The Manufacturing of Microencapsulated Microspheres and Novel Approaches for Characterization

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ABSTRACT

A new process for microsphere coating was developed. For the characterization of these systems we developed some new methods that have, so far, not been used for this purpose. They allow verification of the coating success as well as the characterization of the systems. We used scanning florescence microscopy to distinguish between core and coating of stained particles. To investigate the success of coating for large numbers of particles, we used Electron Spectroscopy (ESCA or XPS) to investigate the surface composition of microspheres. This allowed addressing the problem of the completeness by which the particles were coated and whether some particles failed to be coated. Besides these new techniques we also applied classical techniques like polarized light spectroscopy or scanning electron microscopy (SEM). Polarized light spectroscopy can be a powerful method, requiring little sample preparation. For the investigation of particles by SEM the particles were cut using a cryomicrotome. It turned out that SEM yields the most detailed insight into the systems, but requires time consuming sample preparation.

INTRODUCTION

The application of polymers for the controlled release of substances is a field of steadily growing importance. With a size of less than 100μm, microspheres are a dosage form that allow the parenteral administration of drugs via injection. Besides their medical application(1,2) microspheres are also useful in many other areas. In the film industry for example particles are loaded with light sensitive material(3) and in agriculture they delay the release of herbicides and pesticides(4).

Coating microspheres with a second layer of polymer can serve several purposes. One of the first suggested applications was the suppression of the fast release of drugs at early times of the application also known as "initial burst" (5,6). In this case the coating material acts as a diffusion barrier which controls the release of drug. Other applications include the modification of the release behavior like linear release(7) or the suppression of release for a period of time. The result is a delayed release of the drug. In an extreme case the drug is then released all at once generating a pulsatile release profile(8). Such modifications might be useful for the application of coated microspheres in tumor therapy or for vaccination.

Coated microspheres can be produced by a variety of methods (9-14). In initial approaches microspheres were simply dipped into a polymer solution(11). More advanced methods use the fluidized bed technique which is also an excellent method for coating solids. A major disadvantage however is the relatively large particle size resulting from this technique (12,13) which makes it difficult to use them for parenteral injections. Other methods prepare such particles by dissolving two polymers which are insoluble in each other in an organic solvent and evaporating it. Core and shell are created upon phase separation (14). This method works, however, only for polymers that are

insoluble in each other. Other problems arise from the incorporation of drugs. Drugs that are dissolved together with the two polymers during manufacturing might move into the core or into the coating material, which is hard to control but might be important to achieve a certain type of release behavior (e.g. the rapid release of an initial dose or a delayed pulsed release). Additional problems arise from the encapsulation of water soluble drugs (e.g. proteins and peptides) which are usually encapsulated via the double emulsion technique(15,16). Producing coated microspheres using the phase separation technique might not allow incorporating the drug via an emulsion because the drug would not only be introduced into the core of the particles but also into the coating.

Besides production, the characterization of coated microspheres poses severe problems. Though it might appear trivial it is not easy to verify that particles have been coated completely. Another important question is whether some particles have failed to be coated or what the surface composition of the coating is. It was, therefore, our primary goal in this study to develop techniques that allow the characterization of coated microspheres. Some of these techniques differ substantially from those that have been used for this purpose so far(17,18). Having developed a new coating technique(19), we developed several methods to characterize our system(19). They allow addressing some of the problems involved in the development of coated microspheres in much more detail than previously possible.

MATERIALS

Poly(L-lactic acid) (L-PLA), poly(D,L-lactic acid) (D,L-PLA) and poly(vinylalcohol) (PVA) were obtained from Polysciences, Inc., Warrington, PA. Poly(D,L-lactic-co-glycolic acid (PLA-GA) was obtained from Boehringer Ingelheim, Ingelheim, Germany. Carboxyfluorescein was obtained from Kodak,

Rochester, NY. Nile Red was purchased from Molecular Probes Inc., Eugene, OR. Ethylene-vinyl-acetate-copolymer (EVAC), Elvax 40W (40% Vinylacetate), was obtained from Dupont, Wilmington, DE. EVAC was purified as described earlier(20). The Polyanhydride polymers poly(1,3-bis-p-carboxyphenoxy-propane-co-sebacic acid), p(CPP-SA) 50:50, and poly(fatty acid dimer -co- sebacic acid), p(FAD-SA) 50:50, were provided by Scios-Nova Pharmaceuticals, Baltimore, MD.

METHODS

Particle coating: 100 mg of core microspheres were directly dispersed by vortex mixing into 1ml of organic coating polymer solution. The resulting organic dispersion was emulsified into 2.5ml 1% aqueous PVA solution and finally into 100ml 0.3% aqueous PVA solution. The solvent was allowed to evaporate, the coated microspheres were collected by centrifugation, washed with double-distilled water and freeze-dried. Table I summarizes the various particles that were produced:

Table I: various preparations of coated microparticles

Polymer A (core)	Polymer B (coating)
EVAC	EVAC
p(CPP-SA) 50:50	PLA-GA
p(FAD-SA) 50:50	PLA-GA
p(FAD-SA) 50:50	D,L-PLA
L-PLA	D,L-PLA

Particle analysis by SEM: For investigation by SEM particles were first cut using a cryomicrotome and coated with a 400Å layer of gold.

