Development and Characterization of Microencapsulated Microspheres[5]

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Received February 11, 1994; accepted June 26, 1994

A process for the coating of polymer microspheres with the same or different polymers and the characterization of these particles is described. Coated microspheres were manufactured from degradable and non-degradable polymers. Several physicochemical methods were used to establish that the particles were fully coated. Polarized light microscopy revealed strong birefringence of coated microspheres resulting in the appearance of Maltese Crosses on coated microspheres. After staining the core and the coating of particles using different fluorescent dyes, the uneven distribution of the dyes in the core and on the surface allows one to verify the coating success. After cutting microspheres using a cryomicrotome we were able to assess the microstructure of the coated microspheres. Electron Spectroscopy for Chemical Analysis (ESCA) was used to determine the surface composition of coated microspheres. Determining the carbon and oxygen content of samples we were able to verify the completeness of the coating procedure. To examine the benefit of coating microspheres, the effect of coating on the release of tetanus toxoid from polylactide microspheres was studied as a possible pharmaceutical application.

KEY WORDS: microspheres; coating; microencapsulation; electron spectroscopy; confocal microscopy.

INTRODUCTION

The application of polymers for the controlled release of substances is steadily growing in importance. The use of polymers for drug release in medical applications ranges from birth control(1) to local chemotherapy(2). The use of large implants is undesirable because it requires surgery. To overcome these problems polymer microspheres emerged as an alternative to classical implants(3). With a size of less than 100 μm they allow parenteral administration into tissue via injection through a syringe.

One of the problems involved with the use of microspheres for drug delivery is the fast release at early times, often referred to as the initial burst(4). This fast release of drug depends on the microsphere size and the microencapsulation procedure, and is particularly noticeable for peptides and proteins(5,6). One approach to minimize this effect involves coating microspheres. The coating material acts as an additional diffusion layer, which prevents the drug from being quickly released. There are models that predict the modified release profiles after coating(7). A rare case in which the coating is formed upon erosion can be observed for microspheres which are manufactured from a certain class of polyanhydrides(8). Coatings on microspheres can also serve other purposes. They can be used to suppress the release from microparticles for a period of time or to generate a pulsatile release profile(9) which might be favorable for vaccination or local tumor therapy. Loading the polymers that are used as core and coating material with different drugs can potentially be used for the subsequent release of substances one by one.

There are only a few methods which enable the coating of microspheres (10,11). In initial approaches microspheres were simply dipped into a polymer solution(12). 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 (13,14) 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(15). 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 using the double emulsion technique(16,17). Producing coated microspheres using the phase separation technique would not allow incorporation of the drug via an emulsion, as the drug would not only be introduced into the core of the particles but also into the coating.

To overcome these problems we developed a method that allows the coating of microparticles, without any restriction concerning the polymer type or the polymer solubility. The method allows coating of polymer microparticles with the same as well as different polymer materials and is suited for the incorporation of lipophilic as well as hydrophilic substances. A major problem involved in any kind of coating technique, however, is the characterization of coated particles. Though it might appear trivial it is not easy to verify that particles have been coated completely. Other important questions are whether some particles have failed to be coated or what is the surface composition of the coating. Having developed a new coating technique, we developed several methods to characterize our system. Some of these techniques differ substantially from the standard techniques that have been used for this purpose(18). They allowed us to address some of the problems involved in the development of coated microspheres in much more detail than previously possible. Our main goal in this study was to develop a new encapsulation procedure and to develop methods to characterize coated microspheres. Once such methods are estab-

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0724-8741/94/1100-1568$07.00/0

lished they might become a powerful tool for the characterization of coated microspheres prepared not only by ours but also other methods.

MATERIALS AND METHODS

Materials

The polymers poly(L-lactic acid) (L-PLA), poly(D,L-lactic acid) (D,L-PLA) and the emulsifier poly(vinyl alcohol) (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, Elvax 40W (40% Vinylacetate), was obtained from Dupont, Wilmington, DE and purified as described in (19). The polyanhydride polymers poly(1,3-bis-p-carboxyphenoxypropane-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, purified tetanus toxoid, used as a high molecular weight (150,000) protein model was provided by the Massachusetts Public Health Biologic Laboratories, Boston, MA.

