic natural processes, with interest in clinical applications [116]. The immunoassay, one of the most used analytical method based on the selective affinity of the biological antibody for its antigen, was applied to show that MAPLE-immobilized IgG films can be used as immunosensors for the detection of specific antigens in research or clinical investigations [83]. It is noted that IgG molecules are able to struggle with bacteria and viruses while a quantitative antibody test is clinically indispensable for autoimmune diseases, allergies and recurring infections [117]. To this prospective, the observed morphology change by the content of salts and lipids in MAPLE solution could open the door to reach the best compromise between the IgG content and surface condition over sensing capabilities, an essential step in developing personalized and miniaturized biosensors.

Proteins for Tissue Engineering

Sophisticated synthetic (tissue-engineered) and multi-(bio)functional surfaces or bone scaffolds were proposed to build up a microarchitecture that integrates different biological entities such as proteins, cells and cell processes [118]. An ideal synthetic engineered biomaterial should be biocompatible, biodegradable and to mimic the hierarchical structure of native tissue with the view of promoting actively desirable physiological responses (bioactive). This will avoid additional surgical procedures and reduce risks of infection [5]. In order to accelerate extracellular matrix (ECM) production, enhance cellular activity in the early stage of a material implantation and push its tissue integration, a common approach relies upon the presentation of the arginine-glycine-aspartic acid (RGD) adhesive sequence derived from fibronectin (FN) (glycoprotein present in ECM that interacts with cells to control cell adhesion, cytoskeletal organization and cellular signaling) [119, 120]. More specifically, in the field of biomaterials for bone reconstruction, FN has been proposed for enhancing osseointegration [121].

Deposition of FN by MAPLE from saline buffer-based cryogenic targets was reported. The aim was to transfer and immobilize a high molecular mass protein such as FN (∼450 kDa) on a flat Si surface by a controllable approach in order to obtain a biologically active protein structure. Under these conditions, FN is exposing binding sites that promote intermolecular interactions and cell adhesion and consequently cell proliferation speeding up tissue formation around implant and a faster stabilization. A rather rough surface with a particulate-like morphology was observed. The particulates were uniformly distributed floating on a base film on substrates. The presence of particulates could be beneficial for e.g. orthopedic applications because they increase the specific surface area and thus the binding capacity of MAPLE coated implants to tissues. No noticeable changes were detected of FN structure after the MAPLE transfer. Different organization of intact protein, from small aggregates to fibril-like forms were observed while human osteoprogenitor cells grown on FN thin films exhibited a superior attachment as compared to controls [62] (Fig. 5.7). A similar cytoskeleton morphology was found in osteoblast-like cells grown on intact FN as compared to cells grown on FN fragments [122] suggesting that MAPLE-transferred FN forms patterns with non-denatured and functional cell binding domains. It was also demonstrated that FN adsorption on apatite/nanodiamond films or HA-coated solid substrates improved cellular attachment, adhesion and spreading [123, 124].

A layer-by-layer coating with heparin, growth factor and FN of titanium surfaces were found to improve cell proliferation while multilayer films significantly promoted cell attachment and growth [125].

Three ECM proteins (FN, vitronectin (VN), and collagen I) were in parallel tested and proved that they can play a role in wound and tissue repair [54]. VN is another glycoprotein present in serum and the extracellular matrix which promotes cell adhesion and spreading [126]. It was recently shown that a VN coated Ti implant improved primary fixation in vivo resulting in an increased osteointegration [127]. Human osteoprogenitor cells grown on MAPLE transferred VN on HA coated Ti samples exhibited an improved adherence, spreading and growth compared to cells grown on

