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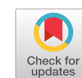
Advantages of infrared transflection micro spectroscopy and paraffin-embedded sample preparation for biological studies



Jie Yao [a,*], Qian Li [b], Bo Zhou [a], Dan Wang [a], Rie Wu [a]

[a] Nanjing Normal University, Nanjing 210023, China
[b] Nanjing University of Chinese Medicine, Nanjing 210023, China

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ABSTRACT

Fourier-Transform Infrared micro-spectroscopy is an excellent method for biological analyses. In this paper, series metal coating films on ITO glass were prepared by the electrochemical method and the different thicknesses of paraffin embedding rat's brain tissue on the substrates were studied by IR micro-spetroscopy in attenuated total reflection (ATR) mode and transflection mode respectively. The Co-Ni-Cu alloy coating film with low cost is good reflection substrates for the IR analysis. The infrared microscopic transflection mode needs not to touch the sample at all and can get the IR spectra with higher signal to noise ratios. The Paraffin-embedding method allows tissues to be stored for a long time for re-analysis to ensure the traceability of the sample. Also it isolates the sample from the metal and avoids the interaction of biological tissue with the metals. The best thickness of the tissues is 4 μm.

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1. Introduction

The use of infrared micro-spectroscopic techniques for the nondestructive analysis of biological specimens is a rapidly expanding research area, with much focus on its utility in cytological and histological diagnosis [1–13]. The techniques combining with intelligent algorithm have potential application in cancer screening and diagnosis. There are still several challenges in the developing application of biospectroscopy with regards to sample preparation, IR modes of measurements, and the selection of substrate materials, etc.

Paraffin-embedding, snap-freezing and air-drying are mainly available state of art methods for tissue sample preparations. Zohdi et al. [14] have systematically examined the spectra for any possible biochemical changes to the native state of the tissue caused by desiccation drying, ethanol substitution or formalin fixation, and have reported the detected changes in infrared absorption band intensities and positions. Thus the tissue preparative effects must be considered when preparing, measuring, and analyzing samples using FTIR spectroscopy. The impact of preparation methods on the chemical composition of biological tissue is still a controversial issue. Liyanage et al. [15] have developed an optimal protocol for cryo-sectioning of biological tissues by varying the temperature of both the cutting blade and the specimen holder. Although FTIR imaging has been used to obtain chemical information from cryo-sectioned sample with no interference of the conventional paraffin-embedding agent and chemicals, the major problem with cryo-sectioning is that the peaks are not well resolved in 8μm tissues section while thin sections (less than 8 μm) wrinkle easily during the preparation. Therefore, repeated sectioning is required in order to obtain good quality sections and consistent results. The paraffin-embedding (FFPE) of tissue section has been a commonly used method in pathological research. There are many discussions about the effects of formaldehyde, ethanol and toluene on the protein, liposome, nucleic acid and collagen in the preparation process. Though the influence of sample preparation on the peaks of infrared spectrum of biological tissue has been confirmed, some scholars still think that this sample preparation method can be applied to the analysis of biological tissue by infrared spectroscopy. FFPE method has been successfully used to differentiate breast cancer tissues from normal tissues and marginal tissues by infrared microscopy [16–18]. The better method for the analysis of infrared imaging remains to be developed. The suitable method should have high quality infrared spectrum and can keep the chemical properties of the original tissue with easy preparation and preservation of the tissues.

The three major Infrared spectroscopy measurement modes are transmission, transflection and Attenuated Total Reflection (ATR) commonly. Each mode offers convenience for some samples and challenges for others. Although there are many advantages of transmission method, but the substrates loaded biological tissue limit its application in analysis biological samples. It requires the substrate must be able to transmit infrared light and water resistant when analysis of fresh biological tissues. Therefore, the choice of the substrate is very limited and the cost of the substrate is higher in transmission mode.

* Corresponding author.
E-mail address: 07111@njnu.edu.cn (J. Yao).

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The use of ATR-FTIR is certainly a tangible accessory for rapid point of care diagnostics. Its operation is simple and requires minimal sample preparation, especially for bio-fluids. However, ATR mode probes samples using the evanescent wave, which penetrates into the sample between 1μm and 10μm depending on the wavelength of the light, angle of incidence and refractive indices of the sample and prism. ATR mode is a contact approach which has the possibility of deforming or damaging the sample such as a tissue.

The transflection mode of measurement preformed on MirrIR substrates draws great attention recently. As the cost of gold-plated slides used for this mode is expensive, many metallic surfaces, such as Aluminum foil, are also useful as substrate for such measurements [19]. Therefore, finding the low-e substrate material is the key to developing this measurement mode. However, there has been considerable debate over the past decade with regard to the validity of FT-IR spectroscopic measurement, as physical/optical, because chemical and physical inhomogeneities introduce spectral artifacts. The so-called electric field standing wave effect (EFCW) has recently been demonstrated to significantly distort FT-IR spectra acquired in a transflection mode, particularly in the field of spectroscopy of biological materials. Whether this mode can be used for biological tissue infrared spectroscopy? The recent work of Wrobel et al. [20] showed that the standing wave effect should be a minor distortion in FT-IR spectroscopy of tissues by simulating models of experimental variability. The model was derived from electromagnetic theory for three-layer system (air, sample, reflective substrate). The study has important implications notably for the validity of the extensive studies which have been performed to date on tissues sample in transflection geometry. So, the preparation of low-e and low-cost substrates is the key for using transflection measurement to study biological tissues.

