pH-Triggered Release of Macromolecules from Spray-Dried Polymethacrylate Microparticles

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Purpose. pH-triggered microparticles release their therapeutic payloads at acidic pH (e.g., in the phagosome), making intracellular drug delivery more efficient. Here we modify lipid-based microparticles that are safe and efficacious in nerve and brain and are potentially inhalable, making them pH-triggerable by incorporating an acid-soluble polymethacrylate, Eudragit E100 (E100).

Methods. Microparticles were produced by spray-drying and characterized by electron microscopy, Coulter counting, density measurement, and release kinetics of fluorescently labeled proteins. In addition, biocompatibility and cellular uptake were observed in rats.

Results. Microparticles were spheroids 3 to 5 μm in diameter with densities of 0.12 to 0.25 g/L. Microparticles with 20% (w/w) or more E100 demonstrated slow release of fluorescently labeled proteins at pH 7.4 but rapid release at pH 5. pH-triggerability was maintained for over 2 weeks in solution. Protein loadings of 0.2–20% (w/w) were pH-triggerable. Histologic examination of particles in rat connective tissue near nerve and muscle demonstrated biocompatibility aside from muscle edema in the cell layers adjacent to the particles and a localized inflammatory reaction with macrophages laden with microparticles.

Conclusions. Microparticles containing E100 were pH-triggerable for many days and were taken up by macrophages, suggesting that they may be useful for intracellular drug delivery.

KEY WORDS: drug delivery; intracellular; biocompatibility; controlled release.

INTRODUCTION

Microparticulate formulations for controlled release of therapeutic agents have been used to achieve both systemic and local drug delivery. However, there are a number of biomedical applications where the desired goal is enhanced delivery into an intracellular compartment. Examples include vaccination, transfection, and the treatment of infections that are located within macrophages (1–3). The encapsulation of drugs in microparticles can facilitate drug delivery via two main mechanisms: (a) the payload is protected from the extracellular environment until the particle is taken up by cells, (b) uptake may be targeted to professional antigen-presenting cells. Macromolecule delivery within cells can be further improved by designing microparticles so that they release their payload instantaneously in response to a low pH so that they would disintegrate following phagocytosis when exposed to the pH (5 to 6.5) in the phagosome, thereby releasing their contents inside the cell (4–7).

We have previously described spray-dried microparticles composed of variable combinations of phospholipids, proteins, simple or complex sugars, and/or drugs with varying physicochemical properties and have demonstrated their safety, biocompatibility, and efficacy for drug delivery to the peripheral and central nervous systems (8–10). Particles of this type can be made to be of a size and density suitable for inhalational drug delivery (8,11). Here, we have developed an approach to rendering these microparticles pH-triggerable by incorporating a polymethacrylate (Eudragit E100, termed E100 here) as a model pH-sensitive material, so that they can be optimized for intracellular drug delivery. E100 is insoluble in aqueous media at physiologic pH but water soluble at acidic pH.

The non–pH-triggered versions of these particles have other properties that may be desirable in this context. They are typically 2 to 5 μm in diameter, thus being of a size that should allow them to be taken up by phagocytosis by immune cells (12) while being too large to be taken up by cells that are not "professionally" phagocytic. Particles of this type produce a transient mild acute inflammatory response, thus potentially attracting the target cell. However, they also have excellent long-term biocompatibility (9,13), partly as a result of the fact that they can be made of excipients that occur naturally in the target milieu. The method of manufacture allows very high maximum loading of the particles with the macromolecule of interest, thus reducing the particulate mass to be injected and hence the associated tissue reaction. The fact that these particles can be easily modified to allow delivery via inhalation is also appealing in the context of the development of methods of providing mucosal immunity (14).

The spray-dried formulations described here may also be desirable when other common particle production methods are not optimal, such as when coencapsulation of certain combinations of excipients (or drugs) with differing solubilities is desired (8,9), or for the production of relatively porous particles (e.g., for inhalational use). In such situations, spray-drying is a useful alternative; its advantages have been reviewed (15).

In addition, we describe the particles' release of fluorescein-labeled albumin (68 kd) and rhodamine-labeled lactalbumin (15 kd) *in vitro*. We also verify the ability of these modified particles to attract immune cells and study their biocompatibility by injecting them at a location where there are many tissue types (muscle, nerve, connective tissue), the sciatic nerve at the hip.