Particle analysis by Scanning Confocal Microscopy: For investigating particles by scanning confocal microscopy, particles were stained with fluorescent dyes which differ by their emission spectrum. Carboxyfluorescein was used to stain the core and Nile Red to stain the coat. Pictures were simultaneously taken at 540nm and 600nm to locate the dyes independently from each other.

Polarized light microscopy: The success of coating for large numbers of individual particles was verified using polarized light microscopy.

Electron spectroscopy (ESCA): The number of non-coated particles was determined using Electron Spectroscopy for Chemical Analysis (ESCA). Integrating the carbon and oxygen signal the ratio was calculated which is specific for each polymer. This allows one to calculate the surface composition of the particles.

Polymer degradation studies: The microspheres were placed in 5ml tubes and incubated in 3ml phosphate-buffered saline, pH 7.4, under agitation in an incubator shaker at 37 °C. Molecular weight distributions of polymers were determined by GPC after 7 and 15 days.

RESULTS AND DISCUSSION

The methods proposed for characterization of coated microspheres can be divided into non-destructive and destructive methods(19). The major advantage of non-destructive methods is not that samples can be recovered, but that in general little sample preparation is needed and investigations can be performed relatively quickly. We used the following methods: Scanning Electron Microscopy (SEM), Polarized Light Microscopy, Electron Spectroscopy (ESCA). Destructive methods, in contrast, have the major disadvantage that "sample preparation" takes substantially more time than with non destructive methods. The preparation techniques include the cutting of microparticles or

their degradation in fluids over days and weeks. We used the following techniques: SEM and light microscopy after cutting the particles and Gel Permeation Chromatography (GPC) during an erosion experiment.

Fluorescence Scanning Confocal Microscopy: In searching for methods that prove the success of coating directly we used scanning confocal microscopy(19) which allows the assessment of pictures from a single x-y plane of a sample. This is often referred to as optical cross sectioning yielding so-called z-sections(21). A major problem of this technique emerges, however, when trying to access deeper layers of the object. Due to the structure of the microspheres and the biodegradable polymers, they are poorly light permeable. This prevents the light from passing into deeper layers hindering the assessment of images. To circumvent these problems, we produced microspheres from EVAC which has the advantage of being perfectly light permeable. The core microspheres were stained with carboxyfluorescein, whereas the coating was stained by adding Nile red to the coating solution. The chemical structure of both dyes is shown in Fig. 1.

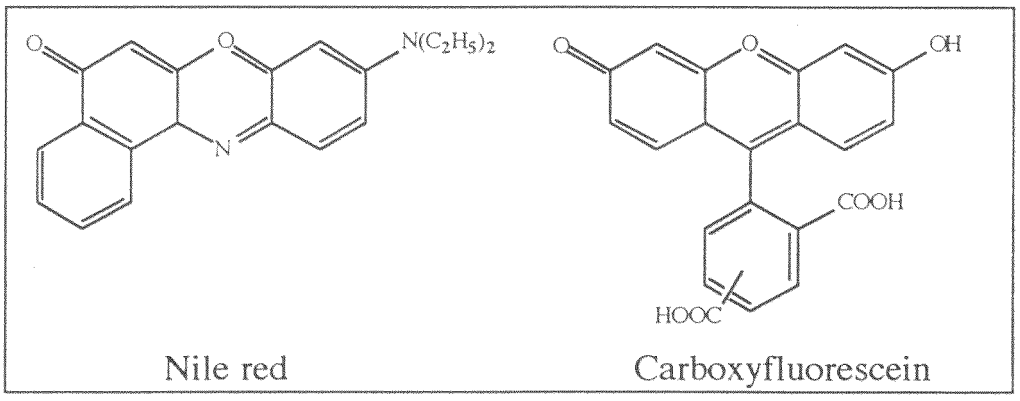

Fig. 1: Chemical Structure of Nile red and Carboxyfluorescein.

The "encapsulated" microspheres were investigated in suspension immediately after the solvent had been extracted. The chromophores were excited at 488 nm and pictures were simultaneously taken at 540nm and 600nm. Figure 2 shows the pictures of a microsphere prior to coating:

