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Chronic Label-free Volumetric Photoacoustic Microscopy of Melanoma Cells in Scaffolds *in vitro*

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ABSTRACT

Visualizing cells in three-dimensional (3D) scaffolds has been one of the major challenges in tissue engineering. Current imaging modalities have limitations. Microscopy, including confocal microscopy, cannot penetrate deeply (> 300 μm) into the scaffolds; X-ray micro-computed tomography (micro-CT) requires staining of the structure with a toxic agent such as osmium tetroxide. Here, we demonstrate photoacoustic microscopy (PAM) of the spatial distribution and temporal proliferation of melanoma cells inside three-dimensionally porous scaffolds with thicknesses over 1 mm. Melanoma cells have a strong intrinsic contrast which is easily imaged by label-free PAM with high sensitivity. Spatial distributions of the cells in the scaffold were well-resolved in PAM images. Moreover, we chronically imaged the same cell/scaffold constructs at different time points over 2 weeks. The number of cells in the scaffold was quantitatively measured from the PAM volumetric information. The cell proliferation profile obtained from PAM correlated well with that obtained using the traditional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We believe that PAM will become a useful imaging modality for tissue engineering applications, especially when thick scaffold constructs are involved, and that this modality can also be extended to image other cell types labeled with contrast agents.

Keywords: inverse opal scaffolds; photoacoustic microscopy; melanoma; tissue engineering; biomedical imaging.

1. INTRODUCTION

Three-dimensional (3D) scaffolds are synthetic porous constructs, which are important in tissue engineering. The aim of tissue engineering is to develop new tissue/organ substitutes for facilitating the restoration and maintenance of biological functions.[1] 3D scaffolds provide physical supports and form adjustable microenvironments for cells. To perform this role, the scaffolds must have proper properties, including biocompatibility, biodegradability, mechanical strength, porosity, pore size, interconnectivity, and among others.

Despite dramatic achievements in tissue engineering, visualizing live cells inside scaffolds is still challenging. Microscopic imaging systems capable of providing volumetric information of cells are quite rare. For example, optical microscopy, including confocal and two-photon laser scanning microscopy, has been widely used for visualizing cells. However, due to strong light scattering, the penetration depth of such a modality is typically limited to several hundred micrometers. Micro-computed tomography (micro-

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CT) based on X-ray can visualize a whole construct with dimensions of several centimeters. However, for cell imaging, it usually requires toxic contrast agents such as osmium tetroxide.[2] Label-free optical coherence tomography (OCT) with a relatively high resolution (~0.9 μm) has been demonstrated for imaging tissue/scaffold constructs.[3] Although OCT could simultaneously resolve the structures of both the tissue and scaffold, it is rather difficult to distinguish between these two. Magnetic resonance imaging (MRI) was also employed to evaluate the differentiation of bone marrow stromal cells in gelatin sponges.[4] However, MRI suffers from low spatial resolution (70-100 μm) and long image acquisition time. Histological analyses can provide excellent details of the sample. Nonetheless, this method is destructive. Therefore, there is still a strong need for a non-invasive imaging modality with high resolution and deep penetration for volumetric information of cells in 3D scaffolds.

The emerging photoacoustic microscopy (PAM) is attractive for imaging cells in a non-invasive manner. PAM detects photoacoustic waves generated from the objects that absorb either pulsed or intensity-modulated laser irradiation.[5] Melanoma cells have a strong intrinsic contrast for PAM with high sensitivity.[6] Additionally, the non-ionizing radiation in photoacoustic (PA) imaging imposes no hazardous effects to tissues, in contrast with ionizing X-rays in micro-CT.[7] Here, we report PAM imaging of melanoma cells seeded in poly(D, L-lactide-*co*-glycolide) (PLGA) inverse opal scaffolds for tissue engineering application. We have successfully demonstrated the capability of PAM to non-invasively image a whole cell/scaffold construct ~ 1.5 mm thick, resolving spatial distribution of cells in a 3D manner. In addition, non-invasive and label-free PAM made it possible to monitor cell proliferation in the same scaffold over time, and to quantitatively analyze the number of cells as a function of time.

