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Expansile Nanoparticles: Synthesis, Characterization, and *in Vivo* Efficacy of an Acid-Responsive Polymeric Drug Delivery System

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Micro- and nanoparticles are being employed for an increasing number of medical applications. In terms of drug delivery applications, these particles are being investigated as a means to increase drug solubility, alter biodistribution, enhance pharmacokinetics, target specific sites, and minimize side effects.[1] These particles, depending on the application, are composed of lipids,[2] proteins,[3] carbohydrate,[4] or hydroxy acid polymers. For example, micro- or nanoparticles composed of poly(lactic-glycolic acid), PLGA, are well studied for drug delivery and represent the prototypical polymer particle.[5] Our efforts are focused on designing new nanoparticle compositions that possess an alternative delivery mechanism whereby a hydrophobic to hydrophilic transition is triggered by a physiologic stimulus resulting in swelling and rapid release of their contents. The synthesis of particles which respond to changes in environmental conditions like pH or temperature is a very promising and active area of research.[6] Here, we report an engineered polymeric nanoparticle that expands several hundred-fold in volume in response to a pH change, going from nanometer to micrometer in diameter and that releases its contents. These expansile nanoparticles loaded with paclitaxel, a poorly water-soluble anticancer drug, prevent establishment of lung cancer *in vivo* and are superior to the conventional drug delivery method for paclitaxel using Cremophor EL/ethanol.

Our approach entails a cross-linked nanoparticle which, in its initial state, is hydrophobic but, upon cellular internalization, transforms to a hydrophilic structure, namely a hydrogel particle, in response to the lower pH of ~5 within the endosome. As such, water enters and the hydrogel[7] particle swells and releases the encapsulant. The potential benefit of this approach is the intracellular release of the drug giving high local concentrations at the site of delivery with low systemic exposure. To develop such pH responsive expansile nanoparticles, we prepared cross-linked nanoparticles from a hydrophobic monomer in which the hydroxyl groups of the resulting polymer nanoparticles are masked by an acid-labile protecting group. At neutral pH the nanoparticles are stable and do not release the encapsulant. A decrease in pH cleaves the protecting group and reveals the hydroxyls, causing the desired hydrophobic to hydrophilic transformation.

The nanoparticles were prepared using a miniemulsion polymerization technique, which combines high-energy emulsification and free radical photopolymerization of an acrylate monomer (see Supporting Information (SI) for details). Specifically, nanoparticles were prepared from monomers **1** and **2**, as shown in Figure 1. Monomer **1** possesses a 2,4,6-trimethoxybenzaldehyde protecting group, which is stable at neutral pH but hydrolyzes at a mildly acidic pH (~5).[6c] In contrast, monomer **2** has a benzaldehyde protecting group that will hydrolyze under very acidic conditions (pH ≤ 1). The monomers were synthesized as follows. 1,1,1-Tris(hydroxymethyl)ethane was reacted with 2,4,6-trimethoxybenzaldehyde or benzaldehyde in the presence of a catalytic amount of *p*-toluenesulfonic acid to afford 5-methyl-2-(2,4,6-trimethoxyphenyl)-[1,3]-5-dioxanylmethanol and 5-methyl-2-(phenyl)-[1,3]-5-dioxanylmethanol, respectively.[8] Next, the remaining primary hydroxyl was methacrylated using methacryloyl chloride and triethylamine dissolved in dichloromethane to give monomers **1** and **2**, respectively. The minimemulsion was created by dissolving the monomer and cross-linker (1,4-*O*-methacryloylhydroquinone, **3**) in a small amount of dichloromethane, adding this organic solution to an aqueous solution of the surfactant sodium dodecyl sulfate and triethanolamine, and sonicating the mixture at 35 W of power for 10 min. Following the miniemulsion step, the photoinitiation system (eosin Y dye and 1-vinyl-2-pyrrolidinone) was added, and the emulsion was photopolymerized using a Xe arc lamp for 20 min while being stirred. The mixture was then stirred overnight while open to the atmosphere to allow the remaining organic solvent to evaporate. The resulting polymeric nanoparticles were then dialyzed against 5 mM, pH 8.5 phosphate buffer over 2 days to remove excess surfactant and salts. Dynamic light scattering (DLS) measurements revealed suspensions of relatively small monodisperse nanoparticles ~100 nm in diameter, prepared from either monomer (see SI). Scanning electron micrographs showed the spherical shape and smooth morphology of the particles (Figure 2A).

