Digest Journal of Nanomaterials and Biostructures Vol. 6, No 1, January-March 2011, p. 29-35

ORMOSILS SCAFFOLDS PRODUCED BY LASER PROCESSING FOR FIBROBLAST CELL GROWTH

A. MATEI[a,b], M. DINESCU[b], E.C. BURUIANA[a], T. BURUIANA[a], I. PETCU[c], C. MUSTACIOSU[c]

[a]*Petru Poni Institute of Macromolecular Chemistry, Grigore Ghica Voda 41A, 6600 Iasi, Romania*

[b]*National Institute for Lasers, Plasma and Radiation Physics, Atomistilor 409, 77125 Bucharest-Magurele, Romania*

[c]*Department of Environmental and Life Physics, National Institute for Physics and Nuclear Engineering 'Horia Hulubei', Bucharest, Romania*

Recently, considerable efforts have been directed towards micro/nanofabrication of two- and three-dimensional devices by two-photon initiated polymerization (2PP). There have been a lot of studies concerning the improvement of the efficiency and precision of 2PP, especially in hybrid methacrylates chemistry (ormosils) capable to generate various compositions with applications in electronics, coatings with tunable properties, drug delivery, biomedical implants and tissue engineering. In this work, substrate adherent and free-standing structures of organic-inorganic polymers were produced via Two Photon Polymerization (2PP) using a Ti: Sapphire laser with the aim to create a suitable scaffold for subsequently tissue engineering applications. The biocompatibility of the resulting materials was tested in L929 mouse fibroblasts to investigate the effect of the polymer structure and its morphology on cells behavior. Cells viability, proliferation and alignment were studied on polymeric structures with different architectures.

(Received November 30; accepted December 8, 2010)

Keywords: Laser, Fibroblast cell; Ormosils; Tssue engineering; Biocompatibility

1. Introduction

Two Photon Polymerization (2PP) is a modern technique which allows the construction of any complex 2D and 3D structures with resolution scaling down to 100 nm [1]. The method is based on the interaction of femtosecond laser radiation with a photosensitive material which induces a highly localized chemical reaction leading to polymerization. 2PP process consists in the simultaneous absorption of two photons, resulting in excitation of the molecules of a photo-initiator [2]. The two photon absorption process exhibits a quadratic dependence on the incident laser intensity [2-3], leading to a subsequently polymerization only in the vicinity of the focal point, where the requirement of high peak power is met [4]. The difference between polymerization via one photon absorption and two photons absorption is that for single photon the polymerization process occurs all the way of the focused beam, whereas for 2PP, the photo-polymerization is highly delimitated at the focused point. There 2PP is used for 3D micro and nanostructuring as a single step procedure.

A standard 2PP system consists in a femtosecond laser, a focusing element (a lens for micronic features or a microscope objective for nano-processing) and a translation XYZ stage. The laser beam can be moved against the sample (typically with galvano-mirrors) which is the faster method, or the sample can be moved in front of the focused laser beam by a translation stage [5].

There are many very promising bio-applications of Two Photons Polymerization (2PP) technique. For drug delivery, in order to decrease the affected zone at the injection site and the

pain of the patient, microneedles of ORMOCER produced by 2PP have been reported [6, 7]. Bone prosthetics and ossicular replacement prosthetics, as well as photonic crystals have also been fabricated with 2PP [5, 8, 9].

For tissue engineering, the ability to produce an arbitrary 3D scaffold structures is very attractive [10-11]. Scaffolds are required for the artificial fabrication of living tissue that will be able to integrate with the host tissue inside the body. 2PP could control the ability to build precise 3D microstructures of the scaffold, allowing modeling and reproducing cellular microenvironment. In addition, there is a need for novel biodegradable scaffolds, as reported by F. Claeyssens [12].

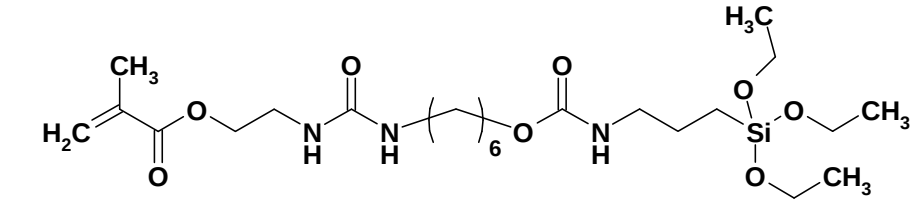
ORMOSILS (organically modified silicates) are hybrid monomers that exhibit strong covalent bonds between the organic and inorganic compounds with high optical quality, good mechanical characteristics and thermal stability. The ormosils show great promise in a wide range of applications, such as optical waveguides [13], coatings with tailored properties [14], biosensors [15], medicine [16].