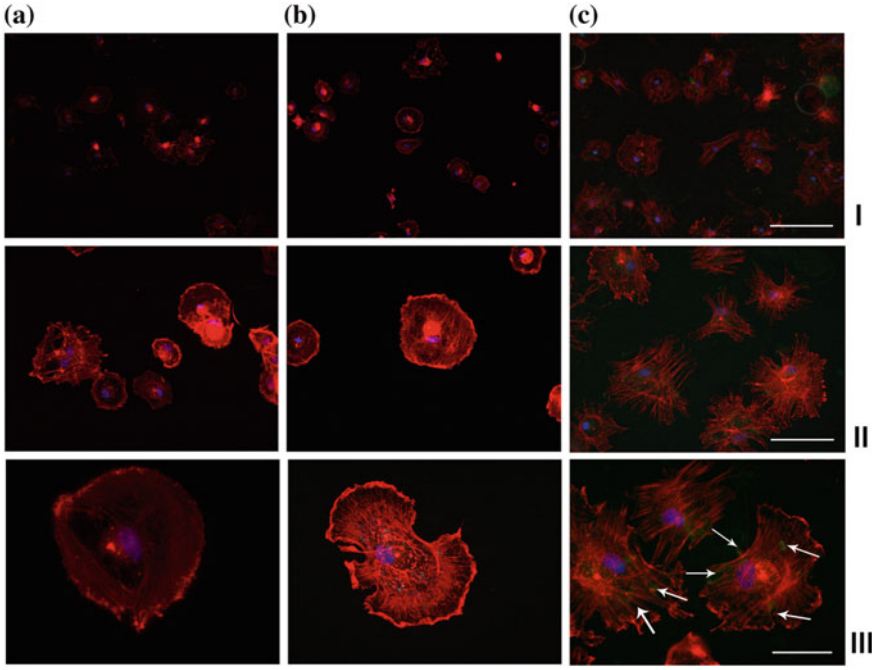

Fig. 5.7 HOP cell actin filament staining on **a** standard cover slips, **b** silicon and **c** FN covered silicon by MAPLE after 3 h in cell culture. Cells were fixed, permeabilized, and stained for actin using Alexa Fluor 594-conjugated phalloidin (*red*). The mounting media contained DAPI (*blue*), which stained cell nuclei. On Fig. 7c FITC conjugated FN are marked in *green* (*arrows*). Scale bars are of 200 (I), 100 (II) and 50 (III) μm respectively. Reprinted with permission from Acta Biomaterialia [62]

Ti/HA samples, supporting a faster cell colonization and proving the physiological VN functionality after laser transfer [63]. Thin films of collagen obtained by MAPLE, with roughness controlled by experimental parameters were also reported [128].

A key advantage of ECM protein coatings obtained by MAPLE versus other simple adsorption methods is the accurate control of the expulsed material and coating thickness. Homogeneous distribution on the surface, in particular for small amounts (μg) of proteins, is beneficial, while the buffer salts (NaCl, TRIS) contained in the starting solution and deposited next to the protein are expected to act as a stabilizer. To enhance protein immobilization, one can easily use calcium phosphate or polymer films already deposited in an unique all-laser procedure [63] which demonstrated beneficial effects as shown in refs [129, 130]. One can thus develop ECM-mimetic biomaterial surfaces that could trigger protein organization into biologically active molecules. Protein-coated calcium phosphate layers, and in particular nanostructured thin films, are expected to provide a synergetic interface for biomimetic implant applications.

5.5.2 Application to Organic–Inorganic Composites

To mimic the multidimensional, hierarchical and complex structure of native tissues (e.g. bone) from the chemical and physical (morphology, structure, composition and functionality) points of view organic-inorganic composite coatings are the best choice to reconstruct the molecular architecture of the local environment and to trigger dynamic biomechanisms. Inorganic materials (e.g. ceramics, metals) are used to render the strength and to provide or compensate for the mineral phase of the tissue. Organic bioactive molecules are able to accelerate tissue integration (as e.g. ECM proteins) or to prevent undesired biological response (as e.g. drugs) in a well-controlled manner [131]. Biodegradable polymers or bioresorbable ceramics are used as scaffold materials as well as matrix carriers for drug release. As a soft laser deposition technique that minimizes the photochemical damage of an organic exposed to the laser light, MAPLE was naturally extended to organic-inorganic composites aiming to create three-dimensional structures for faster cell colonization and tissue regeneration. This could represent a benefit over other techniques including PLD (see Table 5.1) since most of the methods are efficiently applied to either inorganic or organic materials but not to both of them or to composites.

HA-sodium maleate (MP) copolymer thin coatings deposited by MAPLE on Ti substrates were tested in vitro. Osteoblast-like cells showed a higher proliferation when cultivated on these nanocomposite coatings in comparison with the cells grown on Ti coated with HA only (Ti-HA) (Fig. 5.8). This demonstrates that the polymer presence improved surface bio-adhesive characteristics, cell attachment, spreading and proliferation, which recommend the potential of Ti coated with HA-polymer nanocomposites as scaffolds in dental or orthopedic implantology [132].