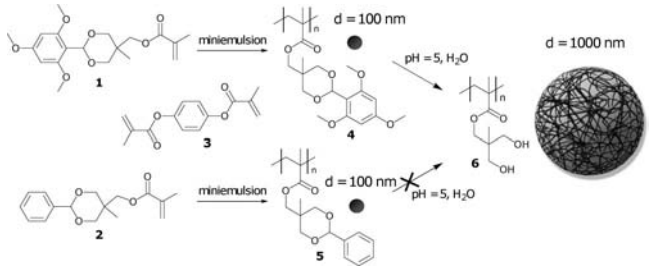

Figure 1. Synthesis of nanoparticles with differing pH responsiveness. The protecting group of nanoparticle **4** but not **5** is cleaved at a pH of ~5. This transformation reveals the hydrophilic hydroxyl groups and formation of nanoparticle **6** with resulting expansion of the hydrogel nanoparticle in water.

A key design feature of these nanoparticles is the hydrophobic to hydrophilic transformation upon exposure to a mildly acidic environment with subsequent swelling. Nanoparticles prepared from monomer **1** or **2** were exposed to buffered aqueous solutions of pH 5 or 7.4, and the diameter of the particles was measured at regular time intervals over 24 h using DLS. Nanoparticle size is shown as a function of time at the two pH conditions in Figure

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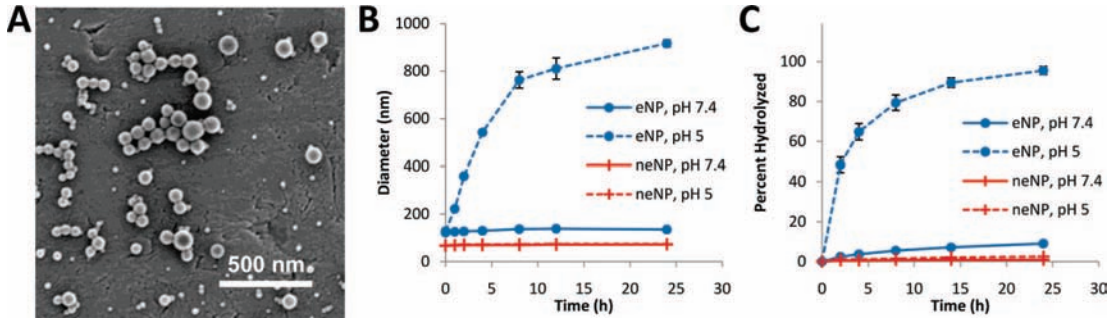

Figure 2. (A) Scanning electron micrograph of expansile nanoparticles (eNP) prepared from monomer **1**. (B) Swelling of the eNP and neNP as a function of pH and time at 37 °C. Data displayed as mean ± SD; $n = 3$. (C) Hydrolysis profile of the protecting group from eNP and neNP as a function of pH and time at 37 °C. Data displayed as mean ± SD; $n = 3$.

2B. Nanoparticles prepared from monomer **1** swelled at pH 5, but not at pH 7.4, and hence are called expansile nanoparticles (eNPs). The change in volume is ~350-fold as nanoparticle **4** transformed into nanoparticle **6** (see SI for count rate and polydispersity data). In comparison, nanoparticles prepared from monomer **2** did not swell at either pH condition and, therefore, are referred to as nonexpansile nanoparticles (neNPs). The loss of the protecting group as a function of pH for the eNPs and neNPs was also monitored. As shown in Figure 2C, the protecting group of the eNP was cleaved only at pH 5 with a similar rate to swelling, but the protecting group of the neNP was not cleaved at pH 7.4 nor 5.

The paclitaxel-loaded expansile nanoparticles (Pax-eNPs) and nonexpansile nanoparticles (Pax-neNPs) were synthesized in an analogous manner described above with the paclitaxel being added prior to emulsification. Paclitaxel was loaded at a concentration of 1 wt %/wt with 85% encapsulation efficiency as determined by HPLC. As shown in Figure 3, the release of paclitaxel in PBS buffer from the loaded expansile nanoparticle was pH dependent and related to the hydrophobic to hydrophilic transformation. Minimal paclitaxel release was observed at pH 7.4 whereas nearly 100% release occurred within 24 h at pH 5. The nonexpansile particles show significant, rapid paclitaxel release at pH 7.4 and 5 within the first 5 h, but the release was not correlated with pH. Thus, the Pax-neNPs are not a negative control for this study.