In this work, the results on creating biocompatible 2D structure of new hybrid monomers, taken alone or in combination with a urethane dimethacrylate containing poly(ethylene oxide) of 400 a.m.u. average molecular weight to improve the biocompatible properties are envisaged. 2PP was used as the processing method for producing compact, substrate adherent or free standing structures.

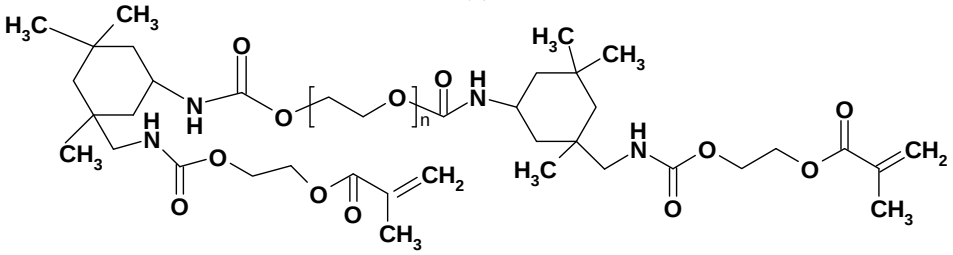
2. Materials and Methods

2.1 Materials

Lab synthesized organic modified silicates were irradiated in the 2PP experiments. The hybrid monomer (methacrylate containing triethoxysilane) used for the subsequent laser processing experiments is N,N'-(methacryloyloxyethyl triehtoxy silyl propyl carbamoyl-oxyhexyl)-urea (SIM 3) (Figure 1). SIM 3 was tested alone or in combination with a multifunctional oligomer of urethane dimethacrylate type (UDA), used as a co-monomer. The chemical structure of UDA is also presented in figure 1.



(a)



(b)

Figure 1. (a) The chemical structure of (a) SIM 3: N,N'-(methacryloyloxyethyl triehtoxy silyl propyl carbamoyloxyhexyl)-ureea and (b) UDA.

2.2 Scaffold preparation

The monomers, SIM 3 or the mixture SIM 3: UDA (1:3 weight ratio), a photoinitiator and a solvent (tetrahydrofuran, THF) were used to prepare a solution that was subsequently uniformly drop-cast on glass substrates. Commercial Irgacure 369 (2-Benzyl-2-dimethylamino-1-(4-

morpholinophenyl)-butanone-1) was used as photoinitiator, as we have used before for similar hybrid monomers [17, 18].

The Two Photon Polymerization set-up for the experiments was a Direct Laser Writing (DLW) workstation coupled with an amplified femtosecond laser Clark CPA-2010 emitting at 775 nm, with pulse duration of 200 fsec and a repetition rate of 2 kHz. The laser beam was moved on the sample using galvanic mirrors. A Z translation stage and a visualization system with CCD camera are used for precisely positioning the sample in the plane of the focused laser.

The processing setup permits the easy change of the focusing element, depending on the desired resolution of the polymerized structure. In our work, a 100 mm lens with the focal spot diameter of 35 μm was used and areas up to 20 mm^2 were processed. As we have mentioned, the aim of this study was to create micronic structures to be tested in fibroblast cells cultures.

The laser fluence, the laser beam scanning velocity, and also the geometry of the designed structures were varied, in order to establish the most appropriate conditions for obtaining a compact and well defined structure, suitable for further experiments as scaffolds for cell cultures.

2.3. Biological tests

Cell culture studies were undertaken on L929 mouse fibroblasts cell line in order to investigate the effect of the polymer on cell behavior. The cytotoxicity was evaluated according to ISO/EN 10993-5 , using the agar diffusion test either on extracts of the polymer films (in complete MEM culture medium), or on polymer films in direct contact with the agar surface. Cells were exposed to the extracts or directly to the presence of the polymer films for 24h. MEM without serum was used as negative control and a solution of 0,45 % phenol was used as positive control.

