pH-Responsive Polymer Microspheres: Rapid Release of Encapsulated Material within the Range of Intracellular pH**

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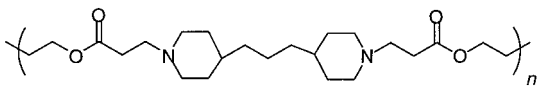
Microparticles formed from biodegradable polymers are attractive for use as delivery devices, and a variety of polymer-based microspheres have been employed for the sustained release of therapeutic compounds.[1, 2] However, for small-molecule, protein, and DNA-based therapeutics that require intracellular administration and trafficking to the cytoplasm, there is an increasing demand for new materials that facilitate triggered release in response to environmental stimuli such as pH.[3] Following endocytosis or phagocytosis, for example, the pH within endosomal compartments is reduced and foreign material is degraded upon fusion with lysosomal vesicles.[4] New materials that release molecular payloads rapidly upon changes in pH within the intracellular range and facilitate escape from hostile environments could have a broad-reaching impact on the administration of therapeutic compounds for intracellular targets.[3, 4]

We recently reported the synthesis of poly(β-amino ester) **1**.[5] The solubility profile of this hydrolytically degradable polymer is directly influenced by solution pH. Specifically, the



poly-**1**

solid, unprotonated polymer is insoluble in aqueous media in the pH range 7.0 to 7.4, and the transition between solubility and insolubility occurs at a pH near 6.5. Based on the differences between extracellular and endosomal/lysosomal pH (7.4 and 5.0–6.5, respectively), we hypothesized that microspheres formed from poly-**1** might be useful for the encapsulation and release of compounds in the intracellular pH range. Choy et al. recently reported that inorganic clays can enhance the trafficking of oligonucleotides to the cytoplasm by dissolution of the inorganic matrix within acidic intracellular environments.[6] This work demonstrates that new materials incorporating such pH/solubility profiles could have an impact on localized intracellular delivery. Herein, we report that microspheres formed from poly-**1** release their encapsulated contents rapidly and quantitatively in the range of intracellular pH values. The size distribution of the spheres considered here (4–6 μm) renders them suitable for delivery to phagocytotic cells such as macrophages, and preliminary evidence suggesting the internalization and processing of poly-**1** microspheres by macrophages is presented.

The encapsulation of water-soluble therapeutic compounds within polymer microspheres is often achieved by employing a double emulsion process.[7] The double emulsion process is well established for the fabrication of microspheres from hydrophobic polymers such as poly(lactic-*co*-glycolic acid) (PLGA), a biodegradable polymer conventionally employed in the development of drug delivery devices.[2] Preliminary experiments demonstrated the feasibility of the double emulsion process for the encapsulation of water-soluble compounds using poly-**1**. Rhodamine-conjugated dextran was chosen as a model for subsequent encapsulation and release studies for several reasons: 1) rhodamine is fluorescent, thus allowing loading and release profiles to be determined by fluorescence spectroscopy, 2) loaded microspheres could be used to monitor cell uptake directly by confocal microscopy, and 3) the fluorescence intensity of rhodamine is relatively unaffected by pH values within the physiological range.[8]

Microspheres encapsulating labeled dextran were fabricated from poly-**1** and compared to controls formed from PLGA. The size distributions of microspheres formed from poly-**1** correlated well with the distributions of PLGA microspheres within the range of 5–30 μm, and average particle sizes could be controlled by variations in the experimental parameters, such as homogenization rates and aqueous/organic solvent ratios.[7] In early experiments, microspheres formed from poly-**1** aggregated extensively during centrifugation and washing steps, and scanning electron microscopy (SEM) images revealed clusters of spheres that appeared to be physically joined or "welded" (data not shown). The observed aggregation was related to the removal of the stabilizing poly(vinyl alcohol) (PVA) employed in the double emulsion procedure (see Experimental Section), as suspensions isolated using 0.5% PVA solutions could be centrifuged/washed as many as four times with no observed aggregation.