Our primary therapeutic interest lies in the prevention of tumor recurrence following surgical removal of cancerous lung tissue.[9a] Of all major cancers, lung cancer has the lowest 5-year survival rate at 15.3%.[9] Moreover, local recurrence following lobectomy for stage I lung cancer occurs in 9% of patients, with documented recurrence rates increased to 24% in patients with poor pulmonary reserve who receive more limited wedge resections.[9] Adjuvant therapies in patients unable to tolerate lobectomy (e.g., radio frequency ablation, external radiation treatment, placement of radioactive seeds, systemic chemotherapy) result in inferior outcomes, and therefore, additional treatment strategies for improved local control of tumor growth following surgery are needed. The local delivery of antineoplastic agents to the resection site at the time of limited surgical therapies (i.e., wedge resections or ablations) is an attractive approach, as it would enhance the local efficacy of chemotherapy while minimizing detrimental systemic side effects that are common with systemic administration. Moreover, a drug delivery system capable of preventing recurrence at the tumor-tissue interface would potentially extend the benefit of surgical therapy to improve the clinical outcomes of patients previously deemed unacceptable candidates for lobectomy.

As a prelude to the *in vivo* studies, we performed cell cytotoxicity experiments with paclitaxel loaded and unloaded nanoparticles against a murine nonsmall cell lung cancer cell line (Lewis lung carcinoma (LLC); see SI). No significant cytotoxicity was observed with the unloaded expansile nanoparticles, with the results being similar to untreated controls (Figure 4). However upon loading the expansile nanoparticles with paclitaxel (1 μg of polymer contains 10 ng of paclitaxel), we observed a dose dependent cytotoxic response with an IC_{50} of ~10 ng/mL. This IC_{50} value is consistent with the value for paclitaxel alone and confirms that the encapsulation procedure does not adversely affect the activity of paclitaxel.

Therefore, to assess the ability of paclitaxel-loaded expansile nanoparticles to prevent establishment of lung cancer in an *in vivo* model mimicking microscopic disease that can remain when the surgical margin is close to the tumor, we evaluated paclitaxel-loaded expansile nanoparticles in a rapidly growing subcutaneous tumor model. Specifically, we assessed the ability of paclitaxel-loaded expansile nanoparticles to prevent establishment of rapidly growing LLC tumors in C57Bl/6 female mice compared to empty expansile nanoparticles, paclitaxel-loaded nonexpansile nanoparticles, and pa-

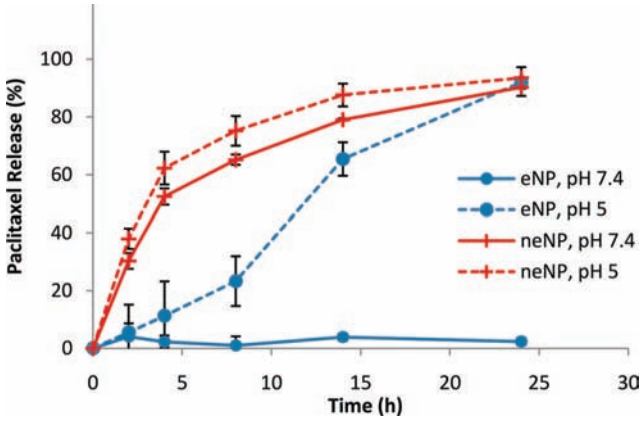

Figure 3. Release of paclitaxel as a function of pH and time for expansile and nonexpansile nanoparticles. Data displayed as mean ± SD; $n = 3$.

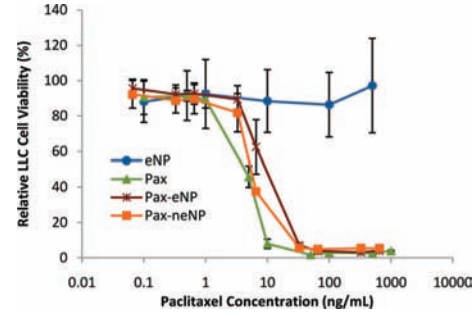

Figure 4. Percent relative viability of LLC cells following 72 h of exposure to paclitaxel, empty expansile nanoparticles, paclitaxel-loaded nonexpansile nanoparticles, and paclitaxel-loaded expansile nanoparticles. Data displayed as mean ± SD; $n = 3$.

