

## TWO-PHOTON FLUORESCENCE EXCITATION CROSS-SECTIONS OF PHOTSENSITIZER THIOXANTHONE TO FABRICATE CELL SCAFFOLD FOR BIOMEDICAL APPLICATIONS

Bibi Safia Haq<sup>1,2</sup>, Hidayat Ullah Khan<sup>3</sup>, Abdul Shakoor<sup>4</sup>, Shahnaz Attaullah<sup>5</sup>, Kausar Shaheen<sup>1</sup>, Khan Alam<sup>3</sup>

### ABSTRACT

*This study investigates the fluorescence emission profile of Thioxanthone in Ethanol due to two photon absorption excitation. Thioxanthone was investigated as Photosensitizer to fabricate cell scaffold in Ethoxylated Bis Phenol A Dimethacrylate for tissue repair in the field of biomedical. Also Incident Laser Power Effects were analyzed and found that the Fluorescence Intensity increases with increasing Incident Laser Power,  $I_0$ , and the emitted fluorescence obeys the square-law dependences. Also when the Photo-Sensitizer was excited at different wavelengths (800nm, 400nm), it was found that Fluorescence Emission Profile did not change but variations occurred only in Fluorescence Emission Intensity.*

**KEY WORDS:** *Two-photon absorption process, Photo-Sensitizer, Two Photon Fluorescence, Cell Scaffold, Tissue Engineering*

### INTRODUCTION

Tissue Engineering approaches are currently being extensively investigated to produce substitutes that can restore, and maintain biological function, or improve functions of a tissue, or a whole organ.

Fabrication of biocompatible materials are quite interesting which can be used as the mechanical framework for attachment of cells and proliferation to enable the integration of living cells<sup>1</sup>. Different materials can be used for the fabrication of scaffold such as ceramics, biopolymers, synthetic polymers, and metals that are not rejected by the body upon implantation. Identification of a suitable material is required to create an engineered tissue for specific application. Also structuring of the scaffold material and cell seeding into the scaffold for cell culturing is required<sup>2</sup>.

For rapid-prototyping of various medical devices such as micro needles for drug delivery, bone replacement prostheses, and tissue engineering scaffolds<sup>3</sup> Two-Photon-Polymerization (TPP) techniques has been used successfully. 3D structures can be fabricated using nonlinear optical micro structuring technique based on two-photon polymerization of organic materials<sup>4</sup>. TPA process, applied to photon polymerization is used for many advanced applications such as fabrication of 3D

microstructures, 3D integrated optical components, optical data storage<sup>5</sup> and also in getting attention because of the potential for two-photon excitation for bimolecular imaging, two-photon photodynamic therapy<sup>6</sup> and two-photon laser scanning fluorescence microscopy<sup>7</sup>.

In two photon absorption phenomena, molecules move from low energy level to high energy level by absorbing two photons simultaneously. To control threshold energy, a femto-second laser system with high peak power, and 800nm wavelength, close to the half of the wavelength of polymerization, and very short pulse width,  $\leq 100$  femto-second (fs), are used for two-photon polymerization processes<sup>8</sup>.

Fabrication of  $\mu\text{m}$  and  $\text{nm}$  scale 3D structures<sup>9</sup> using TPP process is an application of TPA phenomenon. To initiate TPA polymerization processes, chromophores with an efficient TPA property are required as photosensitizer. In the last few years, rigorous work<sup>5,10,11,12</sup> has been undertaken for the development of two-photon chromophores with high TPA cross section to fabricate micron size 3D structures.

In the current study, *Thioxanthone* was investigated as photosensitizer and photoinitiator, and its two-photon excitation fluorescence was recorded. To get the excited fluorescence, femto-second laser beam was allowed

1 Department of Physics, JCW University of Peshawar, Khyber Pakhtunehwa, Pakistan

2 Department of Physics and Mathematics, University of Hull, Hull, HU6 7RX, United Kingdom

3 Department of Physics University of Peshawar, Khyber Pakhtunehwa, Pakistan

4 Department of Materials, SAACME, Loughborough University, LE11 3TU, United Kingdom

5 Department of Chemistry, JCW University of Peshawar, Khyber Pakhtunehwa, Pakistan

to fall on the solutions of the material at 800nm and 400nm wavelength. Emission spectra at 800nm and 400nm wavelength excitation were compared, and their absorption cross sections were calculated.

