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Stimuli-responsive antioxidant nanoprodrugs of NSAIDs

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ABSTRACT

A novel group of α-lipoic acid-containing hydrophobic prodrugs of non-steroidal anti-inflammatory drugs (NSAIDs) was synthesized and transformed into nanometer-sized prodrugs (nanoprodrugs). Three NSAIDs, indomethacin, ibuprofen and naproxen were linked to α-lipoic acid via tetraethylene glycol through hydrolytically degradable ester bonds. The three bifunctional derivatives were dissolved in organic solvents and capable of forming stable nanoprodrugs upon addition of the organic solutions into aqueous phase through the spontaneous emulsification mechanism. Antioxidant property and stimuli-responsiveness of the nanoprodrugs were demonstrated by hypochlorous acid (HOCl) scavenging followed by oxidative destabilization of the nanoprodrugs. The effect of varying NSAIDs on the *in vitro* hydrolytic prodrug activation catalyzed by porcine liver esterase was investigated by monitoring the rates of NSAIDs hydrolysis from the nanoprodrugs. The remarkable feature of these nanoprodrugs is that despite the highly hydrophobic nature of the derivatives NSAIDs were readily hydrolyzed enzymatically from the nanoprodrugs. Furthermore, the rate of hydrolysis was higher when the nanoprodrugs were oxidized and destabilized upon HOCl scavenging suggesting an enhanced activation of the nanoprodrugs in the oxidative environment.

1. Introduction

There is great interest in developing prodrugs that can be activated in response to stimuli and enable specific sustained drug release to reduce side effects (Friedrich et al., 1999; McKenzie et al., 2000; Rodrigues et al., 2003; Fattal et al., 2004; Ulbrich and Šubr, 2004). Stimuli-responsive materials have great potential in drug delivery when they are capable of forming vesicles allowing encapsulation or incorporation of drugs into their vesicular structures. Certain environmental stimuli, such as pH or oxidative molecules (Bellomo et al., 2004; Napoli et al., 2004), can destabilize vesicles allowing the release of drugs at the site of stimulation. This increases the selectivity of the drugs, improves therapeutic efficiency, and reduces adverse side effects. The prodrug strategy offers a similar advantage over parent drugs by introducing bioreversible bonds (Rautio et al., 2008). These bonds undergo selective enzymatic or chemical transformations *in vivo* and yield a significant reduction of adverse side effects.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain, fever, and inflammation. The major mechanism by which NSAIDs exert their anti-inflammatory activity is the inhibition of cyclooxygenase-derived prostaglandin synthesis, which is also responsible for the adverse side effects, such as irritation and ulceration of the gastrointestinal (GI) mucosa (Whittle, 2003). These GI side effects are generally believed to be resulted from the combined effect of the irritation caused by the free carboxylic groups in NSAIDs and blockage of prostaglandin biosynthesis in the GI tract (Dannhardt and Kiefer, 2001). Indomethacin, ibuprofen and naproxen belong to the acidic NSAIDs which are widely used for the treatment of chronic inflammatory conditions. In addition to the side effect which is attributed to their inhibitory effect on the activity of cyclooxygenase, the acidic moiety of these NSAIDs also contributes to the gastrointestinal side effect observed in response to these drugs (Tammara et al., 1993).

Various prodrugs have been proposed which attempt to alleviate the NSAID's adverse side effects as well as to improve their delivery characteristics by masking the carboxylic acid groups through the formation of bioreversible bonds (Bonina et al., 2001; Chandrasekaran et al., 2006; Siskou et al., 2007; Velázquez et al., 2007).

Our goal was to develop a new NSAID prodrug strategy which integrates enzymatic activation of prodrugs with the stimuli-responsiveness to increase the potential for site selective activation. First, α-lipoic acid (ALA)-containing antioxidant derivatives of NSAIDs were synthesized with the aim of obtaining hydrophobic ester prodrugs. Then, the obtained hydrophobic compounds were transformed into the nanoprodrugs using spontaneous emulsification (Bouchemal et al., 2004b).

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The oxidative responsiveness of the nanoprodrugs was studied *in vitro* by oxidative destabilization in the presence of hypochlorous acid (HOCl). The release of parent drugs from the nanoprodrugs was studied *in vitro* by ester hydrolysis catalyzed by pocine liver esterase. The effects of NSAIDs, ALA and oxidation on the rates of hydrolysis are discussed.

2. Materials and methods

2.1. General procedures and materials

Unless otherwise noted, solvents and chemicals were obtained at highest purity from Sigma–Aldrich Chemical Co. (St Louis, MO, USA) and used without further preparation. Chromatographic purification of the synthesized compounds was performed using silicagel (60 Å, 200–400 mesh). All the synthesized compounds were confirmed by thin layer chromatography (TLC)—silicagel plate (Merck 60 F254). Compounds containing ALA were visualized by treatment with a solution of: 1.5 g of $KMnO_4$, 10 g of K_2CO_3, and 1.25 ml of 10% NaOH in 200 ml of H_2O, followed by gentle heating. The derivatives of NSAIDs were visualized under UV light. ^{1}H and ^{13}C NMR spectra were conducted on a Varian 400 MHz spectrometer and chemical shifts (δ) were given in ppm relative to TMS. The spectra were recorded with the solvent $CDCl_3$ at room temperature.

2.2. Synthesis of bifunctional derivatives of ALA and NSAIDs

ALA (10 mmol) and tetraethylene glycol (TEG, 30 mmol) in 50 ml of anhydrous dichloromethane (DCM) were reacted with 4-(dimethylamino)-pyridine (DMAP, 15 mmol) in the presence of a molecular sieve (Fluka, 3 Å, 10–20 mesh beads) for 10 min at room temperature. *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDCI, 10 mmol) was added portionwise over 10 min and the reaction mixture was stirred for 5 h at room temperature in the dark, filtered, and then concentrated under vacuum to reduce the volume. The product ALA-TEG-OH and dimeric byproduct ALA-TEG-ALA were purified using column chromatography by loading the concentrated reaction mixture on the column without prior preparation and characterized as described above. Mono-ALA derivatives of TEG (3.8 mmol) and NSAIDs (4.1 mmol; indomethacin: Ind; ibuprofen: Ibu; naproxen: Npx) in 20 ml of anhydrous DCM were reacted with DMAP (4.1 mmol) in the presence of molecular sieve for 10 min at room temperature. EDCI (4.1 mmol) was added portionwise over 10 min and the reaction mixture was stirred for 5 h at room temperature in the dark, filtered, and then concentrated under vacuum at room temperature. The products were purified using column chromatography and characterized as described above.

2.2.1. ALA-TEG-OH

The column chromatography on silicagel ($CHCl_3$:MeOH 50:1) gave the compound as a yellow oil (63%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.19; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47 (m, 2 × H, H_a), 1.68 (m, 4 × H, H_b), 1.91 (m, 1 × H, H_c), 2.36 (t, 2 × H, H_d), 2.46 (m, 1 × H, H_e), 2.61 (s, 1 × H, –OH), 3.11 (m, 1 × H, H_f), 3.18 (m, 1 × H, H_g), 3.56 (m, 1 × H, H_h), 3.61 (m, 2 × H, H_E), 3.67 (s, 8 × H, H_A), 3.71 (m, 4 × H, H_B), 4.24 (m, 2 × H, H_D). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.55, 28.67, 33.86, 34.54, 38.45, 40.19, 56.31, 61.6, 63.37, 69.11, 70.19, 70.43, 70.45, 70.56, 173.47.

2.2.2. ALA-TEG-ALA

The column chromatography on silicagel ($CHCl_3$:MeOH 90:1) gave the compound as a yellow oil. TLC ($CHCl_3$:MeOH 100:0.5) R_f 0.12; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47 (m, 4H, 2 × Ha), 1.68 (m, 8H, 2 × Hb), 1.91 (m, 2H, 2 × Hc), 2.35 (t, J = 7.5 Hz, 4H, 2 × Hd), 2.46 (m, 2H, 2 × He), 3.15 (m, 4H, 2 × Hf + Hg), 3.57 (m, 2H, 2 × Hh), 3.65 (s, 8H, O–CH_2–CH_2–O), 3.70 (t, J = 4.8 Hz, 4H, 2 × O–CH_2–CH_2–OCO), 4.23 (t, J = 4.8 Hz, 4H, 2 × CO–O–CH_2–). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.56, 28.71, 33.94, 34.56, 38.5, 40.22, 56.33, 63.44, 69.16, 70.56, 173.36.

2.2.3. ALA-TEG-Ind

The column chromatography on silicagel ($CHCl_3$:MeOH 100:0.5) gave the compound as a yellow oil (73%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.33; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48 (m, 2 × H, H_a), 1.69 (m, 4 × H, H_b), 1.92 (m, 1 × H, H_c), 2.33–2.43 (m, 5 × H, H_8 + H_d), 2.47 (m, 1 × H, H_e), 3.15 (m, 2 × H, H_f + H_g), 3.54–3.75 (m, 15 × H, H_7 + H_A + H_B + H_h), 3.86 (s, 3 × H, H_6), 4.27 (m, 4 × H, H_D + H_E), 6.68 (q, 1 × H, H_5), 6.95 (d, 1 × H, H_4), 6.99 (d, 1 × H, H_3), 7.49 (m, 2 × H, H_2), 7.68 (m, 2 × H, H_1). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.4, 24.6, 28.7, 30.2, 33.9, 34.6, 38.5, 40.2, 55.71, 56.3, 63.4, 64.1, 69.1, 69.16, 70.53, 70.58, 101.39, 111.59, 112.50, 114.92, 129.12, 130,65, 130.78, 131.18, 133.91, 135.98, 139.20, 156.03, 168.24, 170.77, 173.41.