2. MATERIALS AND METHODS

2.1 Preparation of inverse opal scaffolds

We fabricated the uniform microspheres of gelatin (Type A; Sigma-Aldrich) and inverse opal scaffolds of PLGA (lactide/glycolide=75/25, Mw≈66,000-107,000, Sigma-Aldrich) by following our recently published procedures.[8]

2.2 Cell culture and seeding

B16 melanoma cells were obtained from the Tissue Culture and Support Center at the Washington University School of Medicine. The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, ATCC, Manassas, VA) and 1% antibiotic antimycotic (ABAM, Invitrogen). Prior to cell seeding, scaffolds were sterilized with 70% ethanol and UV irradiation overnight, washed with PBS (Invitrogen) three times, and stored in a culture medium. During the culture, the media were kept in an incubator at 37 °C under a humidified atmosphere containing 5% CO2, and were changed every other day.

2.3 MTT assay

After PAM imaging of the scaffolds, the culture media were withdrawn, and the scaffolds were collected in a 12-well plate (one scaffold per well). 1 mL 1-propanol was added to each well to completely dissolve the MTT formazan crystals throughout the scaffolds. Optical density was measured at 560 nm using a spectrophotometer (Infinite 200, TECAN). All final data were normalized to the dry weight of each scaffold.

2.4 Scanning electron microscopy

Scanning electron microscopy (Nova NanoSEM 2300, FEI, Hillsboro, OR) was used to characterize both

the PLGA inverse opal scaffolds and the melanoma cells in the scaffolds. Prior to imaging, cells were fixed and samples were dehydrated through a graded ethanol series and sputter-coated with gold for 60 s. Images were taken at an accelerating voltage of 5 kV.

2.5 Photoacoustic microscopy and signal processing

Scaffolds were removed from the culture medium, placed in a PDMS mold containing warm PBS (37 °C), and imaged with PAM. For photoacoustic excitation, a dye laser (CBR-D, Sirah, Kaarst, Germany) pumped by a Nd:YLF laser (INNOSLAB, Edgewave, Wuerselen, Germany) was employed to provide 7-ns laser pulses with a repetition rate up to 5 kHz. The light was coupled into a multimode optical fiber and reshaped by a conical lens to form a ring pattern on the tissue surface. The ring-shaped light pattern was then weakly focused into the sample by an optical condenser, and the optical focus overlapped with the tight detection ultrasonic focus.[6, 9] A focused ultrasonic transducer with 50 MHz central frequency (V214-BB-RM, Olympus NDT, Kennewick, WA) was used to receive photoacoustic waves. The system could achieve 45 μm lateral resolution, 15 μm axial resolution, and more than 3 mm penetration depth.

3. RESULTS

3.1 Scaffolds and melanoma cells culture in the scaffolds

Fig. 1 shows the images of typical inverse opal scaffolds of PLGA. From the scanning electron microscopy (SEM) image in the inset of Fig. 1A (scale bar: 100 μm), it is clear that the inverse opal scaffold had a uniform and well-arranged pore structure. This feature is important for a 3D scaffold, in that it can provide good interconnections throughout the whole scaffold to facilitate cell migration and nutrient/waste transport. Fig. 1B shows the morphology of melanoma cells inside scaffolds with an optical microscope. The inset shows a SEM image of melanoma cells inside a pore (scale bar: 50 μm).

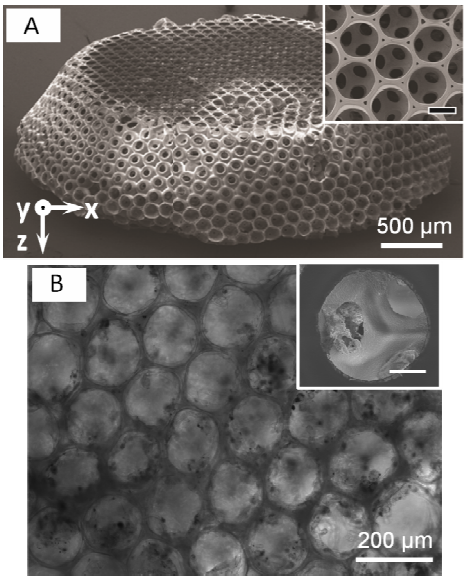

Fig. 1. Optical and SEM images of PLGA inverse opal scaffolds. A Cartesian coordinate is also shown. A) SEM images of a typical inverse opal scaffold. The inset shows an enlarged view, where three windows are visible in each pore. These windows provide good interconnections throughout the whole scaffold. (scale bar: 100 μm). B) A scaffold seeded with melanoma cells acquired at 14 days post-seeding with an optical microscope. The inset shows a SEM image of melanoma cells inside a pore (scale bar: 50 μm).

