International Journal of Heat and Mass Transfer 111 (2017) 163–170

Contents lists available at ScienceDirect

International Journal of Heat and Mass Transfer

journal homepage: www.elsevier.com/locate/ijhmt

Raman microspectroscopic detection of thermal denaturation associated with irreversible electroporation

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ARTICLE INFO

Article history:
Received 16 December 2016
Received in revised form 28 February 2017
Accepted 30 March 2017

Keywords:
Irreversible electroporation
Thermal denaturation
Raman microspectroscopy
Temperature rise
Tissue phantom

ABSTRACT

Irreversible electroporation (IRE) is a less-invasive treatment for ablating tumors. It delivers a train of electric pulses to the cells deep inside the tissue via puncturable electrodes and yields fatal breakdown of the cell membrane around the electrodes. Thermal damage associated with the pulse application is avoidable insofar as the optimal pulse conditions are selected to minimize Joule heating. However, the application of long, intense, and repetitive pulses comes with the risk of a temperature rise around the electrodes. Therefore, the aim of this study was to provide a quantitative evaluation of IRE-induced thermal denaturation at the level of the molecular structure. A tissue phantom containing albumin was observed with a confocal Raman microscope after an IRE protocol. The Raman imaging and subsequent analysis successfully indicated albumin denaturation around the electrodes. The extent of denaturation correlated well with the temperature rise in the tissue that was detected using temperature-sensitive ink. The maximal temperature rise in the experiment also agreed well with that estimated using finite element analysis. Another important finding was that the temperature rise and the consequent denaturation were more significant at the cathode than at the anode, despite the symmetric distribution of the electric field around the electrodes. This implies the occurrence of an unexpected side effect of the IRE.

1. Introduction

Irreversible electroporation (IRE) has been attracting attention as a new minimally invasive therapy for ablating tumors [1–5]. In this therapy, intensive electric pulses give a fatal perforation to the cell membrane [6], and thereby irreversibly necrotize the cells without any thermal damage to the surrounding tissue as long as the appropriate pulse conditions are selected to minimize the effect of Joule heating. The avoidance of thermal damage is of great advantage to healing because the preserved extracellular matrix works as a favorable scaffold for cell migration and consequently enhances rapid tissue regeneration.

In a clinical application of IRE, a train of several kilovolts of electric pulses shorter than several hundreds of microseconds is applied to targeted tissue via a pair of needle-like electrodes. These pulses potentially induce a temperature rise in the tissue because of Joule heating. Hence, it is important to estimate the tissue temperature during the treatment to maximize the advantage of the non-thermal mechanism of cell death. Numerical simulation is one of the methods for calculating the temperature rise associated with the application of the electric pulses. By solving the two-dimensional Pennes bio-heat equation, Davalos and Rubinsky, who are pioneers of IRE therapy, first showed the temperature distribution around needle-like electrodes [7]. Three-dimensional finite element analysis was also performed for more complicated configurations targeting brain [3,8], breast [9], skin [10], and model tissues [11,12]. These studies showed that the application of an electric pulse quickly elevated the tissue temperature around the electrodes; followed, however, by the immediate decrease of temperature after the end of application.

The estimated temperature rise and the accumulation of thermal damage must be validated by comparing the analysis with experiments. However, since the temperature rise occurs adjacent to the electrodes inside the tissue and happens in an extremely short time, it is impossible to monitor the temperature with conventional methods such as the use of thermocouples, thermistors, or other temperature detectors. We recently proposed a new method using a temperature-sensitive ink that exhibits irreversible color change at a temperature higher than a certain threshold [13]. By mixing the ink in a tissue phantom, we demonstrated that a

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http://dx.doi.org/10.1016/j.ijheatmasstransfer.2017.03.115
0017-9310/

temperature rise exceeding 20 K was observed around the electrodes after the application of nine 1-ms-long pulses at 2 kV/cm, whereas it was significantly smaller after ninety 0.1-ms-long pulses at the same electric field. This therefore suggests that the former pulse condition carries the risk of thermal damage to tissue at an initial temperature of 37 °C because the thermal denaturation temperature of proteins is in the range of 55–60 °C [14,15]. However, the thermal damage was not directly examined in conjunction with the temperature measurement.

Denaturation is a result of the change of protein structure that defines the function of the protein. Several methods such as circular dichroism (CD) spectroscopy [16,17], Fourier transform infrared (FT-IR) spectroscopy [18–20], and Raman spectroscopy [21–23] have been used for the identification of the secondary structure of proteins. The CD spectroscopy detects the difference in the absorption between left-handed and right-handed circularly polarized lights. The secondary structure of proteins is identified using the fact that chiral macromolecules absorb each light differently. The FT-IR also detects the absorption of light in the infrared region, which depends on the structure of a protein and exhibits peaks in the infrared spectrum that is specific to the structure of the macromolecules. However, these methods usually require a sample with a special shape. In contrast, Raman spectroscopy is applicable for a wide range of samples without any special pre-machining required. It detects the shift in the frequency of the inelastically scattered light from irradiated macromolecules. Since the frequency modulation is specific to its molecular vibration, the Raman scattering contains information on the material composition and the crystal lattice. The denaturation of protein molecules is therefore detected as a peak shift in the Raman spectra.