Various studies<sup>11,13,14</sup> have revealed that single photon and two photon excited fluorescence are essentially identical processes. Controversy exists, however, about the determination of the collection efficiency and fluorescence quantum yield<sup>15</sup>. This problem can be minimized if the measurement of intensity ratio of single photon to two-photon excited fluorescence is performed, when the physical and geometrical arrangement within the same optical system are identical<sup>11</sup>.

Photo-polymerization initiated by a two photon absorption (TPA) process is used in the present study to fabricate three dimensional (3D) micrometer sized structures and to rapid-prototype tissue engineering scaffolds. In this way, the present investigation explores the use of lasers in such treatments or in the production of structures required for research into new procedures.

## MATERIAL USED

*Photosensitizer Thioxanthone*, used in this study is Type II photoinitiator. *1-chloro-4-propoxythioxanthone* ( $C_{16}H_{13}ClO_2S$ ) is the Chemical Name of *Thioxanthone* with molecular weight 304.79. Its molecular structure is displayed in Figure 1. Compounds that initiate reactions without being consumed are known as Type-II Photoinitiators. They are generally slower than type I photoinitiators<sup>16-18</sup>. To generate a micron scale 3D structure,

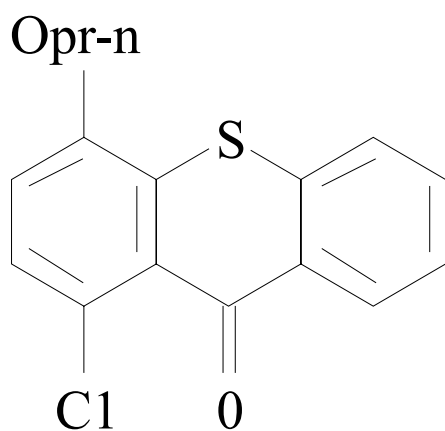


Figure 1. Chemical structure for 1-chloro-4-propoxy-thioxanthone

two-photon polymerization (TPP) of photo-cross-linkable Ethoxylated Bisphenol A Dimethacrylate (EBPADMA) (6EO) monomers was used.

## Experimental arrangements and Methods

To determine the single photon and double photon fluorescence emission spectra for *1-chloro-4-propoxythioxanthone*, the sample solution with  $1.6 \times 10^{-2} M$  was exposed to laser pulses in a 10mm cuvette. The incident laser beam strike the cuvette at the middle passing through irises of different diameters. To record the two photon excited fluorescence spectrum, a fiber coupled spectrometer (USB 4000 Ocean optics) connected to computer was used. To produce single photon excited fluorescence, 400nm wavelength laser light was used under similar experimental set up aforementioned. Fluorescence spectra at 400nm and 800nm wavelength were recorded accompanied by the plots of their normalized intensities. Figure 2 shows solution of the photosensitizer with  $1.6 \times 10^{-2} M$  concentration in ethanol exposed to femto-second laser system with 650mW average laser power and 146fs pulse duration (repetition rate = 1 kHz). The laser beam was passed through attenuation filter and only 200mW at 800nm (or alternatively  $381 \mu W$  at 400nm) was shine on the cuvette through iris without focusing. A strong fluorescence of *1-chloro-4-propoxythioxanthone* was observed at 490 nm wavelength.

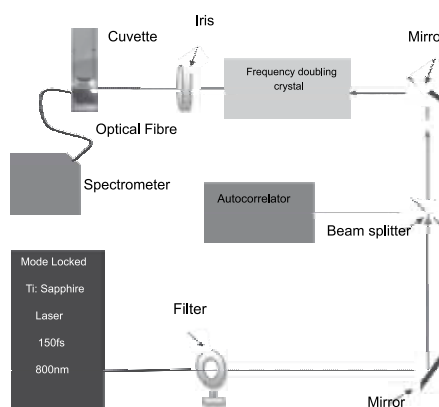


Figure 2 Experimental setup to study Two-photon excitation of photosensitizer

## RESULTS AND DISCUSSIONS

Using a UV/Vis Spectrometer Lambda Bio 10 (Perkin Elmer), absorbance at different concentrations was worked

out for 1-chloro-4-propoxythioxanthone. Laser light was allowed to fall on a solution of the photoinitiator Thioxanthone (0.1M) in ethanol in a 10mm cuvette. Spectrums of the material indicate that the absorbance of the photoinitiator Thioxanthone increases as a function of increase in concentration. The peak absorbance is at 313nm and 385nm wavelength indicated in Figure 3.

