Structural Analysis of Blended Materials Using Multiphoton Autofluorescence and Second Harmonic Generation Microscopy

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ABSTRACT

The purpose of this work is to investigate the microstructure of blended materials using non-invasive, optical imaging modality. Multiphoton autofluorescence and second harmonic generation signals will be used for characterizing and quantifying individual non-linear optical properties of each polymer in pure polymeric thin films. In addition, reflected confocal signals will used to outline the interface of refractive index mismatch. And the phase separation phenomenon of immiscible blended membranes composed of different ratio of nylon and chitosan are analyzed and differentiated using the non-invasive optical information including autofluerescence, second harmonic generation, and reflected signals. We therefore propose the potentiality of using multiphoton autofluorescence and second harmonic generation microscopy complemented with reflected confocal microscopy for studying the synthetic blended polymeric scaffolds and also, in the future, the dynamic, in vivo, cell-matrix interaction in the field of tissue engineering.

Keywords: multiphoton microscopy, second-harmonic generation, reflected confocal microscopy, polymer, nylon, chitosan.

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1. Introduction

Tissue engineering is an emerging technology that attempt to construct and replace the damaged tissue and organ and furthermore to restore the function of target organ[1]. Scaffold-based tissue engineering has been proposed widely in constructing tissue equivalents. The basic function of the implanted scaffolds is to encompass cells, bioactivate molecules, and nutrition necessary for cell growth. The design should therefore fit certain physical and biochemical requirement[2]. All these components are then combined into a construct in order to replace the damaged tissue and to restore the function of the target organ. Conventionally, the techniques for monitoring end-products of engineered tissue equivalents are either through histological examinations by fixation, sectioning, and staining, or through various biochemical analyses of the extraction. Although these approaches may provide useful information about the engineered tissue products, certain degree of destruction of the engineered products may limit the possibility of dynamic monitoring the cell-matix interaction in vivo. Therefore a non-invasive, dynamic monitoring system may be of crucial importance for the field of tissue engineering. Currently reflected confocal microscopy has been promoted as effective imaging modalities in the field of polymeric science and tissue engineering[3-5]. Reflected laser confocal microscopy has been shown to provide superior three-dimensional morphological information than traditional light microscopy, which therefore may be suitable for imaging implanted polymeric scaffolds. However, the limitation of imaging depth within turbid depth may hinder the application for biological imaging. Multiphoton microscopy is another emerging optical technique that has been widely applied in the field of biomedical imaging[6-8]. The nonlinear excitation of fluorescence photons using a ultrafast, near-infrared excitation laser sources provide several advantages as compared with one photon fluorescence excitation imaging system, the decreased of photodamage, increased axial depth discrimination, and increase of imaging depths. The marked deduced photodamage enables multiphoton fluorescence microscope being used in long-term living cell imaging without detectable photodamages. In addition to acquire morphological information, intracellular or extracellular intrinsic fluorescence also allows us to monitor physiological activities of interests. Another nonlinear polarization effect from structures lacking an inversion symmetry that contributes to second harmonic generation signals can provide morphological information from certain biological or synthetic materials, such as collagen, muscle fiber, etc. Since collagen is the most abundant component of extracellular matrix, using multiphoton fluorescence signals in conjunction with the second harmonic generation signals may provide us important information from the cell-matrix interaction, which may therefore be able to be applied as optical approaching system for investigating and monitoring engineered constructs in the field of tissue engineering[8]. In addition to native scaffold, collagen, polymeric materials have also been widely applied as scaffolds in the field of tissue engineering[9]. The plasticity of fabrication the physical and chemical properties of polymeric scaffolds fit the essential requirement for tissue reconstruction, such as capability for supporting cell proliferation, maintaining mechanical strength, and the capability for supplement of nutrition. Moreover, the blended materials provide more plasticity of customizing the physical and chemical properties of scaffolds which may be applied for different types of tissue transplantation. Therefore the capability for differentiation

and identification of individual domain within blended materials may offer us important information about individual cell-matrix interaction.

