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Preparation and characterization of antioxidant nanospheres from multiple α-lipoic acid-containing compounds

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ABSTRACT

The purpose of this study was to prepare and characterize antioxidant nanospheres composed of multiple α-lipoic acid-containing compounds (mALAs). It was found that the nanospheres were remarkably stable under physiologic conditions, maintained the antioxidant property of α-lipoic acid, and could be destabilized oxidatively and enzymatically. The preparations were simple and highly reproducible providing a new strategy for the development of nanometer-sized antioxidant biomaterials. The nanospheres may find applications as antioxidant therapeutics and oxidation-responsive antioxidant nanocontainers in drug delivery for pathological conditions characterized by oxidative stress including cancer and neurodegenerative diseases.

The development of nanostructured biomaterials with antioxidant properties has received significant attention in recent years. The most remarkable property of nanostructured antioxidant biomaterials is their improved bioavailability which can be ascribed to the generation of an enlarged surface area by transformation of bulk antioxidant materials into the nanometer-sized structures.[1,2] It is well known that the surface-to-volume ratio increases with decreasing size of the nanostructures, which improves the bioavailability and enhances the biological efficacy of the materials.[3] The other advantage of nanostructures is that water-insoluble lipophilic antioxidant molecules can be transported more efficiently in the aqueous physiological environment when formed into stable nanostructures.[4]

Several approaches have been developed to prepare antioxidant nanostructures including polymeric and lipid nanoparticles loaded with antioxidant molecules,[5,6] metal nanoparticles or fullerene with surface modification,[7–9] metal–polymer nanocomposites,[10,11] and metal or metal oxide nanoparticles possessing intrinsic antioxidant properties.[12,13] Another possible approach is to form nanospheres using a spontaneous emulsification of hydrophobic antioxidant molecules.[14] The preparation of the water-insoluble antioxidant molecules into nanospheres may produce a large surface area possessing antioxidant activity.

Due to its potent antioxidant activity,[15–17] we have chosen α-lipoic acid (ALA) as the antioxidant component of new nanostructured antioxidant biomaterials. It has been reported that ALA displays prominent antioxidant activity as a scavenger of hydroxyl radicals (·OH),[17] hypochlorous acid (HOCl),[18] trichloromethylperoxyl radical ($CCl_3O_2\cdot$),[19] the reactive nitrogen species peroxynitrite (ONOO),[20] and as a metal chelator by chelating Fe^{2+} and Cu^{+}.[21] Several recent studies have also shown that ALA has a neuroprotective effect against reactive oxygen species (ROS)-induced damages.[16,22,23] In addition, ALA inhibits the formation of β-amyloid fibrils (fAβ), destabilizes the preformed fAβ, and protects neurons against cell death induced by amyloid.[24]

Here, we present the first example of antioxidant nanostructured biomaterials composed of multiple α-lipoic acid-containing compounds (mALAs).

The six hydrophobic mALAs were designed with the goal of constructing antioxidant nanospheres. The esterification of ALA with poly(ethylene glycols) and alkane diols with varying chain length has been reported.[25–27] The synthesis of the compounds by the coupling of ALA to the core polyols via ester bonds is straightforward as described in Scheme 1. The polyols **1**–**6** with two to four hydroxyl groups were reacted with ALA to yield the six mALAs **1a**–**6a**. The purity of the compounds **1a**–**6a** was confirmed by TLC and RP-HPLC. The RP-HPLC elution times of the compounds **1a**–**6a** are summarized in Table S1 (Supplementary Data). The structures were confirmed by ^{1}H and ^{13}C NMR spectroscopy (Supplementary Data: Section 1.2, Figs. S1–S13 and Table S2).