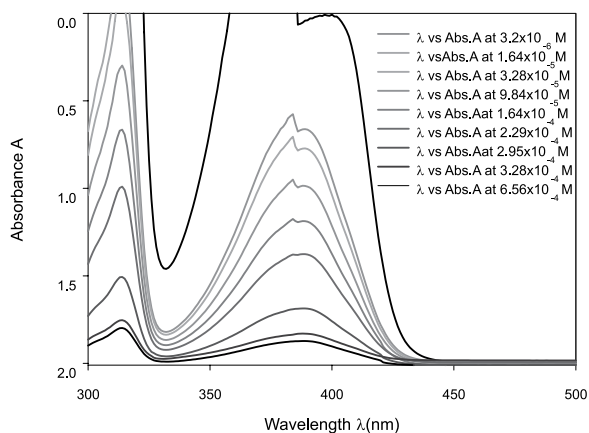


Figure 3. Absorbance spectra at different concentrations of Thioxanthone in ethanol

The appearance of several absorption peaks or shoulders for a given photosensitizer is common<sup>19-21</sup> and may depends on the solvent used as has appeared in Figure 3 where the solvent is ethanol. The molar extinction coefficient  $\epsilon$  from gradient of the graph (absorbance vs. concentrations) at 400nm wavelength was found to be  $2870 \text{ M}^{-1}\text{cm}^{-1}$ , as shown in Fig 4.

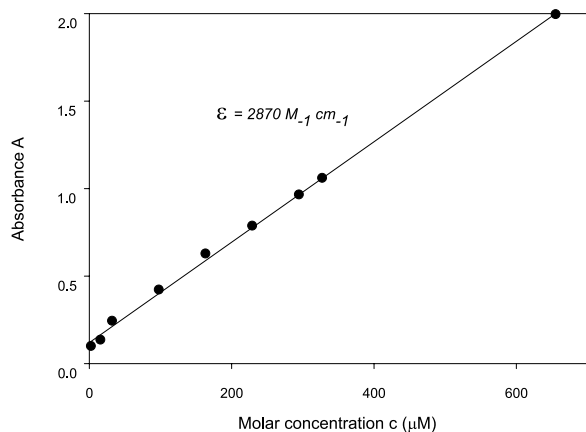


Figure 4. Molar absorption coefficient,  $\epsilon$ , for 1-chloro-4-propoxythioxanthone at 400nm wavelength

Fluorescence spectra emitted at 800nm and 400nm wavelength were plotted as shown in Figure 5. Their normalized curves were also plotted as given in Figure 6. The onset of the centered peaks of the fluorescence spectra excited at 800nm and 400nm at  $\sim 490\text{nm}$  indicated that single photon and double photon absorption populate the same fluorescing excited state<sup>17,18</sup>.

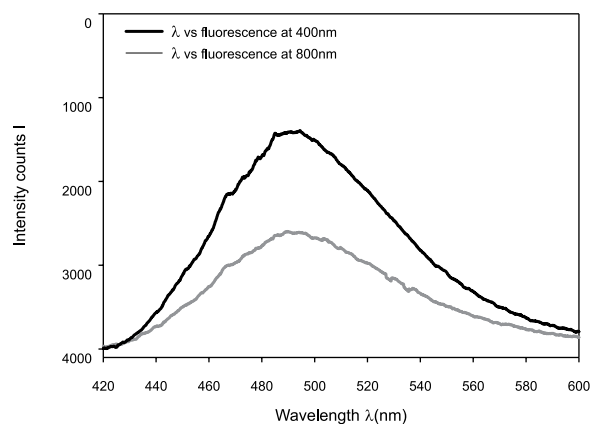


Figure 5. Excitation spectra of Thioxanthone in Ethanol at 400 and 800nm wavelength