We herein proposed using multiphoton autofluorscence and second harmonic generation microscopy with complementary information obtained from reflected confocal microscopy for analyzing the structure of blended materials without additional processing. Nylon/chitosan blended membrane is chosen as experimental system, since this blended system has been shown to be immiscible in microscopic level[10].Hopefully we can differentiate individual polymeric domain with no need of additional histochemical processing, by using intrinsic fluorescence and non linear second harmonic generation signals with complementary reflected confocal signals, and apply this optical imaging modality in the field of tissue engineering as an non-invasive investigating and monitoring system for studying cell-matrix interaction

2. Experimental method

Specimen preparation

The preparation of blended membrane is the same as previous work reported by Wang et al[10]. Nylon (Nylon-66, DuPont Zytel 101, Mn = 87,000 gm/mole), chitosan (Sigma C-646, Mn = 810,000 gm/mole degree of deacetylation = 85%), and nylon/chitosan blended membranes were casted from formic acid solutions. Nylon was used as received without additional purification, which chitosan was purified by filtering chitosan solution through filtering papers. The concentration of polyamide and chitosan within formic acid solution for blending were 20 wt % and 2 wt% respectively. The casting solution was then evaporated in a convection oven at 60^{o}C over 24 hours for preparing membranes. And then the membranes were neutralized in 0.5N NaOH aqueous solution for 24 hours. The samples were prepared in different weight ratios for multiphoton imaging.

Multiphoton microscopy and reflected confocal imaging

The imaging system used in this study is a commercial laser scanning microscope (Meta 510, Zeiss, Germany). The multiphoton autofluorescence and second harmonic generation imaging of the specimens was achieved using the near-infrared excitation source from a titanium-sapphire laser (Tsunami, Spectra Physics, Mountain View, CA) pumped by a diode-pumped, solid state (DPSS) laser system (Millennia, Spectra Physics). The multiphoton excitation wavelength used in this study was 780 nm and then we acquired multiphoton autofluorescence and second harmonic generation signals (centered at 390 nm) from the membrane samples composed of different weight ratio of nylon and chitosan. As for reflective confocal signal, the same laser source with same excitation wavelength of 780 nm was used to acquire reflected confocal signal. In our study, the reflected confocal and multiphoton images were consequently acquired. The instrument set up of the reflective confocal and multiphoton autofluorescence and second harmonic generation microscope is shown in Fig. 1.