3.2 Photoacoustic imaging of melanoma cells in the scaffolds

The PA coronal and sagittal MAP images in Figure 2A clearly show cell distribution in a scaffold, with the capability of penetrating the whole cell/scaffold construct (~1.5mm). Figure 2B shows a 3D depiction of the melanoma cells in the scaffold. Individual cells or cell clusters could be identified (black dots or patches) in both the 2D and 3D images. This penetration depth is rather deep as compared to the conventional microscopy techniques. According to our experience, with the same melanoma cell-seeded scaffold, confocal microscopy and two-photon microscopy could only reach a depth of ~0.2 mm and ~0.3 mm, respectively. PAM system could still provide acceptable resolution at such a deep penetration depth, because the PAM resolution is determined by the ultrasound parameters and ultrasound scattering is much weaker than optical scattering in biological tissues. These images clearly show the advantage of PAM in penetration for characterizing cells in 3D scaffolds. We also imaged a cell-free inverse opal scaffold with PAM. As expected, it was not detectable by PAM because the optical absorption of the scaffold was very low. Hence, we confirmed that the PA signals came from the melanoma cells rather than from the scaffold.

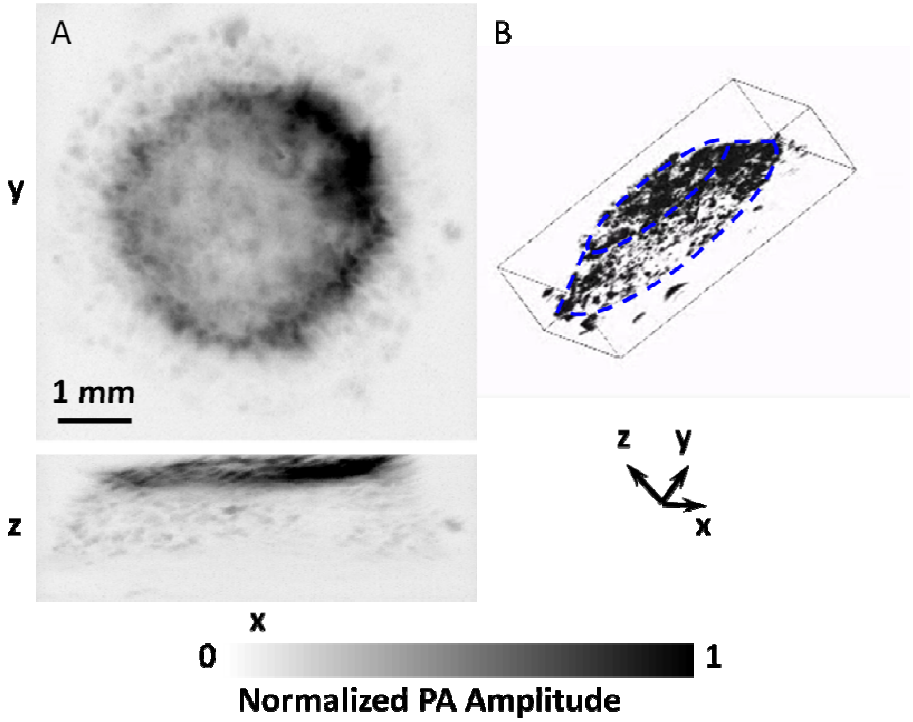

Fig. 2. PA images of melanoma cells in a scaffold acquired at 14 days post-seeding. A) PA coronal (top) and sagittal (side) MAP images. The black dots correspond to melanoma cells. B) 3D depiction of the PA image, where the contour of the scaffold is marked by dotted lines. MAP: maximum amplitude projection.

3.3 Chronic imaging and quantification of melanoma cells

So far, it has been rarely reported to temporally monitor and quantify cell proliferation in a 3D scaffold with relatively high spatial resolution. PAM is a well-suited tool for chronically monitoring and quantifying melanoma cell proliferation in a 3D scaffold because it does not require any exogenous contrast agent. Melanoma cells were seeded into four inverse opal scaffolds with a thickness of 1.5 mm using a spinner flask. At days 1, 7, and 14 post-seeding, the scaffolds with cells were carefully taken out from the media for PAM imaging. Time-course PA coronal MAP images of the entire scaffold clearly show the growth of

melanoma cells inside the scaffolds (Fig. 3A). We further utilized the collected PA volumetric data to quantify the cell numbers, which would be a critical feature of PAM. To elucidate the relationship between the PA signal amplitude and the number of melanoma cells, the scaffolds with different numbers of cells were scanned with PAM under identical conditions. The cells in the scaffolds were then completely released from the scaffolds with AccumaxTM and manually counted with a hemocytometer. The signal amplitude from the PA volumetric data was found to be linearly correlated with the number of cells in the range of investigation ($2 \times 10^4 \sim 6 \times 10^5$).