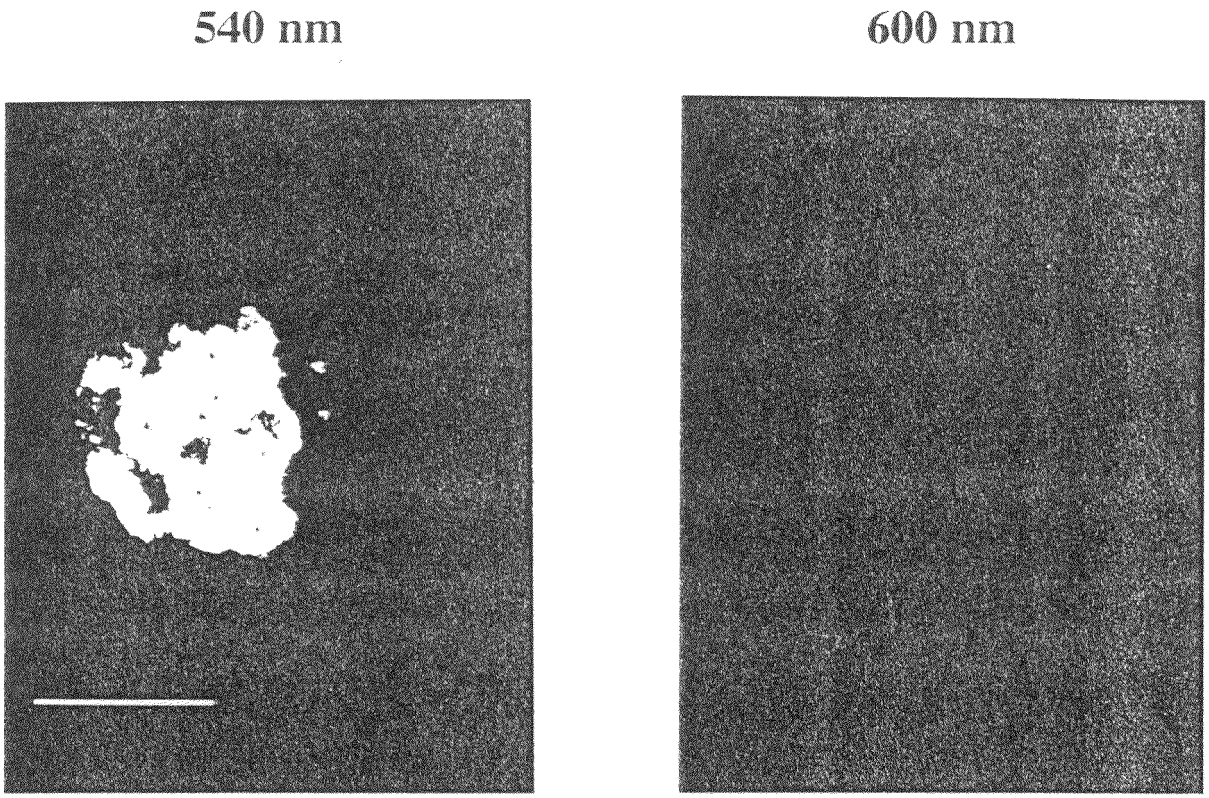

Fig. 2: Picture of a non-coated particle taken by confocal microscopy (scale bar=100μm).

The pictures taken at 600nm from the non-coated EVAC particles are completely dark indicating that Nile red and therefore any coating is absent. Pictures taken from the same particle at 540nm, in contrast show many bright areas, which stem from carboxyfluorescein present in the core.

After coating these particles with EVAC in contrast, fluorescence can be detected in both channels as shown in Figure 3. Nile red appears, thereby, only on the surface and carboxyfluorescein only in the core which proves directly that the particle has successfully been coated

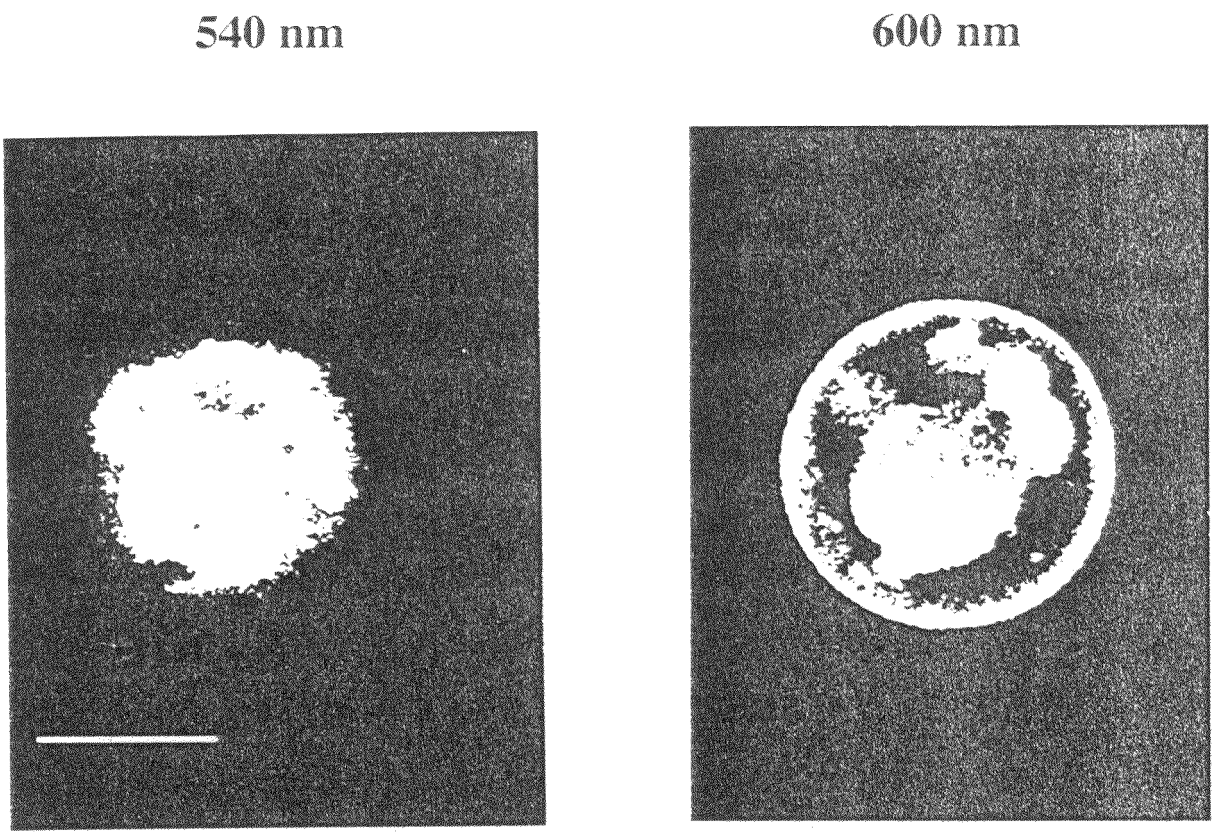

Fig. 3: Picture of coated particle taken by confocal microscopy (scale bar=100μm).

