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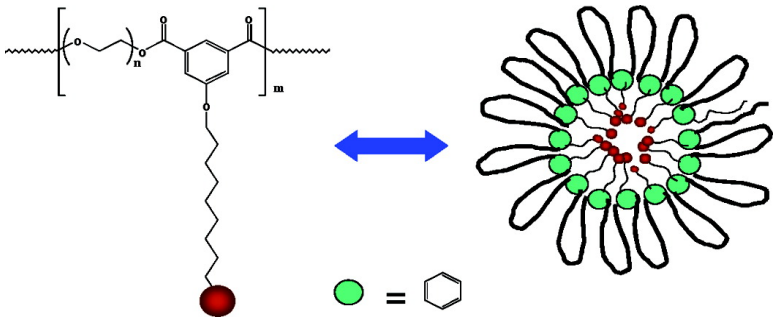

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Supramolecular Assemblies Based on Copolymers of PEG600 and Functionalized Aromatic Diesters for Drug Delivery Applications

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Abstract: A chemoenzymatic approach has been developed to synthesize poly(ethylene glycol)-based amphiphilic copolymers under mild reaction conditions that self-assemble in aqueous media to form polymeric nanomicelles in the range of 20−50 nm. The supramolecular organization of polymeric nanomicelles was studied by ^{1}H NMR longitudinal relaxation time (T_1) and light scattering techniques (static and dynamic). Interestingly, the enzyme novozyme-435 plays an important role in controlling the polymerization and distribution of polymer chains, which is critical for the formation of nanomicelles with unimodal distributions. The methodology developed is highly flexible as it allows the introduction of various functionalities in the polymeric nanomicelles. These self-organized nanomicelles are highly efficient drug delivery vehicles for hydrophobic and partially hydrophilic drugs, both transdermally and orally, as they have the ability to encapsulate guest molecules during self-organization. In vivo studies by encapsulating anti-inflammatory agents (aspirin and naproxen) in these polymeric nanomicelles and by applying topically resulted in significant reduction in inflammation. The % reduction in inflammation using polymeric nanomicelles containing aspirin and naproxen was 62 and 64%, respectively.

Introduction

Polymeric micelles represent a distinct class of micelles and are formed from copolymers consisting of both hydrophilic and hydrophobic segments. Keeping in mind the future pharmaceutical applications of polymeric micelles in drug delivery systems,[1,2] efforts currently concentrate on developing approaches to synthesize materials, which can satisfy a set of requirements. These include high loading capacity, controlled release profile for the incorporated drug, and good compatibility between the core-forming block and the incorporated drug.[3] The recent development of biomolecular devices that function within the living body has required the integration of capabilities for sensing in vivo chemical stimuli, generating detectable signals, and effecting suitable responses into a single molecule or molecular complex.[4] In particular, biopharmaceutical systems that interact with intracellular components or events such as ions, proteins, enzymes, and pH changes are becoming important for implementing programmed functions that respond to signatures of the body.[5−7] Supramolecular chemistry is attracting attention as it offers methods for assembling different constituents capable of structural and dynamic changes into a single molecule.[8]

On the basis of these considerations, we have designed a polymeric system with pendant functional groups such as hydroxyl, carboxyl, and amino groups based on poly(ethylene glycol). However, the reported[9] chemical synthesis of hydroxyl-, carboxyl-, and amino-substituted polyesters required the synthesis of a suitable monomer with a protected functional group

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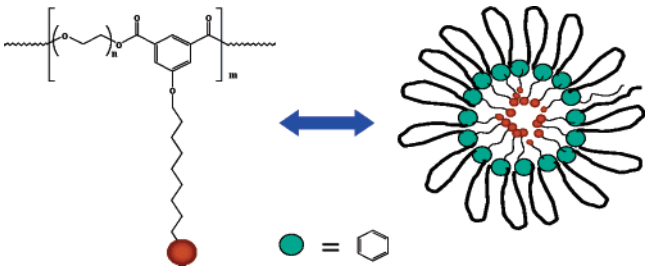