Here, we examined Raman microspectroscopic measurements to quantify the local denaturation of tissue induced by IRE therapy. We proposed a denaturation index using the amount of shift in a Raman spectral peak, and used it to determine the denaturation of a protein-containing tissue phantom. Furthermore, the temperature distribution around the electrodes was measured using temperature-sensitive ink, and was compared to the results obtained from numerical analysis.

2. Materials and methods

2.1. Preparation of native and fully-denatured specimens

Albumin was used as a model protein to quantify the thermal denaturation associated with IRE therapy. Albumin-agar solution was first prepared by dissolving 10% agar (Wako Pure Chemical Industries, Osaka, Japan), 1.8% NaCl, and 0.5% sodium alginate in hot water at 80 °C followed by cooling it down to 60 °C, and then mixing it well with the same volume of 20% albumin aqueous solution (Sigma-Aldrich, St. Louis, MO). The final constitution of the solution was therefore 5% agar solution containing 10% albumin, 0.9% NaCl, and 0.25% sodium alginate. The albumin-agar solution was then gelated in a plastic vessel (20 mm in inner diameter and 42 mm deep) at room temperature and used as a native specimen. To prepare a fully-denatured specimen as a reference, a specimen was sealed with a plastic wrap to prevent evaporation and was incubated in a dry oven at 90 °C for 10 min.

2.2. Reference Raman spectra of albumin

The Raman spectra of native and fully-denatured specimens were obtained as references using a confocal Raman microscope (RAMAN-11, Nanophoton, Osaka, Japan) equipped with a 532 nm Ar-ion laser, a 1200 lines/mm grating, and a 20× Nikon air objective lens (N.A. 0.45). This setting accomplished a spatial resolution of 1 μm/pixel and a spectral resolution of 1 cm^{-1} in the 888–2063 cm^{-1} wavenumber range, respectively. The data were obtained using point-scan mode with the irradiation of the laser at 50 mW for 60 s and were determined by taking an average of 10 repeated measurements.

The spectra of the native specimen showed three prominent peaks in the range from 1500 to 1800 cm^{-1}. They are a C=C stretching band (the peak at 1530 cm^{-1}), an amide II band (1560 cm^{-1}), and a strong amide I band (1660 cm^{-1}) (Fig. 1). However, the spectra of the fully-denatured specimen exhibited the peak of the amide I band at 1670 cm^{-1} while the other two peaks appeared at the same wavenumber as those in the native specimen.

2.3. Spectra analysis and definition of the denaturation index

We assumed that the Raman spectra measured in the experiments are composed of background signals and four basic spectra: a C=C stretching band (1530 cm^{-1}), an amide II band (1560 cm^{-1}), a native amide I band (1660 cm^{-1}) and the shifted amide I band (1670 cm^{-1}) (Fig. 1). We therefore extracted the defined four basic spectra shown in Fig. 2 by applying baseline correction and smoothing process to the raw Raman spectra of native and thermally denatured albumin.

We then deconvoluted the peak intensities from the measured spectra using the polynomial noise reduction proposed by Deguchi et al. [24] as follows:

$$I(x) = \{C_{1530}I_{1530}(x) + C_{1560}I_{1560}(x) + C_{1660}I_{1660}(x) + C_{1670}I_{1670}(x)\} + I_{bg}(x) \quad (1)$$

where $I(x)$ is the measured spectrum at the wavenumber x, $I_i(x)$ is the spectral intensity of each basic spectrum at wavenumber i, $I_{bg}(x)$ is the spectral intensity of the background noise, and C_i is the weight factor. We also assumed that the background noise is expressed by a 7th-order polynomial curve,

$$I_{bg}(x) = a_0 + a_1x_1 + a_2x_2^2 + \cdots + a_7x_7^7 \quad (2)$$

where a_i are the coefficients of the polynomial. All these coefficients and weight factors, twelve in total, were initially set to 1, and then determined using the least squares method.

Thermal denaturation of a tissue phantom was identified by the shift of the spectral peak of the amide I band from 1660 cm^{-1} to 1670 cm^{-1} as a result of conformation change in the α-helix. We

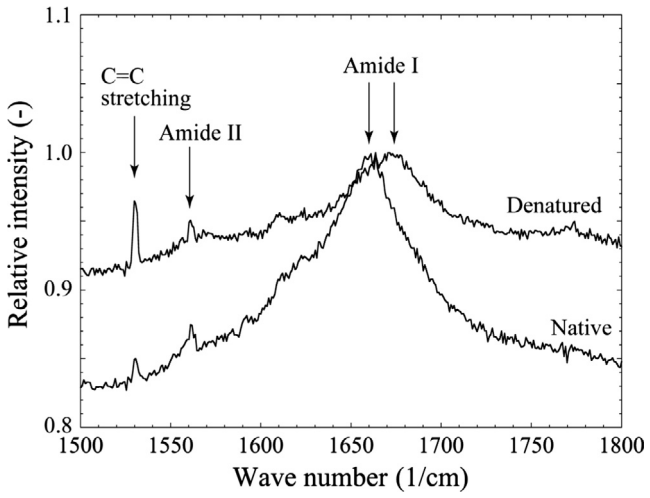

Fig. 1. Typical Raman spectra of native and thermally denatured albumin for wavenumbers ranging from 1500 to 1800 cm^{-1}.