The viability and proliferation of the treated cells were characterizied by a metabolic marker: the mithochondrial reduction of a tetrazolium salt (MTS) into a formazan product, soluble in the culture medium. The “Cell Titer 96Aquous One Solution Cell Proliferation Assay” was used (Promega) and the absorbance of the samples was recorded using TECAN Spectro Fluo Plus reader.

The glass slips covered with polymerized SIM 3, were placed in 24 well plate. 10.000 cells / 50 μl were seeded on each disc and 1 ml complete MEM medium was added. The medium was removed after 24 hours of incubation and 360 μl MEM with MTS assay reagent were added and kept for incubation for 3 more hours. Then, 100 μl solution were moved in triplicate in a 96 well plate and measured at 490 nm.

In order to observe any apoptotic induction in the treated cells a double fluorescent staining was performed with acridin orange and ethidium homodimer–1. The morphology of the cells nuclei were then analyzed using an epifluorescence Olympus microscope with the appropriated filters to observe in the same time the emission of both fluorophores.

3. Results and discussion

Grids and lines systems with different distances between lines (50, 70 and 100 μm) were built. Figure 2 presents the optical images of three laser processed grid structures with line spacing of 50 μm. At first, we examined the image of the polymerized SIM-3 (figure 2.a) and then those of the urethane oligomer UDA (figure 2.b) or of the combination of SIM 3:UDA (figure 2.c) As it can be seen from these images, the morphology of polymerized UDA is different from the other two structures (for instance, the line thickness is about half thinner than for SIM 3). For the monomer combination in figure 2.c, it seems that the UDA presence does not introduce changes in the surface morphology of the polymerized structure, so that the structures a and c look similar, with line thickness of about 10-15 μm.

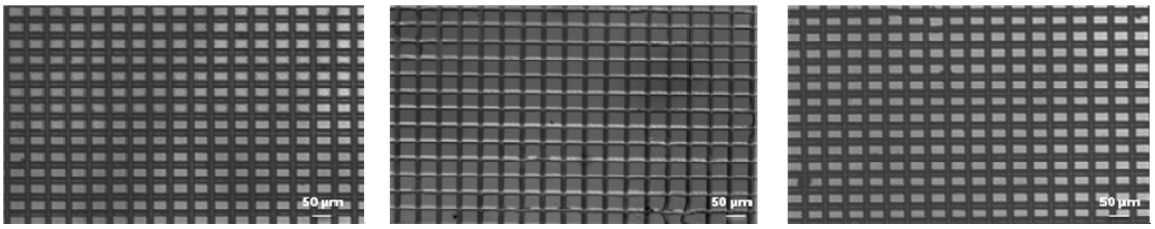


Fig. 2. Optical microscopy images of polymerized (a) SIM 3 (b) UDA (c) SIM 3 +UDA), Photoinitiator = Irgacure 369, Distance between lines = 50 µm, (a) Laser incident power = 15 mW, Scanning velocity =1 mm/s; (b) Laser incident power = 10 mW, Scanning velocity = 3 mm/s; (c) Laser incident power = 15 mW, Scanning velocity = 1 mm/s.

On the other hand, compact structures without defects can be processed over large areas. An example is presented in figure 3. In the 3x3 mm^2 polymerized structure of SIM 3, the lines are about 10-15 µm thick and 10 µm high.