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate-conjugated albumin (FITC-albumin) and rhodamine-labeled lactalbumin (Rho-lactalbumin) were purchased from Sigma Chemical Co. (St. Louis, MO), L-α-dipalmitoylphosphatidylcholine (DPPC) from Avanti Polar Lipids (Alabaster, AL), and USP grade ethanol from Pharmco Products (Brookfield, CT). Eudragit E100 [poly(butyl methacrylate)-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate] = 1:2:1 (termed E100 below) was a gift from Röhm GmbH (Darmstadt, Germany)

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Production of Microparticles

Varying proportions of DPPC and E100, totaling 500 mg of solute, were dissolved in 87.5 ml of ethanol. One milligram of FITC-albumin or Rho-lactalbumin in 37.5 ml of water was added dropwise to this solution. In some experiments 5 to 100 mg of FITC-albumin were used, with a corresponding decrease in the amount of DPPC, while the amount of E100 was kept constant. For example, particles that were 20% (w/w) FITC-albumin, 20% (w/w) E100 were made by incorporating 100 mg FITC-albumin, 100 mg E100, and 300 mg DPPC. The resulting mixture was spray-dried using a Model 190 bench top spray drier (Büchi Co., Switzerland) using the following settings: air flow rate 600 NL/h, aspiration −20 mbar, solvent flow 12 ml/min, inlet temperature 110–120°C, outlet temperature 39–48°C. Particles without E100 were made with the composition 60% (w/w) DPPC, 19.8% (w/w) albumin, 20% (w/w) lactose, as previously described (8,10), with 0.2% (w/w) FITC-albumin added.

Particle Size, Shape, and Density Determination

Particle size was determined with a Coulter Multisizer (Coulter Electronics Ltd., Luton, U.K.), using a 30-μm orifice. Surface characteristics of particles were determined by scanning electron microscopy on an AMR-1000 (Amray Inc., Bedford, MA). Samples were mounted on stubs and given a gold-palladium conductive coating and scanned at 10 kV. Particle density was determined by placing a known weight of particles into a graduated tube and tapping the tube against a benchtop 50 times, after which the density was calculated as the weight divided by the volume.

Release of FITC-Albumin from Microparticles

Five milligrams of each particle type was suspended in 1 ml of 100 mM phosphate-buffered saline pH 7.4 (PBS) and incubated at 37°C. At predetermined time points, the samples were centrifuged, and the supernatants removed for fluorimetry. The pellets were resuspended in PBS. After each time point, the phosphate-buffered saline was replaced with 100 mM sodium acetate pH 5; sample treatment was otherwise unchanged.

Fluorimetry was performed on a PTI system (Photon Technology International, Lawrenceville, NJ) at the following wavelengths (nm): FITC-albumin excitation 485, emission 515; Rho-lactalbumin excitation 560, emission 584.

In Vivo Experiments

Animals were cared for in compliance with protocols approved by the Massachusetts Institute of Technology Committee on Animal Care, in conformity with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). They were housed in groups and kept in a 6 a.m.–6 p.m. light–dark cycle. Young adult male Sprague-Dawley rats weighing 310–420 g were used. Twenty-five milligrams of microparticles suspended in 0.6 ml of carrier fluid [1% (w/v) sodium carboxymethyl cellulose, 0.1% (v/v) Tween 80] was injected at the sciatic nerve under general anesthesia as described (8). Every day after injection, animals were examined for self-mutilation (13,16), a behavior believed to be pain-related, and received a neurobehavioral assessment as described (17,18). In brief, thermal nociception was assessed by a modified hotplate test at 56°C (Model 39D Hot Plate Analgesia Meter, IITC Inc., Woodland Hills, CA). Motor strength was assessed by holding the rat with its posterior above a digital balance and measuring the maximum weight that the rat could bear without its ankle touching the balance. One or 4 days after injection, the sciatic nerves and adjacent tissues were harvested (13) under deep isoflurane anesthesia followed by pentobarbital euthanasia, embedded in paraffin, and stained with hematoxylin and eosin using standard techniques. For subcutaneous injections, the same dose and volume of injectate and animal protocol were used with the exception that the needle was inserted into the loose skin between the shoulder blades, advanced 1 cm parallel to the axis of the animal, and the particle suspension was injected.