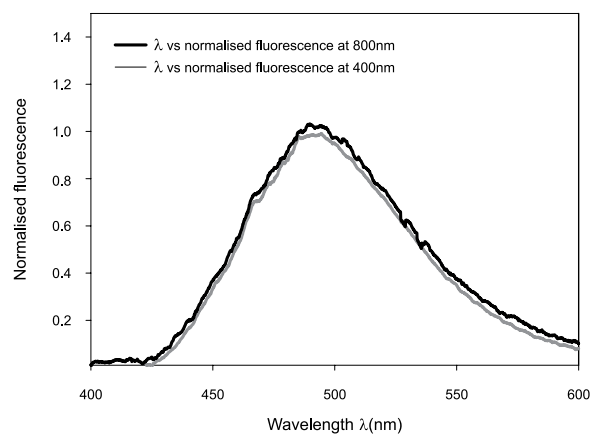
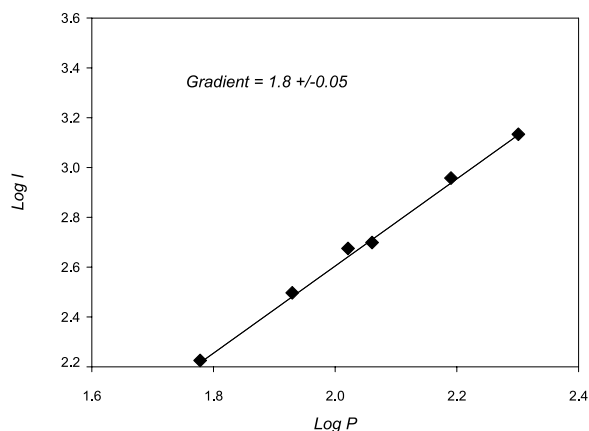


Figure 6. Normalized excitation spectra for Thioxanthone in Ethanol at 400nm and 800nm wavelength

#### 4.1: Two-Photon Absorption phenomenon

For the excitation of fluorophores, laser power was made incident on a fluorophores-containing specimen.

In logarithmic plot of fluorescence verses laser power, it was observed that the fluorescence emitted due to TPA phenomenon dependence on quadratic power. A



**Figure 7. Logarithmic plot of fluorescence as a function of laser power for Thioxanthone shows that linear fit to the data has a gradient of  $1.8 \pm 0.05$ .**

plot on log–log scales shown in Figure 7 indicates that fluorescence intensity depends on the power of incident laser. The fitted slope for *1-chloro-4-propoxythioxanthone* is  $1.8 \pm 0.05$  which confirm squared power dependence.

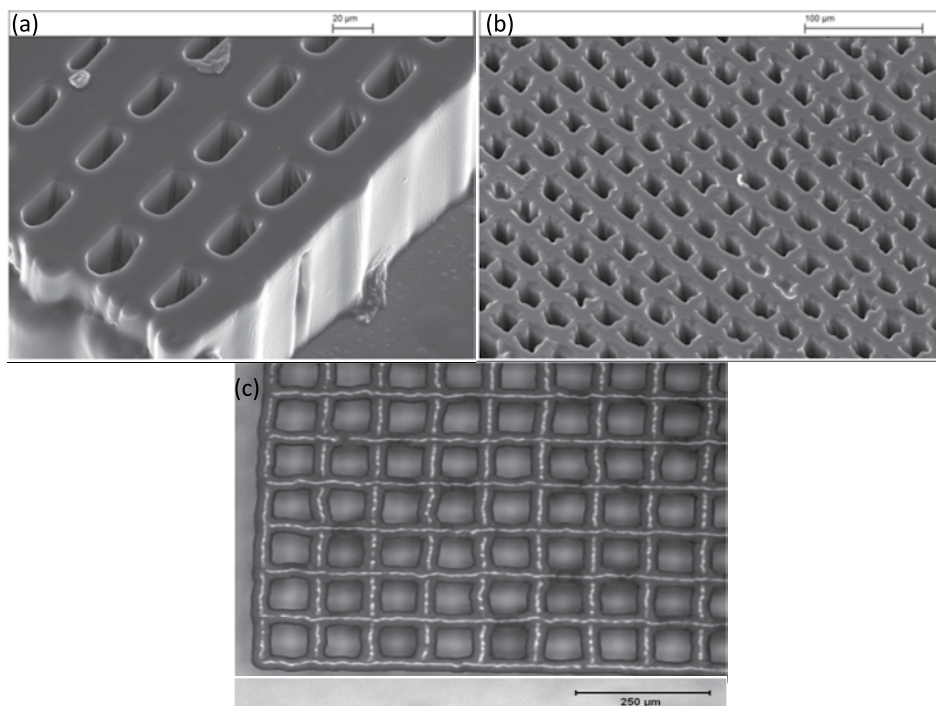
The OPA cross-section, for the  $16 \times 10^{-3}$  molar solution

was based on the measurement of molar absorption coefficient  $\epsilon$  found to be  $2870 \text{ M}^{-1} \text{ cm}^{-1}$  for *1-chloro-4-propoxythioxanthone*. OPA cross section was determined to be  $11 \times 10^{-18} \text{ cm}^2 \text{ molecule}^{-1}$ . A value for the TPA cross-section,  $\delta_{2a} = 7 \times 10^{-50} \text{ cm}^4 \text{ s photon}^{-1}$ , was calculated for *Thioxanthone* in ethanol by comparing the fluorescence signals emitted due to 800nm and 400nm wavelengths excitation respectively<sup>19</sup>.

