Characterizing phase-separated microstructure of polymeric blended membrane using combined multiphoton and reflected confocal imaging

Hsin-Yuan Tan1,2, Ming-Gu Lin3, Wen-Chu Hsiao4, Sung-Jan Lin1,4, Liang-Kun Wen3, Wei-Liang Chen3, Chen-Yuan Dong3*, Tai-Horng Young1,5#

1Institute of Biomedical Engineering, College of Medicine and Engineering, National Taiwan University. No.1, Sec.1, Jen-Ai Rd, Taipei 100, Taiwan
2Department of Ophthalmology, Chang Gung Memorial Hospital, College of Medicine, Chang Gung University. No.5, Fu-Shin St., Kuai-Shang Shiung, TaoYuan 333, Taiwan
3Department of Physics, National Taiwan University. No.1, Sec.4, Roosevelt Rd, Taipei 106, Taiwan
4Department of Dermatology, National Taiwan University Hospital. No.1, Sec.1, Chung-Shang S. Rd., Taipei 100, Taiwan
5Institute of Polymer Science and Engineering, College of Engineering, National Taiwan University. No.1, Sec.4, Roosevelt Rd, Taipei 106, Taiwan

Corresponding Authors:*cydong@phys.ntu.edu.tw
& #thyoung@ntu.edu.tw

Abstract: In this study, we propose a novel, minimally-invasive multimodal optical imaging method which combines multiphoton and reflected confocal microscopy for characterizing three-dimensional phase-separated microstructure of polymeric nylon/chitosan blends. The multimodal image acquisition was performed on a Zeiss LSM 510 inverted microscope system using a ti-sapphire laser source. Differences in nonlinear optical signals between individual homopolymers were used to characterize the phase-separation phenomenon within the polymeric blends. We also used the reflected confocal signals for defining the interfacial boundaries of different refractive indices. Our work demonstrates that the proposed multimodal imaging modality can be used to provide the necessary microstructural information for characterizing the degrees of phase separation within polymeric blends.

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References and links

in fabricating desired materials with physical/chemical properties [2,3]. The bulk morphology commonly unobtainable within homopolymeric materials [1]. For example, blended science. The reason for such interest lies in the desired physical and chemical properties In recent years, polymeric blends have become the subjects of intense interest in material 1. Introduction

In recent years, polymeric blends have become the subjects of intense interest in material science. The reason for such interest lies in the desired physical and chemical properties commonly unobtainable within homopolymeric materials [1]. For example, blended membranes have been successfully introduced in tissue engineering due to the high plasticity in fabricating desired materials with physical/chemical properties [2,3]. The bulk morphology
and phase-separation phenomena, which play key roles in determining the properties of such blends, are worthy of investigation in polymer science. To characterize these novel materials, several imaging approaches such as transmission electron microscopy, atomic force microscopy, fluorescence microscopy, and Raman imaging have been used for analyzing the morphological features of polymeric blends [4-8]. However, the complicated sample preparation procedures and necessity of additional staining in some of these techniques have limited their use. The development of a minimally-invasive imaging technique for analyzing phase-separated morphological characteristics within polymeric blends can be essential for investigating phenomena such as cell-matrix interaction.

Laser scanning confocal microscopy has been introduced for direct observation of microstructures within polymeric blends [9-12]. Using reflected light obtained from interfaces of materials composed of different refractive indices, confocal microscopy is capable of revealing interfacial morphological features [13]. However, the achievable imaging depths may be hindered with increase in specimen turbidity. In such cases, fluorescence labeling combined with multiphoton (MP) fluorescence microscopy can be introduced for three-dimensional visualization enhancement [12, 14]. The MP microscopy is a proven optical imaging modality which has been widely applied in biomedical imaging [15-17]. The nonlinear excitation of fluorescence using an ultrafast, near-infrared excitation laser sources provide several advantages over one-photon imaging techniques. Reduced photodamage, enhanced axial depth discrimination, and increased imaging depths are some of the advantages associated with nonlinear optical microscopy. In addition to MP fluorescence excitation, the nonlinear optical phenomenon of second harmonic generation (SHG) has also been applied in the imaging of a wide variety of materials. Non-centrosymmetric materials such as crystals, polymers, collagen, and muscle have been demonstrated to be efficient generators of second harmonic signals [15, 18-21]. Since the efficiency of SHG depends on chemical structures of polymers, the second harmonic signal may be applied to investigate polymeric blends with heterogeneous structures.

