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Full Length Article

Gradient multifunctional biopolymer thin film assemblies synthesized by combinatorial MAPLE

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ABSTRACT

Combinatorial Matrix-Assisted Pulsed Laser Evaporation (C-MAPLE) was recently introduced to the fast generation of compositional libraries of two biopolymers in a single-step process, for tissue engineering and regenerative medicine applications.

Synchronized laser irradiation of two distinct cryogenic targets, one consisting of Sulfated *Halomonas* Levan and the other of quaternized low molecular weight Chitosan was used to fabricate compositional gradient coatings for surface functionalization. Synthesized coatings preserved the base material composition as confirmed by Fourier Transform Infrared Spectroscopy. Morphological study by Scanning Electron Microscopy, Atomic Force Microscopy and profilometry correlated with water contact angles measurements demonstrated that the obtained thin coatings have improved surface properties with respect to pure material coatings. Fluorescence microscopy validated the compositional gradient, while *in vitro* assays evidenced characteristic responses of mouse fibroblasts (L929 cell line) by distinct deposition surface regions. The coagulation test pointed out good properties for Sulfated *Halomonas* Levan coatings as compared to the case of an increased amount of quaternized low molecular weight Chitosan biopolymer or the control.

The antimicrobial effect of the coatings was demonstrated against *Escherichia coli* and *Staphylococcus aureus* strains, representative for both Gram negative and Gram positive bacterial species, respectively, mainly involved in implant and nosocomial infections. The assembled nanostructures possess variable anti-biofilm activity along the compositional gradient, with a stronger inhibitory effect on the initial adherence phase of both tested microbial strains, but also against mature *Escherichia coli* biofilms.

It was shown that C-MAPLE can generate discrete areas of blended polymeric composition exhibiting improved surface properties for a broad range of biomedicine applications, e.g. the fabrication of thin bioactive and cell-instructive coatings with anti-adherence properties.

1. Introduction

Recently, functional coatings attracted a rapidly growing interest, due to the possibility to explore novel properties of materials at either micro- or nanoscale. With respect to their original protective function, thin films are nowadays in the forefront of sustained advances in fabricating antimicrobial, bioactive and biomimetic, transparent and conductive, thermo-electric, self-healing, self-cleaning and super-hydrophobic coatings, to mention a few applications only. On the other hand, combinatorial research, in its whole spectrum of applications in materials science and chemistry, has proven to drive fast progress in the synthesis of innovative materials or new properties for nanomedicine.

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In the biomedical field, most of the applications are related to the fabrication of bioresponsive surfaces and interfaces able to modulate cells behavior and push forward drug discovery and delivery systems [1,2]. Indeed, combinatorial techniques, correlated with high-throughput screening stand for the best tools to improve the composition-structure and/or properties relationship for both organic and inorganic library coatings [3].

We have recently introduced a single-step combinatorial approach based on Matrix-Assisted Pulsed Laser Evaporation (MAPLE) for both the immobilization and blending of organic and environmental friendly polymeric compounds [4–8]. Later, both experimental and theoretical aspects of C-MAPLE technique were extensively addressed in our studies of different organic, inorganic or composite materials [9,10]. Laser-based synthesis of biopolymers combinatorial coatings is reviewed in Ref. [9].

Chemically modified or natural polysaccharides with sulfate groups have efficient biological characteristics, such as antioxidant and immunomodulation [11,12], antiherpetic [13], antithrombotic and anticoagulant [14,15] action. Levan is a β-2, 6-linked fructose homopolymer synthesized extracellularly from sucrose substrates by various microorganisms. Due to its unique features, it is considered a quite valuable biomaterial with many potential applications in medical, food, pharmaceutical, cosmetics and chemical industries [16]. The Gram negative aerobic bacterial strain *Halomonas smyrnensis* AAD6T was reported as the first halophilic levan producer [17], besides its antioxidant and anticancer activities [18,19]. *Halomonas* Levan (HL) was proved suitable for use as drug carrier systems [20], bioactive thin film blends [21], multilayer adhesive films [22], but also temperature responsive [23] and cytocompatible hydrogels [24]. Recently, a sulfated derivative of HL (SHL) was reported as a heparin mimetic anticoagulant [4,14]. Also, electrospun matrices of SHL with anticoagulant activity were found to have high potential to be used in decreasing neointimal proliferation and thrombogenicity of grafts and prosthesis [25]. SHL was shown to improve the mechanical and adhesive properties of multilayered free-standing films and allow myogenic differentiation and lead to cytocompatible and myoconductive films for cardiac tissue engineering applications [26].

Recent studies reported the characteristics of HL and oxidized HL (OHL) thin films synthesized by MAPLE [27]. HL and OHL were combined and binary gradient films were fabricated using the Combinatorial MAPLE (C-MAPLE) technique. *In vitro* cell culture studies with the SaOS2 bone cell line have shown that the osteoblasts' extracellular signal-regulated kinase signaling was modulated with different propensity. It was suggested that C-MAPLE could indeed serve as a suitable method for the fabrication of new bioactive surfaces controlling the cell response [5].