#### 4.2 :Fabrication of cell-scaffold for Biomedical Applications

To analyze *1-chloro-4-propoxythioxanthone* as photoinitiator in the monomer, its solution (0.1M) was prepared in *Ethoxylated Bis Phenol A Dimethacrylate (EO=6)*. In order to investigate polymerization phenomenon in the monomer, A femto-second

Ti: Sapphire laser source was used to shine laser light on the solution contained in a 10mm cuvette. It was observed that no polymerization effects can be seen in the presence of Thioxanthone derivative when used as



**Figure 8. SEM images (a) tissue scaffold having  $14 \mu\text{m}$  pore sizes with  $10 \mu\text{m}$  ridges suitable for liver as well as fibroblast and (b) 3D structure fabricated for cell scaffolds using two-photon polymerization with small pore size suitable for the cell possession and growth especially for the generation of fibroblast. (c) Optical microscope image of cell scaffold for large pore size.**

Photoinitiator. This revealed that a proper co-initiator is needed to make *1-chloro-4-propoxythioxanthone* an efficient initiator. In the presence of Photosensitizer 4, *4'-Bis (diethylamino) Benzophenon* the resin showed Polymerization and cell scaffold were fabricated for tissue repair as shown in Figure 8. These cell scaffold structures were fabricated to get pores size of approximately 13 $\mu$ m horizontally and 12 $\mu$ m vertically with approximately 10 to 14 $\mu$ m ridges between them as can be seen in Figure 8. Small pore sizes are chosen in order to provide structures where cells might adhere and flourish. Cell scaffolds with such pore size are suitable for the cell possession and growth especially for the generation of fibroblast<sup>20</sup>.

## CONCLUSIONS

This study was aimed to investigate the two photon absorption excitation of *1-chloro-4-propoxythioxanthone* and the incident laser power effects on fluorescence emission profile. Also the photosensitizer was analyzed for two photon polymerization to prepare cell scaffold for tissue repair. To find out two-photon absorption cross-section of *1-chloro-4-propoxythioxanthone*, the relative efficiency of one- and two-photon excited emission spectrum with femtosecond laser pulse trains were used in the vicinity of the Soret band. Fluorescence studies confirm the two-photon excitation of *1-chloro-4-propoxythioxanthone*, exposed to 800nm wavelength excitation. It was found that the fluorescence intensity increases with increasing incident laser power,  $I_0$ , and the fluorescence emitted due to excitations obeys the square-law dependences.

Polymerization effects were not observed in the resin exposed to the laser light, containing Thioxanthone derivatives as Photo-initiator. This indicates that in the absence of a co-initiator, photo-sensitizer used is not an efficient initiator. In the presence of the photo-sensitizer 4, *4'-Bis (diethylamino) Benzo-phenon* or *Camphor-quinone* (CQ) polymerization was observed.

It was observed that fluorescence emitted at different wavelengths (800nm, 400nm) had same emission profile but variations occurred only in the intensity of the emitted fluorescence.

## REFERENCES

1. S. Lin-Gibson, J. A. Cooper, F. A. Landis, and M. T. Cicerone, 2007, "Systematic Investigation of Pore Size and Content on Scaffold Morphometric Parameters and Properties", *Biomacromolecules*, 8:1511-1518.
2. Frederik Claeysens, Erol A. Hasan, Arune Gaidukeviciute, Demetra S. Achilleos, Anthi Ranella, Carsten Reinhardt, et al, 2009, "Three-Dimensional Biodegradable Structures Fabricated by Two-Photon Polymerization", *Langmuir*, 25: 3219-3223.
3. B. Chichkov, 2007, "Two-photon polymerization enhances rapid prototyping of medical devices", *SPIE—The International Society for Optical Engineering*, 1-2.
4. A. Ovsianikov, J. Viertl, B. Chichkov, M. Oubaha, B. MacCraith, I. Sakellari, et al, 2008, "Ultra-Low Shrinkage Hybrid Photosensitive Material for Two-Photon Polymerization Microfabrication", *ACS Nano*, 2: 2257-2262.
5. Schafer, K. J., J. M. Hales, M. Balu, K. D. Belfield, E. W. Van Stryland, and D. J. Hagan, 2004, "Two-photon absorption cross-sections of common photoinitiators", *Journal of Photochemistry and Photobiology A: Chemistry*, 162: 497-502.
6. J. Liu, Y. W. Zhao, J. Q. Zhao, A. D. Xia, L. J. Jiang, S. Wu, L. Ma, et al, 2002, "Two-photon excitation studies of hypocrellins for photodynamic therapy", *Journal of Photochemistry and Photobiology B: Biology*, 68: 156-164.
7. D. X. Cao, Z. Q. Liu, Q. Fang, G. B. Xu, G. Xue, G. Q. Liu et al, 2004, "Blue two-photon excited fluorescence of several D-[pi]-D, A-[pi]-A, and D-[pi]-A compounds featuring dimesitylboryl acceptor", *Journal of Organometallic Chemistry*, 689:2201-2206.
8. K. Lee, D. Yang, P. Prabhakaran, J. Park, S. H. Park, and R. H. Kim, 2006, "Recent developments in the use of two-photon polymerization in precise 2D and 3D microfabrications", *Polym. Adv. Technol*,