Methods

Microsphere preparation: Coated microspheres were prepared by a two step procedure based on the formation of a core composed of drug or dye (e.g. tetanus toxoid or carboxyfluorescein) dispersed in a spherical polymer matrix (e.g. L-PLA or p(FAD-SA)) and subsequent coating with a second polymer (e.g. D,L-PLA or PLA-GA). The matrix core was prepared according to a modified multiple emulsion technique previously described(20). Briefly, a specific amount of drug or dye was dissolved in 50 μl of water and emulsified in 1 mL of methylene chloride containing 200 mg of polymer (e.g. L-PLA). The emulsification was carried out by sonication at output 4 (50 W) for 10 s (ultrasonic probe, Sonic & Materials Inc.). The resulting emulsion was further emulsified in 2 mL of an aqueous solution of PVA (1%) by vortexing for 10 sec and finally added to 100 mL of an aqueous 0.1% PVA solution. This dispersion was then stirred magnetically for 5 min after which 200 mL of a 2% aqueous isopropanol solution was added to extract the solvent to the external aqueous phase and accelerate microsphere hardening. Microspheres were finally collected by centrifugation, washed several times with double-distilled water, and freeze-dried to obtain a free flowing powder.

The coating process was performed in two different ways: Method 1: 100 mg of dried core microspheres were directly dispersed by vortexing for 5 s into 1 mL of ethyl acetate containing 100 mg of the coating polymer (e.g. D,L-PLA). Method 2: The core microspheres, still in aqueous solution after their production, were concentrated by centrifugation to a dispersion of approximately 100mg microspheres per 100μl of water. This dispersion was then emulsified by vortex mixing or sonication (conditions specified above) for 5 s into 1 mL of ethyl acetate containing 100 mg of the coating polymer (e.g. D,L-PLA).

In both cases the resulting organic dispersion was further emulsified into 3 mL of an aqueous 0.3% of PVA solution. The solvent extraction process was carried out in the final suspension by the procedure described above for the core formation. Coated microspheres were finally collected by centrifugation, washed several times with double-distilled water, and freeze-dried. Table I summarizes the different polymer combinations that were produced using our technique.

SEM investigation of morphology using whole microspheres: The surface appearance of microspheres before and after the coating process was examined by Scanning Electron Microscopy (SEM) (Cambridge Instruments 250 MK or Amray 1000 Å).

Investigation of stained whole microspheres using fluorescence scanning confocal microscopy: Particles were prepared from ethylene-vinyl acetate-copolymer (EVAC) according to the protocol described above. Their core was stained by encapsulating a 1.6mM carboxyfluorescein solution, their coating using a 0.2mM Nile Red/polymer coating solution. Particles investigated by this method were visualized immediately after solvent evaporation by dripping some of the dispersion on a glass slide, and covering it with a cover slip. Pictures were assessed by exciting the chromophores at 488 nm. Taking advantage of the different emission maxima of the dyes we assessed pictures at the emission maximum of carboxyfluorescein at 540nm and pictures at the emission maximum of nile red at 600nm simultaneously(21). Thus, we were able to locate both dyes independently from each other and to distinguish between core and coating. All measurements were performed using a MRC 500 scanning confocal imaging system from Bio Rad, Hercules, CA.

Verification of coating by polarized light microscopy using whole microspheres: The success of coating for large numbers of particles was verified using light microscopy. The dried microparticles were observed between crossed polarizers using a Diaphot-TMP Inverted Microscope from Nikon Inc., Tokyo, Japan.