RESULTS

Protein-Containing Particles

Particles were made as described above, containing 0%, 1%, 5%, 20%, 40%, and 80% E100 (w/w), with corresponding proportions of DPPC and an amount of FITC-albumin or Rho-lactalbumin, 0.2% (w/w) unless noted otherwise. Particle yields by weight were generally in the range of 20–40% of the total mass of solute, except for the 1% (w/w) Eudragit particles, where the yield was 10–20%. Particle density varied in inverse proportion to the proportion of Eudragit and protein in the formulation. Particles with 20% (w/w) or less of Eudragit were relatively dense (approximately 0.25 g/ml), whereas particles with 40% (w/w) Eudragit were roughly half as dense (approximately 0.13 mg/ml). Twenty percent (w/w) particles containing 20% (w/w) protein loading had densities roughly one-half those of the corresponding particles with 0.2% (w/w) protein (0.13 and 0.12 mg/ml for FITC-albumin and Rho-lactalbumin, respectively).

A representative scanning electron micrograph of 20% (w/w) E100 microparticles is shown in Fig. 1 In general, particles were spherical or roughly spheroidal, although some

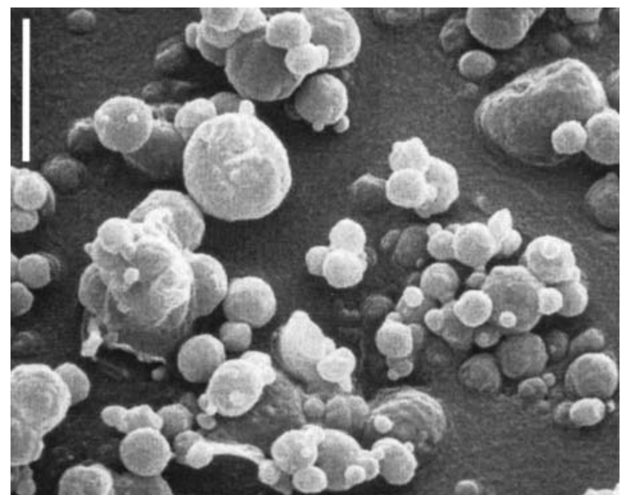

Fig. 1. Scanning electron micrograph of a 20% (w/w) Eudragit E100 particle containing 0.2% (w/w) FITC-albumin. The bar represents 5 μm.

were irregular or concave. The median volume-weighted particle diameters were in the range of 3 to 5 μm by Coulter counting.

We assessed the release of FITC-albumin from the various particle types in 100 mM phosphate-buffered saline, pH 7.4, at 37°C, in which particles suspended readily. Release from these particles was slow (Fig. 2 and 3), particularly compared to particles where the E100 was replaced by other excipients such as albumin and lactose (Fig. 2, 0% Eudragit). In the absence of a triggering stimulus, release proceeded for at least 2 weeks (341 h; Fig. 3).

The effect of resuspending the particle pellet in 100 mM sodium acetate, pH 5, depended on the proportion of E100 in the particles (Fig. 2). The suspension of particles with high proportions of E100, which was cloudy at pH 7.4, became clear at pH 5. In the case of 80% (w/w) E100 particles, there was no solid material left in the test tube after exposure to pH 5. For the other formulations, subsequent centrifugation yielded a pellet of fine white powder, whose size was in inverse proportion to the amount of E100. Particles composed of more than 20% (w/w) E100 showed a large increase in the release rate of fluorescent-labeled proteins on immersion in an acidic environment and showed negligible release thereafter. The release of FITC-albumin from particles containing 5% (w/w) or less E100 did not appear to be affected by pH. The suspension of particles did not become clear in pH 5, and centrifugation yielded a dense pellet with a color reflecting the fluorescent label that was encapsulated.

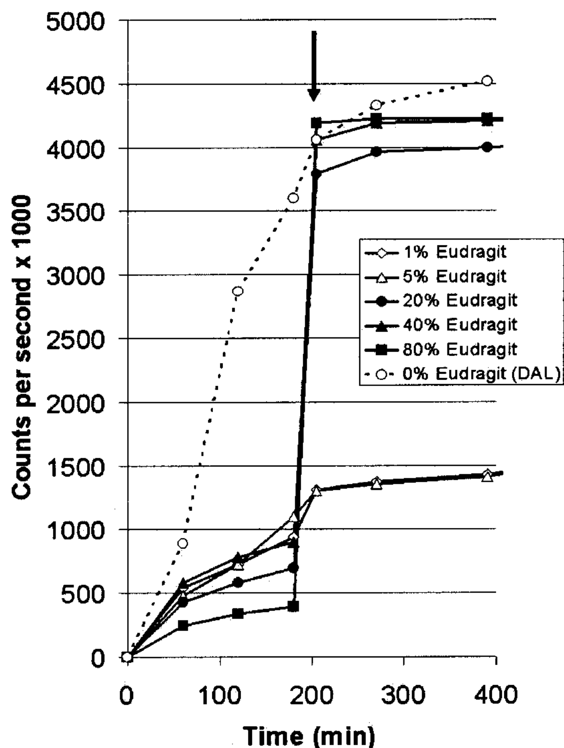

Fig. 2. Representative time courses of pH-triggered release of FITC-albumin from particles containing various percentages (w/w) of Eudragit E100 in phosphate-buffered saline. Arrow indicates change from pH 7.4 to pH 5. The 0% E100 particles are composed of DPPC, albumin, and lactose (DAL), as described in the text.