In this study, a minimally-invasive, multi-modal imaging approach of MP microscopy and reflective confocal microscopy was used for imaging phase-separated microstructures within a blended membrane system. Blended membranes composed of nylon and chitosan were chosen as the experimental model. The selection of this blended polymeric system is motivated by the facts that these materials have been shown to be immiscible at the microscopic level and that they have been successfully applied in culture systems of osteoblast-like cells and melanocytes [2,3]. Since chitosan has been demonstrated to be capable of generating second harmonic and autofluorescence (AF) signals [22-24], one would expect MP fluorescence and SHG microscopy to be effective in resolving chitosan components within the three-dimensional, phase-separated microstructures within polymeric blended membranes. In this work, we intend to use the intrinsic AF and SHG signals in conjunction with reflected confocal (RC) signals to probe structural heterogeneities within blended polymeric materials. If effective, our multi-modal imaging approach can be further developed and applied in polymer science and tissue engineering applications as a minimally-invasive imaging tool.

2. Materials and methods

2.1 Preparation of polymeric membrane samples

The preparation of polymeric blended membrane followed the same procedures as described in previous reports [7,8]. Nylon (Nylon-66, DuPont Zytel 101, \( M_n = 87,000 \) gm/mole), chitosan (Sigma C-646, \( M_n = 810,000 \) gm/mole, degree of deacetylation = 85%), and nylon/chitosan blended membranes were cast from formic acid solutions. Nylon was used as received without additional purification, while chitosan was purified by filtering chitosan solutions through filtering papers. For blending, the concentrations of nylon and chitosan within formic acid solution were 20 wt % and 2 wt % respectively. In preparing the membranes, the casting solution was evaporated in a convection oven at 60°C over 24 hours. The membranes were then neutralized in 0.5 N NaOH aqueous solutions for 24 hours. The
samples were prepared in different chitosan weight ratios of 25% (N75C25), 50% (N50C50), and 75% (N25C75). Each membrane was cut into a 5 mm square block, placed on a slide, and sealed with a No.1.5 coverslip for imaging. For comparison, a set of blended membranes was further processed with etching treatment using m-cresol and chitinase respectively. The additional treatment would remove nylon, chitin and its derivatives from the membrane surface [25].

2.2 Scanning electron microscopy

For comparison, the membrane surface morphology was also examined using scanning electron microscopy (SEM). Dried samples were sputtered with gold under vacuum and imaged with a Hitachi S-2600H scanning electron microscope.

2.3 Multiphoton and reflected confocal microscopic imaging

The multimodal imaging used in this study was performed on a commercial confocal laser scanning microscope system (Meta 510, Zeiss, Germany). The AF and SHG imaging of the specimens was induced with a near-infrared titanium-sapphire laser source (Tsunami, Spectra Physics, Mountain View, CA) pumped by a diode-pumped, solid state (DPSS) laser system (Millennia, Spectra Physics). The MP excitation wavelength used in this study was 780 nm, and the images were acquired in the non-descanned mode. The emitted AF and SHG signals were separated into a two channel detection scheme with a long pass dichroic mirror (440dxru, Chroma Technology, Rockingham, VT) and two band pass filters (HQ390/20m-2p, e700sp-2p-e435, Chroma Technology). For RC imaging, the same 780 nm laser was used as the light source, and the RC signal was acquired in the descanned mode with the pinhole size set to one Airy disk. The 780 nm reflected signal was selected with a 545 nm long pass dichroic mirror in combination with a LP560 long pass filter. Due to the availability of instrument configuration, a narrow band detection scheme for the RC signal was not used for the images shown in this work. Nonetheless, comparison of the RC and AF images suggested that AF contamination of the RC signal was minimal. A high numerical aperture (NA) oil immersion objective (C-Apochromat, 40X/NA 1.3, Zeiss) was used for image acquisition. Each frame of imaging composed of 512 × 512 pixels and is 230 × 230μm² in size. The average laser power at the sample surface was on the order of 100 mW. Three randomly selected regions (around 60μm² each) within one image in homopolymeric nylon and chitosan membranes were chosen for the calculation of the average signal intensity of each material. In addition, a ratiometric approach (AF/SHG) was applied for the characterization of differences in AF and SHG signals between the two materials. A schematic diagram of the instrumentation setup of the MP and RC microscope is shown in Fig. 1.
3. Results

3.1 SEM micrographs

SEM micrographs of nylon/chitosan blended membranes composed of different weight ratio are shown in Fig. 2. In pure nylon membrane, individual particulate domains were clearly visible at the surface [Fig. 2(A)]. In contrast, surface of the pure chitosan membrane specimen was relatively smooth and homogenous [Fig. 2(E)]. Therefore, it is not surprising that the surface topography became smoother as the nylon ratio decreased [Fig. 2(B-D)]. Furthermore, in order to reveal the distribution of individual components in the blended membrane, we applied etching treatment on the blended specimens, and imaged with SEM for comparison [Fig. 2(F-H)]. To be specific, N75C25 was treated by chitinase for chitosan removal, while N50C50 and N25C75 were treated by n-cresol to remove the nylon component. In comparing Fig 2B and 2F, it is evident that the characteristic particulate surface topography of pure nylon membrane was retained in the N75C25 specimen and scattered round coating of chitosan components were found to locate on the particulate nylon domains. Furthermore, SEM micrographs of the N50C50 specimens with and without etching treatment revealed that the characteristic spherical microstructure of nylon domains remained, with smaller diameter, and embedded within homogenous chitosan “matrix” composing smooth surface [Fig. 2(C, G)].
3.2 Multiphoton and reflected confocal imaging

