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Layer-by-Layer Coating of Photoactive Polymers for Biomedical Applications / Chiono, Valeria; Carmagnola, Irene; Gentile, Piergiorgio; Boccafoschi, Francesca; TONDA TURO, Chiara; Ciardelli, Gianluca; Georgiev, G.; Ninov, M.; Georgieva, V.; Pashkuleva, I.; Reis, R.. - ELETTRONICO. - (2009), pp. 798-801. ((Intervento presentato al convegno IEEE Nano 2009 tenutosi a Genova nel 26-30 Luglio 2009.

*Availability:*

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# Layer-by-Layer Coating of Photoactive Polymers for Biomedical Applications

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**Abstract**—The design of advanced, nanostructured materials at the molecular level is of tremendous interest for the scientific community because of their potential in several fields, including medicine, biology and tissue engineering. Layer-by-layer (LbL) assembly is a versatile technique for the realization of multilayered films with tailored characteristics at the nanometer scale. Photoactive nanostructured films were prepared by LbL assembly of photozymes: an amphiphilic zwitterionic copolymer, poly(sodium styrene sulphonate-*stat*-vinyl naphthalene-*stat*-3-dimethyl(methacryloyl)ethyl ammonium propane sodium sulfonate) (PSSS-*stat*-VN-*stat*-DMPAS; code: ZI), and a cationic polyelectrolyte, chitosan-g-fluorescein (code: CHFL), on a crosslinked gelatin substrate. Although successful LbL assembly of photozymes was proven by the performed characterization, gelatin was not an optimal substrate for the coating, due to its low charge density and amphoteric nature. A regular growth of the layers was found after at least 6 layers were deposited. The macromolecules of the zwitterionic photozyme adopted a coiled micellar conformation in solution which was kept during the assembly. The obtained nanostructured films are promising candidates for carrying out efficient electron transfer process within them, responsible for the anti-microbial activity and the osteointegration ability of the coating.

**Keywords:** chitosan-g-fluorescein; gelatin; layer-by-layer; poly(sodium styrene sulphonate-*stat*-vinyl naphthalene-*stat*-3-dimethyl(methacryloyl)ethyl ammonium propane sodium sulfonate); photozymes.

## I. INTRODUCTION

Layer-by-layer (LbL) electrostatic self-assembly of charged polymers has been widely used as a versatile technique for the formation of multilayered thin films with a tailored structure and composition and wide range of electrical, magnetic, biological and optical properties [1, 2]. LbL self-assembly is

based on the alternating exposure of a charged substrate to solutions of positively and negatively charged polyelectrolytes. A rinsing step is included between the two previously described adsorption processes, to remove excess as well as to prevent cross-contamination of the polyelectrolyte solutions. LbL technique allows obtaining of homogeneous monolayer structures and fine control of the coating properties. Additionally, it is applicable to substrates of any shape, it is environmentally-friendly, it allows room temperature processing and low-cost manufacturing. The range of polyelectrolytes used in the LbL assembly is very broad, but the majority of the studies has been utilized commercial homopolymers. There is a limited number of reports on amphiphilic polyelectrolytes [3, 4].

Photozymes are amphiphilic photoactive polymers, composed by fluorophore units such as xanthene dyes (fluorescein, rose bengal, eosin, eritrosin), naphthalene, carbazole, and hydrophilic units, containing strong electrolytes such as sulphonate groups or weak electrolytes such as the primary amino groups of chitosan chains [5-7]. Photozymes may be statistical, block or graft copolymers and they are generally water soluble. Aromatic chromophores undergo strong interactions, which induce the coiling of the macromolecules and the formation of hydrophobic domains surrounded by charged fragments of the chain. The hydrophobic packed domains may dissolve hydrophobic guest molecules and play the role of nanoreactors with photochemical activity [7].

In this work, nanostructured multilayer photoactive films have been produced on crosslinked gelatin substrates via the LbL method, using alternative layers of a *stat*-copolymer photozyme (forming pseudomicellar structures) and a graft-copolymer photozyme (forming compact layers). These innovative nanostructured materials may have important

applications as multifunctional coatings for scaffolds for bone repair: the photosensitizing properties of the different types of photozymes are combined, leading to osteointegration by hydroxyapatite precipitation induced by the graft-copolymer photozyme and anti-microbial action promoted by the stat-copolymer photozyme.