Polarized light microscopy: Scanning confocal microscopy proved to be an excellent method to establish that the particles have been coated. It furthermore allows characterizing such particles in terms of assessing their wall thickness. This method, however, has two disadvantages. One is the need to stain the

particles with fluorescent material, which is not realistic for many pharmaceutical preparations. The other is the need to use transparent polymers in order to avoid excessive scattering and resulting blurred images. In searching for a simple method with less restrictions we used polarized light microscopy. Characteristic of a coated particle is its appearance under crossed polarizers. A coated particle is shown in Fig. 4.

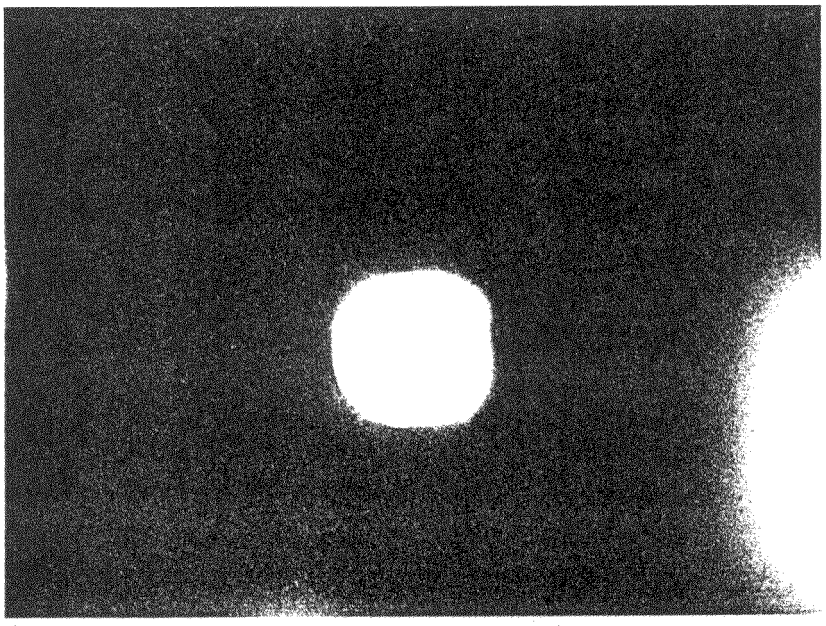

Fig. 4: Picture of a coated particle taken by polarized light microscopy.

The core of these particles consists of p(CPP-SA) 50:50 and was coated with PLA-GA. Characteristic for the coated particle is the Maltese Cross(22) which results from passing polarized light through the spherical polymer/ polymer interface. Polarized light microscopy is, therefore, a useful tool for characterizing coated microspheres. It has the advantage of requiring almost no sample preparation and the necessary equipment is almost standard in any laboratory. Like scanning confocal microscopy, polarized light microscopy

allows again assessing the status of a particle as either coated or non-coated. In contrast to confocal microscopy, however, this method is subject to far less restrictions.

Electron Spectroscopy: Scanning confocal microscopy and polarized light microscopy are useful methods for the investigation of coated microspheres. They allow, however, only individual particles to be investigated. A major problem with coating techniques is posed by particles that fail to be coated. To determine their number, optical techniques can be used, but allow us only to look at individual particles. It would be very cumbersome to investigate large numbers of particles by light microscopy or confocal microscopy. We were, therefore, looking for a method that investigates large numbers of particles all at once and that allows us to determine an "average degree of coating". For assessing such information Electron Spectroscopy for Chemical Analysis (ESCA or XPS) is an excellent method. ESCA allows determining the atomic surface composition of materials. Consisting of carbon, oxygen and hydrogen, the polymers we used as core and coating materials differ in their ratio of carbon to oxygen. If all particles are coated, the carbon to oxygen ratio matches the data of the coating polymer. If there are flaws in the coat, or particles fail to be coated, the carbon to oxygen ratio will have a value between the ratio of the core and coating polymer(19).

To establish the method, the carbon to oxygen ratio of core and coating polymer had to be determined. For that purpose, microspheres were prepared from both materials and their carbon to oxygen ratio determined by ESCA. Fig. 5a shows the spectrum of p(FAD-SA) 50:50, which was used as core material and Fig. 5b shows the spectrum of D,L-PLA.