2.2.4. ALA-TEG-Ibu

The column chromatography on silicagel ($CHCl_3$:MeOH 100:0.5) gave the compound as a yellow oil (69%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.37; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (d, 6 × H, H_7), 1.37–1.48 (m, 5 × H, H_6 + H_a), 1.64 (m, 4 × H, H_b), 1.85–1.95 (m, 2 × H, H_5 + H_c), 2.32 (t, 2 × H, H_d), 2.38–2.45 (m, 3 × H, H_4 + H_e), 3.04–3.18 (m, 2 × H, H_g + H_f) 3.50–3.73 (m, 14 × H, H_3 + H_A + H_B + H_h), 4.20 (m, 4 × H, H_D + H_E), 7.05 (d, 2 × H, H_2), 7.18 (d, 2 × H, H_1). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.59, 22.41, 24.6, 28.71, 30.16, 33.91, 34.58, 38.47, 40.20, 44.98, 45.01, 56.31, 63.43, 63.85, 69.05, 69.16, 70.54, 70.59, 127.18, 129.27, 137.67, 140.44, 173.38, 174.62.

2.2.5. ALA-TEG-Npx

The column chromatography on silicagel ($CHCl_3$:MeOH 100:0.5) gave the compound as a yellow oil (65%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.33; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (m, 2 × H, H_a), 1.54–1.71 (m, 7 × H, H_5 + H_b), 1.88 (m, 1 × H, H_c), 2.33 (t, 2 × H, H_d), 2.43 (m, 1 × H, H_e), 3.05–3.19 (m, 2 × H, H_f + H_g), 3.39–3.67 (m, 13 × H, H_A + H_B + H_h), 3.88 (m, 4 × H, H_4), 4.21 (m, 4 × H, H_D + H_E), 7.12 (m, 2 × H, H_3), 7.40 (q, 1 × H, H_2), 7.68 (m, 3 × H, H_1). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.57, 24.61, 28.73, 33.93, 34.57, 38.48, 40.12, 45.33, 55.32, 56.33, 63.44, 63.96, 69.03, 69.14, 70.53, 105.57, 118.97, 125.99, 126.28, 127.11, 128.91, 129.28, 133.68, 135.63, 157.63, 173.44, 174.59.

2.3. Synthesis of dimeric derivatives of NSAIDs

NSAIDs (6 mmol) and TEG (2.5 mmol) in 40 ml of anhydrous DCM were reacted with DMAP (6 mmol) in the presence of molecular sieve for 10 min at room temperature. EDCI (6 mmol) was added portionwise over 10 min and the reaction mixture was stirred for 5 h at room temperature in the dark, filtered, and then concentrated under vacuum. The products were purified (column chromatography, 100:0.5 CH_3Cl: MeOH) and characterized as described above.

2.3.1. Ind_2TEG

The column chromatography on silicagel ($CHCl_3$:MeOH 100:0.5) gave the compound as a yellow oil (78%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.25; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.35 (s, 6 × H, H_8), 3.56 (m, 8 × H, H_A), 3.64–3.70 (m, 8 × H, H_7 + H_B), 3.80 (s, 6 × H, H_6), 4.25 (t, 4 × H, H_D + H_E), 6.64 (q, 2 × H, H_5), 6.86 (d, 2 × H, H_4), 6.95 (d, 2 × H, H_3), 7.43 (m, 4 × H, H_2), 7.62 (m, 4 × H, H_1). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.4, 30.19, 55.69, 64.13, 69.07, 70.52, 70.57, 101.4, 111.58, 112.51, 114.93, 129.11, 130.66, 130.79, 131.18, 133.93, 135.98, 139.18, 156.04, 168.22, 170.77.

2.3.2. Ibu_2TEG

The column chromatography on silicagel ($CHCl_3$:MeOH 100:0.5) gave the compound as a colorless oil (83%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.54; ^{1}H NMR (400 MHz, $CDCL_3$): δ 0.90 (d, 12 × H, H_7), 1.49 (d, 6 × H, H_6), 1.84 (m, 2 × H, H_5), 2.44 (d, 4 × H, H_4), 3.55 (m, 8 × H, H_A), 3.63 (m, 4 × H, H_B), 3.73 (q, 2 × H, H_3), 4.22 (m, 4 × H, $H_D + H_E$), 7.08 (m, 4 × H, H_2), 7.21 (m, 4 × H, H_1). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.60, 22.42, 30.19, 45.02, 45.04, 63.87, 69.08, 70.57, 70.61, 127.20, 129.29, 137.70, 140.48, 174.67.

2.3.3. Npx_2TEG

The column chromatography on silicagel ($CHCl_3$:MeOH 100:0.5) gave the compound as a colorless oil (75%). TLC ($CHCl_3$:MeOH 50:0.5) R_f 0.46; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.58 (d, 6 × H, H_5), 3.44 (m, 8 × H, H_A), 3.60 (m, 4 × H, H_B), 3.90 (m, 8 × H, H_4), 4.22 (m, 4 × H, $H_D + H_E$), 7.12 (m, 4 × H, H_3), 7.41 (q, 2 × H, H_2), 7.68 (m, 6 × H, H_1).

^{13}C NMR (100 MHz, $CDCl_3$): δ 18.56, 45.33, 55.29, 63.95, 69.02, 70.44, 70.47, 105.56, 118.96, 125.96, 126.27, 127.11, 128.91, 129.27, 133.68, 135.62, 157.63, 174.60.

2.4. High-performance liquid chromatography

RP-HPLC with UV detection was chosen as a simple, fast and effective method for quantification of many NSAID prodrugs and parent drugs (Bundgaard and Nielsen, 1988; Bonina et al., 1996; Redden et al., 1999; Mendes et al., 2002; Zhao et al., 2006). HPLC analysis was performed on a Merck-Hitachi analytical LaChrom D-7000 HPLC/UV detector system (Merck, Darmstadt, Germany) with a CAPCELL PAK, Type SG 120 (Phenomenex, Torrance, CA, USA) C_{18} reversed phase column (250/4.6 mm, 5 μm). The composition of the mobile phase (acetonitrile/water mixture containing 0.1% (v/v) trifluoroacetic acid) was adjusted for prodrugs and NSAIDs in order to provide an appropriate retention time and separation.

Linearity of the calibration curves was tested in the range of 6.25–2000 μg/ml for Ibu_2TEG, Npx_2TEG, Ind_2TEG and ALA-TEG-Ind, 12.5–2000 μg/ml for ALA-TEG-Npx and 25–2000 μg/ml for ALA-TEG-Ibu with good linear relationships ($r^2 > 0.99$). Within this concentration range the amount of the prodrugs could be determined reproducibly.

2.5. Determination of partition coefficients

Partition coefficients of the NSAIDs derivatives were studied using the shake-flask method (Hansch and Elkins, 1971). Briefly, a known amount of NSAIDs derivatives (2 mg) was partitioned between water-saturated *n*-octanol (2 ml) and *n*-octane-saturated water (2 ml) and the mixture was stirred continuously with a magnetic bar for 24 h at room temperature. Following separation of the two phases, concentrations of the NSAIDs derivatives in the *n*-octane and water phase were analyzed using RP-HPLC (Section 2.4). The separation was performed under isocratic condition with a 80/20 mixture of acetonitrile/water (0.1% TFA, v/v) at a flow rate of 1 ml/min. The detection was carried out at 220 nm for Ibu-TEG-Ibu, at 254 nm for Npx-TEG-Npx and ALA-TEG-Ibu and at 330 nm for Ind_2TEG, ALA-TEG-Npx and ALA-TEG-Ind.

2.6. Preparation and characterization of nanoprodrugs

2.6.1. Spontaneous emulsification

Nanoprodrugs were prepared according to the method using spontaneous emulsification (Bouchemal et al., 2004b). Briefly, 25 mg of the compounds were dissolved in acetone (5 ml) containing polysorbate 80 (0.1%, w/v). The organic solution was poured under moderate stirring on a magnetic plate into an aqueous phase prepared by dissolving 25 mg of Pluronic F68 in 10 ml distilled water (0.25%, w/v). Following 15 min of magnetic stirring, the acetone was removed under reduced pressure at room temperature. The suspensions were filtered through 0.8 μm hydrophilic syringe filter (Corning, Part No. 431221, Fisher Scientific Co., Pittsburgh, PA, USA) and stored at 4 °C.

2.6.2. Size measurements

The hydrodynamic size measurement and size distribution of the nanoprodrugs were performed by the dynamic light scattering (DLS) using a Coulter N4-Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL, USA). The nanoprodrugs were diluted in deionized water and the analysis was performed at a scattering angle of 90° and at a temperature of 25 °C. Three separate preparations were analyzed for each nanoprodrug. For each preparation mean diameter and mean polydispersity index (P.I.) of three determinations were calculated. The mean diameter ± standard deviation (S.D.) and P.I. ± S.D. were calculated from the three mean diameters and mean P.I.s. of the three separate preparations.