## II. MATERIALS AND METHODS

A graft-copolymer photozyme, chitosan-g-fluorescein (code CHFL; 0.5 mol.% FL; produced from chitosan FG 90 with Mn:210±10 kDa and Mw/Mn: 2.17, Primex Ingredients ASA) and a stat-copolymer zwitterionic photozyme, poly(sodium styrene-stat-vinyl naphthalene-stat-sulphonate-co-3-dimethyl(methacryloyl)ethyl ammonium propane sodium sulfonate) (PSSS-stat-VN-stat-DMAPS; code ZI; with 20 mol.% VN, 70 mol.% SSA; 10 mol.% DMAPS with Mn: 53.2 kDa and Mw/Mn: 2.34) were synthesized by copolymerization (AIBN, 60°C, DMSO). The behavior of ZI photozyme macromolecules in water solutions with various concentration, pH and ion strength was studied by Dynamic Light Scattering, DLS and zeta potential measurements (Zetasizer Nano ZS). Before any applications, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cytotoxicity test was carried out on ZI and CHFL solutions using an osteoblast cell line SaOs-2 osteoblasts. The photo-bioactivity of CHFL was studied by tests in simulated body fluid (SBF) under irradiation and in the dark (control). The photosensitizing activity of ZI was shown by standard tests using probe molecules [6]. Type A gelatin from porcine skin (code G) was supplied from Sigma-Aldrich. Genipin (Challenge Bioproducts) was used as a crosslinker for G. All solvents used were of analytical grade and used without further purification. Cast films on glass slides (1.2 cm diameter) were obtained from GP-crosslinked G (code G\_GP; 2.5 wt.% GP) [8] as a substrate for LbL assembly. LbL was realized by alternative incubation into 0.1% (w/v) photozyme solutions (pH 5.5) with intermediate washing steps (pH 4.5). The procedure was repeated and 10 layers were deposited. At the end, samples were further rinsed in bi-distilled water. LbL coatings on G\_GP were characterized by scanning electron microscopy-energy dispersive x-ray analysis (SEM-EDX; FEI QUANTA INSPECT 200 apparatus, equipped with EDAX Genesis Software), FTIR-ATR analysis (Perkin Elmer Spectrum One Spectrometer), UV-VIS spectroscopy (Perkin Elmer), static water contact angle (CAM 200 KSV Instrument).

## III. RESULTS

DLS is a non-invasive well-established technique for measuring the size of molecules and particles typically in the submicron region. A bi-modal size distribution was determined for the ZI aggregates (with 40nm and 340nm medium size), regardless the ZI solution concentration (0.25-2.5 mg/ml). While the size of 40 nm is consistent with an isolated ZI macromolecule, the size of 340nm indicates the presence of macromolecular aggregates. This speculation was confirmed by the observation that the quantity of the smaller aggregates increases at higher ion strength of the solution. ZP is one of the main forces that mediate inter-particle interactions. Particles with a high ZP of the same charge sign, either positive

or negative, will repel each other. Conventionally a high ZP can be high in a positive or negative sense, i.e. <-30mV and >+30mV. For molecules and particles that are small enough, and of low enough density to remain in suspension, a high ZP will confer stability, i.e. the solution or dispersion will resist aggregation. ZP and DLS measurements confirmed the ability of ZI to assemble into stable nano-aggregates, with high ZP values (<-30 mV) even at elevated ion strength (2.5% KCl). However, a tendency for decreasing ZP was observed with increasing the photozyme concentration (0.25-2.5 mg/ml), which is a sign for possible decreasing stability of the dispersion in terms of aggregation abilities. ZP was only slightly affected by the solution pH in the 4-10 pH range. The solution ion strength highly influenced ZP, which decreased with increasing the ion strength (0-2.5% KCl).

A previous work on LbL assembly of stat-copolymer photozymes has shown that their coiled micellar conformation is preserved in the layer-by-layer assembled film [9]. Here, the behavior of surface wettability of LbL coated films as a function of layer number could indirectly suggest a micellar assembly of ZI photozyme in the LbL coating layers (Fig. 1). Contact angles of coated surfaces were similar to the value measured for G\_GP up to the 2nd layer which is consistent with the sensibility of this surface characterization technique. Then, the contact angles increased for the 3<sup>rd</sup> and the 4<sup>th</sup> coating layers. The observed changes can be associated not only with changes of the surface chemistry but also with the increased roughness generated during the coating process.

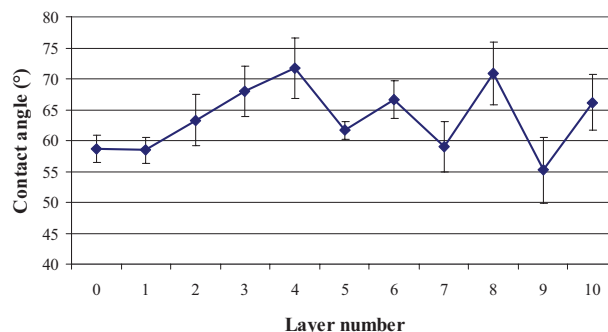


Figure 1. Contact angle as a function of the layer number. Reported data are the average values whereas the bars are the standard deviation (n=5).