We considered the possibility that residual solvent contained within poly-**1** microspheres could contribute to the observed aggregation. Although isolation was carried out below the glass transition temperature (T_g) of poly-**1** (T_g = 49.8 °C),[9] this parameter does not apply directly under the conditions to which the microspheres are exposed during fabrication and isolation. In the later stages of the "hardening" process, for example, the microspheres may be assumed to contain residual CH_2Cl_2 or small amounts of water that could act to increase the tackiness of the polymer matrix. Extended drying times and the application of a dynamic vacuum to ensure complete removal of the organic solvent

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prior to centrifugation did not eliminate particle aggregation. However, aggregation could be essentially eliminated if centrifugation and washing were conducted at reduced temperatures (4 °C). Aqueous suspensions of poly-**1** microspheres isolated under these conditions consisted primarily of single, unaggregated microspheres, as determined by optical and fluorescence microscopy. Microspheres in the range of 4–6 μm were essentially free of cracks, holes, and other defects, as determined by SEM (Figure 1).

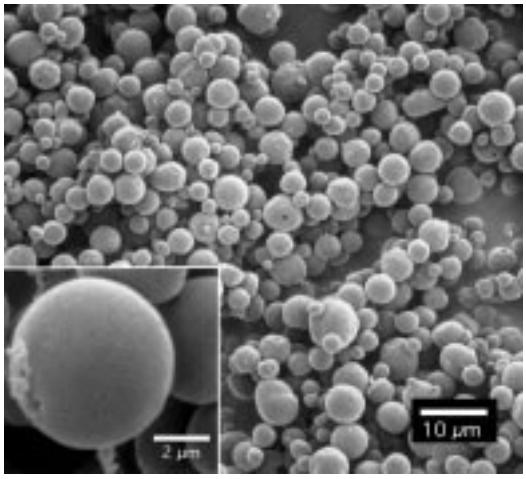
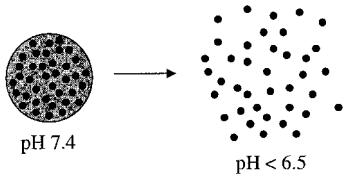


Figure 1. SEM image of rhodamine/dextran-loaded microspheres fabricated from poly-**1**.

Poly-**1** microspheres suspended at pH 7.4 remained intact and stable toward aggregation (by visual inspection), but the spheres dissolved instantly when the pH of the suspending medium was lowered to between 5.1 and 6.5 (Scheme 1). The



Scheme 1.

release of labeled dextran from poly-**1** microspheres was determined quantitatively by fluorescence microscopy (Figure 2). The release profile at pH 7.4 was characterized by a small initial burst in fluorescence (7–8%) which reached a limiting value of about 15% after 48 h. This experiment demonstrated that the degradation of poly-**1** was relatively slow under these conditions and that 85–90% of encapsulated material could be retained in the polymer matrix for suitably long periods of time at physiological pH values.

The application of poly-**1** microspheres to the triggered release of encapsulated drugs in the endosomal/lysosomal pH range was examined by conducting a similar experiment in which the pH of the suspension medium was changed from 7.4 to 5.1 during the course of the experiment. As shown in Figure 2, the microspheres dissolved rapidly when the suspension buffer was exchanged with acetate buffer (0.1M,

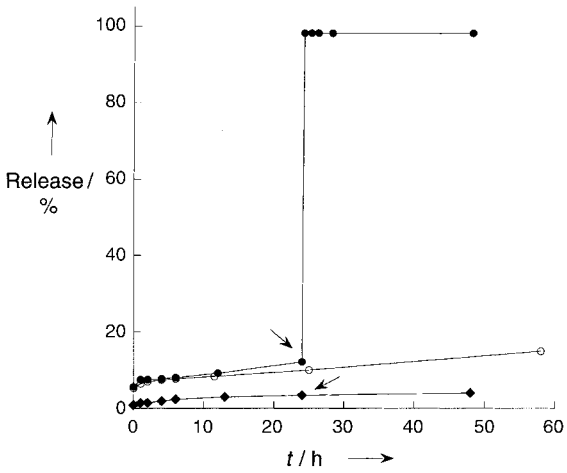


Figure 2. Release profiles of rhodamine/dextran from poly-**1** and PLGA microspheres at various pH values (○, poly-**1** (pH 7.4); ●, poly-**1** (pH 7.5/5.1); ◆, PLGA (pH 7.4/5.1)). The arrows indicate the points at which HEPES buffer (pH 7.4) was exchanged with acetate buffer (pH 5.1) (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazininyl]ethanesulfonic acid).