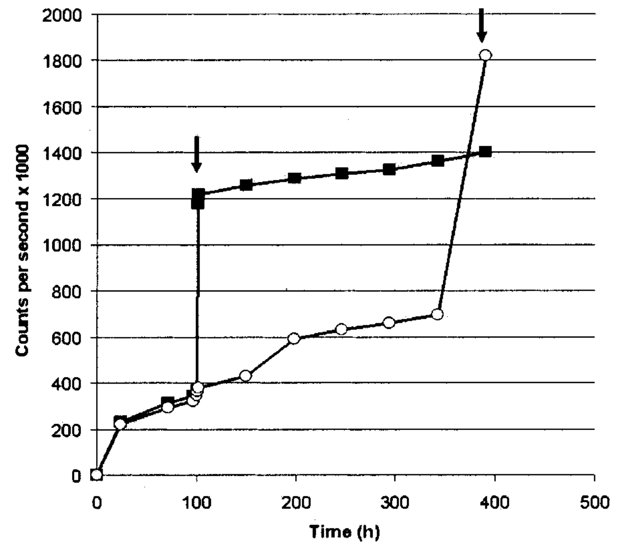

Fig. 3. Representative time courses showing prolonged release and triggerability of FITC-albumin from 20% (w/w) Eudragit E100 particles. Arrows indicate change from pH 7.4 to pH 5. Particles were exposed to pH 5 either 100 h (■) or 390 h (○) after initial placement in suspension.

The following controls were performed to verify that the increased fluorescent counts seen with acidification resulted from the release of the proteins of interest and not of E100 from the particles at pH 5: (a) aqueous solutions of E100 in 100 mM sodium acetate, pH 5, at concentrations as high as 10 mg/ml, did not cause fluorescence above baseline; (b) when blank (no labeled proteins) 80% (w/w) E100 particles were placed in an acidic environment and then centrifuged, the supernatants did not contain increased fluorescence over baseline.

The capacity to release FITC-albumin in response to pH changes was retained for at least 390 h (16.25 days) after immersion in phosphate-buffered saline (Fig. 3). The capacity for prolonged release and pH triggering was also seen in particles loaded with Rho-lactalbumin (Fig. 4). A larger burst release was noted with Rho-lactalbumin than with FITC-albumin.

The protein loading in the particles could be increased greatly. We produced particles that contained 1%, 10%, or 20% (w/w) FITC-albumin or Rho-lactalbumin and 20% (w/w) E100. These particles had release characteristics similar to those with 0.2% (w/w) protein content, except that they had a large initial burst release (Fig. 5). They displayed a marked release of FITC-albumin on exposure to pH 5 but retained the coloration of their fluorescent label after pH triggering, albeit to a much diminished degree.

In Vivo Studies

To verify the potential of these particles to attract phagocytic (immune) cells and to assess their biocompatibility, six animals were injected at the sciatic nerve with 20% (w/w) E100 particles containing 0.2% (w/w) albumin. There was no evidence of self-mutilation at any time after injection in any animal, and the neurobehavioral exam of all animals was nor-

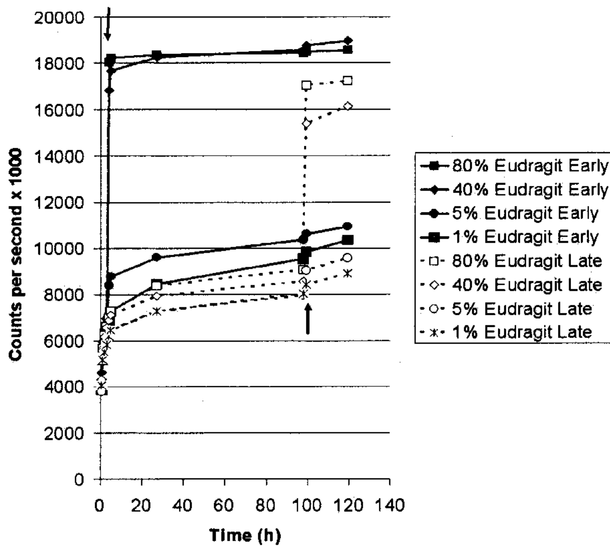

Fig. 4. Representative time courses showing release of Rho-lactalbumin (Rh) from particles containing various percentages (w/w) of Eudragit E100. Arrows indicate change from pH 7.4 to pH 5. Particles were exposed to pH 5 either 4 h (solid symbols) or 99 h (open symbols) after initial placement in suspension.