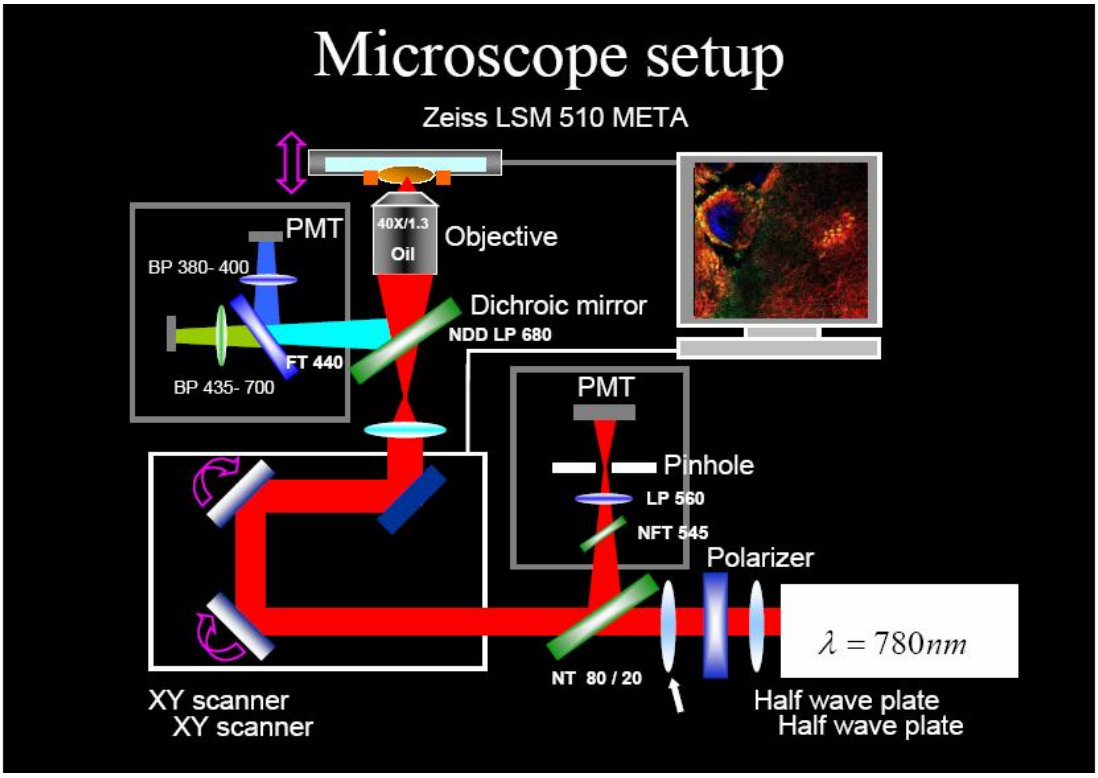

Fig. 1. The instrumentation setup of combined reflective confocal microscopy and multiphoton microscopy.

Scanning Electron microscopy

The morphology of the membrane was also examined using scanning electron microscope for comparison. The dried samples were sputtered with gold in a vacuum and viewed using a scanning electron microscope (Hitachi S-2600H).

3. Results

The morphology of pure nylon and chitosan membrane was first demonstrated with multiphoton autofluorescence and second harmonic generation imaging only. (as shown in Fig.2 and 3 respectively). In pure dense nylon membrane, we can identify that the nylon domains are constructed into a particulate morphology at the surface of membrane, which can be outlined clearly by autofluorescent signals, while the second harmonic generation signals in pure nylon membrane are relatively weak so that the architecture cannot be visualized as clear as autofluorescence image. As for the pure chitosan membrane, there is no such spherical domains existed. In stead, both the autofluorescence and second harmonic generation signals are homogeneously distributed within membrane, without particular distributing pattern can be demonstrated.

The intensity of autofluorescence and second harmonic generation signals obtained from pure nylon and chitosan membrane were further compared. The intensity of both autofluorescence and second harmonic generation signals of

pure chitosan membrane are significant stronger than pure nylon membrane, which may therefore be applied as an effective differentiating markers in immiscible nylon/chitosan blended membrane.

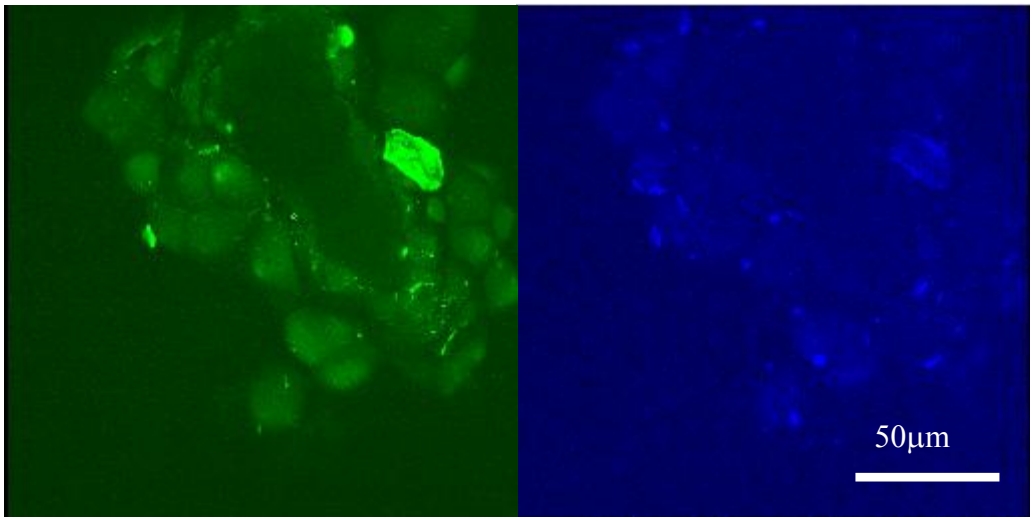

Fig. 2. Multiphoton autofluroescence and second harmonic generation imaging of pure nylon membrane. Spherical domains can be identified both in autofluorescence and second harmonic generation imaging. (Left: autofluorescence imaging; right: second harmonic generation)