pH 5.1), and resulted in instantaneous and quantitative release of the labeled dextran remaining in the polymer matrices. By contrast, the release from dextran-loaded PLGA microspheres did not increase for up to 24 h after the pH of the suspending medium was lowered (Figure 2). Figure 3 shows fluorescence microscopy images of a sample of dextran-loaded microspheres at pH 7.4, and a sample to which a drop of acetate buffer was added at the upper right edge of the microscope coverslip. The rapid release of rhodamine-conjugated dextran was visualized as streaks extending from the dissolving microspheres in the direction of the diffusion of

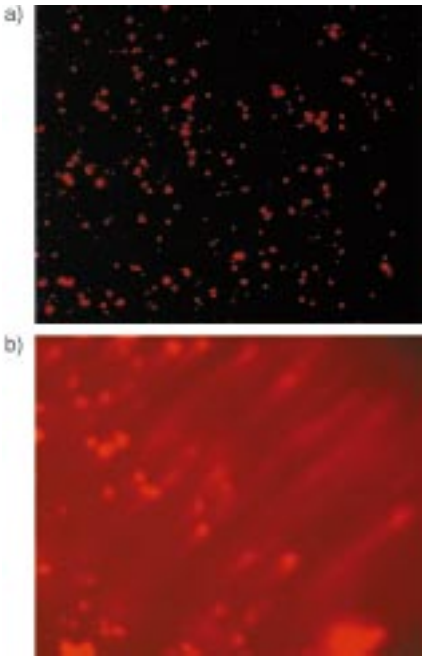


Figure 3. a) Representative fluorescence microscopy image of rhodamine/dextran-loaded poly-**1** microspheres suspended in HEPES buffer (pH 7.4). b) Sample of loaded poly-**1** microspheres at pH 7.4 after addition of acetate buffer (pH 5.1). The direction of diffusion of acid is from the top right to the bottom left of the image (elapsed time: ca. 5 s).

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added acid and an overall increase in background fluorescence.

Both cell membrane translocation and escape from acidic intracellular vesicles represent substantial obstacles to efficient delivery by endocytosis or phagocytosis.[4] One recent approach to facilitating endosomal and/or lysosomal escape is the incorporation of weak bases, or "proton sponges", which are believed to buffer acidic endosomal environments and disrupt membranes by increasing the internal osmotic pressure within the vesicle.[10] Poly-**1** microspheres release encapsulated material in the endosomal pH range through a mechanism (dissolution) that involves the protonation of amines in the polymer matrix. Thus, it is possible that poly-**1** microspheres could also incorporate a membrane-disrupting means of endosomal escape and prolong the lifetimes of hydrolytically unstable therapeutics.

The microspheres investigated in this current study fall within the size range commonly used to target delivery to macrophages,[1c] and we have initiated particle uptake experiments employing the P388D1 macrophage cell line. The confocal microscopy image in Figure 4a demonstrates that particles formed from poly-**1** can be internalized by phagocytosis and suggest that particle rupture occurs more readily (Figure 4b, taken 18 h after particle addition) inside the cell than for PLGA microspheres (no particle rupture observed after 18 h, data not shown).