mal, with no difference between the injected and contralateral extremities. On dissection 1 (n = 2) and 4 (n = 4) days after injection, well-demarcated pockets of particles were noted at the site of injection. The tissues appeared slightly edematous in the immediate vicinity of those pockets. On hematoxylin-eosin–stained sections of tissues harvested from those animals, there was evidence of acute inflammation with neutrophils and macrophages (Fig. 6A), many of which appeared to be laden with particles (Fig. 6B). Inflammation was restricted to the immediate vicinity of the particles, with some infiltration of the adjoining muscle tissue. There was some interstitial edema in the muscle cell layers that were directly adjacent to the area of inflammation, but the myocytes themselves appeared intact (Fig. 6C). Similarly, histologic examination of the sites of subcutaneous injections revealed acute

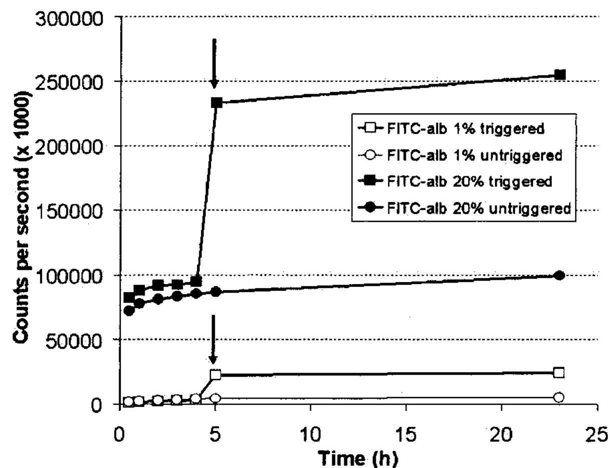

Fig. 5. Representative time courses showing prolonged release and triggerability of 20% (w/w) Eudragit E100 particles containing increased loading (w/w) with FITC-albumin. Arrows indicate change from pH 7.4 to pH 5.