Figure 1. Self-assembly of the amphiphilic polymers **7**−**9** in aqueous media to form polymeric nanomicelles.

prior to the polymerization step. Therefore, the overall chemical synthesis of the functional polyester may involve a complex multiple-step reaction pathway. Enzymatic synthesis has tremendous potential[10,11] in addressing certain fundamental problems in polymer preparations, particularly for multifunctional monomers, and provides a powerful methodology where regioselection of functional groups can allow the synthesis of linear polymers from such monomers while circumventing much of the protection/deprotection chemistry. Enzyme-catalyzed polycondensation reactions have been extensively studied during the past decade as a possible alternative to chemical polymerizations. In particular, lipase-catalyzed polyester synthesis has received much attention from many researchers as an environmentally compatible methodology because of the use of nontoxic enzyme catalysts and mild reaction conditions.[12,13]

We describe here the first chemoenzymatic synthesis of functionalized amphiphilic copolymer system, which self-assembles in aqueous media to form polymeric nanomicelles (Figure 1). The extraordinary ability of enzymes to catalyze reactions with a high degree of regio- and stereoselectivity results in direct synthetic routes to well-defined polymers by reducing the need for traditional approach, which involves tedious protection/deprotection chemistry. These self-organized nanomicelles are highly efficient drug delivery vehicles for hydrophobic drugs both transdermally and orally as they have the ability to encapsulate guest molecules during self-organization. In vivo studies by entrapping anti-inflammatory agents (aspirin and naproxen) in these polymeric nanomicelles resulted in significant reduction in inflammation. The % reduction in inflammation using nanomicelles containing aspirin and naproxen was 62 and 64%, respectively. In addition, the functionality of the polymeric nanomicelles can be modified simply by changing the chemical structure of the amphiphilic copolymers, which in turn helps in encapsulating not only hydrophobic drugs but partially hydrophilic drugs as well and thus increases the therapeutic potential of the drugs.

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Experimental Section

Materials. Novozyme-435 (*Candida antarctica* Lipase B), an immobilized enzyme, was a gift from Novozymes Inc., Denmark, and was dried over P_2O_5 under vacuum prior to use. Poly(ethylene glycol) was dried under vacuum for 24 h before use, and acetone was dried by distillation over fused potassium carbonate. All other chemicals and solvents were of analytical grade and were used as received unless otherwise noted.

Animals for Anti-Inflammation Studies. Seven-week-old male CD-1 mice (Charles River Laboratories, Wilmington, MA), a strain very susceptible to inducing inflammation, were used in this study. CD-1 mice (mean body weight 25−28 g) were housed in standard polycarbonate cages ($33 \times 23 \times 12$ cm^3) under controlled conditions of temperature (22 ± 0.5 °C), relative humidity (50%), and 12/12 light/dark cycle. Mice had free access to water and rodent chow (Ralston Purina, St. Louis, MO) and were allowed to adapt to laboratory housing for one week before the commencement of the study.

Statistical Analyses for Animal Studies. Origin 6.0 and Microsoft Excel software were used for all statistical evaluations. A one-way analysis of variance (ANOVA) was used to analyze all data. When statistical significance was found by ANOVA, the Student−Newman−Keuls separation of means was used to determine group differences. All values were expressed as mean ±SD, and statistical significance was set at the minimum $p < 0.05$(3).

Instrumentation. Gel permeation chromatography (GPC) was used to determine the molecular weights and molecular weight distributions, M_w/M_n of polymer samples. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Instrument Inc. DPX 500 spectrometer at 500 and 125 MHz, respectively. Static light scattering data were collected on a laser light scattering photometer (Wyatt Technology DAWN model F) equipped with a 632 nm He−Ne laser as the light source. Dynamic light scattering was performed on these miceller solutions using a 50 mw He−Ne Laser, an avalanche photodiode detector BI-APD, a digital time correlator BI-9000 and software from Brookhaven Instruments Corporation, and dynamic light scattering software CONTIN and DOUBLE EXPONENTIAL, etc.