- 17:72-82.
9. S. Passinger, 2008, "Two-Photon Polymerization and application to Surface Plasmon Polaritons", Cuvillier Verlag, Göttingen.
  10. C. Xu and W. W. Webb, 1996, "Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm", *Journal of the Optical Society of America B: Optical Physics*, 13:481-491
  11. J. M. Song, T. Inoue, H. Kawazumi, and T. Ogawa, 1999, "Determination of two photon absorption cross section of fluorescein using a mode locked titanium sapphire laser", *Analytical Sciences*, 15: 601-603.
  12. H. V. W. Martinus, N. Nerambourg, D. Pelegry, Y. L. Grand, and M. Blanchard-Desce, 2005, "Action cross sections of two-photon excited luminescence of some Eu(III) and Tb(III) complexes", *Photochemical & Photobiological Sciences*, 4:531-538.
  13. M. Atif, 2005, "Fluorescence dynamics studies of PDT photosensitizer", *Fluorescence dynamics studies of PDT photosensitizer*, PhD thesis, Department of physical Sciences (physics), University of Hull.
  14. M. Atif, P. E. Dyer, T. A. Paget, H. V. Snelling, and M. R. Stringer, 2007, "Two-photon excitation studies of m-THPC photosensitizer and photodynamic activity in an epithelial cell line", *Photodiagnosis and Photodynamic Therapy*, 4: 106-111.
  15. R. Signorini, C. Ferrante, D. Pedron, M. Zerbetto, E. Cecchetto, M. Slaviero et al, 2008, "Effective Two-Photon Absorption Cross Section of Heteroaromatic Quadrupolar Dyes: Dependence on Measurement Technique and Laser Pulse Characteristics", *The Journal of Physical Chemistry A*, 112:4224-4234.
  16. D. K. Balta, S. Keskin, F. Karasu, and N. Arsu, 2007, "Quinoxaline derivatives as photoinitiators in UV-cured coatings," *Progress in Organic Coatings*, 60: 207-210.
  17. S. Jockusch, Q. Zheng, G. S. He, H. E. Pudavar, D. J. Yee, V. Balsanek et al, 2007, "Two-photon Excitation of Fluorogenic Probes for Redox Metabolism: Dramatic Enhancement of Optical Contrast Ratio by Two-photon Excitation" , *Phys. Chem. C*, 111: 8872-8877.
  18. T. Wakebe and E. V. Keuren, *Jpn.*, 1999, "The excitation spectra of two photon induced fluorescence in xanthenes dyes", *Jpn. J. Appl. Phys.* 38: 3556-3566.
  19. B. S. Haq, H. U. Khan, K. Alam, M. Ajmal, S. Attaullah, and I. Zari, 2015, "Determination of two-photon absorption cross sections of photosensitizers and its implications for two-photon polymerization", *Applied Optics*, 54: 132-140.
  20. J.-W. Choi, R. Wicker, S.-H. Lee, K.-H. Choi, C.-S. Ha, and I. Chung, 2009, "Fabrication of 3D biocompatible/biodegradable micro-scaffolds using dynamic mask projection microstereolithography", *Journal of Materials Processing Technology*, 209: 5494-5503.
  21. D. Skrtic and J. M. Antonucci, 2007, "Dental Composites Based on Amorphous Calcium Phosphate – Resin Composition/Physicochemical properties study", *Journal of Biomaterials Applications*, 21: 375-393.