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Mechanical properties of extracellular matrix/hydroxyapatite composites

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An increasing interest in biomimetics – design of materials based on natural biological structures - has led to the nanomechanical characterization of biomaterials. In this regard, nanoindentation has been used in conjunction with the determination of the mechanical properties of the extracellular matrix (ECM) proteins that are known as ligands in reactions with cell surface receptors involved in bone physiology. The aim of the presented work is to investigate the - and nano-scale mechanical properties of laser designed extracellular matrix/hydroxyapatite composites. The osteoblast-like cell line SAOS-2 synthesised and assembled its own ECM on the solid substrates under standard cell culture conditions. After selective removal of cells, thin films of ECM on substrates of stainless steel (SS), silicon (S) and silica glass (SG) were obtained. One group of samples was soaked in simulated body fluid (SBF) and another was obtained by simultaneous immersion in the SBF and treatment by laser irradiation. As a result, a hydroxyapatite (HA) crystal layer was grown on the surfaces. The mechanical properties of the obtained composites, such as elastic modulus (E) and indentation hardness (H), were analysed. It was observed that by applying a typical working force in the range 200 μN to 600 μN and a displacement range of 0-60 μm, E increased for all composites obtained by the laser process (for samples immediately removed from the SBF). Surface scanning along the direction centre of the sample to the laser treated area showed a decrease in the Young's modulus, to values similar to those in the human bone.

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1. Introduction

Extracellular matrix (ECM) is an intracellular substance made by the tissue and has diverse functions, depending upon the cell type [1]. For example, in bone cartilaginous connective tissue, ECM supports the function of the tissue. ECM is a complex of proteins: mainly collagen, elastin, etc., known as ligands in the reaction with cell-surface receptors involved in bone physiology. We investigated the influence of native ECM proteins as representatives of the organic counterpart in the processes of biomineralization on the growth and ordering of hydroxyapatite (HA) [2]. In our previous study, HA layer were grown on the surfaces of various substrates (stainless steel, silicon and silica glass) coated with ECM. It was shown that the substrate surface, coated by ECM, induced the growth of a HA layer consisting of regular spherical aggregates with an average size of 5 μm, grouped homogeneously in a network [3]. It was concluded that the ECM proteins facilitated the mineralization on the various surfaces by serving as an *in-vitro* matrix for the HA growth and contributing to its crystallization [1-3]. Little is known about the mechanical properties of ECM/HA composite coatings on different substrates. Better bonding between the ECM/HA and the substrate surface can be easily obtained using laser surface processing [4]. On the other hand, it was shown that novel liquid laser solid interaction process (LLSI) can stimulate the HA growth [3] and enhance mechanical properties of the ECM/HA composite. A nanoindentation technique has been used to measure the mechanical properties of the microstructural features in bone and teeth, investigate variations in mechanical properties with changes in tissue organisation or composition in mineralised and soft tissues, and map the mechanical properties spatially in complex biomaterials [5].

This paper describes a nanoindentation study of the mechanical properties of ECM/HA composites obtained on stainless steel, silicon and silica glass substrates.

2. Experimental

Three types of materials were used as substrates: silicon (S), stainless steel (SS) and silica glass (SG) [1]. The ECM coating was prepared by plating SAOS-2 cells (DSMZ) on the substrates [3]. Following confluence, the medium was changed to a differentiation medium (α-MEM with 15% FBS, 50 μg/ml ascorbic acid, and 891 μg/ml β-glycerophosphate). After 4 days, the samples were exposed to 1000 μl of a 15 mM NH_4OH solution for 6 min, to remove cells from the substrates. Samples were washed with 1000 μl buffered saline phosphate. Two groups of samples were prepared: (1) SS/ECM, S/ECM, SG/ECM samples soaked in a SBF for 4 and 24 hours, and (2) SS/ECM, S/ECM, SG/ECM samples immersed in the

SBF and simultaneously irradiated by the laser beam a CuBr vapor pulsed laser equipped with a scanning system (λ= 578.2 nm, 330 mW [3]). The laser beam was directed perpendicularly to, and focused on, the substrate surface, and the interaction time was within 5 min. By scanning the surface, a design of seven squares at a distance of 200 μm was created at the edge of each sample. Thus, the centre of the substrate was not irradiated. After the end of the LLSI process, some of the samples were immediately taken out of the SBF, and the rest were subsequently soaked in the irradiated SBF solution for 4 and 24 hours.

The mechanical properties of the composites were studied by a nanoindentation technique (Nanoscan). The structure of the grown layer was analysed by Raman micro-spectroscopy (HR800, Yobin Yvon Horiba, λ=532.14 nm, backscattering mode). The surface topography was observed by coherence probe microscopy (Leitz Linnik interferometer).