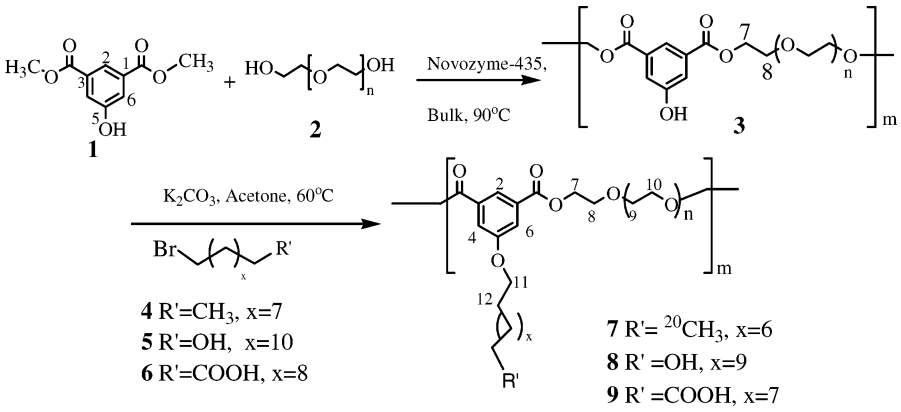
Polymerization. Dimethyl 5-hydroxyisophthalate (**1**, 1.0 mmol) and PEG 600 (**2**, 1.0 mmol) were placed in a round-bottom flask. To this mixture was added the enzyme (10 wt % wrt monomers), and the reaction vial was then placed in a constant temperature oil bath maintained at 90 °C under vacuum. The reaction was allowed to proceed for 48 h, after which it was quenched by adding water and filtering off the enzyme and any unreacted monomer **1** under vacuum. The filtrate was dialyzed using membrane (MWCO 6000). After the completion of dialysis, the product polymer **3** was obtained as a semisolid by freeze-drying.

Poly[(poly(oxyethylene-600)-oxy-5-hydroxyisophthaloyl] 3. ^{1}H NMR data ($CDCl_3$): δ 3.6−3.79 (bs, methylene protons of PEG main chain), 3.86 (t, 2H, C-8H), 3.96 (s, $COOCH_3$ end group), 4.50 (t, 2H, C-7H), 7.75 (s, 2H, C-4H and C-6H), and 8.24 (s, 1H, C-2H); M_n-(GPC) 18 000 Da, PD = 1.8, isolated yield 80%.

Coupling of Bromodecane (4), 12-Bromododecanol (5), and 11-Bromoundecanoic Acid (6) with Poly[(poly(oxyethylene-600)oxy-5-hydroxyisophthaloyl] (3). Equimolar quantities of **3** and **4** (or **5** and **6**) were dissolved in dry acetone, and to the resultant solution was added equimolar amount of anhydrous potassium carbonate. The reaction mixture was refluxed, and progress of the reaction was monitored by TLC using ethyl acetate in petroleum ether (30%). After completion, the potassium carbonate was removed by filtration, and the solvent was removed under vacuum to give the products **7**−**9**.

Poly[(polyoxyethylene-600)-oxy-5-decanyloxyisophthaloyl] 7. ^{1}H NMR data ($CDCl_3$): δ 0.90 (t, 3H, CH_3), 1.30−1.40 (bm, CH_2 protons of side chain), 1.81 (m, 2H, C-12H), 3.66−3.69 (bs, CH_2 protons of PEG main chain), 3.87 (t, 2H, C-8H), 3.96 (s, $-COOCH_3$ end group), 4.10 (t, 2H, C-11H), 4.51 (t, 2H, C-7H), 7.77 (m, 2H, C-4H and C-6H), and 8.30 (s,1H, C-2H); M_n(GPC) 18 730 Da, isolated yield 85%.