2.6.3. Stability of nanoprodrugs in simulated gastric fluid and simulated intestinal fluid

The stability of the nanoprodrugs was assessed in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8). Briefly, 1 ml of freshly prepared nanoprodrugs was dispersed in 10 ml of freshly prepared SGF and SIF and incubated at 37 °C on a rotatory shaker for 3 days. SGF and SIF were prepared as described in Carafa et al. (2006): SGF was composed of 0.2% (w/v) sodium chloride, 0.32% pepsin (w/v) and 0.7% (w/v) hydrochloric acid. SIF was composed of 0.067 M mixed sodium and potassium phosphate buffer ($Na_2HPO_4{\cdot}7H_2O/KH_2PO_4$-Sorensen's buffer). The stability of the nanoprodrugs were evaluated on the basis of the size and quantification of the intact prodrugs after 3 days. The recovery yield of the NSAIDs prodrugs was assessed by RP-HPLC as follows: the suspensions of nanoprodrugs were sedimented by centrifugation at 20,000 × *g* for 10 min, the pellets were dissolved in acetonitrile and analyzed using RP-HPLC as described (Section 2.5). The recovery yield was calculated as follows:

$$\text{recovery yield } (\%) = \frac{\text{amount of prodrugs after incubation}}{\text{amount of prodrugs before incubation}} 100$$

2.7. Oxidative destabilization of nanoprodrugs

The nanoprodrugs were purified by centrifuging three times at 20,000 × *g* for 10 min at 25 °C and resuspending each time in the same volume of deionized water. The purified nanoprodrugs were resuspended in phosphate buffered saline (PBS, pH 7.4) to give the final concentration of 250 μM NSAID derivatives. The concentration of HOCl in the diluted commercial sodium hypochlorite solution was determined spectrophotometrically ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$) (Morris, 1966). HOCl was added to the suspension of nanoprodrugs to give the final concentration of 25–1000 μM and the reduction in turbidity was measured immediately using ultraviolet/visible spectrometer (Bio-Rad SmartSpec™ 3000, Hercules, CA, USA) at room temperature with a wavelength λ = 500 nm.

2.8. Assay for HOCl scavenging

HOCl scavenging was monitored according to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay as described (Ching et al., 1994). Briefly, 2-nitro-5-thiobenzoate (TNB) was formed by raising the pH of a 0.5 mM aqueous solution of DTNB to 10 and then readjusting it to 7.4 (Riddles et al., 1983). After 2 min of HOCl treatment, the nanoprodrugs were centrifuged at 20,000 × *g* for 10 min. The supernatant (50 μl) was added to a reaction tube containing 950 μl of TNB solution (70 μM). After 5 min, the absorbance at 412 nm was

measured. The absorbance of TNB after addition of 0–25 μM HOCl served as control.

2.9. Enzymatic destabilization of nanoprodrugs

The nanoprodrugs were purified as described above and suspended in PBS (pH 7.4) to give the final concentration of 500 μM NSAID derivatives. Esterase (porcine liver, Sigma, code E3019) was added to the final concentration of 5 U/ml. For the nanoprodrugs from the three bifunctional prodrugs, ALA-TEG-Ibu, ALA-TEG-Npx and ALA-TEG-Ind, the reduction in turbidity was monitored using ultraviolet/visible spectrometer (Bio-Rad SmartSpec™ 3000) for 60 min at room temperature with a wavelength $\lambda = 500$ nm and 1 min acquisition interval. For the nanoprodrugs from the three dimeric prodrugs, Ibu_2TEG, Npx_2TEG and Ind_2TEG, the samples were taken during the incubation for 12 h at room temperature with appropriate time interval and the reduction in turbidity was measured.

To determine the amount of enzymatically hydrolyzed NSAIDs, samples were centrifuged for 10 min at 20,000 × *g* and the supernatants were analyzed by RP-HPLC using a C_{18} reversed phase column (Section 2.5). The separation was performed under isocratic condition with a 60/40 mixture of acetonitrile/water (0.1% TFA, v/v) at a flow rate of 1 ml/min and the hydrolyzed NSAIDs were detected at 254 nm for indomethacin and naproxen and at 220 nm for ibuprofen.

The concentration of the NSAIDs was determined using calibration curves generated in the concentration range of 25–500 μM. Error bar represents ± S.D. calculated from triplicate determinations. The stability of the nanoprodrugs under the experimental conditions in the absence of esterase was evaluated as follows. The purified nanoprodrugs were suspended in PBS (pH 7.4) and incubated for 12 h at room temperature. The amount of the intact prodrugs was determined using RP-HPLC as described (Section 2.6.3).

2.10. Sequence of enzymatic hydrolysis

Sequence of the enzymatic hydrolysis of ALA and NSAIDs from the nanoprodrugs was evaluated by measuring NSAIDs and other hydrolyzed species in the supernatant using RP-HPLC. The nanoprodrug containing 500 μM of ALA-TEG-Ibu, ALA-TEG-Npx or ALA-TEG-Ind (PBS, pH 7.4) was incubated in the presence of esterase (5 U/ml) at room temperature for 5 min and removed by centrifugation for 10 min at 20,000 × *g*. The separation of the hydrolyzed species in the supernatant was performed under isocratic condition at a flow rate of 1 ml/min with a 60/40 mixture of acetonitrile/water (0.1% TFA, v/v) for ALA-TEG-Ibu, with a 50/50 mixture for ALA-TEG-Ind and a 35/65 mixture for ALA-TEG-Npx. NSAIDs were detected at 254 nm for indomethacin and naproxen and at 220 nm for ibuprofen. The supernatant was incubated for 30 min and analyzed as described above.

2.11. Influence of oxidation on enzymatic prodrug activation

2.11.1. Recovery of intact prodrugs from oxidized nanoprodrugs

The recovery yield of intact prodrugs was determined for oxidized and non-oxidized nanoprodrugs. The nanoprodrugs prepared from ALA-TEG-Ibu, ALA-TEG-Npx and ALA-TEG-Ind (250 μM in PBS, pH 7.4) were incubated in the presence of HOCl (500 μM) for 2 min. The nanoprodrugs were sedimented by centrifugation at 20,000 × *g* for 10 min and the pellets were dissolved in acetonitrile and analyzed for intact prodrugs using RP-HPLC (Section 2.6.3).

2.11.2. Recovery of NSAIDs from oxidized nanoprodrugs

The recovery yield of NSAIDs was determined for oxidized and non-oxidized nanoprodrugs. The nanoprodrugs prepared from ALA-TEG-Ibu, ALA-TEG-Npx and ALA-TEG-Ind (250 μM in PBS, pH 7.4) were incubated in the presence and absence of HOCl (500 μM) for 2 min. Esterase (2 U/ml) was added and mixed by pipetting up and down. In order to determine the total amount of available NSAIDs from the oxidized and non-oxidized nanoprodrugs, the hydrolysis was carried out overnight at 37 °C. The reaction mixture was centrifuged for 10 min at 20,000 × *g* and the hydrolyzed NSAIDs in the supernatant were quantified using RP-HPLC (Section 2.9). Error bar represents ± S.D. calculated from triplicate determinations.

2.11.3. Effect of nanoprodrug oxidation on the rate of enzymatic hydrolysis

In order to evaluate the effect of prodrug oxidation on the rate of enzymatic hydrolysis, we determined the initial hydrolysis rate of NSAIDs from the oxidized and non-oxidized nanoprodrugs. The nanoprodrugs prepared from ALA-TEG-Ibu, ALA-TEG-Npx and ALA-TEG-Ind (250 μM in PBS, pH 7.4) were incubated in the absence and presence of HOCl (500 μM) for 2 min. Esterase (2 U/ml) was added and mixed by pipetting up and down. The reaction mixture was centrifuged immediately after addition of esterase and the supernatant was quickly transferred into a new tube and incubated further to complete hydrolysis. The hydrolyzed NSAIDs in the supernatant were quantified using RP-HPLC as described above (Section 2.9). Error bar represents ± S.D. calculated from triplicate determinations.

To evaluate the sequence of hydrolysis after oxidation, the nanoprodrugs were incubated in presence of HOCl (500 μM) for 2 min and esterase (2 U/ml) was added. The reaction mixture was mixed by pipetting up and down and immediately centrifuged for 10 min at 20,000 × *g*. The supernatant was analyzed using RP-HPLC as described (Section 2.10).