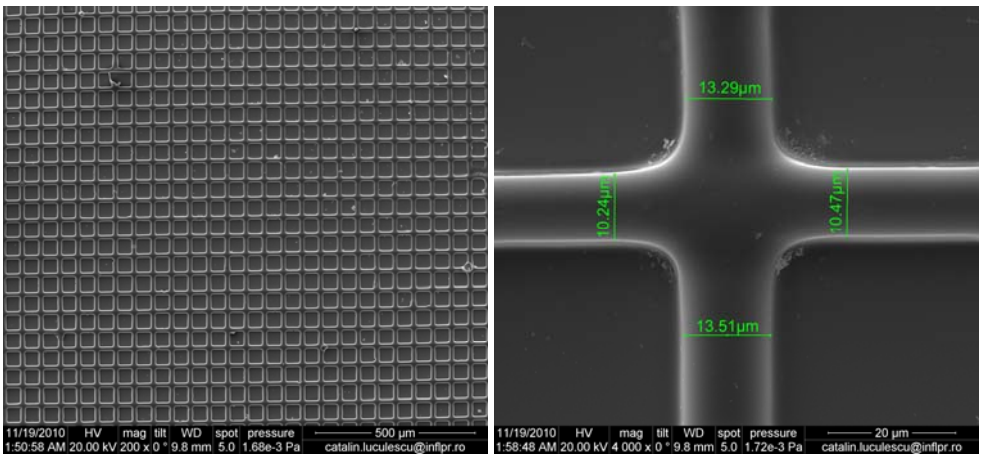
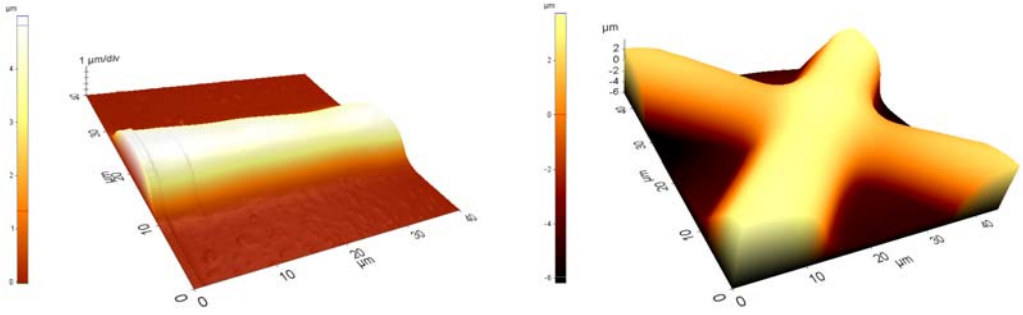


Fig. 3. SEM images of a 60x60 lines polymerized SIM 3 sample, Photoinitiator = IRG 369, Distance between lines = 50 µm, Scanning velocity = 1 mm/s, Laser incident power = 15 mW.

AFM images on two structures, a line and a grid of polymerized SIM 3 are presented in figure 4. For the first case, the line is 5 µm high (with a variation of about 15%) and about 17 µm thick (with a variation of about 15%). For the second case, the lines are about 10 µm high, and the thickness is around 15 µm, as these images suggested.



(a) (b)

Fig. 4. AFM images of (a) a line of polymerized SIM 3; (b) a crossing of a polymerized SIM 3; Scanning velocity = 1 mm/s, Laser incident power = 15 mW.

Similar structures (grids with different spacing between lines) can be grown and detached from the glass substrate in order to create a free-standing scaffold. Figure 5 presents a 30x30 lines

laser processed structure, with 100 µm spacing between lines. These structures have been detached from the glass substrate and kept in alcohol or milli Q water. The scaffolds were very compact and defect free. Moreover, they could be easily manipulated, without being deteriorated.

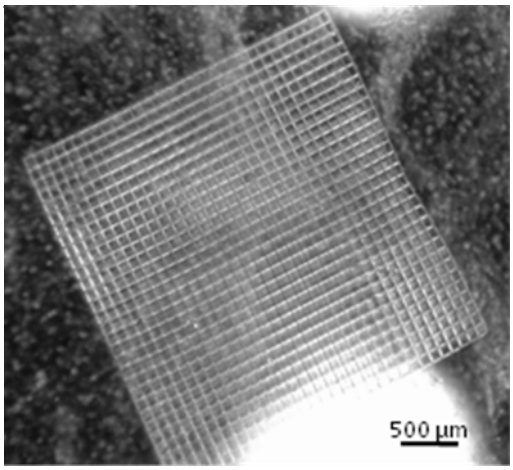


Fig. 5. Optical image of a free-standing structure 3x3 mm^2 generated by the mixture SIM 3 and UDA. Spacing between lines 100 µm; Scanning velocity = 1 mm/s, Laser incident power = 15 mW.

