Communications to the Editor

Quantitative Analysis of the Staining of a Polyisoprene-*block*-polystyrene

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The staining of polymer systems by OsO_4 is a well-established method to generate or increase the contrast of samples used for transmission electron microscopic investigations. OsO_4 staining is very specific for polymers containing double bonds and therefore can be used to stain block copolymers such as polyisoprene-*block*-polystyrene (SI).[1] The staining method, which was originally developed for biological samples, was first applied to polymer materials by Andrews et al.[2] and Kato.[3] Although the method is now well-established, little is known about the details of the reaction itself. In this report, we present a quantitative analysis of the staining reaction of OsO_4 with a polyisoprene-*block*-polystyrene copolymer using electron energy loss spectroscopy (EELS), which can determine the sample thickness of the ultrathin cut and the ratio of oxygen atoms, i.e., absorbed OsO_4, to all of the carbon atoms, i.e., polystyrene and polyisoprene, contained in the sample.

The SI block copolymer has a 42/58 (mol %/mol %) composition and a molecular weight M_n of 1.2×10^4. The synthesis was done via anionic polymerization with *sec*-*tert*-butyl lithium as initiator and cyclohexane as a solvent. The block copolymer was solvent-cast from toluene (5 wt %) in a Petri dish from which the solvent was evaporated at room temperature for 1 week. The remaining solvent was removed under high vacuum for 2 days. The thus obtained bulk sample was

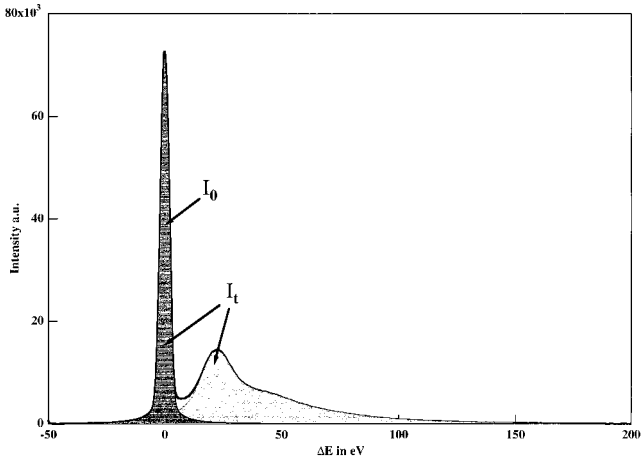

Figure 1. Sample EEL spectrum in the zero loss area of the unstained SI block copolymer with a thickness of about 40 nm. I_t is the sum of the dark gray area and the bright gray area. I_0 corresponds to the dark gray area.

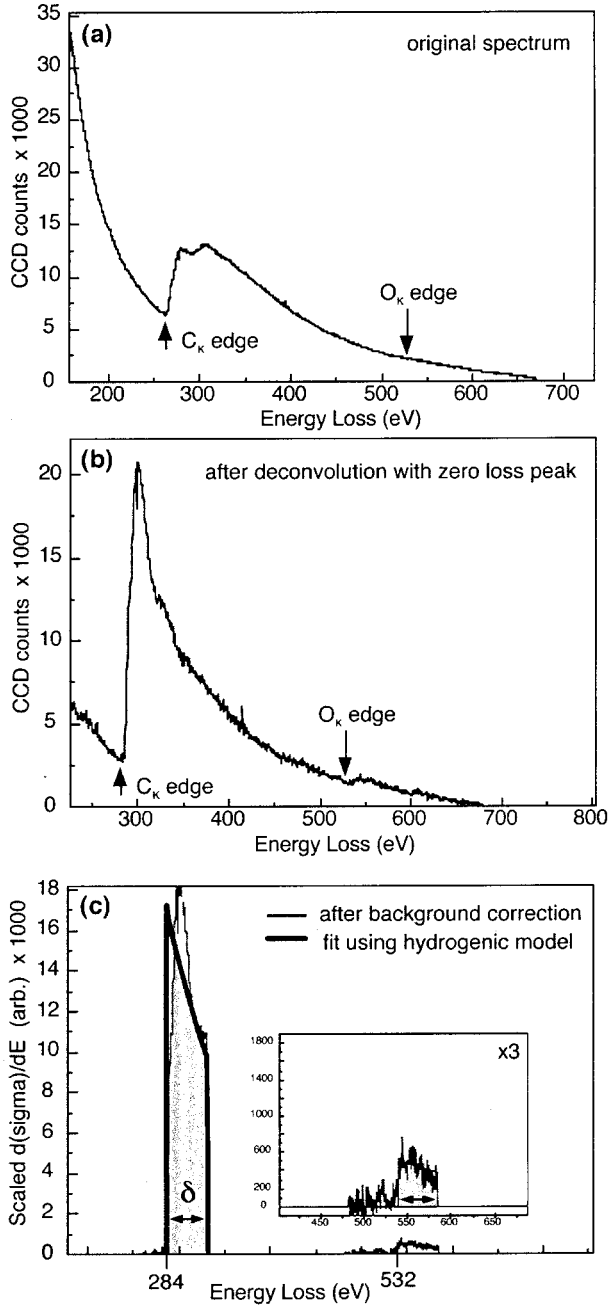

Figure 2. EEL spectra in the area of the carbon and oxygen K-edge of an SI sample stained with OsO_4 for 1 h: (a) original spectrum, (b) Fourier ratio deconvolution, and (c) background subtraction and fit using hydrogenic model.