2.12. Statistical analysis

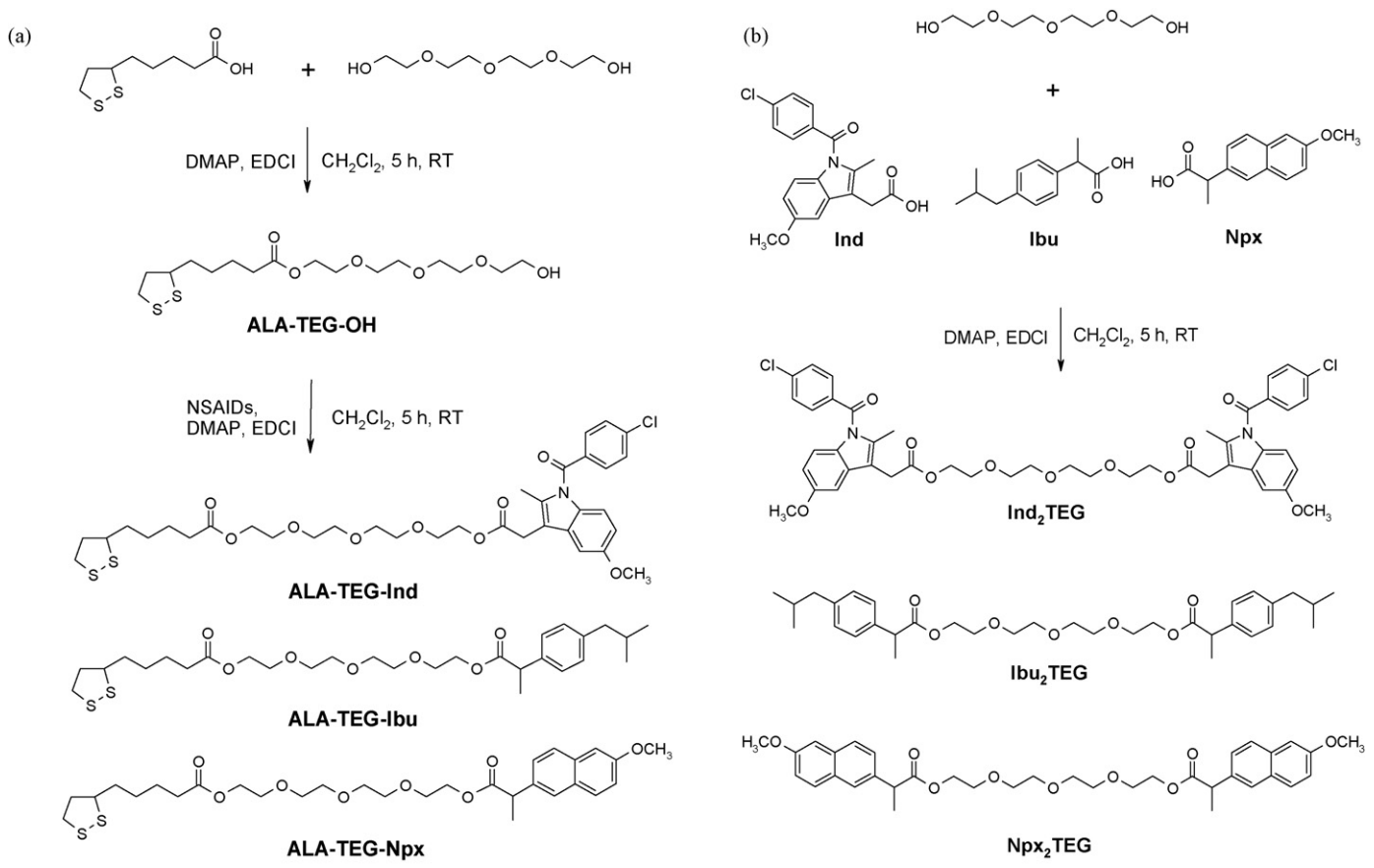
The results were analyzed and expressed as mean ± standard deviation (S.D.).

Statistical analysis of the results was carried out using Student's *t*-test. For all tests, differences with a $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Synthesis and characterization of hydrophobic derivatives of NSAIDs

Since the overproduction of reactive oxygen species (ROS) is associated with the inflammatory conditions and pathophysiology of many diseases (Finkei and Holbrook, 2000), NSAIDs prodrugs would be exceptionally valuable if they could scavenge ROS. Gastrointestinal ulceration is the major undesired side effect of almost all NSAIDs which is related mainly to the suppression of prostaglandin synthesis via inhibition of cyclooxygenase activity. In addition, it has been shown that the production of ROS is increased after NSAIDs treatment and the resulting oxidative damage has been also considered to be an important pathogenic component of gastrointestinal ulceration (Kusuhara et al., 1999; Basivireddy et al., 2004; Asensio et al., 2007). Meanwhile, it has been evidenced that the combination of antioxidant and anti-inflammatory activity could benefit the treatment of various inflammatory diseases by reducing ROS related side effects (Hassan et al., 1998; Kourounakis et al., 2000; Detsi et al., 2007; Ineu et al., 2008).



Scheme 1. Synthesis of hydrophobic derivatives of NSAIDs.

In order to combine the concept of prodrug, antioxidant, and stimuli-responsiveness, we have made use of nanometer-sized prodrugs (nanoprodrugs). Design and synthesis of hydrophobic NSAIDs prodrug molecules that are capable of forming stable nanoprodrugs, scavenging ROS, and being degraded to the parent drugs, were central to obtaining oxidant-responsive nanoprodrugs.

To achieve sensitivity to oxidation, we designed a series of hydrophobic derivatives containing ALA and NSAIDs. Due to its potent antioxidant activity and beneficial effects on the prevention and treatment of oxidative stress-related diseases (Packer et al., 1995; Biewenga et al., 1997), ALA provides a rational foundation to the development of a new antioxidant and oxidation-responsive prodrug. The bifunctional compounds which combine ALA and NSAIDs into one molecule were synthesized using a two-step synthesis as described in Scheme 1a. Tetraethylene glycol was converted to a mono-ALA derivative (ALA-TEG-OH), which was followed by the esterification of NSAIDs. To reduce the formation of dimeric byproduct of ALA (ALA-TEG-ALA), a 3-fold molar excess of TEG was used. Due to its more hydrophobic nature, the dimeric byproduct was easily separated by column chromatography. The dimeric byproduct was identified as ALA-TEG-ALA using ^{1}H and ^{13}C NMR.

The dimeric derivatives of NSAIDs consist of a core diol molecule TEG and two NSAID molecules (indomethacin: Ind; ibuprofen: Ibu; naproxen: Npx) which are coupled to the diol through esterification (Scheme 1b). The structures were confirmed by ^{1}H (Fig. 1) and ^{13}C NMR spectroscopy. The ^{1}H NMR data indicate that the resulting spectra are essentially a composite of the NSAIDs and the core molecule. The integration values of the protons of ALA and NSAIDs relative to those of the core molecule allow us to unambiguously identify the compounds.

The synthetic method presented here provides a simple and reproducible procedure to prepare large quantities of diverse hydrophobic compounds from NSAIDs and ALA. The purity of each synthesized compound was analyzed by thin layer chromatography and RP-HPLC (Table 1).

3.2. Partition coefficient determinations

Partition coefficient determination using the shake-flask method was not successful for all the six NSAID derivatives, as they were too hydrophobic for distribution in the aqueous phase to be measured with HPLC.

3.3. Preparation and characterization of nanoprodrugs of NSAIDs

3.3.1. Nanoprodrug formation through spontaneous emulsification

The nanoprodrugs were prepared using the spontaneous emulsification mechanism developed for the formation of stable nanocapsules (Bouchemal et al., 2004a,b). The hydrophobic derivatives of NSAIDs in organic solvents spontaneously formed nanometer-sized prodrugs (nanoprodrugs) upon the addition of their organic solvents into an aqueous solution containing hydrophilic surfactants. The principle of nanocapsule formation by a spontaneous emulsification process is well described in publications (Chouinard et al., 1991; Bouchemal et al., 2004a,b). The size of nanocapsules depends on multiple factors, such as the nature and concentration of the compounds in organic solvents, the nature and concentration of surfactants in organic and aqueous phase, the ratio of organic solvent to water, and the rate of diffusion of organic phase into aqueous phase

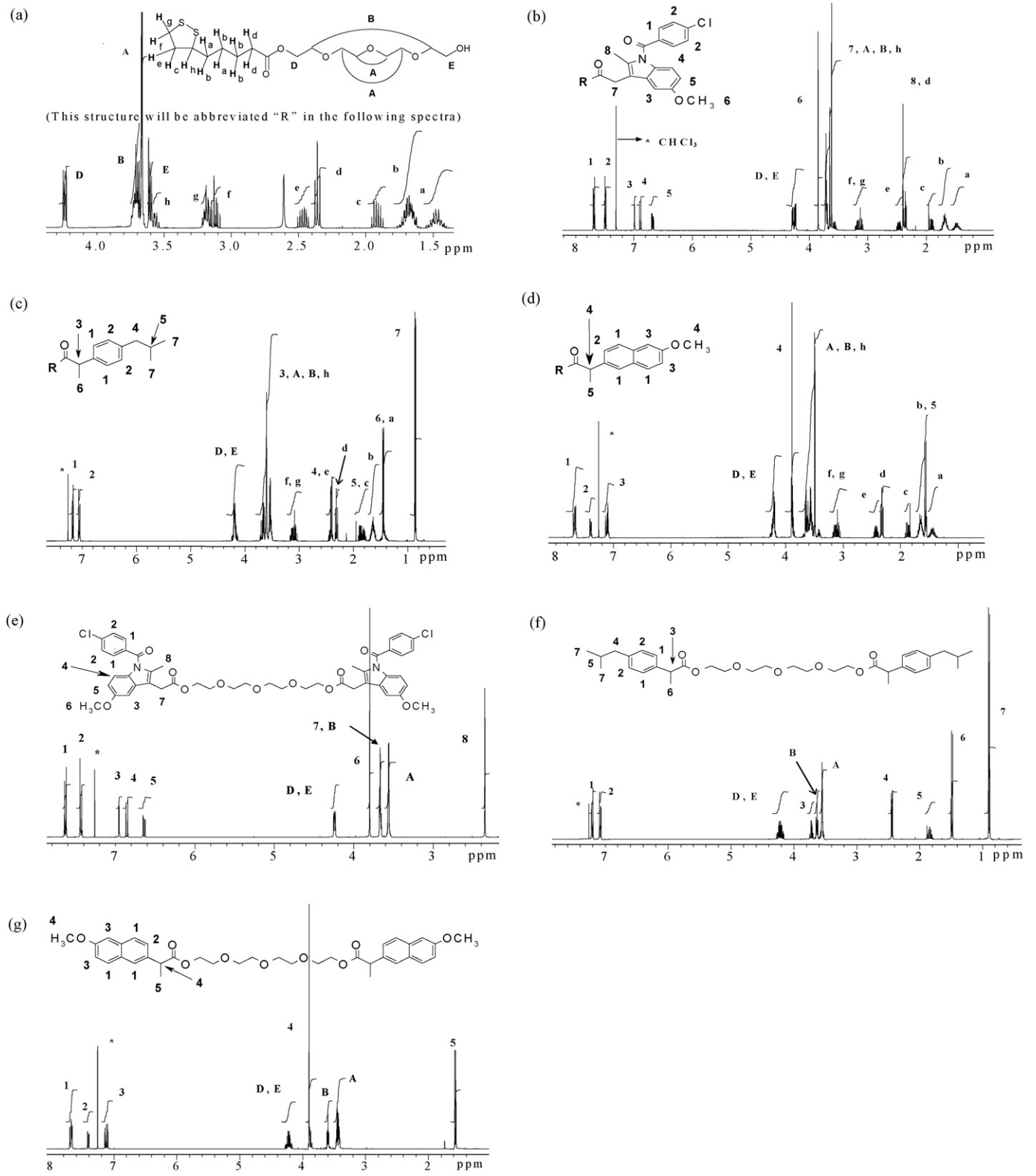


Fig. 1. ^{1}H NMR spectra of the NSAID derivatives. (a) ALA-TEG-OH; (b) ALA-TEG-Ind; (c) ALA-TEG-Ibu; (d) ALA-TEG-Npx; (e) Ind_2TEG; (f) Ibu_2TEG; (g) Npx_2TEG.