In this paper, series metal coating films on ITO glass were prepared by the electrochemical method and the different thicknesses of paraffin embedding rat's brain tissue on the substrates were studied by IR microspetroscopy in attenuated total reflection (ATR) mode and transflection mode separately.

2. Experimental

2.1. Materials

All reagents used in this work were purchased from Sinopharm Chemical Reagent Co., Ltd. Indium-tin oxide (ITO) glass with sheet resistance 25 Ω was obtained from Conduc Optics & Electronics Co., Ltd.

2.2. Preparation of Different Metal Films on ITO

2.2.1. Cleaning of ITO Glass

The ITO glass was cleaned in acetone, ethanol and deionized water with ultrasonic vibration for 10 min to remove lipid and other impurities, then boiled in mixed solution (H_2O_2:NH_3H_2O:H_2O = 1:1:5, V/V) to activate ITO substrate. Finally, the substrate was cleaned in deionized water with ultrasonic vibration for 10 min twice.

2.2.2. Preparation of Metal Films on ITO

The experiment was carried out in a three electrodes system (platinum electrode as the counter electrode, SCE as the reference electrode, ITO conductive glass as the working electrode). A potentiostat/galvanostat (CS150) was controlled with a computer potentiostatically.

2.2.2.1. Preparation of Co-Ni-Cu Alloy Film on ITO. Co-Ni-Cu alloy films were electrodeposited from the electrolyte containing 0.018 mol/L cobalt sulfate, 0.18 mol/L nickel sulfate, 0.002 mol/L copper sulfate, 0.4 mol/L boric acid, 3.25 wt% glucose at −1.0 V.

2.2.2.2. Preparation of Ni Film on ITO. Nickel films were electrodeposited from the electrolyte containing 0.1 mol/L nickel sulfate, 0.01 mol/L nickel chloride, 0.05 mol/L boric acid, 0.3756 mol/L diethanolamine at 1.3 V.

2.2.2.3. Preparation of Cu Film on ITO. Copper films were electrodeposited using constant potential electrolysis with 0.1 mol/L CuSO4 solution by adding certain amount of surfactant diethanolamine (0.371 mol/L) at 2V.

2.2.3. Clearing of Deposition Films

After deposition, the substrates were rinsed and immersed in ethanol for 5 min to discontinue the reaction and to remove all unreacted electrolyte. The substrates were stored in desiccators for characterizations.

2.3. Preparation of Paraffin-Embedded Sample

The brain tissue of rats was collected and fixed with 4% paraformaldehyde for 3 days. It was rinsed with water to remove excess paraformaldehyde and then dehydrated with 80% ethanol for 2h, 100% ethanol I for 2h, 100% ethanol II for 1.5h. Further the tissue was dehydrated with xylene I for 1.5h and xylene II for 1 h. Finally, it was embedded with paraffin.

2.4. Infrared Spectroscopy

The IR ATR spectra of the rat's brain tissues were recorded on Nicolet NEXUS 670 Fourier transform infrared (FT-IR) spectrometer, using a deuterated triglycine sulfate (DTGS) detector. All spectra were recorded with a resolution of 4 cm^{-1}, 32 scans in the spectral range between 650 cm^{-1} and 4000 cm^{-1}. The background spectrum of atmosphere was measured as a single beam and was used as reference.

The micro IR transflection spectra of the samples were collected on Bruker Hyperion infrared microscope, using Liquid Nitrogen cooled HgCdTe (MCT) Infrared detector. All spectra of the rat's brain tissues, recording over the range 4000–400 cm^{-1} at 4 cm^{-1} spectral resolution and 64 co-added scans. The corresponding metal film on ITO was used as the background spectrum.

The map analyzed here was recorded with a Bruker microscope LUMOS using Liquid Nitrogen cooled HgCdTe (MCT) Infrared detector. The images were recorded in transflection mode over the range 4000–600 cm^{-1} at 4 cm^{-1} spectral resolution and 64 co-added scans. The gold mirror was used as the background spectrum.

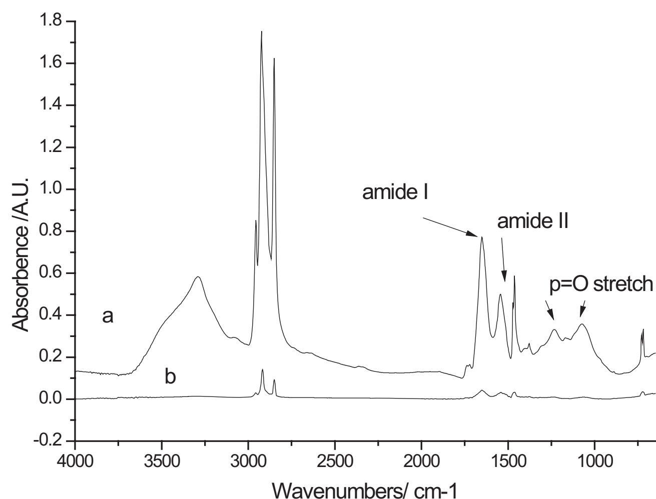

Fig. 1. The spectra of rat's brain tissue. a. transflection. b. ATR.

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