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Raman Imaging of PLGA Microsphere Degradation Inside Macrophages

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One of the key issues in successful application of biomaterials for tissue engineering and drug delivery is a well-characterized in vivo biodegradation behavior. Poly(lactic-*co*-glycolic acid) (PLGA) has been used for a wide variety of medical applications, from resorbable sutures to bone screws and microspheres for drug delivery. Degradation of PLGA in vitro as well as in vivo mainly takes place through either hydrolysis of the ester linkages and/or enzymatic degradation (see the chemical structure in Figure 1).[1,2]

It has been reported that devices of PLGA degrade in a heterogeneous manner.[3] A model describing this process and based on work by Vert and co-workers was proposed by Park,[4] in which the degradation proceeds more rapidly in the center than at the exterior.[3,5,6] This phenomenon is thought to be caused by autocatalytic action of the carboxylic acid end groups of the degrading material trapped in the internal milieu. It has been questioned whether this model is also valid when dealing with microspheres.[7] A more homogeneous microsphere degradation, has been postulated on the basis of observation of homogeneous degradation of small devices such as thin films.[8]

In many cases, implantation of a biomaterial results in a foreign body reaction involving macrophages, which phagocytose small particles of the degrading materials. After phagocytosis by these cells, degradation may continue inside the phagosome, which plays a crucial role in degradation of internalized materials. The in vivo response to implanted biomaterials is in general studied by histology, which examines the tissue reaction by light microscopy. The limitation of these studies is that, although morphological changes can be studied, no information about the chemical composition of the degrading materials can be obtained from such measurements. Raman spectroscopy allows one to study the chemical bonds involved in degradation of these polymers by detecting intensity and wavelength changes in the vibrational bands of these bonds. We studied the degradation of PLGA microspheres, after macrophage phagocytosis in vitro, by nonresonant confocal Raman spectroscopy and imaging (see Figure 2 and 3). The PLGA microspheres (50:50 glycolide/lactide by weight) were produced by a so-called salting-out procedure described before.[9,10] Scanning electron microscopy (SEM) showed that their size varied between 1 and 10 μm in diameter and that they were of solid nature (Figure 3E). The microspheres were sterilized in 70% ethanol for 15 min and then washed in sterile PBS. After washing, the material was opsonized for 30 min using human serum. The microspheres were then added to a macrophage cell line (RAW 264.7) cultured on poly-L-lysine-coated CaF_2 slides, in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, and antibiotics. The cells were cultured for 1 and 2 weeks at which point they were fixed using 4% paraformaldehyde for 30 min. The samples were washed and placed in PBS for Raman measurements, which were performed using a home-built confocal Raman microscope as described previously.[11] A pinhole of 15 μm diameter was employed, providing an axial resolution (FWHM) of 1.5 μm. During Raman imaging, we scanned an intracellular area of 7.5 × 7.5 μm^2, using a signal accumulation time of 1 s per pixel at 100 mW 647.1 nm excitation.

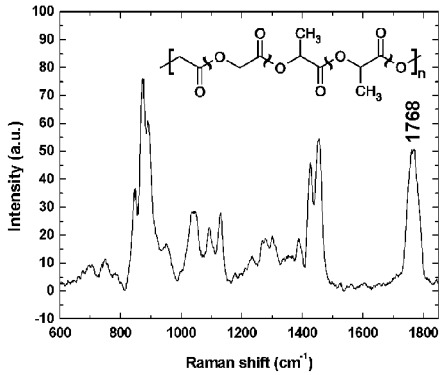

Figure 1. Raman spectrum and chemical structure of PLGA. In the structure, two glycolic acid units are shown on the left, whereas lactic acid units are displayed on the right. The ester bonds (marked "∼"), which are hydrolyzed upon degradation, are characterized by a carbonyl stretching vibration at 1768 cm^{-1}.

The internalized microspheres showed signs of intracellular degradation already after 1 week of cell culture. Light microscopy revealed what appeared to be a cavity in the center of the microspheres at 1 and 2 weeks of cell culture (Figure 3, A and C). Confocal Raman spectroscopy and imaging of degraded PLGA spheres confirmed these observations. In contrast, PLGA microspheres incubated under identical conditions, but in the absence of macrophages (insets in C and D of Figure 3), did not show any signs of degradation. Raman images of phagocytosed microspheres constructed in the 1768 cm^{-1} band specific for the ester groups of PLGA clearly show a low intensity of this band in the internal area of several particles already after 1 week (Figure 3B). This indicates the loss of PLGA ester bonds from the center of these microspheres. We scaled both the pure PLGA spectrum (Figure 1) and averaged spectra taken from the high- and low-intensity regions of the PLGA microsphere in Figure 3D to the 875 cm^{-1} band, which is assigned to the C−COO stretch vibration of lactic acid[12] and is not affected by hydrolysis. Analysis of the spectroscopic data after Raman imaging revealed that 2 weeks after phagocytosis a ∼30% reduction in the ester bond intensity was found in both the low- and high-intensity area of the internalized microsphere, compared to that in pure PLGA. This is demonstrated by the negative band in Figure 2, A and B, at 1768 cm^{-1} after subtraction of the scaled spectra of pure PLGA. Moreover, the difference spectrum in Figure 2A shows bands specific for the cell cytoplasm. On the basis of