Scheme 1. Chemoenzymatic Synthesis of Functionalized Amphiphilic Polymers



Poly[(polyoxyethylene-600)-oxy-5-(12-hydroxydodecanyloxy)-isophthaloyl] 8. ^{1}H NMR data ($CDCl_3$): δ 1.31−1.38 (bm, CH_2 protons of side chain), 1.54−1.57 (m, 2H, C*H*$_2$CH$_2$OH), 1.82 (m, 2H, C-12H), 3.43 (t, 2H, CH$_2$C*H*$_2$OH), 3.67−3.74 (bs, CH_2 protons of PEG main chain), 3.86 (t, 2H, C-8H), 3.95 (s, −COOCH$_3$ end group), 4.05 (t, 2H, C-11H), 4.51 (t, 2H, C-7H), 7.76 (bs, 2H, C-4H and C-6H), and 8.28 (s,1H, C-2H); M_n(GPC) 19 220 Da, isolated yield 78%.

Poly[(polyoxyethylene-600)-oxy-5-(10-carboxydecanyloxy)-isophthaloyl] 9. ^{1}H NMR data ($CDCl_3$): δ 1.32−1.43 (bm, CH_2 protons of side chain), 1.68−1.81 (bm, 4H, C-12H and C*H*$_2$COOH), 3.68 (bs, CH_2 protons of the PEG main chain), 3.86 (t, 2H, C-8H), 3.96 (s, 3H, −COOCH$_3$ end group), 4.13 (t, 2H, C-11H), 4.50 (t, 2H, C-7H), 7.77 (bs, 2H, C-4H and C-6H), and 8.30 (s,1H, C-2H); M_n(GPC) 15 000 Da, isolated yield 82%.

Method for Encapsulation of Hydrophobic Drugs by Nanomicelles. The copolymer **7** and the hydrophobic drug aspirin were dissolved in chloroform to obtain 1:5 drug/polymer w/w ratios, and they were mixed for 15 min. Organic solvent was removed under vacuum, and the highly viscous mixture of drug and polymer obtained was dissolved with an extensive vortexing in water to form nanomicelles. Nonincorporated aspirin was separated by filtration of the prepared nanomicelle suspension through a 0.2 μm filter (aspirin crystals as well as the crystals of other insoluble drugs under normal circumstances cannot pass through the filter unless the drug is solubilized by nanoparticles). The filtrate obtained was freeze-dried to determine the overall efficiency of the encapsulation and found to be ~80%. The % of aspirin in nanomicelles estimated by UV spectroscopy (λ 277 nm) using a calibration curve for aspirin in methanol was 20%. In the absence of nanomicelles, less than 1% of aspirin was found in the filtrate when dispersed at the same concentration as in nanomicelles containing samples, indicating the drug that passes the filter in the presence of polymer is encapsulated by nanomicelles. The preparation and estimation of % encapsulation of naproxen-loaded nanoparticles were performed as described above for aspirin and found to be 7%.

Methods for Animal Studies of Inflammation. Animals were anaesthetized with a combination of ketamine (100 mg/mL) and xylazine (20 mg/mL). In the present study, auricular thickness of the appropriate ear surface was measured using a micrometer caliper before and after the application of croton oil and post anti-inflammatory treatment at 6 h. For the purpose of inducing inflammation, a 2% solution of croton oil (Sigma 6719) was dissolved in 1 mL of acetone. The inner surface of the right auricles (ears) of 22 CD-1 mice was exposed to 50 μL of the above preparation for 2 h as reported by Lopez et al.[14] The animals were randomly divided into four groups of five, and the inner surface of the right auricle exposed to 1 mL of empty nanospheres or nanospheres containing aspirin or naproxen at the end of 2 h was measured. Two animals served as untreated controls. Animals were sacrificed at 6 h post croton oil using 50% CO_2, and auricular thickness was remeasured and compared to previous measurements.