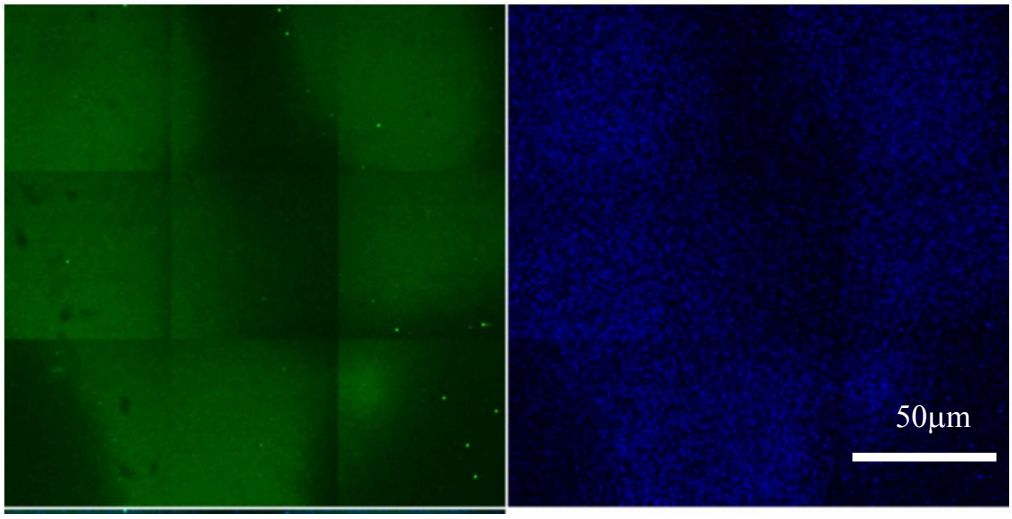

Fig. 3. Multiphoton autofluroescence and second harmonic generation imaging of pure chitosan membrane. Homogenous distribution of autofluorescence and second harmonic generation signals within membrane was noted. (Left: autofluorescence imaging; right: second harmonic generation)

We therefore analyzed multiphoton autofluorescence and second harmonic generation imaging, in conjunction with reflected confocal imaging of blended nylon/chitosan membrane which different weight ratio. Take

nylon75%chitosan25% for example, as shown in fig. 4, we can identify focal high-intensity autofluorescent particles coated on spherical domain with lower intensity of autofluorescence. And the distribution of high-intensity autofluorescence is compatible with the area with higher second harmonic generation signals, and also the area with increased reflected confocal signals. Similar distribution can also be demonstrated within three-dimensional imaging (Fig. 5). As compared with our previous analysis of pure nylon and chitosan membrane, we can proposed the area with higher intensity of autofluroescence and second harmonic generation signals may be chitosan domain, while the spherical domain with less autofluorescence and second harmonic generation signals will be nylon domains.