HA-sodium maleate-vinyl acetate copolymer coatings were synthesized on Ti surfaces for specific biological investigations. Human primary osteoblasts spread and proliferated onto modified surface and formed groups of cells which during biosynthetic activity expressed osteoblast markers [133]. PMMA-bioglass composites were obtained by MAPLE as uniform thin layers onto chemically etched Ti from targets of mixtures containing PMMA reinforced with either 6P57 (lower silica content) or 6P61 (higher silica content) bioglass powders [134]. Osteoblast-like cells were found in both cases to entirely cover the MAPLE coatings with which they strongly interact, as proved by the pseudopodia deeply infiltrating into the composite material. The difference in density proves that cells find a more friendly living medium on glasses with lower silica content. In addition, the corrosion characteristics of these glass-polymer composite coatings on titanium were investigated [135]. An unexpected self-arrangement of a double layer nanostructure after immersion in SBF consisting of an inner barrier (polymer) and an outer porous layer (bioapatite) was revealed with potential effects for osseointegration capacity of the Ti implants. The authors suppose that the immersion in SBF triggered an intense ion exchange process between coating and solution leading to the formation of a rapidly increasing bioapatite layer, which proved very efficient in protection against corrosion. The process evolved faster in case of nanostructured 6P57+PMMA coatings but a better

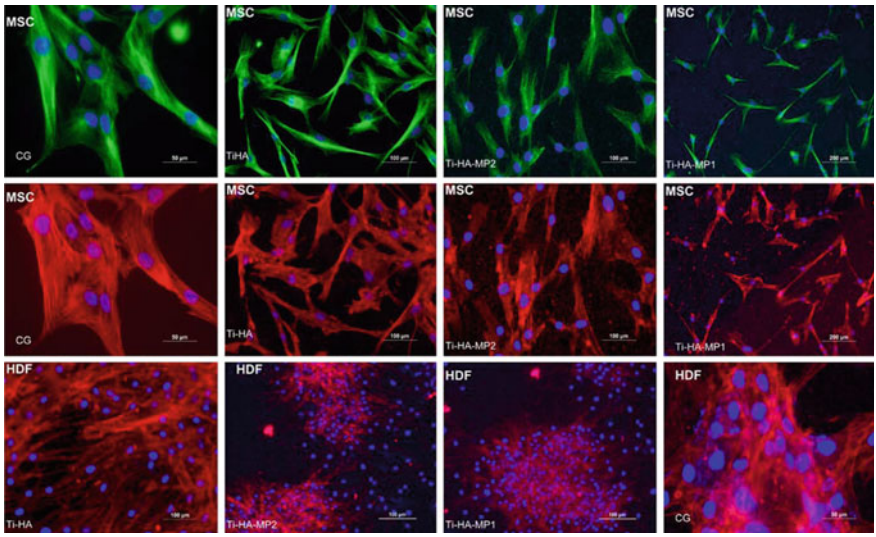

Fig. 5.8 Cytoskeleton organization of mesenchymal stem cells and human dermal fibroblast cultured on different surfaces modified by MAPLE. Cells were grown for 24 h in direct contact with: Ti-HA, Ti-HA-MP1, or Ti-HA-MP2 (Ti-HA-MP1 and Ti-HA-MP2 corresond to solutions with 0.2 % and 1 % HA-MP powder); standard borosilicate cover glass (CG). Fixed cells were stained for actin (*red*), microtubules (*green*) and nuclei (*blue*) and analyzed by fluorescence microscopy; Reprinted with permission from J Mater Sci: Mater Med [132]

protection was reached for 6P61+PMMA coating when the corrosion was almost completely stopped.

MAPLE was also applied to obtain thin coatings of alendronate-hydroxyapatite composites [136] after nanocrystals' synthesis in aqueous medium with increasing bisphosphonate content (3.9, 7.1 % wt) [137]. For control, MAPLE was conducted with pure HA (0 % wt biphosphonate content) as well. The presence of alendronate in the MAPLE synthesized HA thin films had a positive effect on osteoblast viability and differentiation while inhibited osteoclast proliferation and differentiation, causing their apoptosis [136]. Similarly, a comparison was carried out between MAPLE deposited films of pure HA and silk fibroin mixed with HA thin structures for biomimetic implants [138]. The best results from physico-chemical and biological points of view were found for the composite HA-silk fibroin in comparison with MAPLE deposition of pure HA or fibroin films [138]. These were the first attempts to deposit HA by MAPLE technique to difference of PLD which is usually applied in this case [139]. We mention that in all cases the composite films presented superior mechanical and biological characteristics as compared to the films obtained by MAPLE from the respective pure materials.