Determination of the degree of coating using ESCA: The degree of coating was determined by Electron Spectroscopy for Chemical Analysis (ESCA). ESCA allows the investigation of the atomic surface composition of a material. We took advantage of the fact that the investigated polymers have a different carbon and oxygen content. By determining the carbon to oxygen ratio for the core and the coating polymer it is possible to determine the surface composition of a coated particle just from measuring its carbon to oxygen ratio by ESCA. To exclude the effect of surface topology three types of particles were prepared: microspheres made of core polymer, microspheres made of coating polymer and coated microspheres. After coating, the particles were centrifuged at 3000 rpm for 10 minutes and the clear supernatant

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

was decanted. The sediment was redispersed in 1mL of demineralized water and placed into a Pasteur pipette. This concentrated dispersion was dripped onto a 1cm × 1 cm metal strip that served as a sample holder and dried for 24h in a desiccator over phosphorous pentoxide. The samples were investigated using a Model 5100 x-ray photoelectron spectrometer from Perkin Elmer, Newton, MA. The instrument was equipped with magnesium anodes and run at 15kV and 300W.

Cutting of microspheres using a cryomicrotome: For further investigation by SEM and light microscopy, particles were cut using a cryomicrotome, Minot Microtome, Damon/International Equipment Company, Bedfordshire, England. Best results were obtained from samples that were embedded in ice. Therefore, some drops of water were placed on the sample holder pin of the microtome and kept at −18°C. When freezing started, small quantities of dried microspheres were sprinkled over the surface of the freezing water, where they were kept in place due to the surface tension of the water as well as adsorbed air. After the water was completely frozen, the particles were cut into pieces of 8μm thickness. The brittle flakes were placed on a glass slide and dried for 24h in a desiccator over phosphorous pentoxide.

Investigation of cut particles by Scanning Electron Microscopy (SEM): For investigation by SEM, cut particles were transferred from the glass slides to aluminum pins using double sided adhesive tape. Pressing the taped surface of the pin on the glass slide and peeling it off again transfers the cut particles almost quantitatively from the slide to the pin.

Polymer degradation studies: Molecular weight distributions of polymers forming the core and coating of the microspheres were determined on a Perkin-Elmer GPC system using a refractive index detector. Samples of coated L-PLA/D,L-PLA microspheres were analyzed immediately after microsphere production and after different degradation times (7 and 15 days). For degradation studies the microspheres were placed in 5-mL tubes and incubated in 3 mL phosphate-buffered saline, pH 7.4, under agitation in an incubator shaker at 37 °C. Samples were dissolved in chloroform, filtered and then eluted with chloroform through a Phenogel column (linear 0-10,000 K, mixed bed) (Phenomenex) at a flow rate of 1 mL/min. The number average molecular weight and polydispersity of the distribution was determined relative to polystyrene standards with a molecular weight range of 1,250-233,000 from Polysciences, Warrington, PA.

Protein release studies: Protein release experiments were conducted simultaneously to the polymer degradation experiments under the conditions specified above. At desired times, the samples were collected and centrifuged for 20 min at 6000 g using a Sorvall RC-5B centrifuge from Du Pont Instruments. 2 mL of the release medium were assayed for protein release and replaced by 2 mL of the fresh buffer to maintain sink conditions (i.e. the concentration of dissolved protein was not higher than 10% of the protein solubility). The protein concentration in the release medium was determined using a microBCA protein assay (Pierce, Rockford, IL). Release experiments were done independently in triplicate.

RESULTS AND DISCUSSION

The methods that we propose for characterization of coated microspheres can be divided into non-destructive and destructive methods. Non-destructive methods have the advantage that in general little sample preparation is needed and investigations can be performed relatively quickly. Destructive methods, in contrast, have the major disadvantage that "sample preparation" can take substantially more time. The preparation techniques include the cutting of microspheres or their degradation in fluids over days and weeks for experiments concerning molecular weight changes or drug release. In addition to the characterization of particles, we performed drug release studies using tetanus toxoid loaded microspheres.