In order to analyze the three-dimensional phase-separation microstructures in a minimally-invasive fashion, we performed MP AF and RC imaging of the same set of pure and blended nylon/chitosan membranes. In the following images, AF, SHG, and RC signals are shown in the pseudo colors of green, blue, and red, respectively.

3.2.1 Multiphoton and reflected confocal imaging of pure nylon and chitosan membranes

Shown in Figs. 3 and 4 are the two-dimensional and three-dimensional images of pure nylon and chitosan membranes, respectively. In the pure nylon membrane, the particulate morphology of individual nylon domains can be visualized with AF and the relative weaker SHG signals. Diameters of nylon domains at the membrane surface was measured to be around 30~40\(\mu\text{m}\), which is similar to the SEM result [Fig. 2(A)]. Furthermore, the surface can be outlined more clearly by the RC signal derived from the membrane surface (Fig. 3). In imaging the pure chitosan membrane, both AF and SHG signals were more evenly distributed within the membrane (Fig. 4). This observation is consistent to the smooth surface topography observed in the SEM micrographs [Fig. 2(E)]. In addition, we compared AF and SHG intensities detected from pure nylon and chitosan membranes (Table 1). Both the AF and SHG signals of the chitosan membrane were substantially stronger than those of the nylon membrane. Compared to the AF signal, detected SHG signals within both pure chitosan and nylon membrane were relatively weak. For additional analysis, we used a ratiometric approach to determine the AF/SHG ratios of nylon and chitosan. The AF/SHG ratio was higher in chitosan than that in nylon. Our results suggest that differences in the detected AF and SHG signals may be used for the effective differentiation of immiscible polymeric domains nylon/chitosan blended membrane system.
Fig. 3. Combined multiphoton (MP) and reflected confocal (RC) imaging of pure nylon membrane in two-dimensions (A) and three-dimensions (B). Spherical domains can be identified in reflected confocal (RC), autofluorescence (AF), and SHG images. The weaker SHG signal prevents a sharp definition of the domain boundaries.

Fig. 4. Combined MP and RC imaging of pure chitosan membrane in two-dimensions (A) and three-dimensions (B). Relatively uniform AF and SHG signals are observed within full-thickness of the membrane while the RC signal is most intense at the interfaces.

Table 1. Comparison of the average intensity of AF and SHG signals of pure nylon and chitosan membranes.

<table>
<thead>
<tr>
<th></th>
<th>AF</th>
<th>SHG</th>
<th>AF/SHG</th>
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<tbody>
<tr>
<td>Nylon</td>
<td>35.26 ± 7.75</td>
<td>10.76 ± 4.85</td>
<td>3.82±2.06</td>
</tr>
<tr>
<td>Chitosan</td>
<td>157.56 ± 5.01</td>
<td>21.48 ± 2.85</td>
<td>7.44±1.14</td>
</tr>
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3.2.2. Multiphoton and reflected confocal imaging of blended nylon/chitosan membranes

MP and RC imaging of blended membrane materials (N75C25, N50C50, and N25C75) was performed and the results shown in Figs. 5-7. We found that the particulate substrates composed of intense AF and SHG signals coated on large spherical domains with less intense AF and SHG signals. The interfacial domains can be visibly outlined by the RC signal (Fig.
5). We also found that the surface topography is irregular in the N75C25 material. This is evident in the XY-sectional image which shows the presence of different domains. In Fig. 5(A), highly autofluorescent chitosan particles coated on a spherical nylon domain were outlined by a white dashed circle. In comparison, the yellow dashed circle delineates the inner structure of a nylon spherical domain without embedded chitosan components. In addition, three-dimensional imaging [Fig. 5(B)] reveals the presence of particles composed of stronger AF and SHG signals (chitosan domain) distributed randomly on the surface of a large particulate domain with lower AF and SHG intensity (nylon domain). Furthermore, the cross-sectional view [Fig. 5(C)] shows that chitosan distributed mostly on the membrane surface. This suggests that chitosan molecules were preferentially located at the surface during the membrane formation process. Clearly, the uneven domain distribution within the blended materials in three-dimensions cannot be easily analyzed by SEM techniques. Furthermore, diameters of nylon spherical domains on the N75C25 membrane surface were estimated. Compared to the pure nylon substrate, diameters of the nylon domains increased significantly to 70~80 μm, a result consistent with the SEM result [Fig. 2(B)]. This indicates that chitosan had a plasticizing effect during the incorporation of chitosan molecules into the nylon domain.