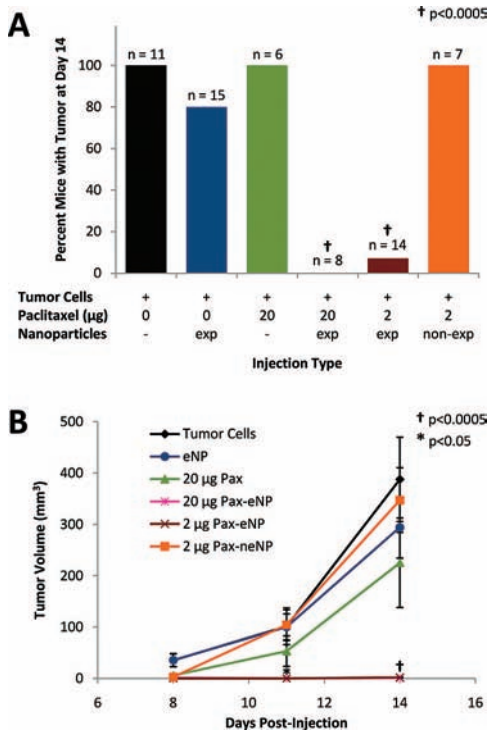

Figure 5. (A) Paclitaxel-loaded expansile nanoparticles prevent tumor growth *in vivo*, whereas paclitaxel-loaded nonexpansile nanoparticles, empty expansile nanoparticles, and paclitaxel do not. $^{\dagger}p < 0.0005$ vs control. (B) Tumor growth over time for animals receiving the different treatment groups. Data displayed as mean ± SEM. Day 11 $^{*}p < 0.05$ vs control and day 14 $^{\dagger}p < 0.0005$ vs control.

clitaxel alone. In these experiments, 750 000 LLC tumor cells plus paclitaxel-loaded expansile nanoparticles containing a total dose of 2 or 20 μg of encapsulated paclitaxel were injected subcutaneously into the flank of a mouse. The contralateral flank received a second injection of 750 000 LLC cells alone or LLC cells mixed with an equivalent dose of empty expansile nanoparticles, paclitaxel-loaded nonexpansile nanoparticles, or paclitaxel solubilized with 1:1 Cremophor EL/ethanol as used clinically. The two doses of paclitaxel investigated, 2 and 20 μg, are 100- and 10-fold lower, respectively, than a single dose typically used in multidose regimens for murine tumor studies in vivo.[6a] Animals were monitored clinically, and tumors were measured twice a week without knowledge as to the treatment received. At 14 days, large tumors were noted at the site where LLC cells were coinjected with media alone, empty expansile nanoparticles, paclitaxel-loaded nonexpansile nanoparticles, or paclitaxel alone (Figure 5). In contrast, sites receiving LLC cells plus paclitaxel-loaded expansile nanoparticles showed a significantly reduced incidence of tumor and tumor burden (Figure 5). Importantly large tumors were present in animals treated with paclitaxel alone, despite the local adminstration of an equivalent dose of paclitaxel. In addition, systemic toxicity was not observed with delivery of the nanoparticle. The observation that animals receiving all other treatment regimes besides the paclitaxel-loaded expansile nanoparticles rapidly developed large tumors suggests that nanoparticles possessing the responsive expansile characteristic are an effective delivery vehicle for paclitaxel. In fact, a single 100× smaller dose of paclitaxel can be used with the eNP compared to the standard dose with Cremophor EL/ethanol.

In summary, pH-responsive polymeric nanoparticles that expand in response to a mildly acidic pH have been synthesized, characterized, and evaluated *in vivo*. The hydrophobic anticancer drug, paclitaxel, was encapsulated within these nanoparticles and is released upon a pH triggered hydrophobic to hydrophilic transition. The successful *in vivo* studies using paclitaxel loaded expansile nanoparticles to prevent establishment of lung cancer demonstrates the effectiveness of this approach, the requirement for the responsive property, and superiority over the standard-of-care method for paclitaxel delivery using Cremophor EL/ethanol. This new delivery approach and vehicle allow localized delivery of low doses of drug resulting in high efficacy, low systemic exposure, and reduced side effects. Continued development and evaluation of chemically responsive nanotechnologies will afford new treatment options for the local control of a wide variety of tumors, which currently limit patient survival.

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Supporting Information Available: Synthesis, characterization data, and experimental procedures. All animal experiments were in accordance with an IACUC approved protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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