(Fessi et al., 1989; Chouinard et al., 1991; Bouchemal et al., 2004b).

In this study, formulation parameters were kept constant as described in Section 2.6.1 to evaluate the influence of the different NSAIDs derivatives on the size and stability of the nanoprodrugs. We prepared six nanoprodrugs from the three bifunctional derivatives of ALA and NSAIDs and from the three dimeric NSAID derivatives. Upon addition of the solutions of the derivatives in acetone into the aqueous phase, nanoprodrugs formed spontaneously. The hydrodynamic size was within the range of 150–300 nm and found to be compound specific (Table 2). The size of the nanoprodrugs was significantly larger when prepared from the naproxen derivatives

Table 1
RP-HPLC retention time of the derivatives of NSAIDs.

NSAIDs derivatives	Retention time (min)	Detection (nm)
ALA-TEG-Ind	7.84	330
ALA-TEG-Ibu	9.63	254
ALA-TEG-Npx	6.51	330
Ind_2TEG	10.3	330
Ibu_2TEG	17.2	220
Npx_2TEG	6.80	254

($p < 0.05$). The size of the nanoprodrugs from the dimeric derivatives was significant smaller ($p < 0.01$) than the size of the nanoprodrugs from the bifunctional derivatives, suggesting that a more compact steric arrangement of the symmetrical dimeric derivatives led to the formation of the smaller nanoprodrugs. We also found that, except for the nanoprodrug from Ind_2TEG, the size decreased with increasing retention time in RP-HPLC (Table 1), suggesting a dependence of the size on the hydrophobicity of the prodrug molecules. The retention time in RP-HPLC may be useful to assess the hydrophobicity of the compounds (Hammers et al., 1982; Hafkenscheid and Tomlinson, 1984). Considering the combined results of the retention time in RP-HPLC and size measurement, it can be assumed that the size decreases with increasing hydrophobicity of the compounds, probably due to a more stronger hydrophobic interaction between the molecules.

3.3.2. Stability of nanoprodrugs in SGF and SIF

The stability of all the nanoprodrugs studied was examined in SGF and SIF. The samples were incubated at 37 °C on a rotatory shaker for 3 days. To evaluate the stability, the hydrodynamic size and amount of intact prodrugs were measured after 3 days. In SIF at pH 6.8, the size slightly increased except for the nanoprodrugs from ALA-TEG-Ibu and Ibu_2TEG (Fig. 2a). In SGF at pH 1.2, the increases were significant larger than those in SIF ($p < 0.05$). Nevertheless, the size of the nanoprodrugs increased in both SIF and SGF, indicating that the nanoprodrugs tend to swell rather than disintegrate.

In order to maintain the antioxidant activity and oxidation responsiveness of the nanoprodrugs, the dithiolane ring moiety of the ALA should remain intact. It has been reported that the reactivity and instability of the 1,2-dithiolane moiety of ALA result in a considerable intermolecular polymerization (Wagner et al., 1956). Thus, the maintained functionality of the dithiolane ring moieties along with the physical stability of the nanoprodrugs is the basis for the development of the antioxidant and stimuli-responsive nanoprodrugs. The amount of the intact prodrug molecules was quantified after 3 days of incubation in SIF and SGF. As shown in Fig. 2b, 70–90% of the compounds remained intact after the incubation. The difference between SIF and SGF was not significant ($p > 0.05$). In the supernatant, no prodrugs were detected, which is in agreement with the observed insolubility of the prodrugs in aqueous solution (Section 3.2). Considering the results of the size measurement and chemical stability, it is concluded that the method of spontaneous emulsification produces nanoprodrugs that are stable in SIF and SGF. It is believed that the observed chemical and physical stability of the nanoprodrugs can be ascribed to the strong assembly of the hydrophobic prodrug molecules which reduces the interaction with water, consequently increasing the structural integrity of the nanoprodrugs and thus decreasing the chemical degradation of the prodrugs in the aqueous environment.

Table 2
Size and polydispersity index (P.I.) of the nanoprodrugs ($n = 3$, ±S.D.).

NSAIDs derivatives	Size (nm)	P.I.
ALA-TEG-Ind	253 ± 25	0.09 ± 0.05
ALA-TEG-Ibu	251 ± 13	0.10 ± 0.03
ALA-TEG-Npx	298 ± 6	0.05 ± 0.01
Ind_2TEG	159 ± 10	0.06 ± 0.03
Ibu_2TEG	186 ± 11	0.13 ± 0.02
Npx_2TEG	259 ± 9	0.06 ± 0.02

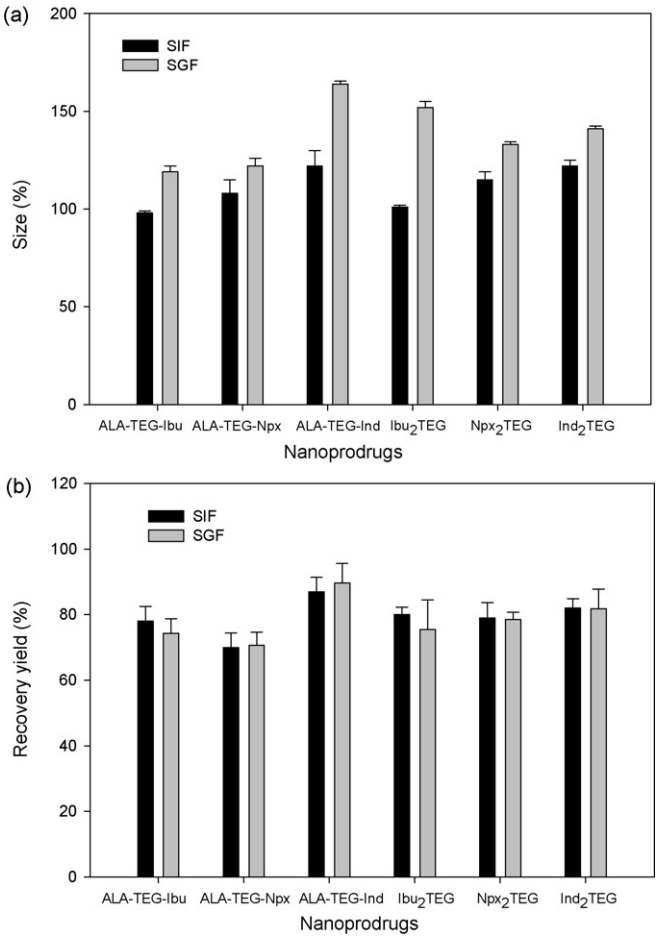


Fig. 2. Stability of nanoprodrugs in SIF pH 6.8 and SGF pH 1.2. The results are calculated as the percentage of size (a) and prodrugs (b) with 100% equal to the size and amount of prodrugs before incubation. The results are the mean ± S.D. of three experiments.

3.4. Oxidative destabilization of nanoprodrugs

The antioxidant properties and oxidation-responsiveness of the nanoprodrugs are attributed to the dithiolane ring system of ALA. The ring system can scavenge a variety of ROS, which leads to the formation of thiosulfinate and thiosulfonate (Biewenga et al., 1994; Trujillo and Radi, 2002). Therefore, it was expected that the oxidation would make the ALA-containing NSAID prodrugs less hydrophobic, which would lead to the destabilization of the nanoprodrugs.