After the 4th layer, contact angle displayed alternate values suggesting the beginning of the layer-by-layer assembly. Literature data report that contact angles of LbL films generally oscillate between the values for pure components. A similar result was not found for the photozyme coatings: in this case, the contact angle of each layer varied between 65°-70° which is only higher than the CHFL contact angle (60°) and a lower value (around 55°), which is much higher than the typical ZI contact angle (around 18°). The assembly of ZI photozyme into nano-aggregates (pseudomicelles) on the corresponding layers produced nano-roughness on the surface which could be the cause for the increased surface wettability of the ZI layers. Atomic force microscopy measures are in progress to study the ZI morphology onto each layer.

Moreover, contact angle measurements are sensitive to both surface nano- and micro-roughness. SEM analysis showed an increase of surface roughness with increasing the number of layers (Fig. 2). On the other hand, as LbL coatings are expected to have nanometer thickness, it was not possible to distinguish the deposited layers by SEM images of the fractured surfaces.

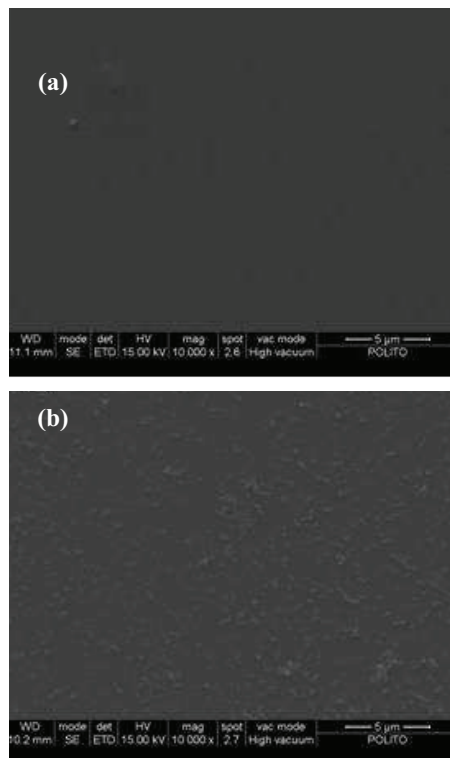


Figure 2. SEM images of surfaces of LbL coated G<sub>GP</sub> with alternate ZI/CHFL layers: (a) 1 layer; (b) 7 layers.

FTIR-ATR analysis showed changes in the chemical characteristics of the surfaces of coated G<sub>GP</sub> cast films as compared to the control, particularly in the 1400-900 cm<sup>-1</sup> wavenumber range. The main absorption bands of ZI photozyme were present in the FTIR-ATR spectra of the coated surfaces, precisely asymmetric (1125 and 1180 cm<sup>-1</sup>) and symmetric (1036 cm<sup>-1</sup>) stretching of O=S=O groups and S-O-C stretching (1009 cm<sup>-1</sup>). On the other hand, the CH-FL absorption bands were not detected in the FTIR-ATR spectra of coated G<sub>GP</sub>. Similar results have been reported for multilayers of poly(styrene-sulphonate)/chitosan [10], for which FTIR-ATR analysis only evidenced the absorption bands of the poly(styrenesulphonate) polyanion.

EDX analysis allows the investigation of a surface layer with a micrometer thickness. In our case, when the analysis was performed on the upper surface of coated cast films, EDX spectra included the elemental composition of both the G<sub>GP</sub> substrate and the coatings. EDX spectra of the upper surfaces of coated G<sub>GP</sub> showed the presence of S due to the occurred deposition of the anionic photozyme (ZI). The peak due to S was particularly intense on surface agglomerates. In conclusion, both FTIR-ATR and EDX analyses confirmed that ZI was successfully assembled onto G<sub>GP</sub> films.

On the other hand, UV-VIS spectroscopy performed on LbL coated films showed the typical absorbance peak of FL dye at around 500 nm, which intensity increased with increasing the layer number (Fig. 3) and the characteristic absorption peak of G<sub>GP</sub> at around 605-610 nm.