Non-destructive Methods

SEM: We used SEM to compare the appearance of microspheres before and after coating. Fig. 1A shows L-PLA microspheres loaded with tetanus toxoid before coating. The particles appear spherical and have a smooth surface. Fig. 1B shows that after coating with D,L-PLA the microspheres have lost to some extent their spherical shape and that the surface is substantially rougher than with the non-coated particles. These changes yield some evidence that the coating of the microspheres was successful. SEM investigations with microspheres before and after coating are, however,

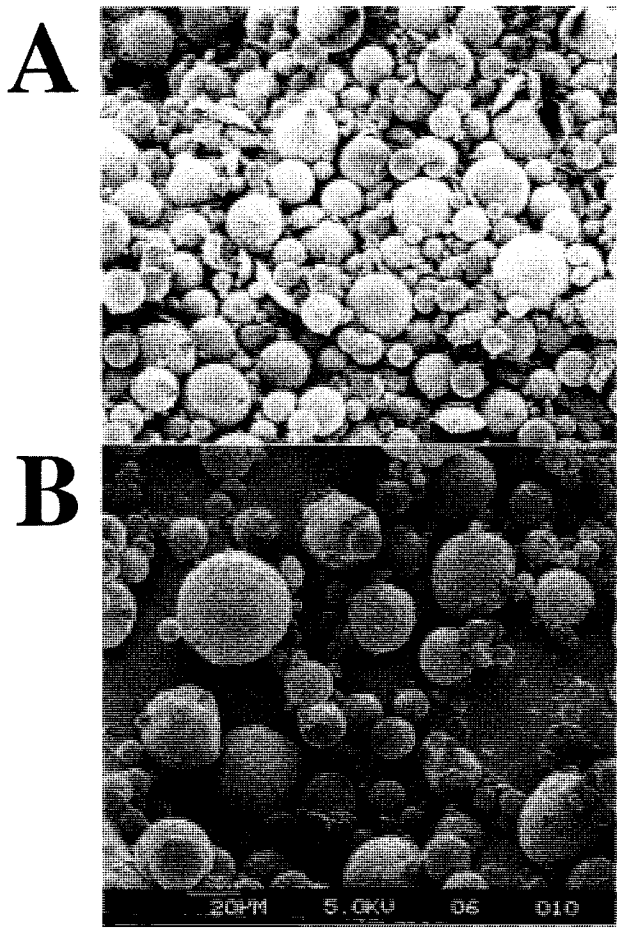

Figure 1: SEM picture of tetanus toxoid loaded microspheres: A Core microspheres made of L-PLA, B After coating with D,L-PLA.

only an indirect method to establish that coating was successful.

Fluorescence scanning confocal microscopy: We used scanning confocal microscopy because it allows assessment of pictures from a single x-y plane of a sample(22). A major problem of this technique emerges, however, when trying to access deeper layers of an object. Because of the structure of the microspheres and the biodegradable polymers, they are poorly light permeable and do not allow assessing confocal images. To circumvent this problem, we produced microspheres from EVAC which is light permeable. The core microspheres were stained with carboxyfluorescein, whereas the coating was stained by adding Nile Red to the coating solution. The "encapsulated" microspheres were investigated in suspension immediately after the solvent had been extracted. The pictures taken at 600 nm are dark, indicating that Nile Red is absent. Pictures taken at 540 nm in contrast show many bright areas, which stem from carboxyfluorescein.

The appearance of a coated microparticle is shown in Fig. 2A and B. The picture taken at 540 nm shows an uneven distribution of the dye in the perfectly round particle due to the double emulsion technique that was used for the production of these spheres. The picture taken at 600nm now indicates the presence of Nile Red, which is located on the outer areas of the cross sectional picture. This proves directly that the particle has successfully been coated.

Polarized light microscopy: Scanning confocal microscopy turned out to be an excellent method to establish that the particles have been coated and allows assessing particle wall thickness. This method, however, has two disadvantages. One is the need to stain the particles with fluorescent material and the other is the need to use more or less transparent polymers. In searching for a simple method with less restrictions we used polarized light microscopy. A non-coated particle is shown in Fig. 3A. Coated particles are shown in Fig. 3B. The core of these particles consists of p(CPP-SA) 50:50 and was coated with PLA-GA. Characteristic for a particle after coating is the Maltese Cross(23) 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 equipment in any laboratory.