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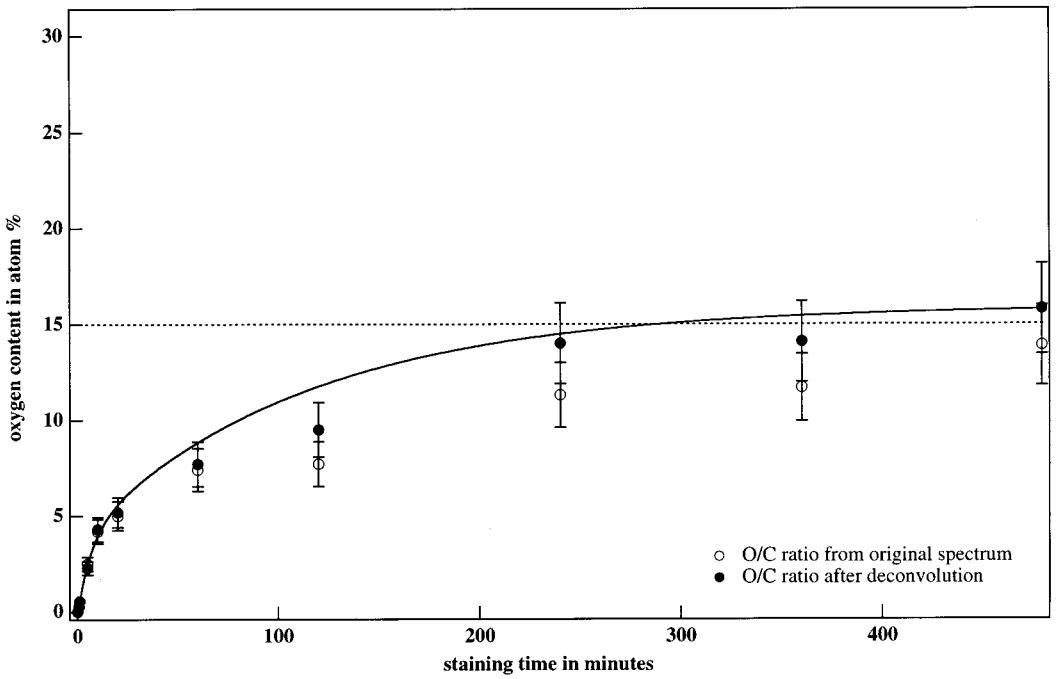


Figure 3. Oxygen content in percent, 100 $N^{oxygen}/(N^{oxygen} + N^{carbon})$, as a function of time spent for staining calculated using eq 2.

ultramicrotomed using a Reichert /Nissei FCS and diamond knife at −80 °C without prior embedding or staining. The ultrathin cuts were allowed to warm to room temperature and the sample thickness was determined by EELS. For staining, the grids were put into a glass chamber (about 125 cm^3 volume) saturated with OsO_4 vapor for different time periods. These samples were then used to determine the oxygen/carbon ratio after different staining times. For TEM/EELS studies a JEOL 2000FX TEM equipped with a GATAN electron energy loss filter operating at 200kV was used.

The sample thickness had no direct influence on the determination of the oxygen/carbon ratio of the OsO_4 stained samples for our experiment. The film thickness only affects the rate of the staining, meaning that a thicker sample will take longer for staining, as the diffusion time of the OsO_4 to the center of the film will become longer. To avoid this problem only samples with the same thickness were used. As it was impossible to obtain samples with always the same sample thickness via ultramicrotomy, areas on the sample having identical local thickness, i.e., the area where the sample composi-tion should be determined later, were determined byEELS. Using eq 1, the sample thickness can be determined by measuring the integral of the zero loss peak and the whole spectrum.

$$t = \lambda \ln \frac{I_t}{I_0} \tag{1}$$

where t is the absolute sample thickness in nm, I_t the intensity under the entire spectrum, I_0 the intensity under the zero loss peak, and λ the mean free path in nm. λ can be estimated at an acceleration voltage of E_0 = 200kV as 160 nm.[4] For our purpose, we do not need an exact value of λ because we are not interested in the absolute sample thickness but in finding samples with the same local thickness, so that even if λ has a large absolute error, it has no influence on the determination of the oxygen/carbon ratio. A typical spectrum of a sample with 40 nm thickness is given in Figure 1. For the measurement of the oxygen/carbon ratio, only areas of a ultrathin cut indicating a thickness of 40 nm were chosen.