Results and Discussion

The novozyme-435 (immobilized *C. antarctica* lipase B) catalyzed condensation of **1** and PEG (M_n 600) (**2**) under solventless conditions gave the polymer **3** in 80% isolated yield (Scheme 1). The structure of the repeating units of the polymer **3** was identified using ^{1}H and 2D (COSY) NMR experiments. In the ^{1}H NMR spectrum, the appearance of the signal at δ 4.50 (for the C-7 protons) indicated the formation of the ester linkages in the polymer. This was further confirmed by the disappearance of the signal because of the methoxy protons of dimethyl 5-hydroxyisophthalate. In the ^{1}H−^{1}H correlation spectrum, the signal at δ 4.50 showed coupling with the signal at δ 3.86, i.e. the C-8 protons of the PEG end group, which further confirms the ester linkage between the alcoholic hydroxyl group of PEG unit and the methoxy carbonyl of **1**. The lipase-catalyzed reaction is highly chemoselective as there was no reaction with phenolic hydroxyl group. The ^{1}H and ^{13}C NMR spectra of the product **3** did not indicate any trace of transesterification between the phenolic hydroxyl and main chain ester units. The number average molecular weight of the polymer **3** is 18 000 Da (P_d 1.8), as determined by GPC.

The polymer **3** was further functionalized by coupling with bromodecane (**4**), 12-bromodocecanol (**5**), and 11-bromoundecanoic acid (**6**) using anhydrous potassium carbonate and acetone to give the alkylated copolymers **7**−**9**, in 78−85% isolated yields. The structures of these polymers were established by ^{1}H NMR, ^{13}C NMR, and ^{1}H−^{1}H correlation spectra. The ^{1}H−^{1}H NMR correlation spectra of the synthesized polymers **7**−**9** showed a signal at δ 4.05−4.13 for the C-11 protons, which also showed coupling with C-12 protons at δ 1.82, thus indicating the functionalizations at phenolic OH group of **3**. The degree of functionalization was determined by comparing the intensity of the signal at δ 4.10 (C-11 protons) with one at δ 4.50 (C-7 protons) in the ^{1}H NMR spectrum of the polymers **7**−**9** and was found to be >95%.

The process of micellization of the amphiphilic copolymers **7**−**9** was observed using ^{1}H NMR longitudinal relaxation time (T_1) studies, because the formation of micelles, viz. aggregation of the hydrophobic segments in water, may influence the proton spin relaxation. Figure 2 shows the comparison of the T_1 vs concentration correlation of protons 4, 6, and 20 (the protons

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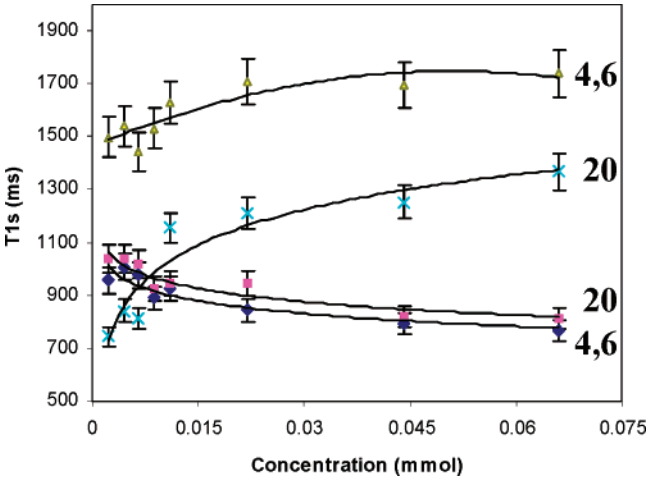

Figure 2. T_1-concentration correlation of protons 4, 6, and 20 of **7** in D_2O (bottom two lines) and $CDCl_3$.