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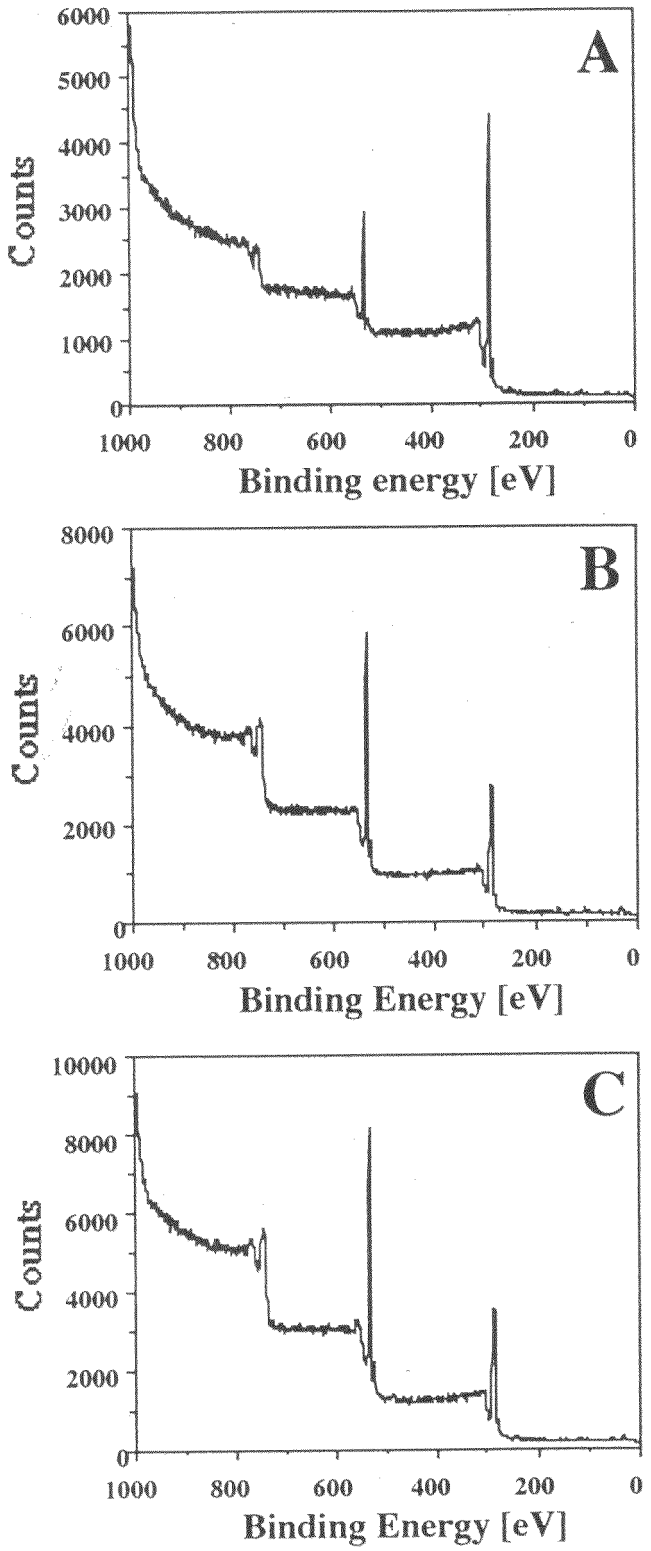


Figure 5: XPS spectra of p(FAD-SA) 50:50 microspheres (**A**), D,L-PLA microspheres (**B**) and D,L-PLA coated p(FAD-SA) 50:50 microspheres (**C**).

The peaks around 535eV are due to electrons that stem from oxygen atoms, whereas the peaks around 288eV are due to electrons that stem from carbon atoms. It is clearly visible that the height and, therefore, the area of these peaks is different for both polymers. The carbon to oxygen ratio of a polymer is calculated by integrating the two peaks and calculating the ratio. The values are shown in Table II.

Table II: Carbon to oxygen ratio of polymers determined by ESCA

Polymer	Carbon to oxygen ratio
p(FAD-SA) 50:50	5.54
p(D,L-LA)	1.61

The surface composition of microspheres made of p(FAD-SA) 50:50 and coated with D,L-PLA was then determined measuring the carbon to oxygen ratio using ESCA. Figure 5c shows the spectrum that we obtained for such particles. We obtained a value of 1.57 which is close to the value obtained for D,L-PLA. This establishes that the spheres have completely been coated. Additional useful information can be extracted from these spectra by having a closer look at the carbon peaks(23). Figure 6a, b and c show the shape of the carbon peak for the p(FAD-SA) 50:50 core, the D,L-PLA coating and the coated microspheres respectively.