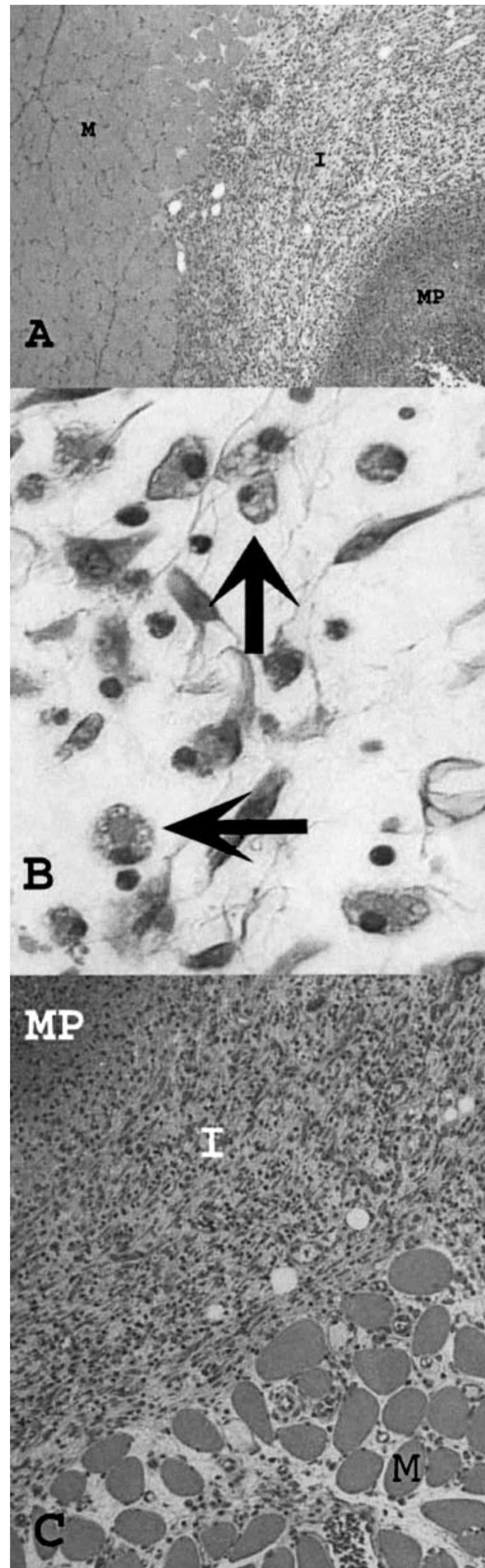

Fig. 6. Tissue reaction to 20% (w/w) Eudragit E100 particles containing 0.2% (w/w) albumin 4 days after injection. MP, microparticles; M, muscle; I, inflammation. (A) Acute inflammatory response surrounding a pocket of microparticles. ×100. (B) Macrophages laden with particles (arrows). (C) Edematous muscle with separated fibers adjacent to a pocket of microparticles.

inflammation with neutrophils and macrophages. The inflammatory reaction was restricted to the loose connective tissue at the site of injection.

DISCUSSION

The formulations described above provided pH-triggered release of macromolecules at pH 5 across a range of loadings of E100 greater than 20% (w/w). The ability to trigger was not impaired by high protein loadings.

Another benefit of the E100 was that it extended the duration of release of the proteins examined from less than 2 h (in particles that did not contain E100) to more than 16 days (the last time point examined). The capacity to trigger was also maintained during that period. These features may be useful *in vivo* because the arrival of phagocytic cells to a given site (e.g., subcutaneous depot) often accrues over many days, and we observed that particles were still present in the tissue 4 days after injection.

In selecting this particle type as a candidate delivery system for intracellular drug delivery, we considered that inducing inflammation to attract immune cells to the site of injection would be a crucial element in determining their effectiveness, as that would lead to the particles' uptake. Our results supported that assumption. The acute inflammatory reaction to these particles is consistent with the pattern that is seen at this time in reaction to foreign material and is similar to what has been described with injected microparticles, including biocompatible microspheres composed of poly(lactic-co-glycolic) acid and lipid-protein-sugar particles similar to the particles described in this report (13,19,20). The presence of macrophages that appeared to be laden with particles suggests that these particles can be taken up by phagocytosis.

E100 is commonly used for enteric coating or flavor-masking of pharmaceutical preparations, but it is not biodegradable, and its fate when delivered parenterally is not known (Rohm USA, personal communication). For this reason, we chose to perform injections into a location that included many tissue types, so as to be able to better assess biocompatibility. The tissue injury was mild and did not extend far outside the pockets of particles. The fact that there was no evidence of animal distress, self-mutilation, or neurologic deficit when the particles were injected at the epineurium (immediately outside the nerve sheath) is also reassuring.

These particles were produced by spray-drying. One advantageous property of that process is that it allows potentially high loadings of the excipients or active molecules of choice. As seen here, particles could be made of 1–80% (w/w) E100. Similarly, we achieved 20% (w/w) loading of albumin, and loadings in excess of 60% are easily feasible (data not shown); we have previously described particles that were 36% (w/w) albumin (8). In principle, such high loadings of DNA could also be possible; we have produced particles that are 4% (w/w) DNA (unpublished observation) but did not attempt higher loadings because of the prohibitive cost. The ability to produce particles with very high loadings of macromolecules is not shared by some more conventional methods of encapsulation into polymeric microspheres (21). Another appealing aspect of this production method is the flexibility it affords in terms of potential excipients, active agents (drugs), and adjuvants. Although this report focused on E100 as a model pH-sensitive material, the technique presented here could in principle be applied to any material with similar properties, such as recently described biopolymers that are both pH-sensitive and biodegradable (22). Another appeal of the spray-drying process is that it is easy to scale up.

Particles of this type may be useful for stimulating mucosal immunity, particularly in the airway. The lack of effective mucosal antigen delivery is believed to be a major obstacle in the targeting of vaccines to such sites (23,24). Because the immune response is generally strongest at the site of vaccine delivery (14), it may be advantageous for induction of mucosal immunity in the tracheobronchial tree to be able to deposit the antigen or DNA of interest in the airway. The particles described here could be modified so that their aerodynamic properties are suitable for inhalational delivery (8,11); they are already of an appropriate size for that purpose, and as we have seen their density is readily lowered by changing the excipients.

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