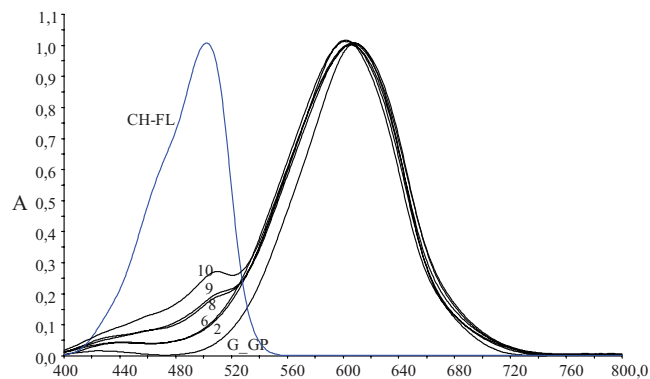


Figure 3. UV-VIS spectra of CHFL, G-GP and LbL coated G<sub>GP</sub> with alternate ZI/CHFL layers.

CHFL absorption peak was not detected for a number of layers lower than 8, probably due to the low intensity of the CHFL peak which was masked by the absorption of G<sub>GP</sub>.

Several cytotoxicity tests, such as MTS or MTT, are well accepted for an initial screening of a material biocompatibility. Cytotoxicity tests using osteoblasts cell line (SaOs-2) were performed with pure photozymes. The MTS results for ZI are presented in Fig. 4. ZI solutions in culture medium did not show any cytotoxic behavior against SaOs-2 osteoblast like cells. A slight decrease of cell viability with increasing the material concentration was observed. However, the cell viability calculated for all the samples was above 80%, which indicates a good biocompatibility of the photozyme.

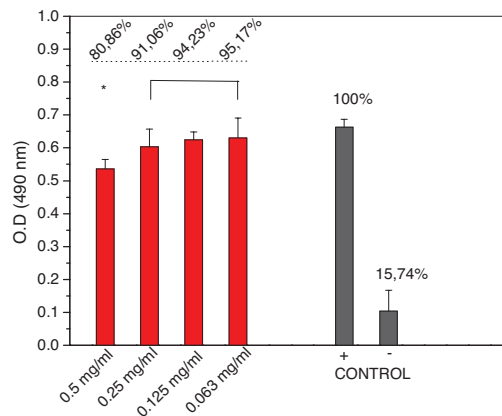


Figure 4. Viability of SaOs-2 cells cultured for 24 h in solutions of ZI photozyme at different concentrations.

Similarly, CHFL dissolved in culture medium at 0.01-2 mg/ml concentrations was found not to be cytotoxic towards osteoblast-like cells.

CHFL was also found to have photo-bioactivity, i.e. the ability to deposit HA crystals under visible light irradiation in a suitable ion medium (SBF) (Fig. 5).

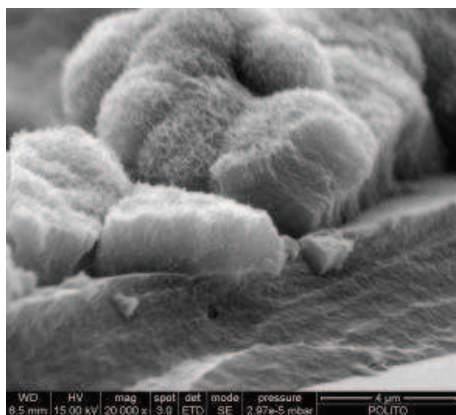


Figure 5. SEM image of the surface of CH-FL cast films after incubation in SBF for 14 days under irradiation.

This property was probably a consequence of light excitation of CHFL, leading to singlet oxygen formation, variation of the material surface charge and electrostatic attraction of the SBF solution ions with HA nucleation and further growth. However, the study of the mechanisms for the deposition of HA is in progress.

The ability of the ZI photozyme towards singlet oxygen formation was also demonstrated by a UV-VIS spectroscopy using probe molecules, such as perylene or methylene blue: this property could be exploited for tailored photochemical reactions, e.g. with the purpose to obtain materials able to kill bacteria [11], increasing the well-known anti-fouling properties of zwitterionic polymers.

#### IV. CONCLUSIONS

The layer-by-layer deposition of photoactive molecules (photozymes) was demonstrated by an accurate physico-chemical characterization. Further studies are in progress to analyze the morphology of the layers at the nano-scale. The studied LbL films showed interesting properties for future applications as coatings on scaffolds (or prostheses) to be implanted for bone regeneration purposes. The alternate deposition of ZI and CHFL photozymes allows the combination of their properties for the obtainment of materials

with decreased risk of infection (due to ZI antimicrobial and antifouling properties) and able to stimulate the light-switchable deposition of HA (due to CHFL photo-bioactivity).

#### ACKNOWLEDGMENT

This work was supported by the European Union within the project "Photonanotech, Photozyme Nanoparticle Applications For Water Purification, Textile Finishing, Photodynamic Biomaterialization And Biomaterial Coating" (Contract Number: 033168).

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