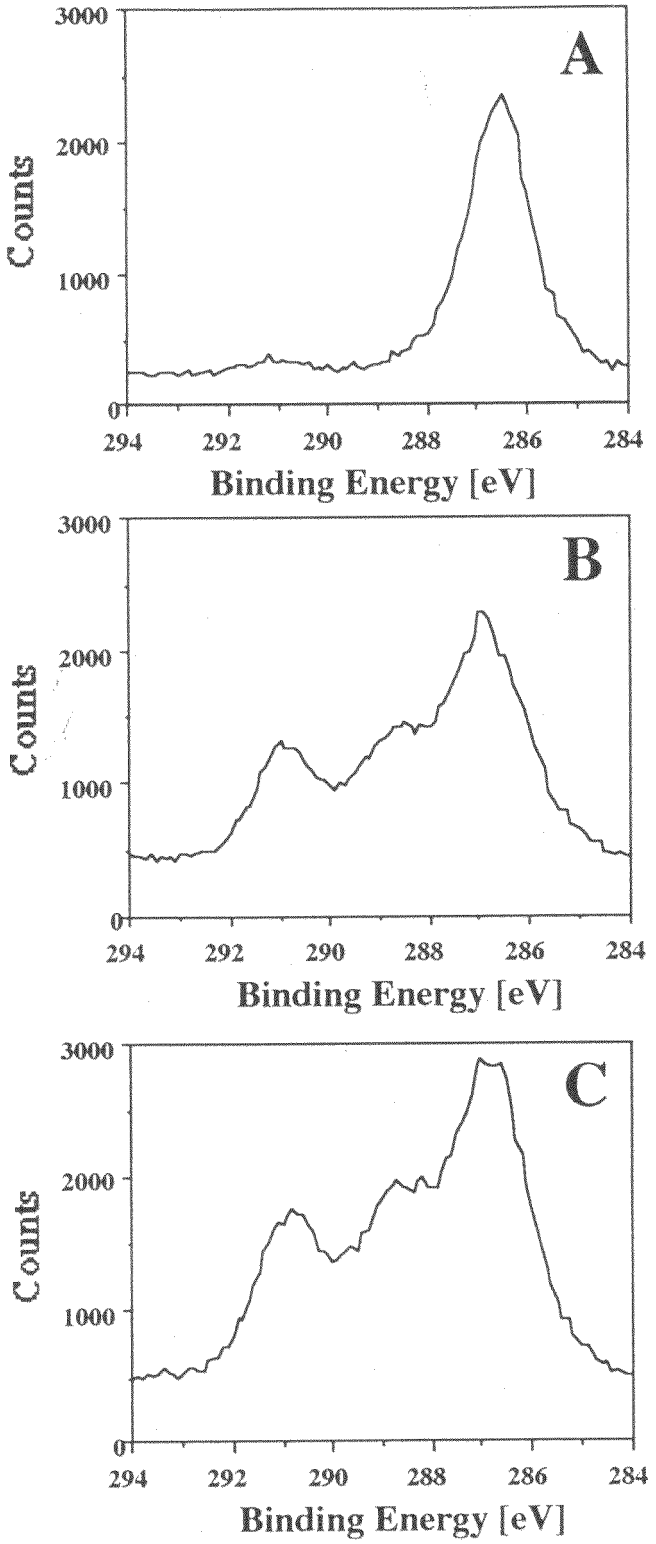


Figure 6: Shape of the carbon peaks taken by XPS: p(FAD-SA) 50:50 microspheres (**A**), D,L-PLA microspheres (**B**), D,L-PLA coated p(FAD-SA) 50:50 microspheres (**C**).

The appearance of the peak group is the result of overlapping individual peaks that stem from the single carbon atoms present in the polymer structure. These peaks differ in their x-axis position according to the type of ligands they have(23). The peak shape of the coated microspheres matches exactly the structure of the coating material which is another indication that the coating consists of the desired polymer only. The peak group is the result of the overlapping individual peaks that stem from the single carbon atoms present in the polymer structure. These peaks differ in their position according to the type of ligands they have. Providing such information, ESCA could be a useful method to investigate microencapsulated microspheres produced by other techniques. With methods that are based on the immiscibility of two polymers and where particles are coated by phase separation during the solvent evaporation(23) it has to be assessed which of the two polymers resides on the microsphere surface and which is in their core. If the polymers are partially miscible, questions concerning the coating composition might arise. ESCA may be a promising method for addressing such problems.

Investigation of cut microparticles by SEM: SEM investigations with cut microspheres yield probably the most detailed information on a system's structure. Cutting microspheres is, however, not easy and can be time consuming. Even though hundreds of particles are cut all at once with the technique that we used, most of them failed to be cut properly. Many of them were crushed or broken irregularly and it became very laborious to find particles that reveal the true structure of the microspheres. An additional problem arose when we tried to use this method to establish that the particles had successfully been coated. Fig. 7a shows schematically a particle which is cut through its core and the coating.