Table 1. Critical Micelle Concentration, R_g, R_h, and R_g/R_h for Polymers **7**−**9**

polymer	CMC (mmol)	R_g (nm)	R_h (nm)	R_g/R_h
7	0.035	17.3 ± 2.1	9.56 ± 1.2	1.87
8	0.040	12.4 ± 4.9	6.56 ± 1.8	1.89
9	0.056	21.6 ± 2.5	13.5 ± 2.4	1.60

on the hydrophobic chain) of the copolymer **7** in D_2O and $CDCl_3$. It was observed that in D_2O, with an increase in concentration, the T_1 decreases and after a particular concentration, levels off (the lower two lines). This is because in D_2O, hydrophobic segments were forced to aggregate in the core of the micelles because of the hydrophobic interactions and thus result in the restriction of the mobility of side chains and stabilization of the local magnetic field. The T_1's, which were intrinsically influenced by the chemical and physical interactions between the nuclei, especially protons, thus decrease with increasing concentration and finally stabilize in D_2O. On the contrary, when the copolymers were dissolved in $CDCl_3$, strong hydrophobic interactions did not exist in the solution, and this led to a lot of fluctuations in the magnetic environments which in turn resulted in an increase in the T_1's (top two lines in Figure 2) with the increasing concentration. On the basis of these studies, we have observed that in water, the hydrophobic segments have strong tendency to aggregate and become the core of the micelle, while the hydrophilic segments extend themselves in the bulk solvent water.

Further, we have studied the supramolecular organization of the amphiphilic polymers **7**−**9** in water by light scattering techniques (static and dynamic) as they readily dissolved in water. The critical micelle concentration (CMCs) values of these polymers in aqueous media were determined by static light scattering technique; these were found to be about 0.035−0.056 mmol/mL (Table 1). A radius of gyration (R_g) of 17.3 ± 2.1 nm (concentration 0.63 mg/mL) was obtained for **7**, whereas under similar conditions for the copolymers **8** (having hydroxyl) and **9** (having carboxyl) with different functionalities at the end of the hydrophobic side chain, R_g was 12.4 ± 4.9 and 21.6 ± 2.5, respectively, indicating no major change in the particle size with side chain functionality. Similarly with different functionalities in the side chains, different micelle arrangements and shapes are expected, whereas the ratio of R_g/R_h (where R_h was determined under same conditions by dynamic light scattering)

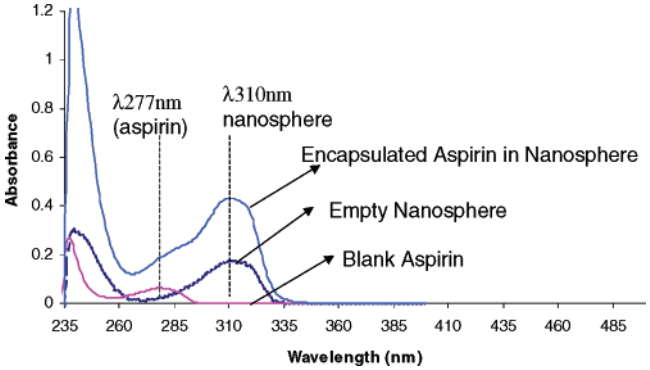

Figure 3. UV absorption spectra of (a) aspirin, (b) empty nanosphere, and (c) aspirin in polymeric nanosphere **7**.

for all the copolymers was very close (Table 1). The ratio of R_g/R_h for micelles formed by copolymers **7**, **8**, and **9** suggests that the micelles have a shape between a hollow sphere and a star-like sphere (theoretical values for hard uniform spheres are 0.775 and for hollow spheres are between 1 and 3).[15]

To demonstrate the potential of the nanospheres formed by the amphiphilic polymers **7**−**9** as drug carriers, we have performed detailed studies on the possibility to load the nanospheres formed by **7** with some model hydrophobic anti-inflammatory agents such as aspirin and naproxen. The formation of the nanospheres in the presence of drugs was monitored by static light scattering technique, and the R_g after encapsulation of aspirin and naproxen was found to be 19.5 ± 1.5 and 21.6 ± 2.5 nm, respectively, as compared to 17.3 ± 2.1 for the empty nanosphere formed by **7**, suggesting that the drugs were really encapsulated and not simply adsorbed on polymer. Figure 3 shows the absorption spectra of aspirin, polymeric nanospheres, and aspirin encapsulated in nanospheres **7**. Nanoparticles with aspirin encapsulated show absorption having a maxima at 285 nm for aspirin in addition to a maxima at 310 nm for the empty nanospheres on comparison with aspirin and empty nanospheres indicating the encapsulation. The concentration of the aspirin and naproxen after encapsulation was determined by UV spectroscopy and found to be 20 and 7 wt %, respectively, wrt nanosphere weight.