Chitosan (CH) is the deacetylated product of the well-known natural biopolymer chitin. Its chemical reactivity is attractive due to the presence of $–NH_2$ groups [28]. Chitin and Chitosan are highly basic unlike most other natural polysaccharides [29]. Chemical properties of CH include the linear polyamine structure, reactive amino and hydroxyl groups and possibility to chelate transitional metal ions. CH is a natural, biocompatible, biodegradable, safe, non-toxic polymer [30,31]. It has hemostatic, anti-thrombogenic, antibacterial, fungistatic, spermicidal, immunoadjuvant, antitumor, anti-cholesteremic biological properties and may accelerate bone formation and so can be used as central nervous system depressant [32,33]. Because of diverse bioactivities, CH can be applied in a wide range of biomedical applications such as wound healing or tissue engineering [31,34], implant coatings [35–38] and drug delivery systems [28,31,39,40].

CH molecular weight exerts a major influence upon its biological and physical-chemical properties. Thus, the crystallinity, degradation, tensile strengths and moisture content have been shown to be dependent on the molecular weight [30,41–44]. As known, commercially available CH has a high molecular weight. Accordingly, methods have been developed using ultrasound, heat, enzymatic or chemical hydrolysis to depolymerize CH. Depolymerization of CH using nitrous acid (HNO_2) was imposing as a preferred technique, because it is rapid, cost-effective and can be monitored to produce CH of a pre-selected size [45–49]. The way to improve or add new properties to CH [50] is to chemically modify the chain by adding functional groups to the main one, non-altering the original structure and properties. The primary amine and hydroxyl groups (OH) are responsible for reactions such as e.g. quaternization. Moreover, the presence of these reactive primary amino groups adds special properties to CH which make them very appropriate in pharmaceutical applications [51–53]. There are several studies in literature on CH quaternization, reporting the improvement, even under basic conditions, of properties such as the antimicrobial activity [53–56], moisture-retention capacity [57,58], bio-adhesivity, permeation enhancing effect and antifungal activity [56,58].

The possibility to deposit two natural, biocompatible, non-toxic polysaccharides, i.e. Sulfated *Halomonas* Levan (SHL) and quaternized highly deacetylated low molecular weight CH (QCH) on Si, glass or Ti substrates is explored in this study. We mainly aimed to fabricate thin coatings able to offer a suitable interface for cells leading to an improved osseointegration and simultaneously exhibiting good antimicrobial properties. To this purpose, C-MAPLE method was applied to transfer and deposit SHL and QCH in a single process in order to produce new organic thin libraries with improved properties.

To the best of our knowledge, this is a first attempt to synthesize *in situ* SHL and QCH thin coatings for the selection of best bioactive surface able to control cells behavior and ensure protection against microbial colonization and biofilms formation.

2. Materials and methods

2.1. Synthesis of combinatorial functional coatings by C-MAPLE

The preparation of quaternized highly deacetylated and low molecular weight CH (QCH) was carried out via established protocols described in literature [21,40,49,58–60] (and given in detail in Supplementary Information (SI) section). The sulfonation of *Halomonas* Levan (SHL) was achieved following the procedure described in Refs. [14,25,26] (also detailed in SI).

First, 25 mg of SHL were dissolved in 5 mL of dimethyl sulfoxide (DMSO) to obtain a homogeneous solution. DMSO was selected as solvent because it does not chemically interact with SHL, and efficiently absorbs the laser wavelength (248 nm) used for the evaporation of the target in frozen state [27]. A concentration of 2% QCH dissolved in deionized H_2O (dH_2O), was used for the preparation of the second C-MAPLE target. The two solutions were transferred into distinct compartments of a ring-like concentric copper holder. This holder has been designed and manufactured to accommodate in each compartment between 5 and 10 mL of solution to be used in C–MAPLE experiments. The holder containing the solutions was carefully immersed in liquid nitrogen (LN) to get the two cryogenic targets. Next, the holder was mounted inside the cooler, which was under the continuous supply of LN, to keep the target frozen.

The schematic representation of the C-MAPLE experimental set-up is depicted in Fig. 1. The laser beam generated by a KrF* excimer laser source (λ = 248 nm, τ_{FWHM} = 25 ns), operating at a repetition rate of 10 Hz is divided by an optical splitter. The two beams are directed and focused onto the two concentric targets. The distance between the centers of the two laser spots is set at 2 cm. The evaporated polymers were collected either onto glass, Si (1 0 0) or Ti substrates placed parallel to the targets, at a separation distance of 5.5 cm. Before deposition, all substrates were successively cleaned in an ultrasonic bath for 15 min in acetone, alcohol and dH_2O. For the growth of each SHL to QCH coatings, 40.000 laser pulses have been applied. One obtains in this configuration the polymer co-deposition onto a 4 cm long sample, the edges containing 100% SHL at one end and 100% QCH at the other one. A continuous gradient of SHL–QCH blended composition is reached in