3. Results and discussion

Fig. 1 shows the elastic modulus curves as a function of the load, for SS/ECM, S/ECM and SG/ECM composites, obtained by the LLSI process and immediately removed from the SBF solution. For this group of samples, the value of the elastic modulus is higher for SG/ECM composites than that for SS/ECM and S/ECM. S/ECM composites show a greater curve slope than those for SG/ECM and SS/ECM. It can be concluded that the elastic modulus of the composites is influenced by the type of substrate.

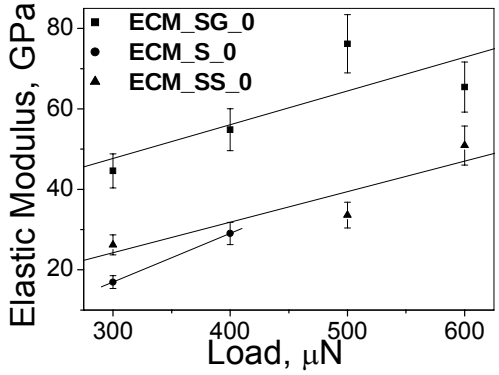
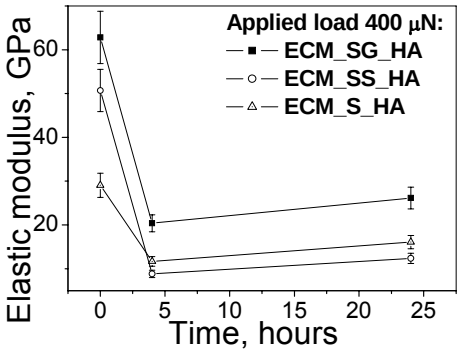


Fig. 1. Elastic modulus curves as a function of load, for SS/ECM, S/ECM, SG/ECM composites obtained by the LLSI process and immediately taken from the SBF solution. Error bar 9.5 %.

For the soaking process, the three groups of samples showed an increase of the elastic modulus (E) with increased duration of soaking, as shown in Fig. 2. The first point in each curve represents data for the samples coated with ECM only. The next two points in each curve represent values for the elastic modulus of the SG/ECM, SS/ECM and S/ECM composites soaked for 4 and 24 hours in the SBF. HA grown on the ECM matrix by soaking for 4 hours in the SBF have a low elasticity and lead to a minimum in the curve.



Fg. 2. Elastic modulus curves for SS/ECM/HA, S/ECM/HA, SG/ECM/HA composites as a function of the duration of the soaking process (4 and 24 hours). Error bar 9.5 %.

An increase in the thickness of the HA layer after 24 hours leads to a slight increase in the elastic modulus values. According to our previous studies [2], the obtained HA layer is thicker and homogenous for SG/ECM/HA composites, comparable to S/ECM/HA and SS/ECM/HA composites. It is known that the elastic modulus increases with increasing layer homogeneity [5].

Nanoindentation can serve as a complementary characterization tool to other techniques that assess the composition or structure with high spatial resolution, such as Raman spectroscopy. In this regard, this technique has played a pivotal role in defining the structure-property relationships.

In Fig. 3, typical Raman spectra for SS/ECM/HA, S/ECM/HA and SG/ECM/HA composites, after LLSI and subsequent soaking for 24 hours in SBF are displayed. Well resolved peaks at 980-1066 cm^{-1} were characteristic for the ν_1 and ν_3 P-O symmetric and asymmetric stretching of PO_4^{3-}, and peaks at 560 and 600 cm^{-1} were ascribed to ν_4 and ν_2 P-O stretching [2].

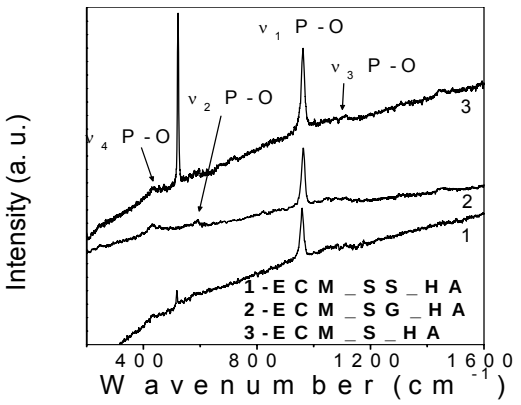


Fig. 3. Typical Raman spectra for SS/ECM/HA, S/ECM/HA and SG/ECM/HA composites after LLSI and subsequent soaking for 24 hours in SBF.

Raman spectra (not show here) present, in the case of applying the LLSI process and samples immediately taken out from the SBF, the formation of a very thin calcium phosphate layer. This step was regarded as the very fast (within a few minutes) formation of CaP nuclei, which was not feasible by simple soaking [3].