From the calibration curve, the average cell numbers at days 1, 7, and 14 were calculated to be 4.9×10^4, 3.4×10^5, and 2.7×10^5 per scaffold, respectively. The cell numbers were plotted against different time points (Fig. 3B). A parallel cell MTT viability experiment was conducted. MTT assay is a typical method for accurately evaluating cell proliferation. Interestingly, the overall profile obtained from MTT assay had a trend similar to that from PAM, with a slight difference at the initial stage. The average cell number at days 1, 3, 7, and 14 in the MTT group were calculated to 7.1×10^4, 3.3×10^5, and 2.6×10^5, respectively. The results showed the effective quantification by PAM.

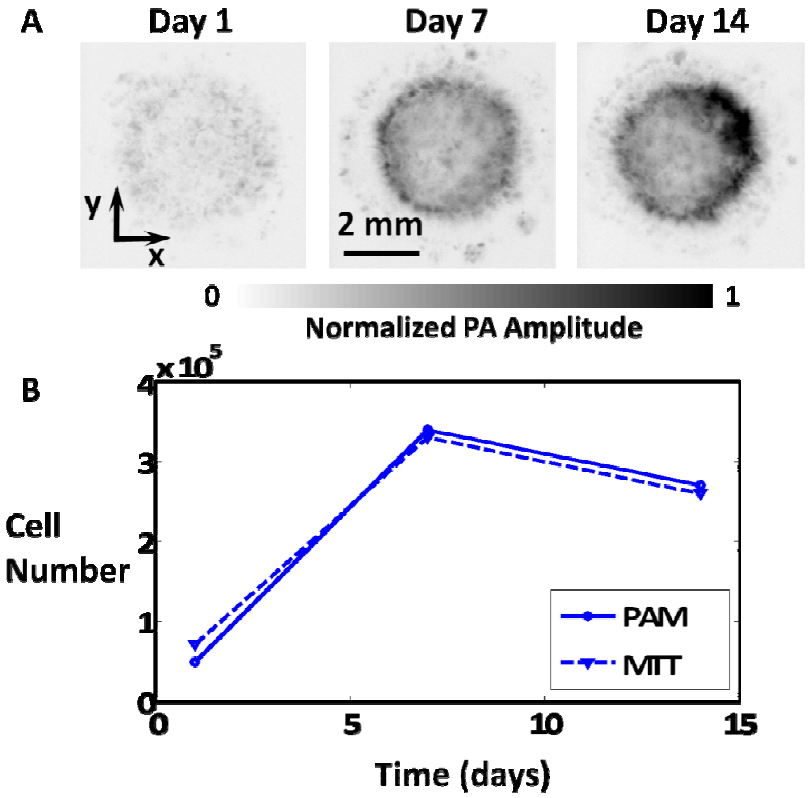

Fig. 3. A) Time course PA images (coronal MAP of the entire volume) of melanoma cells in a typical scaffold at 1, 7 and 14 days post-seeding. B) Quantitative analysis of melanoma cells in scaffolds derived from both PA imaging and MTT cell viability analysis.

4. CONCLUSION

We demonstrate photoacoustic microscopy (PAM) of the spatial distribution and temporal proliferation of cells inside three-dimensionally porous scaffolds with thicknesses ~1.5 mm in a non-invasive manner. Melanoma cells were used as a model cell line due to their intrinsic dark pigment. It is worth noting that we were able to chronically image the same cell/scaffold construct at different time points by PAM. The

number of melanoma cells in the scaffold was quantified by PAM and agreed well with traditional MTT assay. We believe that PAM will become a useful technique as an imaging modality for tissue engineering applications, especially when thick cell/scaffold constructs are involved, and this modality can also be extended to image other cell types labeled with contrast agents such as organic dyes.

5. ACKNOWLEDGMENTS

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