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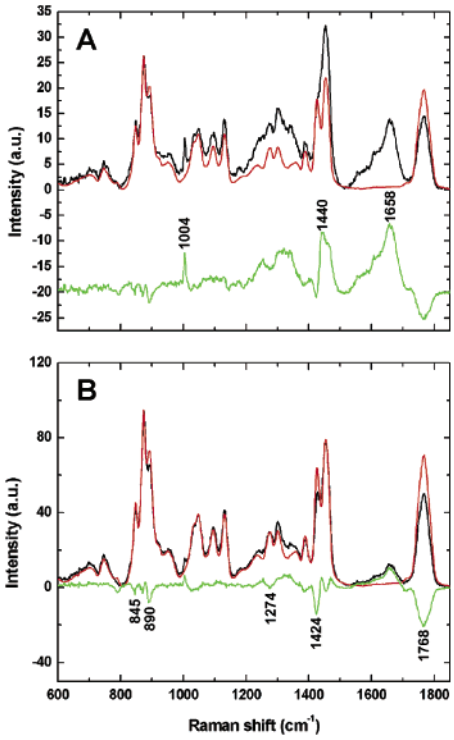

Figure 2. Raman spectra (in black) of the low- (A) and high-intensity (B) regions of the PLGA microsphere in Figure 3D. Pure PLGA is shown in red, and difference spectra (after scaling to the 875 cm^{-1} band) in green. Note the negative band at 1768 cm^{-1} (indicating a decrease in the number of ester bonds) and the negative bands specific for glycolic acid at 845, 890, 1274, and 1424 cm^{-1}.[11]

the presence of bands at 1004 cm^{-1} (phenylalanine) and 1662 cm^{-1} (amide I) and the 1440 cm^{-1} band assigned to CH_2 groups predominantly found in lipids, we conclude that both proteins and lipids are present in the degradation-induced void present in the microspheres. It is unlikely that these molecules have diffused through the intact outer shell of the microspheres, since this particular composition of PLGA is rather hydrophobic. Proteins and lipids have probably traveled through one or more pores formed by degradation which connect the cavity in the center to the phagosomal milieu. Such a mechanism is also indicated by the collapsed microsphere in the top right corner of the Raman image in Figure 3B. Using either 3D Raman imaging or observation of the microsphere surface by SEM after isolation from the macrophages will probably resolve these issues.

The outcome of our study adds not only histological but also chemical data to the model proposed by Park[4] and Vert,[3,5,6] describing heterogeneous degradation of PLGA starting from the center and going outward. The degradation of PLGA leading to the induction of concentric cavities in the microspheres after uptake by macrophages, as described here, is in favor of that model. However, analysis of the spectroscopic data strongly suggests that degradation takes place throughout the whole microsphere by hydrolysis of the ester bonds preferentially related to the glycolic acid block in the polymer, as indicated by the negative bands in Figure 2, A and B. This finding is more related to the suggestion by Li of homogeneous degradation when dealing with microspheres.[7] As existing models do not take into account the influence of the cell on the microsphere degradation process, they must be different from a phagosomal degradation mechanism. Our results indicate that the PLGA degradation behavior shown here is a cell-mediated process, caused by either the low pH (~5.5) and/or the presence of hydrolytic enzymes in the phagosome, since control

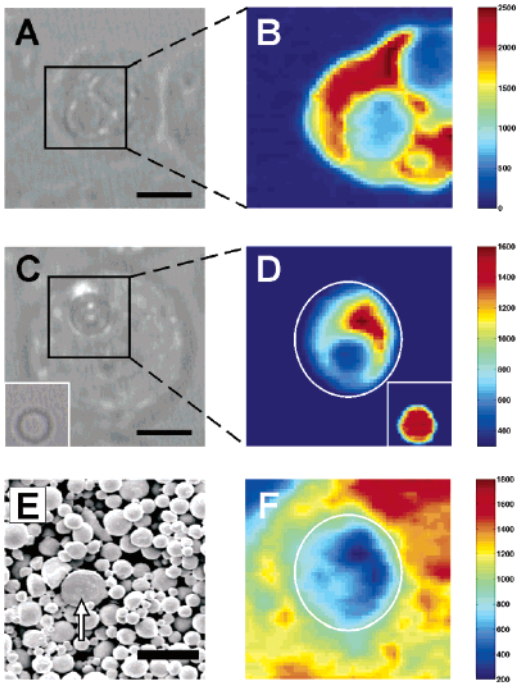

Figure 3. (A and C) Light micrographs of macrophages containing phagocytosed beads cultured for 1 and 2 weeks, respectively (scale bar 5 μm). The Raman images (7.5 × 7.5 μm^2) of these beads are shown in B and D. The scale in B and D shows the relative intensity of the 1768 cm^{-1} band used for imaging (specific for PLGA ester bonds). Insets in C and D show controls (image size 7.5 × 7.5 μm^2). The Raman image in 3F depicts the intensity of the 1527−1714 cm^{-1} region, which contains bands from cytoplasmic species. (E) Electron micrograph of PLGA microspheres after freezing and crushing in liquid nitrogen. A cross-section of a broken sphere, showing the solid center, can be seen (arrow) (scale bar 10 μm).

samples lacking macrophages did not show any degradation after 1 and 2 weeks (insets, Figure 3).

In conclusion, we have demonstrated that confocal Raman spectroscopy and microscopy are unique, label-free tools to study intracellular microsphere degradation after in vitro phagocytosis. These techniques allow detailed information about the chemical composition of the degrading polymer particles to be obtained. In addition, they will enable studies directed at the chemical investigation of biomaterial degradation in vivo.

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