We have performed the animal testing results with freshly prepared nanospheres and compared them with the same dose of aqueous preparation of commercially available naproxen sodium and aspirin (acetyl salicyclic acid). Application of these loaded nanospheres to the right auricle of the mice for 2 h after treatment with the pro-inflammatory compound, croton oil, resulted in a significant reduction of inflammation. The % reduction in inflammation using nanospheres containing aspirin and naproxen was 62 and 64%, respectively; however, the empty nanospheres also exhibited some anti-inflammatory activity (~18%). The study also showed that our nanosphere-mediated delivery of same dose (800 μg) of aspirin and naproxen has significantly better results as compared to same dose (800 μg) aqueous preparation of commercially available naproxen sodium (34%) and aspirin (32%) (Figure 4a,b). The nanosphere-mediated delivery increased the efficacy by 1.8−2.0 times. In view of the inflammatory component of several diseases, such

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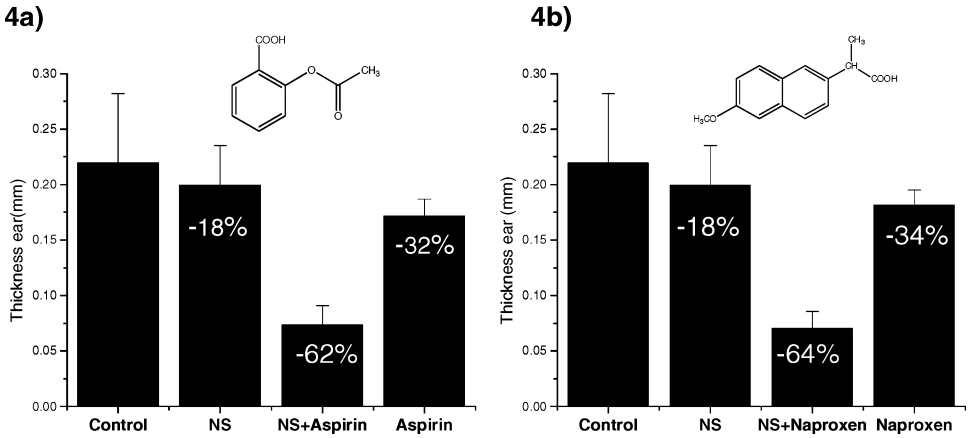

Figure 4. Anti-inflammatory properties of PEG nanospheres containing (a) aspirin and (b) naproxen.

as rheumatoid and osteoarthritis, asthma, and more recently cardiovascular diseases,[16] the ability to deliver anti-inflammatory agents topically or transdermally via nanoparticles, as opposed to the oral route, reduces the side effects of many anti-inflammatory products (no cream or lotion preparation based on aspirin or naproxen is available). With fewer side effects as a result of nanosphere-mediated topical delivery, aspirin will not only continue to provide therapeutic benefits for the inhibition of thrombosis but also for reduction in inflammation[17] during asthma attacks[18] and act as potential mediators of cardiovascular risk due to smoking.[19] Similarly, topical delivery of naproxen via nanoparticles will allow for more efficient delivery and better management of inflammation with this drug as reported during intraocular lens implantation,[20] osteoarthritis,[21] and rheumatoid arthritis.[22] Our ability to topically deliver anti-inflammatory agents using the novel polymeric nanospheres formed by amphiphilic copolymers generated through chemoenzymatic routes offers additional advantage of "Green appeal" and further enhances its widespread application potential as an important strategy.

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