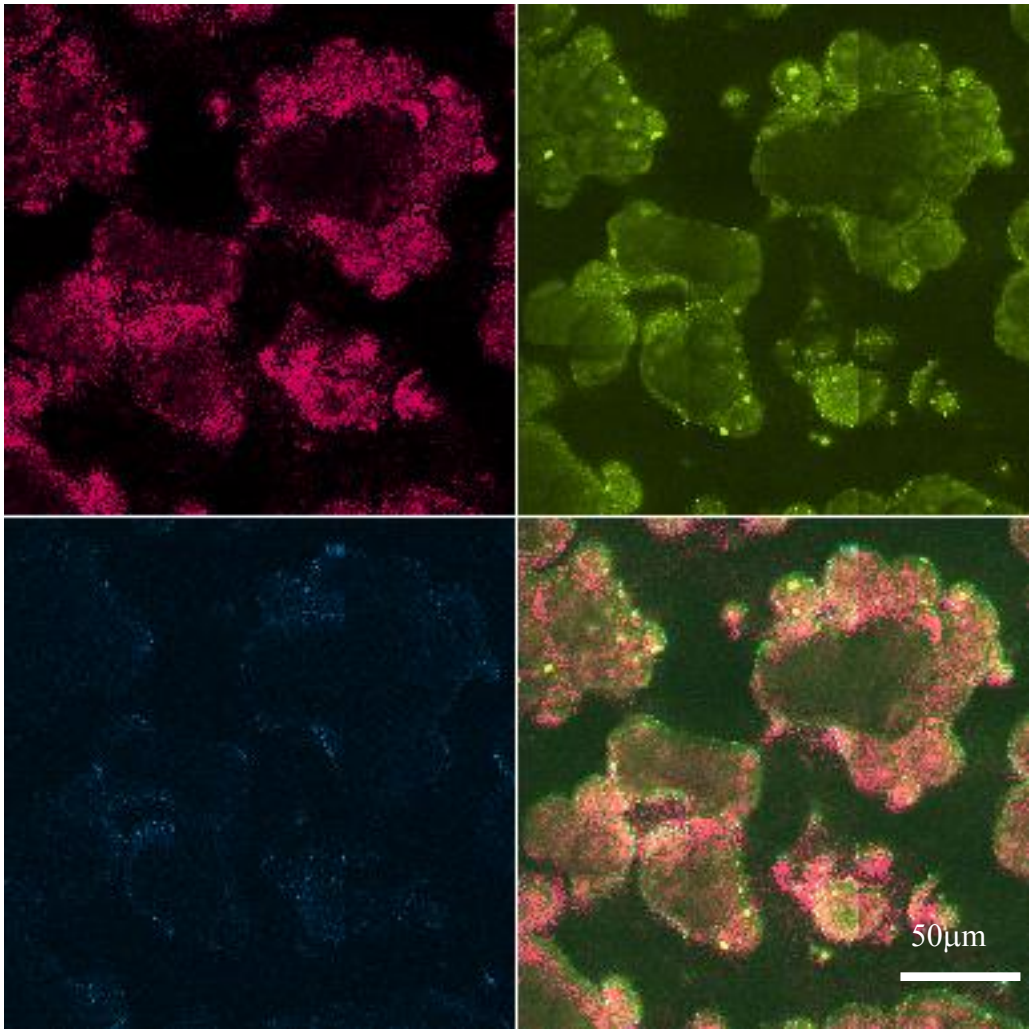

Fig. 4. The multiphoton autofluorescence and second harmonic generation imaging combined with reflected confocal imaging of blended nylon/chitosan membrane which composed of 75% of nylon and 25% chitosan. (Left Top: reflected confocal imaging; Right Top: autofluorescence imaging; Left lower: second harmonic generation imaging; Right lower: combined imaging.) Autofluorescence and second harmonic generation signasl are higher at the outermost of spherical domain composed of less autofluorescence and minimal second harmonic generation signals, and reflected confocal signals provided complementary information from the interface.