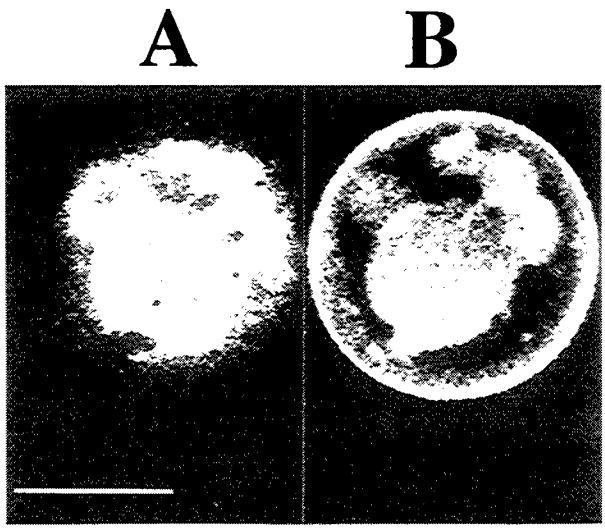

Figure 2: Picture of an EVAC microsphere coated with the same material. Core stained with carboxyfluorescein, coating with Nile Red (scale bar = 100μm); A Picture from the green channel (550nm) B Picture from the red channel (600nm).

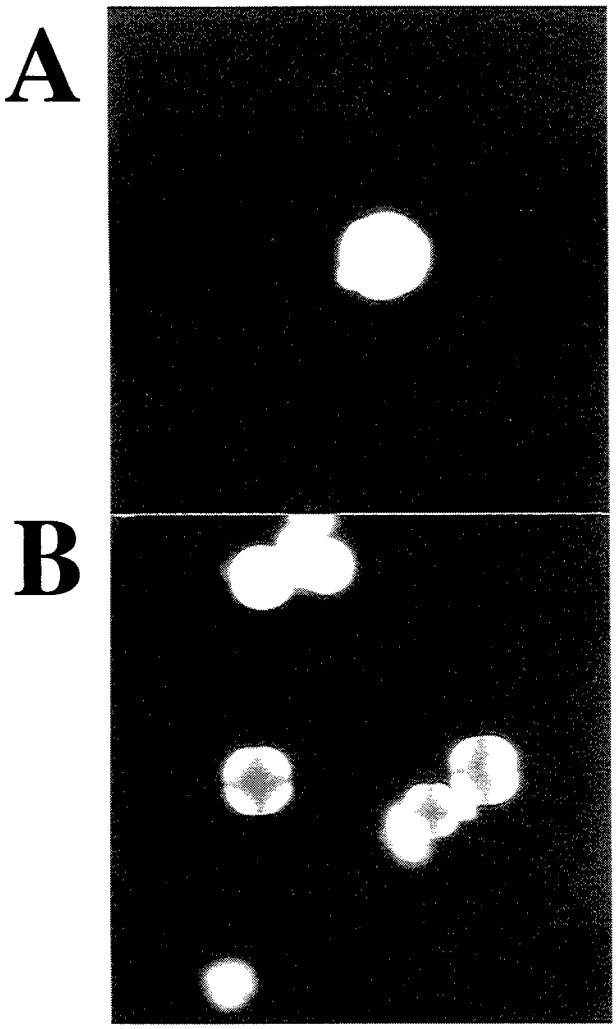

Figure 3: Appearance of microspheres as viewed by polarized light microscopy: A Non-coated particle made of p(CPP-SA) 50:50 B After coating with PLA-GA 50:50.

Electron spectroscopy: Scanning confocal microscopy and polarized light microscopy allow only individual particles to be investigated. One of the major questions arising from any kind of coating technique is whether particles fail to be coated. It would be very cumbersome to investigate large numbers of particles by light microscopy. We were, therefore, looking for a method that investigates large numbers of particles all at once and that determines an "average degree of coating." 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.