In this study, we used hypochlorous acid (HOCl) as the oxidant to elucidate the antioxidant properties and oxidative destabilization of the nanoprodrugs. In the presence of physiological concentration of chloride ions, H_2O_2 is efficiently halogenated by the inflammatory enzyme myeloperoxidase (MPO) to yield HOCl (Krasowska and Konat, 2004; Malle et al., 2007). H_2O_2 is not particularly toxic as it is not acutely reactive against many biologically important molecules, but the cytotoxicity was greatly enhanced by converting of up to 80% of the H_2O_2 generated by activated neutrophils into the highly reactive HOCl (Weiss et al., 1982; Foote et al., 1983; Babior, 2000; Hussien et al., 2002). HOCl is by far the most abundant oxidant generated by activated phagocyte cells and is a powerful oxidizing

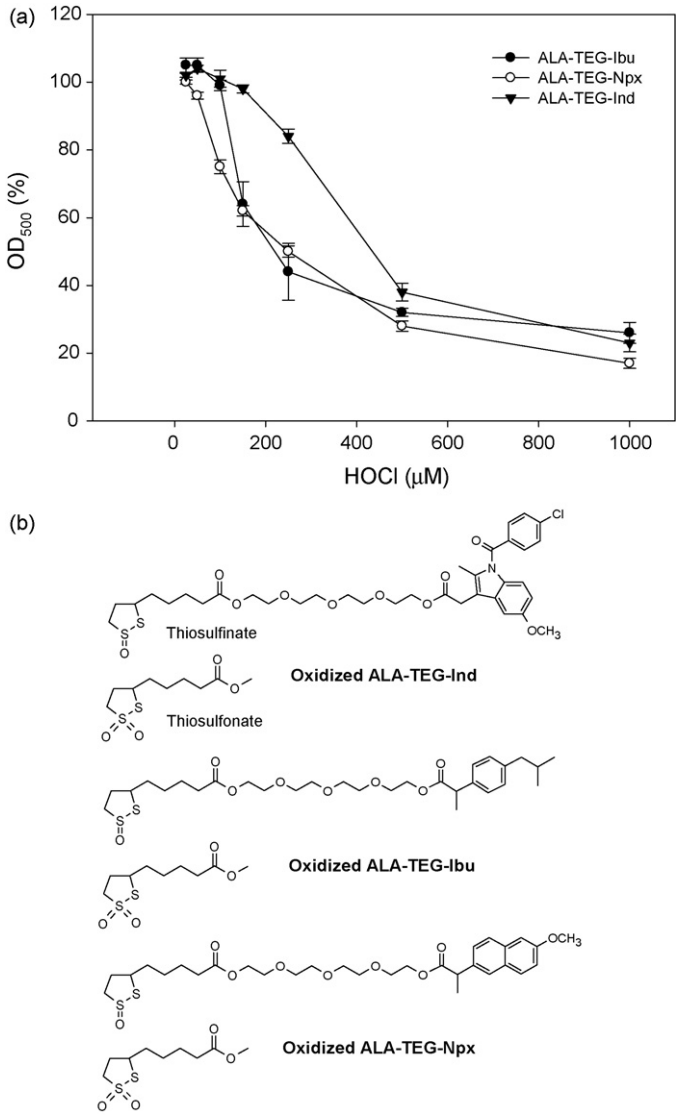


Fig. 3. Oxidative destabilization of nanoprodrugs. The results are calculated as the percentage of prodrugs with 100% equal to the amount of prodrugs prior to the addition of HOCl. The results are the mean ± S.D. of three experiments.

agent that can react with many biological molecules (Winterbourn, 2002; Krasowska and Konat, 2004; Messner et al., 2006; Spickett, 2007; Yap et al., 2007). It has been also reported that HOCl can be easily converted into a hydroxyl radical (•OH) by a reaction with superoxide radical ($O_2^{\bullet -}$) and ferrous iron (Fe^{2+}) (Candeias et al., 1993).

To demonstrate the antioxidant properties and oxidative destabilization of the nanoprodrugs, the optical density of the nanoprodrug suspension (250 μM) was measured in the presence of HOCl (25–1000 μM) at 500 nm (OD). In the absence of HOCl, no changes in OD were observed during an incubation for 24 h (data not shown). In addition, the nanoprodrugs lacking ALA (Ind_2TEG, Ibu_2TEG, and Npx_2TEG) did not show any changes in OD (data not shown).

As expected, because of the oxidation of the dithiolane ring system of ALA unit in the bifunctional derivatives of NSAIDs a concentration-dependent reduction in turbidity was observed (Fig. 3a). At lower HOCl concentrations, the reduction was smaller, progressing to a stronger reduction in the higher HOCl concentrations. This can be explained by an induction phase in which the prodrug molecules on the surface scavenge HOCl, which is not sufficient to elicit a destabilization of the nanoprodrugs. After the induction phase, the nanoprodrugs begin to destabilize as the surface molecules scavenge further HOCl to become more and more hydrophilic. The increase in hydrophilicity is ascribed to the formation of thiosulfinate and thiosulfonate after scavenging one and two HOCl molecules per dithiolane ring system, respectively (Biewenga et al., 1994; Napoli et al., 2004). Fig. 3b shows the proposed structures of the oxidized prodrugs. The formation of thiosulfinate would increase the hydrophilicity, which may be not enough to destabilize the nanoprodrugs. Upon scavenging the second HOCl molecule and formation of the thiosulfonate, the more increased hydrophilicity and modified molecular structure of the prodrugs may cause a rapid disintegration of the oxidized prodrugs from the surface, accounting for the burst effect observed after the induction phase.

The amount of HOCl for the three nanoprodrugs to overcome the induction phase was increased in the order of ALA-TEG-Npx, ALA-TEG-Ibu, and ALA-TEG-Ind. The induction phase is less apparent for the nanoprodrug of ALA-TEG-Npx and a rapid reduction was observed after each additional HOCl. This may be ascribed to the relatively lower hydrophobicity of ALA-TEG-Npx which can be assessed from the retention time in RP-HPLC (Table 1). However, comparing the two nanoprodrugs prepared from ALA-TEG-Ibu and ALA-TEG-Ind, the nanoprodrug of ALA-TEG-Ind showed the longest induction phase and the overall reduction in turbidity was less and slower, although it showed a shorter retention time in RP-HPLC and thus was assessed to be less hydrophobic. This is not in agreement with the assumption that the nanoprodrug prepared from less hydrophobic prodrug may destabilize more quickly upon oxidation.

The oxidative destabilization process may involve the disintegration of the oxidized prodrugs from the surface of nanoprodrugs. The additional increase in hydrophilicity after each oxidation is assumed to be the same for all the three prodrugs. Therefore, in order to explain the observed discrepancy, the steric nature of the oxidized and non-oxidized prodrugs has been taken into account. As shown in Fig. 3b, the structural changes of the prodrugs are related to the formation of thiosulfinate and thiosulfonate, which may cause a steric hindrance and thus enhance the disintegration process of the oxidized prodrugs. Although the same structural changes are introduced to the prodrugs and thus the changes in their steric nature are of same magnitude, the relative effect of these changes could be different between the prodrugs because of the different structure of the NSAIDs. Based on this consideration, it can be hypothesized that the slower destabilization in case of ALA-TEG-Ind may be due to a smaller relative increase in steric hindrance which can be ascribed to the dominant contribution of the bulkier indomethacin to the steric nature of the molecule, making the effect of the ALA oxidation less pronounced (Fig. 3b).

3.5. Hydrolytic activation of nanoprodrugs

In order to evaluate potential applications of the NSAIDs prodrugs as nanoprodrugs, the water-insoluble hydrophobic prodrugs were formed into nanoprodrugs and the enzymatic reconversion of the prodrugs into the parent drugs was investigated. The formation into the nanoprodrugs may generate a large surface area on which the interaction between hydrolytic enzymes and prodrugs can take place (Huang et al., 2003; Heckert et al., 2008). This interaction would be otherwise impossible due to the insolubility of the prodrugs in aqueous media (Section 3.2).

According to the molecular design based on ester bonds, the derivatives were expected to be degraded by enzymatic hydrolysis. However, due to the water-insolubility of the compounds, enzymatic hydrolysis could not be measured in an aqueous solution. A

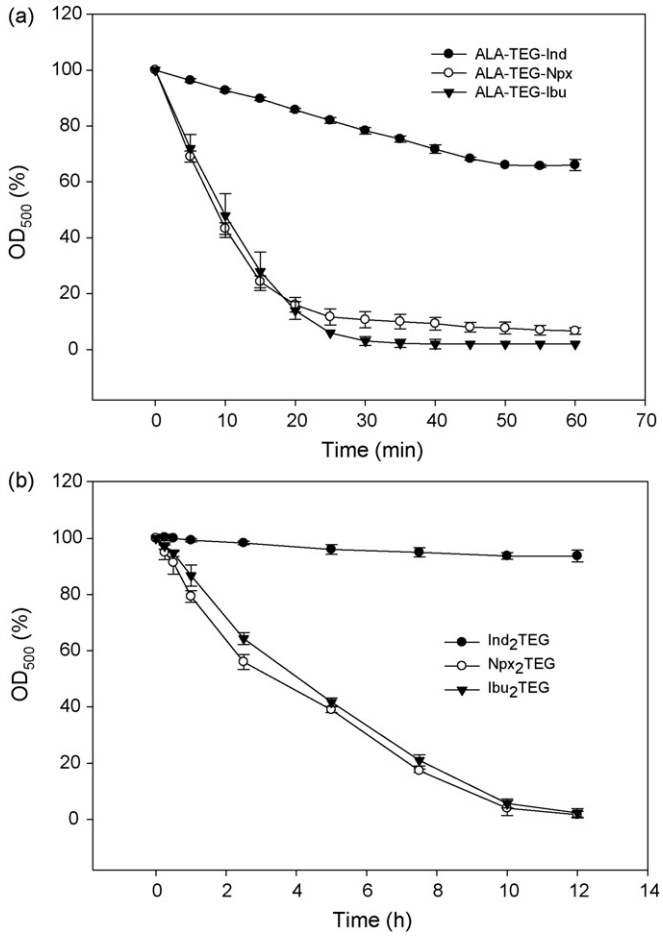


Fig. 4. Enzymatic destabilization of nanoprodrugs. The results are calculated as the percentage of OD with 100% equal to the OD prior to the addition of esterase. The results are the mean ± S.D. of three experiments.

key feature of the nanoprodrugs is that water-insoluble prodrugs can be transported through the aqueous physiological environment once transformed into stable nanoprodrugs.

We expected that the NSAIDs would be released by enzymatic ester hydrolysis of the prodrugs from the surface, which would erode the nanoprodrugs gradually, and ultimately destabilize the nanoprodrugs. Porcine liver esterase was used to establish the *in vitro* susceptibility of the prodrugs because it is stable and capable of hydrolyzing a wide range of esters (Foroutan and Watson, 1999). This enzyme has been widely used to estimate the *in vitro* enzymatic activation of ester prodrugs of NSAIDs (Redden et al., 1999; Bonina et al., 2001; Velázquez et al., 2007).