Figure 6 shows the MP and RC images of the N75C25 membrane after etching treatment. Compared to the images of the membrane without treatment, deposits of intense AF and SHG signals disappeared after the etching process. This observation confirms locations of chitosan domains within the blended membrane. Other artifacts such as the presence of regions with high-AF signals at the edges do not interfere with the morphological characterization of the blended membrane. The presence of these artifacts may be attributed to the incomplete reaction of chitinase. Similar finding of the incomplete dissolution of chitosan was also found in the SEM micrograph [Fig. 2(F)].

Two-dimensional and three-dimensional morphological imaging of N50C50 membrane is shown in Fig. 7. In this case, undulating surface topography disappeared. Instead, a relatively flat surface similar to that found on pure chitosan membrane was found. Similar to the SEM result, scattered small nylon spheres composed of weaker AF and SHG signals are found to embed within the chitosan matrix with stronger AF and SHG signals. In this case, diameters of nylon spherical domains decreased significantly to below 10 μm.
Fig. 6. Two-dimensional (X-Y plane) MP and RC images of blended N75C25 membrane with etching treatment for chitosan removal. The presence of hollow regions (white dashed circle) may be due to etching of the autofluorescent and second harmonic generating chitosan domains. The remaining autofluorescent substrates are likely the results of the incomplete dissolution of chitosan during the etching process.

Fig. 7. Combined MP and RC imaging of blended N50C50 membrane in two- (A) and three- (B) dimensions. Compared with that of the N75C25 blend, the surface topography is relatively smooth. Spherical domains composed of weaker AF and SHG signals (nylon domains), are embedded within the matrix of stronger AF and SHG signals (most likely the chitosan domains).

4. Discussion

In this work, we demonstrated phase-separation phenomena in an immiscible nylon/chitosan blended membrane system using a multimodality optical microscopy imaging approach. The 3D distribution of different domains of homopolymers within membrane can be identified and differentiated with the characteristic nonlinear optical signatures of each material. In addition, we also found that the interfaces of the polymer materials can be outlined with reflected
confocal imaging. The ability of our technique to resolve nylon and chitosan domains has been further confirmed by the etching processing and SEM micrographs. The surface segregation of individual homopolymers at the outermost layers was visible in 3D imaging (Figs. 5 and 7). The characterization of the membrane surface topography is significant in that numerous applications using the membrane materials depend heavily on the structural and chemical properties of the surfaces. In the case of cell culture applications, it is important to directly observe the preference of cell attachment and understand the behaviors of different cells seeded on different homopolymeric domains. Furthermore, the 3D distribution of individual homopolymeric domains was demonstrated in the polymeric blends. In nylon/chitosan blended system, nylon tends to aggregate into spherical domains with varying diameters according to the weight percentage of nylon. In the N75C25 system, nylon spherical domains increase in diameter, which may be related to the plasticizing effect of the incorporated chitosan. As the weight ratio of nylon decreases to 50% or below, the nylon spherical domains decreased in size significantly and were embedded in the homogenous chitosan matrix. Since the applications of polymeric blends have increased markedly due to their versatility in areas such as tissue engineering, the availability of a minimally-invasive, optical imaging system for investigating phase-separation phenomena may be of significant value. In this preliminary work, the detailed spectroscopic analysis of this blended polymeric system has not been address. The AF and nonlinear optical signals within polymeric systems have been shown to be related to molecular structures, functional groups, and also intermolecular interaction, including the extent of crosslinking [22,24]. Further ongoing works with additional spectroscopic analysis may be helpful for characterizing the source and the alterations of signals within blended polymeric structures in which complicated intermolecular interactions may occur.

In this work, we combined MP and RC microscopy for imaging phase-separation phenomena in polymeric blends. The possibility of simultaneously obtaining complementary structural and functional information has made the multimodal imaging approach a popular choice in biomedical imaging applications [26-28]. In this work, nonlinear optical signals provide intrinsic molecular and structural information which helped to differentiate homopolymeric domains within polymeric blends. In addition, RC imaging enhanced and highlighted the interfaces with mismatched refractive indices. The application of our approach enabled the phase-separation phenomena within immiscible polymeric blends to be resolved in a minimally-invasive fashion. In conclusion, we report the first application of applying MP and RC imaging for analyzing the 3D phase-separation phenomena in immiscible nylon-chitosan blends. The full potential of our multimodal imaging approach as a powerful tool for studying polymeric blends in polymeric science and tissue engineering remains to be explored.

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