After the films were exposed to OsO_4 vapor, EEL spectra in the region of the carbon−K edge at an energy loss ΔE_k^{carbon} at 284 eV and the oxygen−K edge at ΔE_k^{oxygen} at 532 eV were acquired (see Figure 2a). The atomic ratio of oxygen and carbon can be determined by using eq 2.

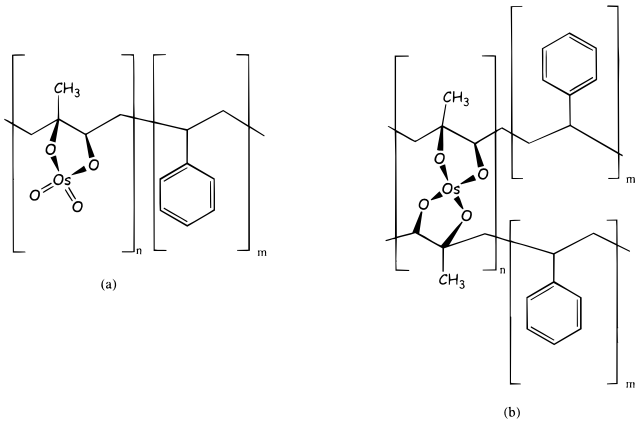
$$\frac{N^{oxygen}}{N^{carbon}} = \frac{\sigma_k^{carbon}(\beta,\delta) I_k^{oxygen}(\beta,\delta)}{\sigma_k^{oxygen}(\beta,\delta) I_k^{carbon}(\beta,\delta)} \tag{2}$$

N^{oxygen} and N^{carbon} are the number of atoms in the selected sample area. I_k^{oxygen} and I_k^{carbon} are the integrals under the carbon−K edge and oxygen−K edge at an energy width δ (here, δ = 50 eV) and a collection aperture angle β, the angle limiting objective aperture (here, β = 6.79 mrad). σ_k^{oxygen} and σ_k^{carbon} are the cross sections of the carbon and oxygen K-shell at E_0 and can be estimated with an error of about 10% using the hydrogenic model.[4−6]

The original spectrum of the sample stained for 1 h is show in Figure 2a. Figure 2b shows the spectrum after Fourier ratio deconvolution[4,5] using the zero loss peak to remove multiple scattering. After background subtraction (Figure 2b; for details regarding the background subtraction see Appendix A and the literature[4,5]), a peak is obtained which obeys the fit using the hydrogenic model much better (Figure 2c).

In Figure 3, the oxygen content in percent is plotted against the staining time, which also include the values without Fourier ratio deconvolution. The values obtained without deconvolution are always smaller than the values obtained with deconvolution but are in the same range. As we can see, the oxygen content is increasing very rapidly in the first minutes but is slowing down to level off at an oxygen content of about 15% after about 2 h. Assuming that the OsO_4 is only

Scheme 1. Quantitative Analysis of the Staining of a Polyisoprene-*block*-Polystyrene



able to undergo a reaction with the double bonds of the polyisoprene, one OsO_4 molecule has the option to react with one or two double bonds. If only one OsO_4 reacts with one double bond of the polyisoprene, it would lead to the product seen in Scheme 1a. In this case, a saturation of all double bonds would lead to a OsO_4/double bond ratio of 1:1 or an oxygen/carbon ratio of 4:13.8 (one OsO_4 is absorbed by one double bond until saturation) corresponding to a 29% content of oxygen atoms. In case of Scheme 1b, unreacted oxygen atoms react with another double bond, leading to a cross linking of the polyisoprene and the structure seen in Scheme 1b. In this case, the OsO_4/double bond ratio would be 1:2 or an oxygen/carbon ratio of 2:13.8 corresponding to a 14.5% content of oxygen atoms. As visible from the graph given in Figure 3, the oxygen content saturates at a value around 15%, which is in good agreement with the predicted value for a double OsO_4 reaction and clearly inconsistent with the 29% oxygen content predicted for a single OsO_4 reaction, suggesting that cross linking is the dominant mechanism during the staining procedure.

Appendix A The slope of the EEL-spectrum before an element edge obeys eq 3.

$$I = Ae^{-r\Delta E} \tag{3}$$

A and r are determined by fitting. Therefore, the background can be determined by fitting using eq 3 and extrapolating through the element edge.

References and Notes

(1) For example, see: Inoue, T.; Soen, T.; Kawai, H.; Fukatsu, M.; Kurata, M. *J. Polym. Sci., Polym. Lett. Ed.* **1968**, *6*, 75. Inoue, T.; Soen, T.; Hashimoto, T.; Kawai, H. *J. Polym. Sci.* **1969**, *7*, 1283.
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