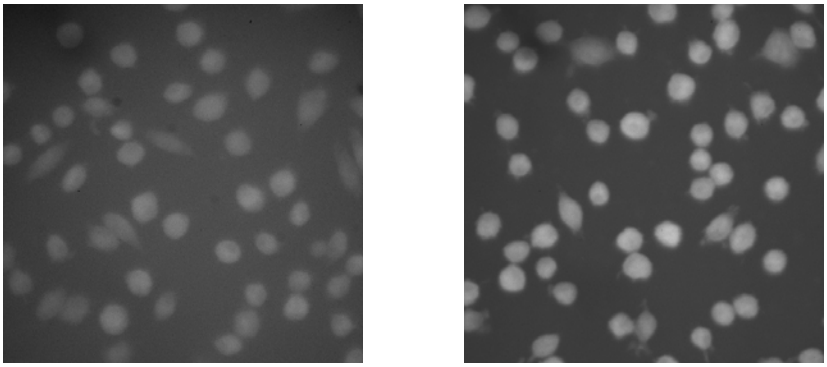
The cells viability was tested on glasses covered with polymeric thin films. The influence of the polymeric lines spacing was also investigated. The calculated cells viability rate after 24 hours was 0.87 for those in contact with the polymeric films and 1 for those in contact with the glass slides or the control well. After 48 hours, the viability rate decreased for cells in contact with the polymer to 0.81 and for cells in contact with the glass substrate to 0.94, compared to the control well where the viability rate was considered as 1.

The biocompatibility test performed on the polymer films revealed the non-cytotoxicity of the investigated materials.

Table 1: Biocompatibility of the polymer films assessed by Agar Diffusion Test (ISO/EN 10993-5)

Parameters	Negative control (MEM)	Positive control (0..45% phenol)	Extract of the polymer film	Polymer film in contact with agar	Glass slide in contact with agar
Zone index	0	2	1	0	0
Lysis index (% lysis)	1 (12%)	3 (49%)	1 (19%)	1 (15%)	1 (13%)
Cytotoxicity	Non cytotoxic	Moderately cytotoxic	Non cytotoxic	Non cytotoxic	Non cytotoxic

For the apoptosis experiments, the cells grown on polymeric films, marked and analyzed in fluorescent microscopy proved to be viable cells. An example of such viable treated cells is presented in figure 6.



(a) (b)

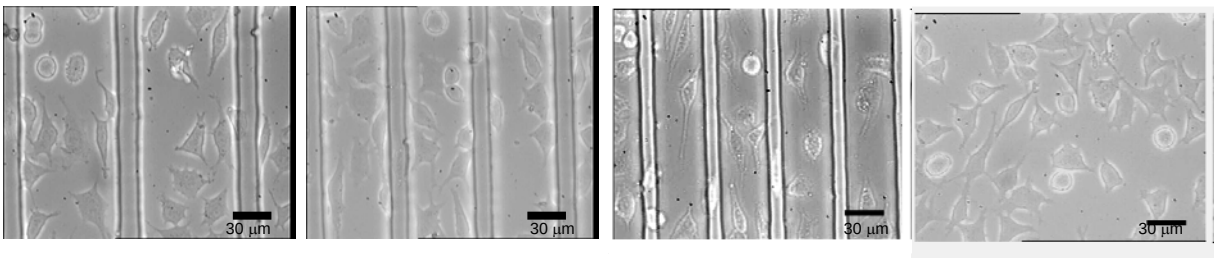
Fig. 6. L929 mouse fibroblasts seeded on (a) a film of polymerized SIM 3 and (b) on glass control (b)

Polymerized SIM 3 structures consisting of lines were used for cell culture experiments. As we have mentioned before, the investigated cells were fibroblasts L929, for which polymeric lines of SIM 3 with different spacing between lines (50, 70 and 100 μm) were tested.

At 30 minutes after seeding, the cells start to form processes and after 4 hours, the shape of the cells becomes polygonal. The results given in table 2 sustain that some differences in cells behavior appeared for different configurations (line spacing). It is very interesting to note that, for all the investigated structures, the cells proliferate and show a tendency to align along the polymeric lines, as it can be seen from figure 7, where the control surface is also presented.

Table 1. Cells shape on lines structures with different spacing between lines

Cells shape		Control (%)	Spacing between lines 100 μm (%)	Spacing between lines 70 μm (%)	Spacing between lines 50 μm (%)
0.5 h	Round	94	92	98	97
	Round with starting processes	6	7	2	3
	Polygonal	-	-	-	-
4 h	Round	20	17	20	16
	Round with starting processes	30	43	52	59
	Polygonal	50	40	28	25



(a) (b) (c) (d)

Figure 7. Fibroblasts culture on polymerized SIM 3 with line spacing of 100 μm (a), 70 μm (b), 50 μm (c), control surface

The 2PP procedure confirms again that this is a relatively simple method of preparing ormosils structures, which could be used as scaffolds for tissue engineering applications.