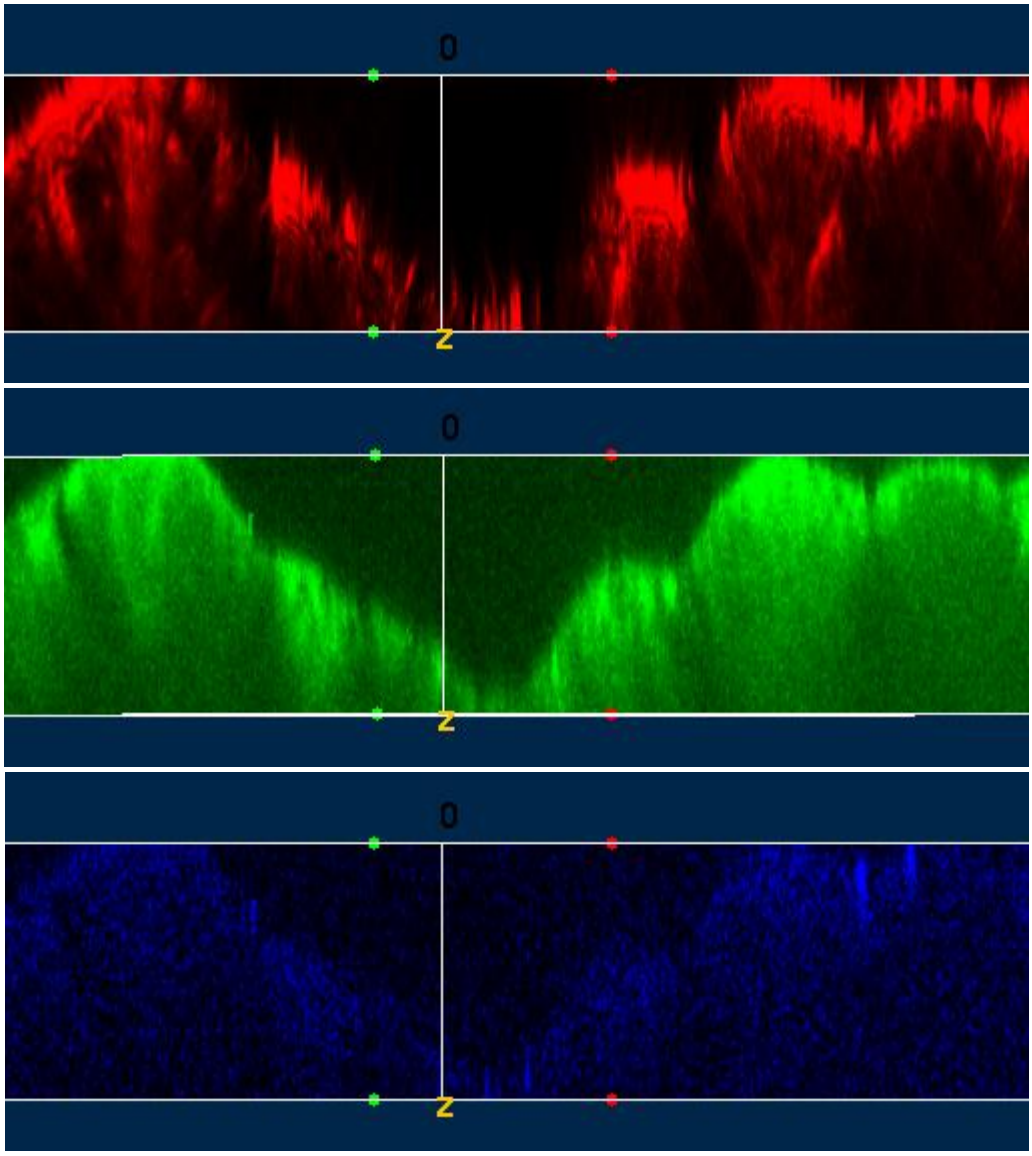

Fig. 5. Sectioning View of multiphoton imaging of nylon75%chitosan25% blended membrane. (Top: reflected confocal imaging; Middle: autofluorescence imaging; Lower: second harmonic generation imaging.) Both autofluorescence and second harmonic generation signals are stronger at the surface of spherical domains, and is compatible with the area with higher reflected confocal signals.

4. Conclusion

We herein demonstrated the ability of multiphoton autofluorescence and second harmonic generation microscopy with complementary reflected confocal information in resolving polymeric domain within immiscible nylon/chitosan blends. Using individual specific nonlinear optical properties, in conjunction with the supplementary information from

reflected confocal signals, we can obtain three-dimensionally morphological information of polymeric scaffolds with no need of additional processing. Therefore we proposed the potential application of this combined optical modality, multiphoton fluorescence and second harmonic generation microscopy with supplementary information from reflected confocal microscopy, for in vivo imaging of the dynamic cell-matrix interaction in scaffolds-based tissue engineering.

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References

1. Stock UA, Vacanti JP. Tissue engineering: current state and prospects. Annu Rev Med. **2001**;52:443.
2. Hutmacher DW. Polymeric scaffolds in tissue engineering bone and cartilage. Biomaterials. **2000**;21:2529-2543.
3. Ribbe AE. Laser scanning confocal microscopy in polymeric science. TRIP. **1997**;5:333-337.
4. Jinnai H, Kitagishi H, Hamano K, Nishikawa Y, Takahashi. Effect of confinement on phase-separation processes in a polymer blend observed by laser scanning confocal microscopy. Phys Rev. **2003**;67:021801.
5. Lin ASP, Barrows TH, Cartmell SH, Guldberg RE. Microarchitectural and mechanical characterization of oriented porous polymer scaffolds. Biomaterials. **2003**;24:481-489.
6. Denk, W. et al. 2-Photon laser scanning fluorescence microscopy. Science **2006**; 248, 73-76.
7. So, PTC. et al. Two-photon excitation fluorescence microscopy. Annu. Rev. Biomed. Eng. **2000**;2, 399-429.
8. Zoumi, A. et al. Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. P. Natl .Acad. Sci. USA. **2002**; 99, 11014-11019.
9. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. European Cells and Materials. **2003**;5:1-16.
10. Wang JH, Wei CW, Liu HC, Young TH. Behavior of MG-63 cells on nylon/chitosan-blended membranes. J Biomed Mater Res **2003**;64:606-615.