To establish the method, microspheres were prepared from both materials and their carbon to oxygen ratio was determined by ESCA. Fig. 4A shows the spectrum of p(FAD-SA) 50:50, which was used as core material and Fig. 4B the spectrum of D,L-PLA. The peaks around 535 eV 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 clear that the height 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. 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. Fig. 4C shows the spectrum that was 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 been coated completely. Additional useful information can be extracted from these spectra by having a closer look at the carbon peaks(24). Fig. 5A shows the shape of the carbon peak for p(FAD-SA) 50:50 core, Fig. 5B for the D,L-PLA coating and Fig. 5C for the coated microspheres. 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.

Table II. Carbon to Oxygen Ratio of Polymers Determined by ESCA

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

Destructive Methods

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. An additional problem arose when we tried to use this method to establish that the particles had successfully been coated. When viewing the cross section 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. In some instances, however, particles that have been cut at the very top through their coating, have the same appearance when viewed from the top, owing to the fact that the diameter of the cross section 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 45°. Fig. 6 shows a cut particle with a core of p(FAD-SA) 50:50 and a coating of PLA-GA 1:1 taken at 180° and 135°. The pictures illustrate the benefit of tilting the samples.

Gel permeation chromatography (GPC) during an erosion experiment: First we investigated the molecular weight

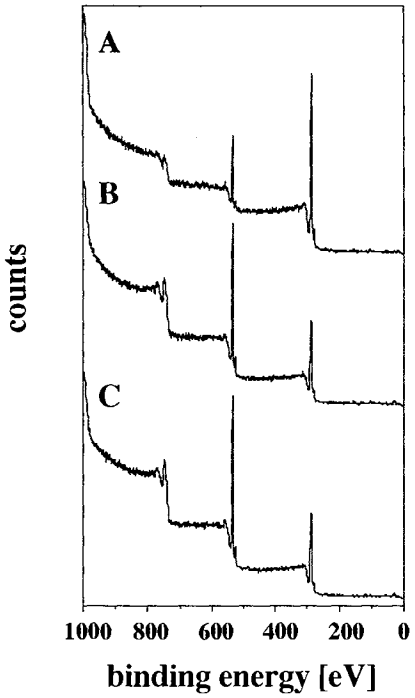

Figure 4: XPS spectra of: A p(FAD-SA) 50:50 microspheres, B D,L-PLA microspheres, C D,L-PLA coated p(FAD-SA) 50:50 microspheres.

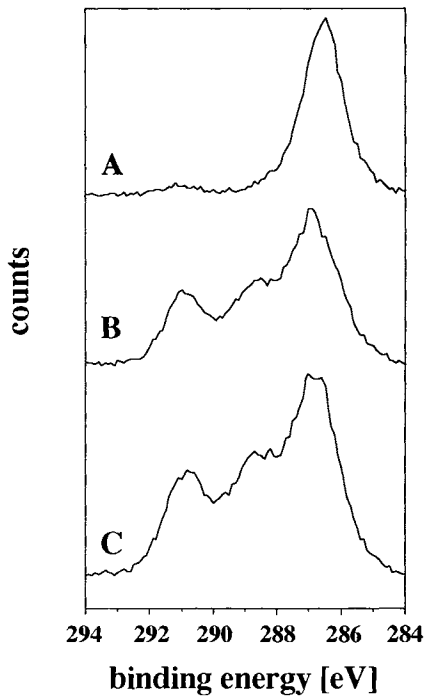

Figure 5: Shape of the carbon peaks taken by XPS: A p(FAD-SA) 50:50 microspheres, B D,L-PLA microspheres, C D,L-PLA coated p(FAD-SA) 50:50 microspheres.