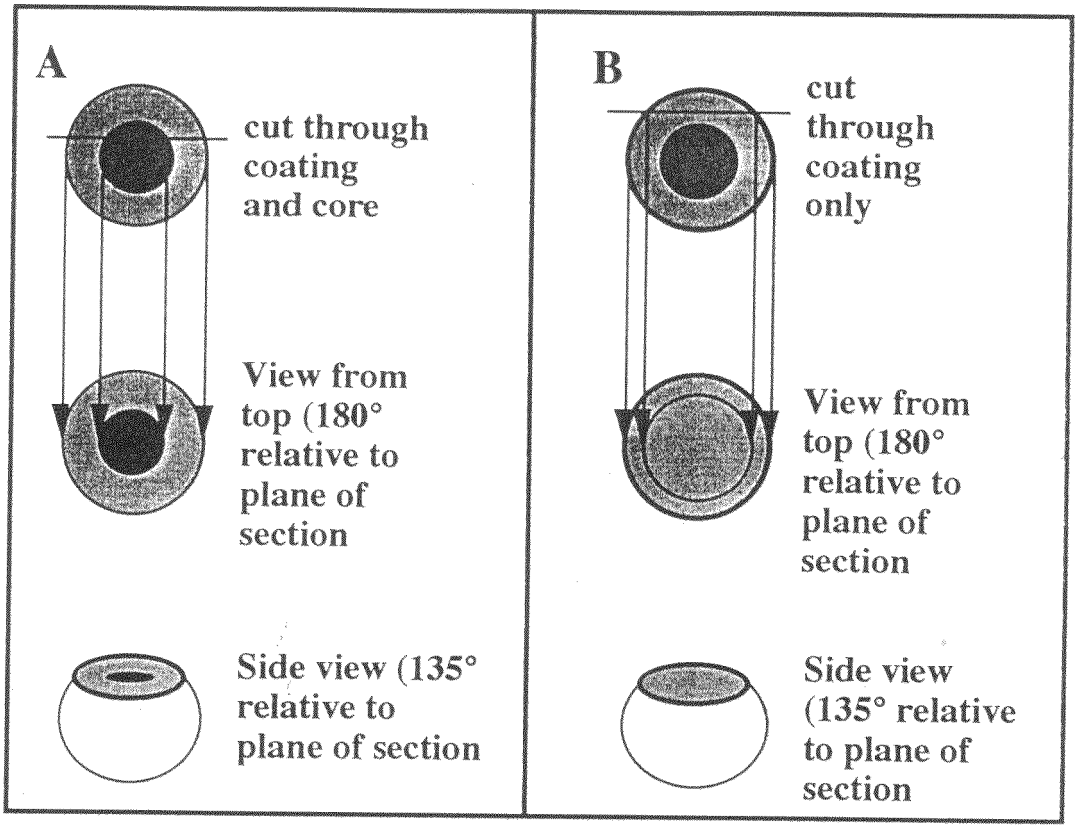
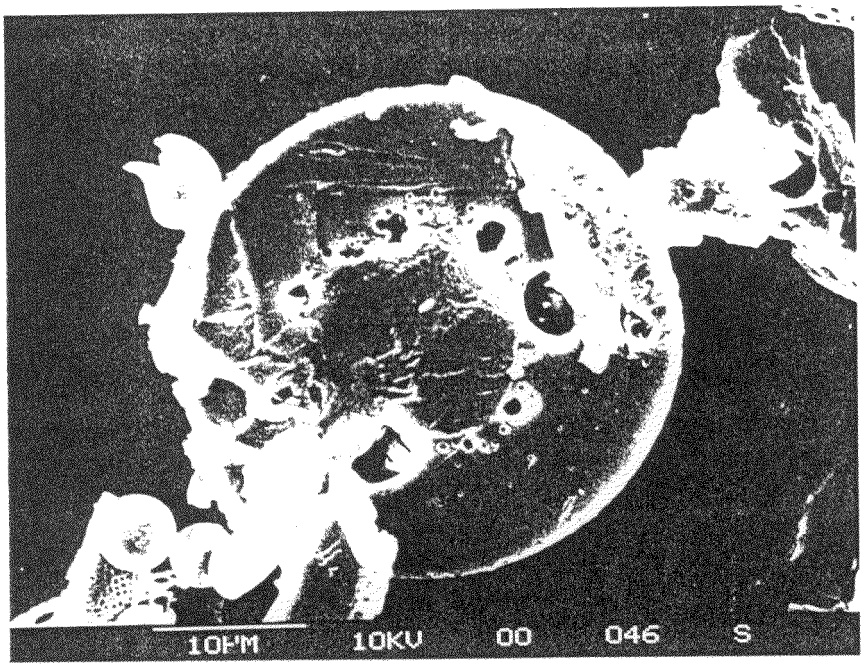


Figure 7: Schematical illustration of microspheres cutting and resulting microscopical appearance of cross sections:
A Particle cut through core and coating,
B Particle cut through coating only.

When viewing the crosssection from above at an angle of 180° relative to the cross section, we expect to find that one circular structure, the core polymer, is enclosed in a larger circular structure, the coating polymer, as shown in Figure 7a. In some instances, however, particles that have been cut at the very top through their coating (Figure 7b), have the same appearance when viewed from the top, owing to the fact that the diameter of the crosssection is smaller than the total diameter of the cut particle. In this case the microsphere wall appears falsely as a "coating". To overcome this problem we always viewed cut

particles not only from the top (180°) but also from the side (135°) by tilting the sample holder by 45°.