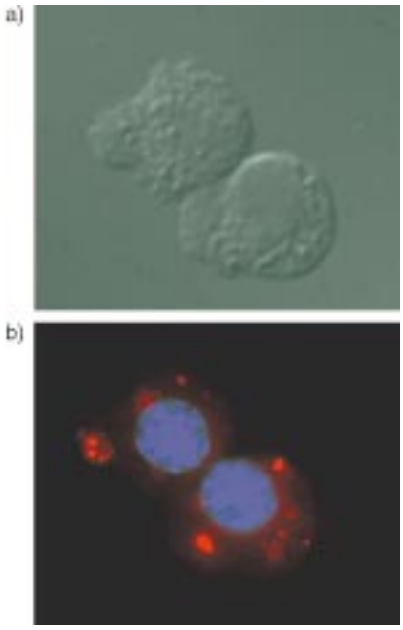

Figure 4. Confocal microscopy images of P388D1 macrophages 18 h after addition of poly-**1** microspheres. a) Differential interference contrast image of fixed cells, and b) overlayed confocal microscopy images (red = rhodamine/dextran; blue = DAPI nuclear stain).

The proof-of-concept experiments described above demonstrate that poly-**1** can be used to encapsulate a water-soluble polymer, retain the material at extracellular or cytoplasmic pH values, and release the encapsulated contents in the range of endosomal/lysosomal pH values. Preliminary data also suggest that these microspheres can be internalized and "digested" by macrophages. The incorporation of degradable, pH-sensitive materials such as poly-**1** could be useful in the design of new DNA-based vaccine formulations targeted to macrophages, several of which currently employ PLGA as an encapsulating material.[11] To this end, we are pursuing studies related to the encapsulation of DNA employing poly-**1**. The incorporation of additional design criteria (for example, smaller particle sizes, surface characteristics, targeting moieties) may yield particles useful for the targeting of smaller, somatic cells that internalize particles by endocytosis. Such particles could represent an important addition to the arsenal of materials applied to intracellular drug delivery, such as pH-responsive polymer/liposome formulations.[3]

Experimental Section

Fabrication of microspheres: The optimized procedure for the fabrication of microspheres was conducted in the following general manner: An aqueous solution of rhodamine-conjugated dextran (200 μL of a 10 μg μL^{-1} solution, $M_n \approx 70$ kD) was suspended in a solution of poly-**1** in CH_2Cl_2 (200 mg of **1** in 4 mL of CH_2Cl_2, $M_n \approx 10$ kD), and the mixture was sonicated for 10 s to form a primary emulsion. The cloudy pink emulsion was added directly to a rapidly homogenized (5000 rpm) solution of poly(vinyl alcohol) (50 mL, 1% PVA (w/w)) to form the secondary emulsion. The secondary emulsion was homogenized for 30 s before adding it to a second aqueous PVA solution (100 mL, 0.5% PVA (w/w)). Direct analysis of the microsphere suspension using a Coulter microparticle analyzer revealed a mean particle size of approximately 5 μm. The secondary emulsion was stirred for 2.5 h at room temperature, transferred to a cold room (4 °C), and stirred for an additional 30 min. The microspheres were isolated at 4 °C by centrifugation, resuspended in cold water, and centrifuged again to remove excess PVA. The spheres were resuspended in water (15 mL) and lyophilized to yield a pink, fluffy powder. Characterization of the lyophilized microspheres was performed by optical, fluorescence, and scanning electron microscopies as described in the text.

Confocal microscopy imaging: P388D1 macrophages were seeded onto fibronectin-coated cover slips in a 6-well cell culture plate at an initial seeding density of 200000 cells per well, and the cells were incubated overnight. Poly-**1** microspheres and PLGA microspheres encapsulating rhodamine-labeled dextran (average diameter = 4–6 μm) were added to each well to give final microsphere concentrations of 5 and 10 μg mL^{-1}. Samples were incubated for 18 h, washed three times with PBS buffer, and fixed with formalin. Each cover slip was mounted on a glass slide using mounting medium containing DAPI as a nuclear stain, and the samples were analyzed by confocal microscopy.