Furthermore, after subsequent soaking for 4 and 24 h, of the laser irradiated samples, 2.3 and 1.3 times thicker HA layers, respectively, have been grown on the composites. After applying the laser irradiation and subsequent short-time soaking process (i.e. 4 hours in the SBF), different CaP phases were obtained (graphs not presented). Their formation was more significant in the case of layers grown on the ECM/SS samples. With increasing duration of the soaking process, only one phase (HA) was present [1, 2]. Also, the increasing intensity of the ν_1 characteristic peak and the appearance of other, less intensive, peaks showed that that layer thickness increased after 24 hours soaking in the SBF. Fig. 4 reveals the surface topography of a composite, treated with LLSI, where the laser stripe can be observed. Laser irradiation treatment led to melting of the surface, which increased the surface roughness, as shown by CPM roughness measurements. The root mean square (rms) roughness inside and along the laser stripe was in the order of 0.52 to 2.9 μm.

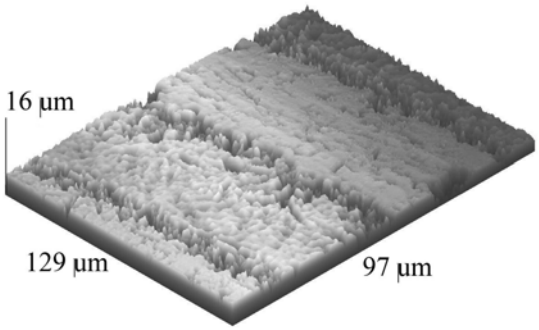


Fig. 4. Typical topography of a laser treated composite, measured by CPM.

Nanoindentation mapping of the surface across the laser stripes for elastic modulus measurements in Fig. 5a. The nanoidentation test consisted of twelve subsequent steps, each consisting of five measurements points in the range of a 5 μm square area, as shown in Fig. 5b.

SS/ECM and S/ECM composites have comparatively high elastic module (Fig. 6). Points disorder display big differences in the surface morphology across the laser stripes. It is shown that points disorder for SS/ECM composite comparable to S/ECM. The dependence has a negative curve slope, which reveals a decreasing modulus in the irradiated area. The calculated E standard deviation for the SS/ECM composites is in a wider range compared to S/ECM. The reason for this phenomenon is thermo-conductivity of the two materials. Therefore, SS/ECM is easily affected by the laser beam, posses inhomogeneously layer and different E in irradiated and non-irradiated areas.

Measured by the NanoScan hardness H (load 400 μN) for SG/ECM, SS/ECM and S/ECM composites obtained by LLSI and immediately taken from SBF are 2.55, 6.75, and 2.14 GPa respectively.

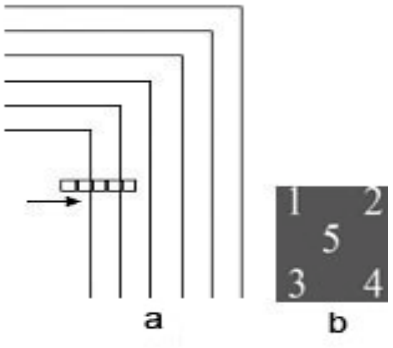


Fig. 5. Nanoindentation mapping of the scanning from the centre to the edge through the laser modified spiral-shaped zone. The scanned square areas (b) are 5 x 5 μm; the arrow shows the direction of scanning.

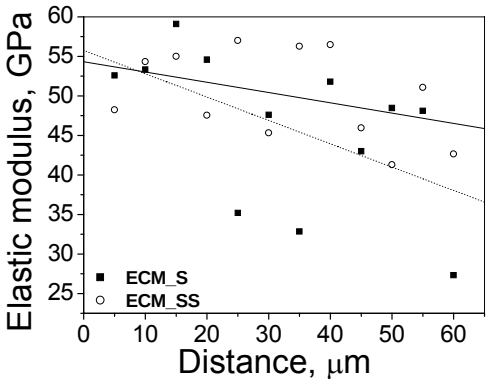


Fig.6. Nanoindentation mapping of the elastic modulus on SS/ECM and S/ECM composites across the laser stripes. The straight line is for a SS/ECM composite and the dotted one is for S/ECM. Error bar 9.5 %.

3. Conclusions

The mechanical property measurements of SS/ECM, SG/ECM and S/ECM composite layers were observed. Composite, helps HA crystal growth and influence its mechanical properties. The measured E for SS/ECM, SG/ECM and S/ECM composites is in the range of 50, 60 and 30 GPa respectively, hence the type of substrate affects the composite's E value.

Soaking in SBF for 4 hours of SG/ECM/HA S/ECM/HA, and SS/ECM/HA reduces the E value. Prolonging the soaking process up to 24h increases the thickness of the HA, measured by Raman and CPM spectroscopy, and leads to a slow increase in the E value in the range of 26.16, 16.10 and 12.36 respectively.

Nanoindentation mapping of the elastic modulus across the laser strip shows considerable disorder of the E values, as a result of increasing surface roughness. This technique can provide effective information about biomaterials that are hierarchical in structure, allowing measurement of properties at small length scales that can be used to better understand or model the macroscale behaviour.

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