5.5.3 Application to Inorganics

In view of reconstructing gradient inorganic layers or inorganic-organic multilayers by a single-experiment process, a significant attention was paid during the last 5 years to MAPLE application to inorganic compounds since the method was shown to produce nanoparticulate inorganic films [140, 141]. The single-experiment process of inorganic-organic multilayers could represent an actual advantage of MAPLE technique in respect with PLD which cannot be applied to sensitive organic polymers or proteins. Moreover, by using different solvents and deposition temperatures one can control the film assembling (growth and surface morphology) on substrate. When increasing the specific surface area a nanostructured or nanoparticulate film aspect (very often the MAPLE deposited coatings exhibit a high density of nano- and micron-sized droplets) could boost surface properties for specific applications such as sensors, drug delivery systems or biomimetic implants where a larger contact area is advantageous.

Following the application of MAPLE to organics, then to organic-inorganic composites, the method turned naturally to inorganics. Although the biological evaluation demonstrated that the composite films are presenting improved performances than pure inorganic coatings, as presented in Sect. 5.5.2, the opportunity to obtain inorganic-organic multilayers in a single-experiment process pushed the research toward experiments of MAPLE application to inorganics. Thus, MAPLE was employed to the deposition of calcium phosphates, in particular octocalcium phosphate (OCP) thin films, on titanium substrates [64] which is a challenge to OCP coatings fabricated by PLD [142]. It was demonstrated that the milder conditions of a MAPLE process ensured a higher degree of OCP crystallization with respect to PLD. This was in accordance with the presence of crystal fragments to the difference of OCP coatings deposited by PLD, which consisted of cauliflower-like aggregates and droplets only [64]. Next, Mg and Sr-doped OCP were deposited by MAPLE [64]. A remarkably uniform dopant distribution in films was evidenced. An enhanced cellular proliferation and differentiation on SrOCP and MgOCP in comparison with OCP films demonstrated that ion-doping improved the effect of OCP on bone cells, suggesting that such biomimetic coatings could be usefully applied for orthopedic use.

Nanoparticulate films obtained by MAPLE exhibited a good sensitivity in sensing, in particular, a good electrical response to acetone and ethanol vapors was evidenced for MAPLE deposited TiO2 coatings starting from nanoparticle powders suspended in deionised water [140]. The good sensitivity was attributed to the nanoscale dimensions of the TiO2 particles in the deposited films [141]. Recently, MAPLE was used to deposit single-wall carbon nanotubes functionalized with oxygen-containing groups without any alteration of the starting material [143].

5.6 Conclusion and Perspectives

During the last 15 years, MAPLE developed and improved fast and became a significant competitor to other deposition methods in the field of nanotechnology, in particular, for biomimetic applications such as drug delivery systems, biosensors or advanced implants. The method can ensure the control of film uniformity and thickness on either rough or flat substrates and permits the synthesis of coatings with increased adhesion.

MAPLE was initially proposed as an alternative to PLD for transferring and depositing thin organic materials. Extracellular matrix proteins, enzymes and polymers were transferred by MAPLE onto facing collectors and found to significantly improve their biological characteristics. It was possible to fabricate inorganic–organic composite coatings with improved bioactivity and controlled action in view of local release of some drugs to promote bone formation and prevent bone resorption. MAPLE turned recently unexpectedly to application of inorganic materials and transformed into a cryogenic PLD, a real competitor for PLD. Thus, MAPLE was for the first time applied to deposit inorganic coatings of ion-substituted OCP thin films. The obtained structures were found to support the growth and differentiation of osteoblast-like cells. In particular, an enhanced activity was demonstrated when cells were grown on ion-doped OCP coatings in comparison with pure OCP.

Because MAPLE was efficiently applied to either organic or inorganic material deposition it possesses actual advantages over other deposition techniques since most of them do not apply to both organic and inorganic or to composite materials. One single-experiment process of inorganic-organic multilayer deposition could be approachable as well by MAPLE technique. One can therefore foresee the potential use of appropriate masks in MAPLE experiments to manufacture well-controlled microsized, single or multilayer, organic-inorganic samples for advanced biomimetic applications.

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