In order to demonstrate the enzymatic hydrolysis, we monitored the changes in OD of the nanoprodrug suspensions from the three bifunctional derivatives of ALA and NSAIDs and from the three dimeric derivatives of NSAIDs. The amount of released drugs was determined at the end of the incubation using RP-HPLC as described in Section 2.9. Because of the different structures of the three prodrugs, a different rate of enzymatic hydrolysis and changes in OD were expected. As shown in Fig. 4a, the nanoprodrug from the indomethacin derivative was more stable compared with the nanoprodrugs prepared from the derivatives of ibuprofen and naproxen. The concentrations of the released drugs were 194 μM, 417 μM and 454 μM for the nanoprodrugs containing ALA-TEG-Ind, ALA-TEG-Ibu and ALA-TEG-Npx, respectively, corresponding to 39%, 83% and 91% in drug release. This relationship was repeated in the nanoprodrugs from the three dimeric derivatives of NSAIDs (Fig. 4b). Obviously, the rates of hydrolysis for the relatively smaller prodrugs of ibuprofen and naproxen were greater than the rate of hydrolysis for the bulkier indomethacin prodrugs, indicating that steric hindrance was important (Redden et al., 1999).

Interestingly, the enzymatic destabilization of the nanoprodrugs from Npx_2TEG and Ibu_2TEG (Fig. 4b) was much slower when compared to those of the nanoprodrugs from ALA-TEG-Npx and ALA-TEG-Ibu (Fig. 4a), suggesting that the replacement of one ibuprofen and naproxen with ALA has a profound effect on the enzymatic hydrolysis. After a 1 h-incubation, less than 10% of parent drugs were hydrolyzed from the nanoprodrugs of Npx_2TEG and Ibu_2TEG. In comparison, approximately 90% of drugs were released from the nanoprodrugs of ALA-TEG-Npx and ALA-TEG-Ibu. After a 12 h-incubation, the concentrations of the released drugs from Npx_2TEG and Ibu_2TEG were comparable with those from ALA-TEG-Npx and ALA-TEG-Ibu after a 1 h-incubation. According to the assessment of the hydrophobicity based on the retention time in RP-HPLC (Table 1), the dimeric derivatives are more hydrophobic than the bifunctional derivatives. Therefore, the increased rate of hydrolysis may be attributed to the less hydrophobic nature of the bifunctional prodrugs (Iley et al., 1997).

The stability of all the nanoprodrugs studied was examined in the absence of the enzyme to determine whether the nanoprodrugs were subjected to non-enzymatic degradation or auto-oxidation. The nanoprodrugs were incubated and analyzed under the same condition except for the omission of enzyme. All the nanoprodrugs were found to be stable for the length of the assay (data not shown). Considering the combined results of the enzymatic destabilization and the stability in SIF, SGF and PBS, it can be concluded that the prodrugs are considerably resistant to chemical degradation in aqueous solutions, and enzyme-mediated activation might be required to generate a significant level of parent drugs *in vivo*.

3.6. Sequence of enzymatic hydrolysis

In order to elucidate the different rates of hydrolysis between the nanoprodrugs observed in Fig. 4, the sequence of enzymatic hydrolysis from the nanoprodrugs was studied. We hypothesized that the increased rate of hydrolysis may be due to the less hydrophobic nature of the bifunctional derivatives.

The sequence of the enzymatic hydrolysis was evaluated by measuring the released NSAIDs and other hydrolyzed species in the supernatant using RP-HPLC (Section 2.10). The total amount of NSAIDs and thus the peak intensity were kept constant in the first and second chromatogram by separating the intact nanoprodrugs from the reaction mixture and taking the first and second chromatogram from the same supernatant with an interval of 30 min. In this way, the obtained chromatograms showed a comparable peak intensity and, more importantly, the further hydrolytic degradation of the same set of hydrolyzed species could be analyzed.

In Fig. 5a–c, the first chromatograms (solid line) were taken from the supernatant after incubation for 5 min and separation of the intact nanoprodrugs from the reaction mixture. The composition of the supernatant is similar to the original mixture except for the hydrolyzed species and omission of the intact nanoprodrugs. After taken first chromatogram, the supernatant was incubated for an additional 30 min and the second chromatogram (dotted lines) was taken. As depicted schematically in Fig. 5d, ALA was hydrolyzed first (pathway B) followed by ibuprofen, naproxen and indomethacin, indicating that ALA is more accessible to the enzymes. The results suggest that the replacement one NSAID molecule with ALA leads to the increase in hydrophilicity and decrease in steric hindrance towards the enzymes, consequently increasing the rate of hydrolysis of the prodrugs (Iley et al., 1997; Redden et al., 1999).

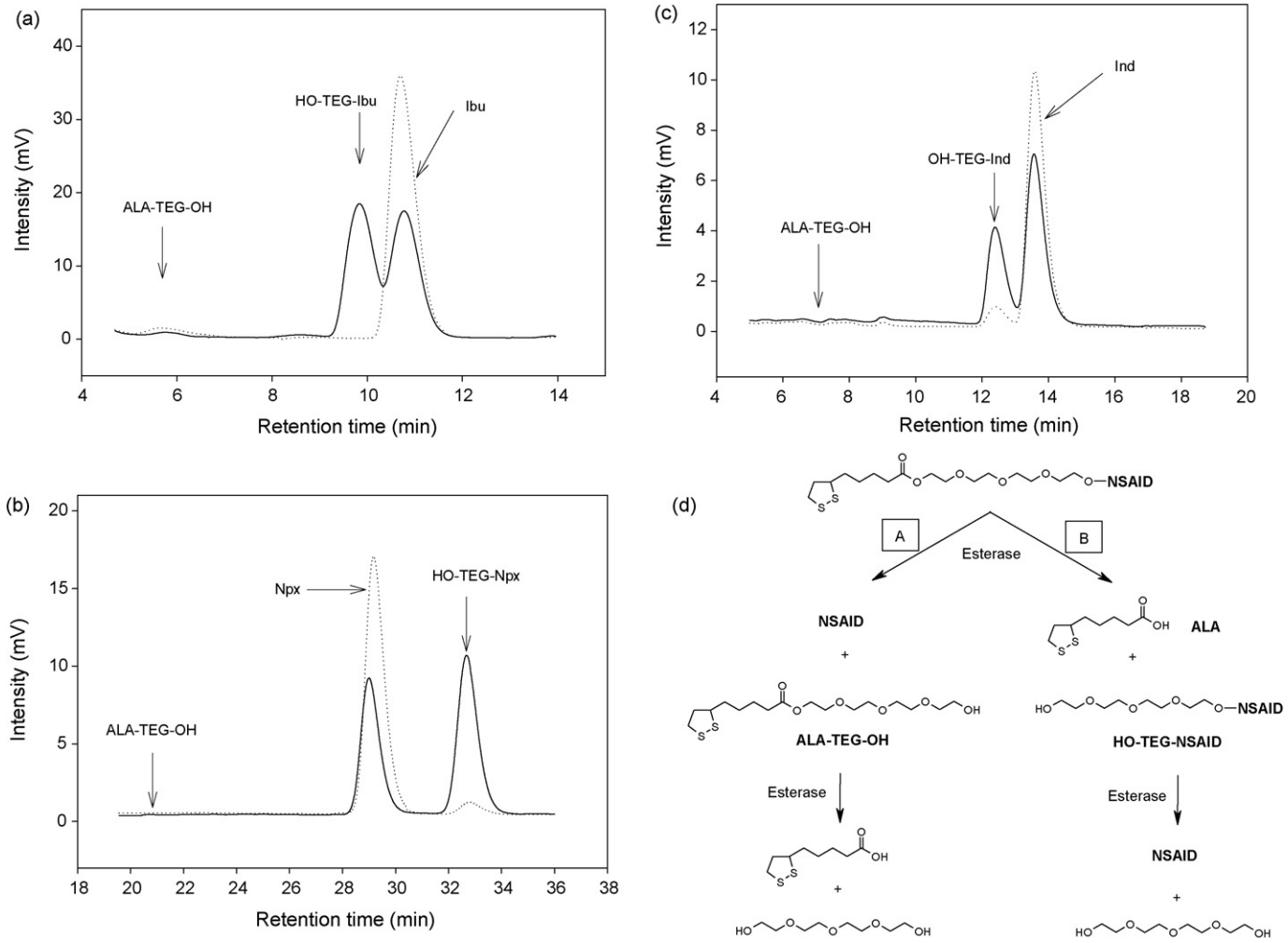


Fig. 5. Sequence of enzymatic hydrolysis.