4. Conclusions

Two-dimensional structures starting from organic/inorganic hybrid monomers were obtained by two photon polymerization. The laser processed structures acting as scaffolds are biocompatible and easily processable, making them suitable for tissue engineering applications.

The polymeric films are biocompatible; the cells adhered and proliferated on the laser processed surface. On the polymeric lines, the fibroblast cells extended and aligned along the lines.

Acknowledgements

Two authors (Andreea Matei and Tinca Buruiana) acknowledge the financial support of European Social Fund – „Cristofor I. Simionescu" Postdoctoral Fellowship Programme (ID POSDRU/89/1.5/S/55216), Sectoral Operational Programme Human Resources Development 2007 – 2013. The authors acknowledge Dr. Marian Zamfirescu, for helping with the 2PP set-up.

References

[1] H.-B. Sun, S. Kawata, Adv. Polym. Sci. **170**, 169 (2004)
[2] S.D. Gittard, R.J. Narayan, Rev. Med. Devices **7**(3), 343-356 (2010)
[3] T. Weiß, G. Hildebrand, R. Schade, K. Liefeith, Eng. Life Sci. **9**(5), 384 (2009)
[4] K. D. Belfield, X. Ren, E. W. Van Stryland, D. J. Hagan, V. Dubikovsky, E. J. Miesak, J. Am. Chem. Soc., **122**(6), 1217 (2000)
[5] Ovsianikov, A; Ostendorf, A; Chichkov, BN, Appl. Surf. Sci., **253** (15), Pp: 6599-6602 (2007)
[6] R. J. Narayan, C. Jin, A. Doraiswamy, I. N. Mihailescu, M. Jelinek, A. Ovsianikov, B. N. Chichkov, D. B. Chrisey, Adv. Eng. Mater. 7, no. 12, pp. 1083-1098 (2005).
[7] M. R. Prausnitz, Adv. Drug Deliv. Rev. 56, no. 5, pp. 581-587 (2004).
[8] A. Ovsianikov, B. Chichkov, P. Mente, N. A. Monteiro-Riviere, A. Doraiswamy, R. J. Narayan, Int. J. Appl. Ceramic Technol. **4**(1), 22 (2007)
[9] R. Houbertz, P. Declerck, S. Passinger, A. Ovsianikov, J. Serbin, B. N. Chichkov, Phys. Stat. Sol. (a), **204**(11), 3662 (2007)
[10] M. Malinauskas, P. Danilevičius, D. Baltriukienė, M. Rutkauskas, A. Žukauskas, Ž. Kairytė, G. Bičkauskaitė, V. Purlys, D. Paipulas, V. Bukelskienė, R. Gadonas, Lithuanian J. Phys., **50**(1), 75 (2010)
[11] A. Ovsianikov, S. Schlie, A. Ngezahayo, A. Haverich and B. N. Chichkov1, J Tissue Eng Regen Med **1**, 443 (2007).
[12] F. Claeyssens, E. A. Hasan, A. Gaidukeviciute, D. S. Achilleos, A. Ranella, C. Reinhardt, A. Ovsianikov, X. Shizhou, C. Fotakis, M. Vamvakaki, B. N. Chichkov and M. Farsari, Langmuir, **25**(5), 3219 (2009).
[13] B.L. Wang, L.L. Hu, Ceram Int, **32**(1), 7 (2006)
[14] G. Palmisano, E. Le Bourhis, R. Ciriminna, D. Tranchida, and M. Pagliaro, Langmuir, **22**(26), 11158 (2006)
[15] P. C. Pandey, S. Upadhyay, I. Tiwari, G. Singh, V. S. Tripathi, Sensor Act. B: Chem, **75**(1-2), 48 (2001)
[16] M. Manzano, A.J. Salinas,M. Vallet-Regí, Progress in Solid State Ch, **34**(2-4), 267 (2006).
[17] A. Matei, M. Zamfirescu, F. Jipa, C. Luculescu, M. Dinescu, E.C. Buruiana, T. Buruiana, L.E. Sima, S.M. Petrescu, American Institute of Physics (AIP) proceedings, to be published
[18] A. Matei, M. Zamfirescu, C. Radu, M. Dinescu, E.C. Buruiana, T. Buruiana, L.E. Sima, S.M. Petrescu, Appl. Phys. A, to be published.