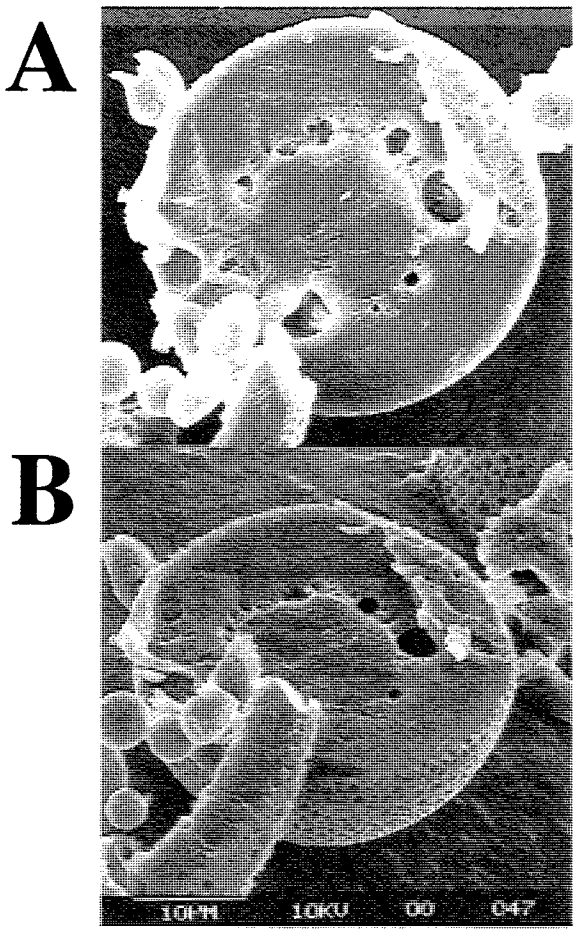

Figure 6: Cross section through a PLA-GA coated p(FAD-SA) 50:50 microsphere: A Sample holder in 180° position, B Sample holder in 135° position.

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. 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. 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.

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)

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)

This is an indirect proof that the coating polymer protects the core from being degraded and that all particles have been coated. It must, however, be mentioned that GPC is only an indirect method which depends on the detectability of polymer degradation and is by far less sensitive than ESCA.

Drug release studies: We studied the effect of coating on drug release using tetanus toxoid loaded microspheres. Fig. 7 shows the drug release from non-coated and coated microspheres prepared by the two different procedures. Under the conditions in this study both coating methods cannot completely prevent an initial burst, but they suppress it substantially which shows the effectiveness of coating. Using dried core microspheres for the encapsulation procedure seems, thereby, less effective than using "freshly prepared" ones. More formulation work needs to be done to evaluate the applicability of microencapsulated microspheres.

SUMMARY AND CONCLUSIONS

We developed a new method for the preparation of coated microspheres and evaluated it using destructive and non-destructive methods. Destructive methods involving cutting microspheres and microscopy are useful but laborious. For non-destructive methods we applied new techniques to characterize such systems, such as fluorescence scanning confocal microscopy and electron spectroscopy. These methods require minimal supply preparation and allow for the success of coating to be checked quickly.

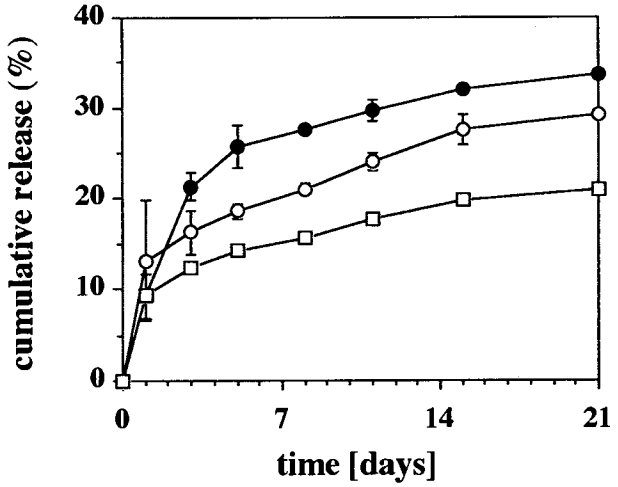

Figure 7: Release of tetanus toxoid from various preparations of microspheres. ● non-coated, ○ coated (method 1), □ coated (method 2).

ACKNOWLEDGMENTS

We thank Scios Nova for providing us with polyanhydrides and Dupont for providing us with EVAC. Thanks are also due to the Massachusetts Public Health Biologic Laboratories for providing the tetanus toxoid. This study was supported by NIH grants GM 26698 and AI 33575.

We also want to thank Joachim Seidel from ETH Zurich and Dr. Steve Schwendeman from MIT, for assistance.

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