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Merging of Hard Spheres by Phototriggered Micromanipulation**

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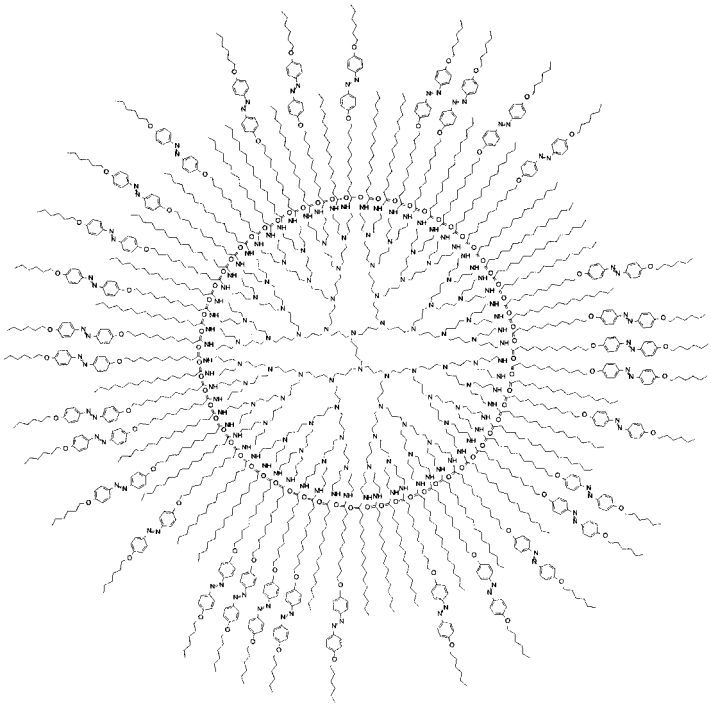
Amphiphilic dendrimers, namely those that carry both hydrophobic and hydrophilic regions within one molecule, tend to self-assemble into a large variety of different aggregates depending on their structure. The dendritic amphiphiles investigated so far include unimolecular micelles,[1] bolaamphiphiles,[2] dendronized polymers,[3] superamphiphiles,[4] and various other AB and ABA block copolymers.[5] Poly(propyleneimine) dendrimers modified with aliphatic end groups proved to be extremely flexible. Protonation of the amine core (for example, by water) resulted in these dendrimers acting as amphiphiles in which all (64 for the fifth generation) the aliphatic tails point to one side while the dendrimer core adjusts its conformation to form a nearly flat structure.[1f] Recently, the use of photoresponsive (dendritic) building blocks, obtained through the incorporation of azobenzene units, led to the formation of photoswitchable monolayers, whose macroscopic properties could be completely controlled by irradiation.[6] Here we describe how the use of amphiphilic azobenzene-modified dendrimers can give rise to the formation of photoresponsive supramolecular assemblies. With the aid of micromanipulation techniques, these well-defined supramolecular aggregates of dendrimers were subsequently used as secondary building blocks in the formation of even larger objects, with dimensions in the micrometer regime. Consequently, the structures ultimately obtained are the result of tuning at three different length scales.

We will focus on the fifth generation azobenzene-containing alkyl-modified poly(propyleneimine) dendrimer **1** (Scheme 1), an amphiphilic blockcopolymer with a random shell structure that carries on average 32 azobenzene groups and 32 palmitoyl groups.[7] Although a statistical distribution of the two different groups is obtained, as determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analyses, which implicates a random positioning of these groups, the dendritic scaffold remains monodisperse.



Scheme 1. Schematic representation of azobenzene-modified dendrimer **1**, to illustrate the random character of the attachment of the azobenzene and palmitoyl units at the rim of the dendrimer.

Dendrimer **1** is molecularly dissolved in organic solvents such as tetrahydrofuran (THF) or chloroform, and displays reversible *cis*–*trans* isomerization upon excitation with light with a wavelength of 365 nm. Injection of concentrated solutions of **1** in THF into water (pH 1–8) at 333 K results directly in the formation of opalescent solutions containing vesicles. These vesicles were subsequently investigated by transmission microscopy (TM), scanning fluorescence microscopy (SFM), confocal scanning fluorescence microscopy (CSFM),[8] atomic force microscopy (AFM),[9] scanning electron microscopy (SEM), and cryo-transmission electron microscopy (Cryo-TEM).[10] Transmission microscopy reveals

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