180°



135°

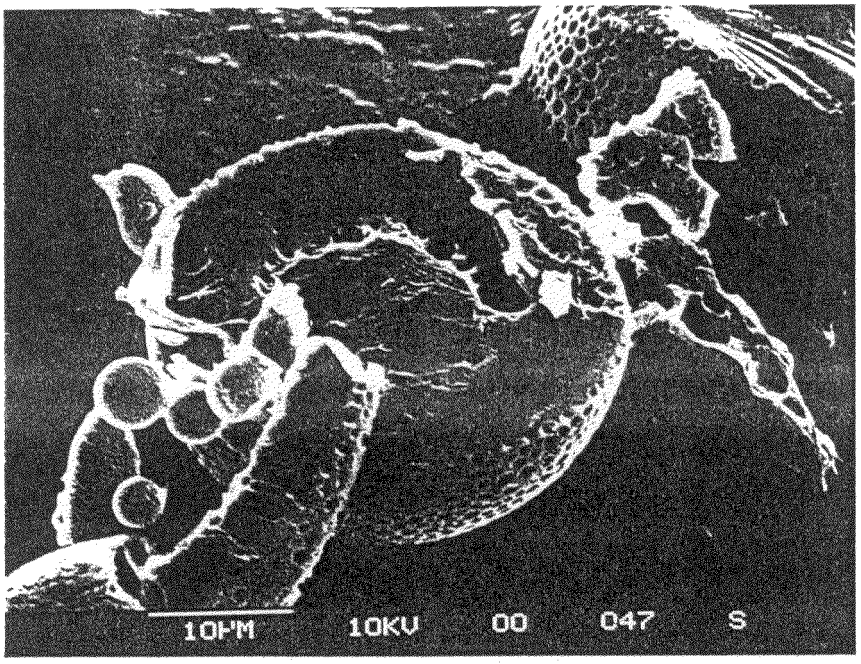


Figure 8: Cross section through a PLA-GA coated p(FAD-SA) 50:50 microsphere with sample holder in 180° and 135° position.

Figure 7a and b illustrate that it is possible from this second angle to decide whether the viewed particle is truly a coated one. Figures 8a and b show a cut particle with a core of p(FAD-SA) 50:50 and a coating of PLA-GA taken at 180 and 135°. Similar pictures were obtained from cut particles made from p(CPP-SA) 50:50 and PLA-GA. The pictures illustrate the benefit of tilting the samples.

Investigation of cut particles by light microscopy

A cross section through a particle consisting of a p(CPP-SA) 50:50 core and a PLA-GA coating can be seen figure 9. The core and coating can clearly be distinguished. This technique might be used to assess the average diameter of the core as well as the coating, but has some limitations due to the low resolution of the light microscope.

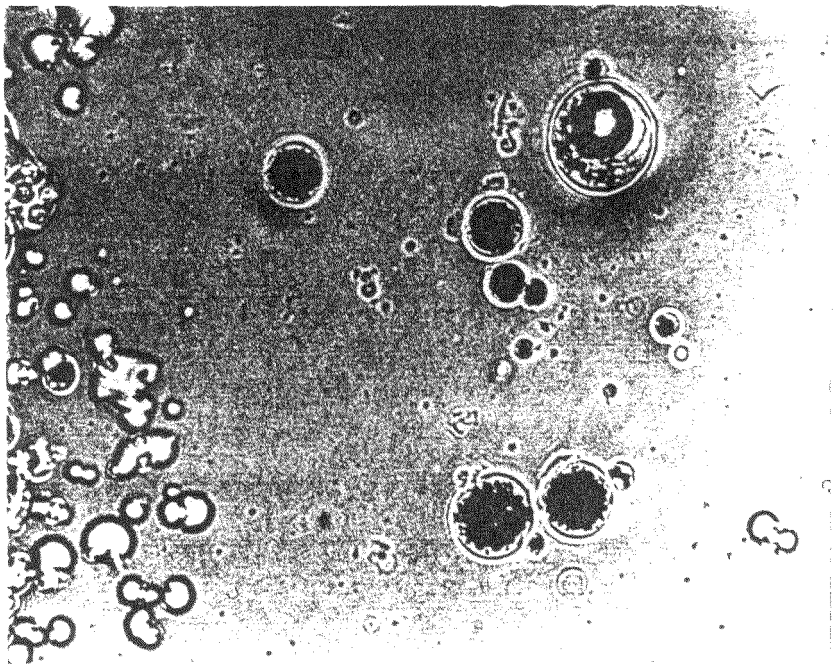


Figure 9: Cross section through a PLA-GA coated p(CPP-SA) 50:50 microsphere seen by light microscopy.

Gel Permeation Chromatography (GPC) during an erosion experiment

First we investigated the molecular weight of the polymers prior to the production of coated microspheres and then immediately after microsphere preparation. The values for L-PLA and D,L-PLA are shown in Table III.

Table III: Number average molecular weight (and polydispersity) of L-PLA and D,L-PLA

Polymer	MW
L-PLA	2,900 (1.40)
D,L-PLA	142,000 (1.08)

The polymers were chosen in such a way, that the resulting differences in retention time would allow distinguishing between both species in the chromatogram. Once the microspheres were eroding we took polymer samples and examined for changes in molecular weight. Table IV shows the result from these investigations.

Table IV: Number average molecular weight and polydispersity of L-PLA microspheres coated with D,L-PLA during erosion

Time [days]	High molecular weight peak	Low molecular weight peak
0	145,000 (1.09)	2,500(1.36)
7	78,800 (1.28)	2,400 (1.12)
15	76,400 (1.25)	2,600 (1.16)

Throughout the experiment we had a high and a low molecular weight peak owing to the molecular weight of the core and the coating polymer. The high molecular weight peak drops from 145,000 to 78,800 within a week, whereas the low molecular weight peak retains almost its initial value. This indicates clearly, that the high molecular coating polymer is degraded substantially faster than the low molecular weight core polymer. This is an indirect proof that the coating polymer protects the core from being degraded.

CONCLUSIONS

We applied a variety of methods to characterize coated microspheres. They allow to prove the coating success and to characterize the spheres. Especially useful were non-destructive methods, as they require little sample preparation. Polarized light microscopy, scanning confocal microscopy and Electron Spectroscopy (ESCA) were found to be easy to apply and yield precious information on the particle structure.

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