3.7. Influence of oxidation on enzymatic prodrug activation

3.7.1. Recovery of NSAIDs from oxidized nanoprodrugs

After oxidative and enzymatic destabilization of the nanoprodrugs were studied separately, in next step, we evaluated the influence of the oxidation of nanoprodrugs on the enzymatic prodrug activation. First, we examined the chemical stability of the NSAID prodrugs in the presence of HOCl. The oxidative degradation of the prodrugs was determined by measuring the amount of intact prodrugs after HOCl treatment. After treatment of the nanoprodrugs with a two-fold molar excess of HOCl in PBS (pH 7.4), no intact prodrugs were detected by RP-HPLC. HOCl scavenging by

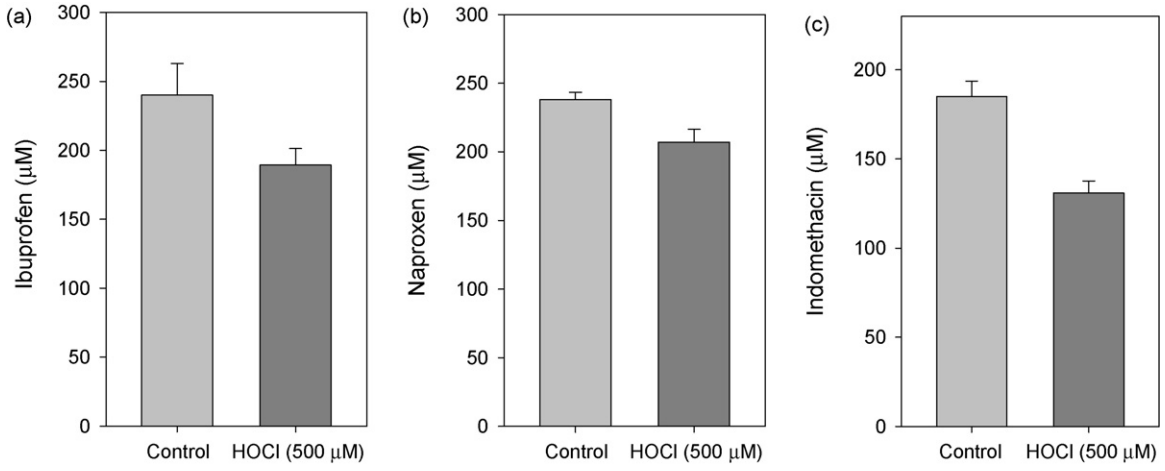


Fig. 6. Enzymatic hydrolysis of NSAIDs from oxidized nanoprodrugs. The results are the mean ± S.D. of three experiments.

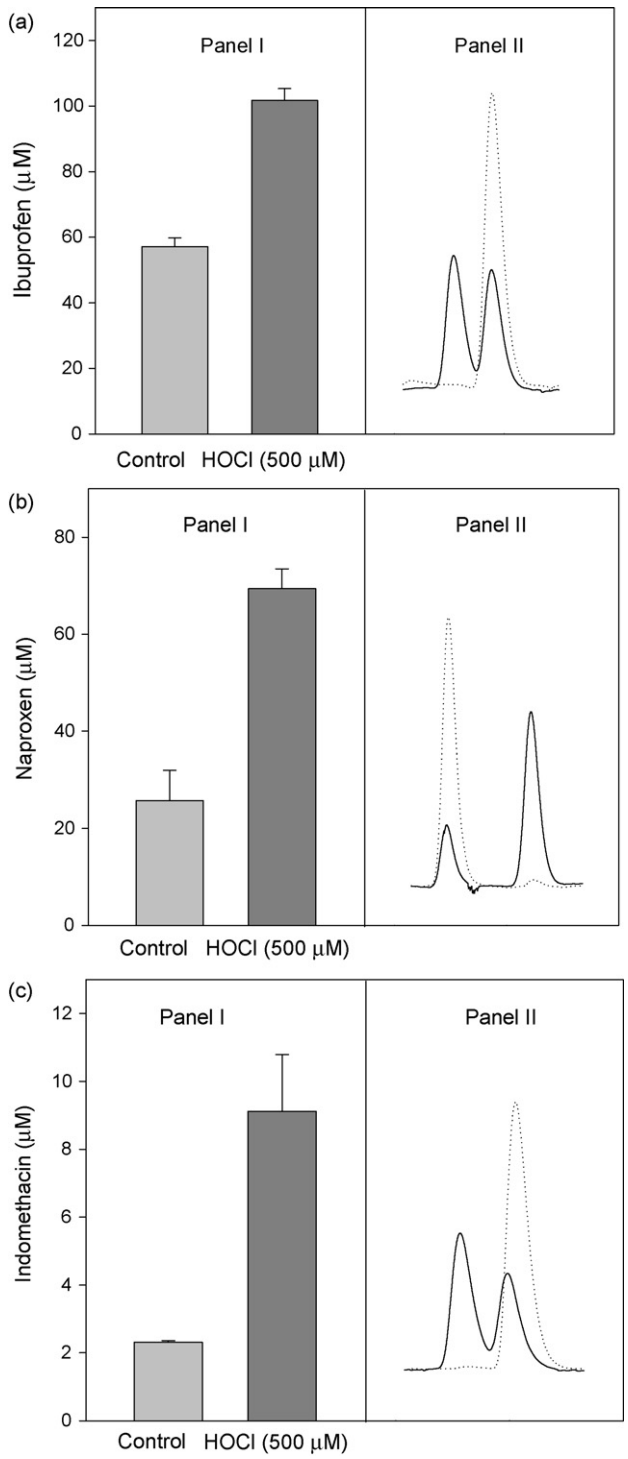


Fig. 7. Influence of oxidation on the rate (panel I) and sequence (panel II) of enzymatic hydrolysis. The results are the mean ± S.D. of three experiments.

the nanoprodrugs occurred instantaneously and completely as evidenced by DTNB assay (Section 2.8). After incubation for 2 min with a twofold molar excess of HOCl, no remaining HOCl was detected, indicating that one ALA moiety scavenged at least two molecules of HOCl (Biewenga et al., 1994). To see whether these completely oxidized prodrugs are still available as prodrugs of the NSAIDs, the HOCl-treated nanoprodrugs were incubated in the presence of esterase. In comparison with non-oxidized controls, the released parent drugs from the oxidized nanoprodrugs were 80% ($p < 0.05$), 87% ($p < 0.01$) and 71% ($p < 0.001$) for the nanoprodrugs from ALA-TEG-Ibu, ALA-TEG-Npx and ALA-TEG-Ind, respectively (Fig. 6). This study shows that the ALA-containing derivatives of NSAIDs can serve as prodrugs even after the ALA moieties have been oxidized completely.

3.7.2. *Effect of nanoprodrug oxidation on the rate of enzymatic hydrolysis*

Obviously, the oxidation of the prodrugs results in the destabilization of the nanoprodrugs which is attributed to the decreased hydrophobicity of the oxidized prodrugs, consequently increasing the solubility in an aqueous environment. It can be hypothesized that the oxidation of the prodrugs on the surface and their increased hydrophilicity may lead to an expulsion of the oxidized prodrugs and make the otherwise tightly assembled surface structure loose. This may change the morphology and surface structure of the nanoprodrugs, which strongly influences the interaction between the oxidized prodrugs and hydrolytic enzymes.

The effect of oxidation on the rate of enzymatic hydrolysis is shown in Fig. 7. Although the total amount of available prodrugs was higher in the non-oxidized nanoprodrugs (Fig. 6), the amount of hydrolyzed drugs from the oxidized nanoprodrugs was significant higher when it was determined immediately after the addition of esterase and separation of the intact nanoprodrugs from the reaction mixture. The amount of hydrolyzed drugs was increased approximately two, three and four times for ALA-TEG-Ibu ($p < 0.001$), ALA-TEG-Npx ($p < 0.001$) and ALA-TEG-Ind ($p < 0.01$), respectively. Noteworthy, the selectivity for the oxidized nanoprodrugs increased with the decreasing rate of hydrolysis for the non-oxidized nanoprodrugs. The amount of hydrolyzed drugs from the non-oxidized nanoprodrug of ALA-TEG-Ind is about 10 and 25 times less than those of ALA-TEG-Ibu and ALA-TEG-Npx, respectively, which makes the effect of the oxidation more pronounced for ALA-TEG-Ind. It has been also found that the sequence of hydrolysis was similar to those of the non-oxidized prodrugs (Panel II).

These findings confirm the previous assumption that the oxidation of the ALA-containing prodrugs support the enzymatic hydrolysis probably by making the oxidized prodrugs more accessible to the enzymes, which can be attributed to the increased hydrophilicity and water-solubility (Napoli et al., 2004) and less compact assembling of the oxidized prodrugs.

It should be noted that the observed pattern and rate of the hydrolysis under the *in vitro* condition may not reflect *in vivo* fate of the nanoprodrugs. Considering the rate of hydrolysis in the absence of the oxidant, it can be expected that a considerable amount of nanoprodrugs might be hydrolyzed before they have reached the site where ROS are overproduced. In this regard, the more selective nanoprodrug of ALA-TEG-Ind would be an appropriate candidate for *in vivo* study. The results also provide a rational approach for further development of prodrugs, based on the hydrophobicity, in order to obtain nanoprodrugs with desired stability, rate of hydrolysis and selectivity.

4. Conclusion

In this work, novel stimuli-responsive antioxidant nanoprodrugs of NSAIDs were prepared by spontaneous emulsification of hydrophobic derivatives of NSAIDs. We demonstrated their antioxidant activity, oxidant responsiveness and enzymatic activation. Despite the highly hydrophobic nature of the derivatives, NSAIDs were readily hydrolyzed enzymatically from the nanoprodrugs, and the hydrolysis was accelerated when the nanoprodrugs were destabilized upon ROS scavenging. The unique interaction between the oxidative destabilization and enzyme reactivity characterizes this novel family of ROS-sensitive anti-inflammatory nanopro-

drugs. The nanoprodrugs may be used as anti-inflammatory and antioxidant drug delivery vehicles. Whenever the drug combination is favorable to the treatment of diseases, the antioxidant and anti-inflammatory properties of the nanoprodrugs may increase the therapeutic effect of the delivered drugs and reduce ROS-related adverse effects. Notably, the design and synthesis of water-insoluble hydrophobic prodrugs and their preparation into nanoprodrugs may create a new paradigm in the prodrug strategy.

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