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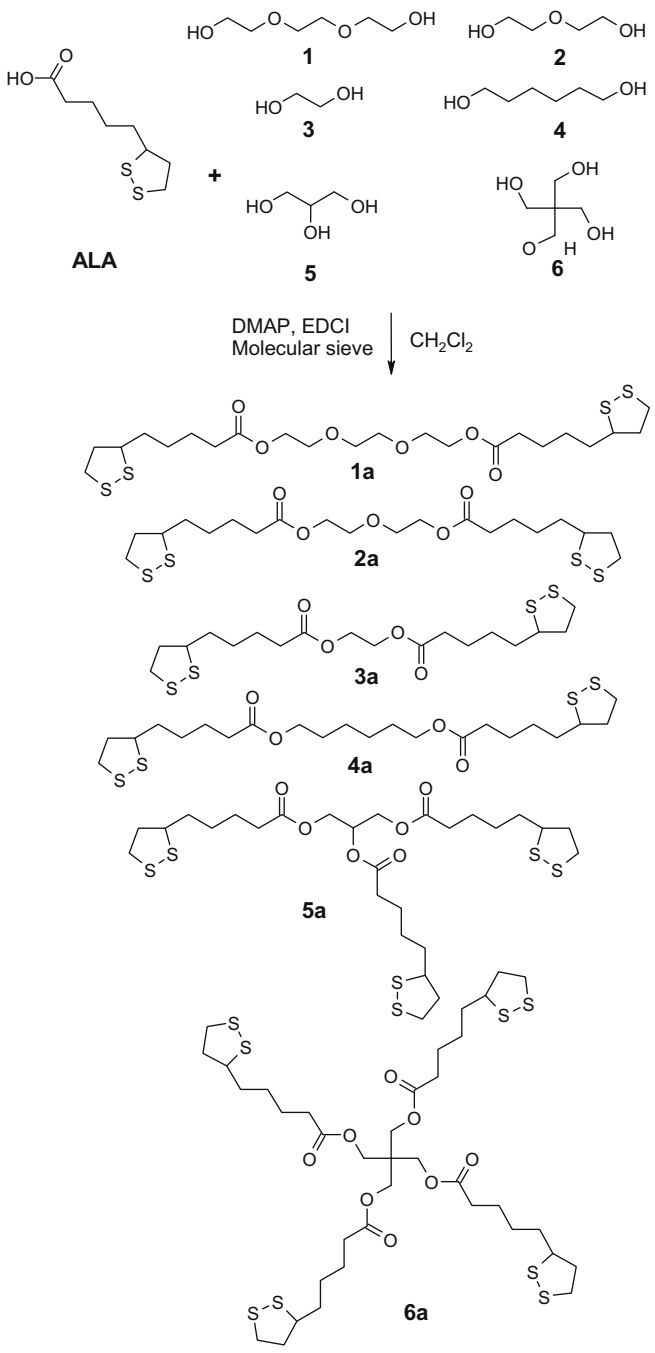

Scheme 1. Synthesis of the multiple α-lipoic acid-containing antioxidant compounds (mALAs) **1a**–**6a**.

Nanospheres were prepared according to a method which utilizes spontaneous emulsification with slight modifications.[14,28] Experiments were performed to investigate the formation of nanospheres from the six mALAs, and the influence of hydrophobicity on the size and stability of the nanospheres. The size was within the range of 200–600 nm and found to be compound specific (Supplementary Data: Section 1.3 and Table S3). In general, the effective hydrodynamic size depended on the hydrophobicity of the compounds. We found that the size decreased with increasing hydrophobicity of the compounds which could be assessed from the retention time in RP-HPLC.[29,30]

To assess the physical and chemical stability of the nanospheres in physiologic conditions, the nanospheres were incubated in phosphate buffered saline (PBS, pH 7.4) at 37 °C for 2 weeks and the size as well as the amount of the intact mALAs were measured. The size decreased for the nanospheres **1**–**3** and slightly increased for the nanospheres **4**–**6**. This suggests that the nanospheres from less hydrophobic compounds tend to erode from the surface leading to the decrease in size whereas the nanospheres from more hydrophobic compounds tend to swell (Fig. 1). The results may indicate that the nanospheres made from more hydrophobic compounds are more stable and compact.

In order to maintain the antioxidant activity of the nanospheres, the dithiolane ring moiety of ALA should remain intact. The functionality of the dithiolane was quantified by measuring the amount of intact compounds **1a**–**6a** in the nanospheres after 7 and 14 days of incubation in PBS at 37 °C. Compounds (50–70% and 30–60%) remained intact after incubation of 7 and 14 days, respectively (Fig. 2).

The physical stability of the nanospheres along with the maintained functionality of the dithiolane provides a particularly attractive basis for the development of antioxidant drug delivery devices.

The antioxidant properties and oxidation-responsiveness of the nanospheres are attributed to the capability of the dithiolane ring system to scavenge a variety of ROS leading to the formation of more hydrophilic thiosulfinate and thiosulfonate.[18,31,32] It was expected that this oxidation would make the mALAs less hydrophobic which induces destabilization of the nanospheres. To

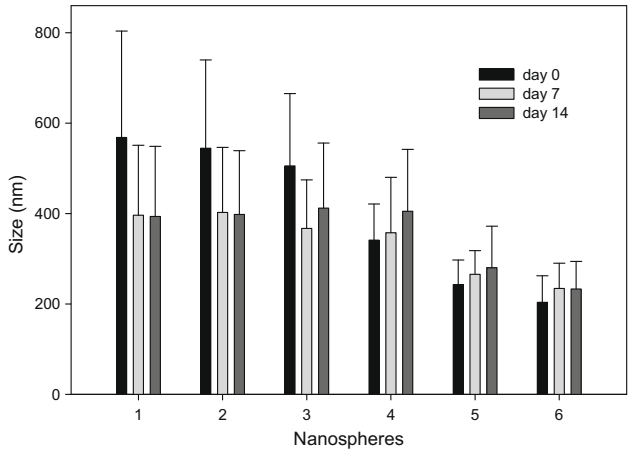

Figure 1. Size of the nanospheres after incubation at 37 °C for 7 and 14 days. Error bar represents ±SD of the size determined in triplicate.

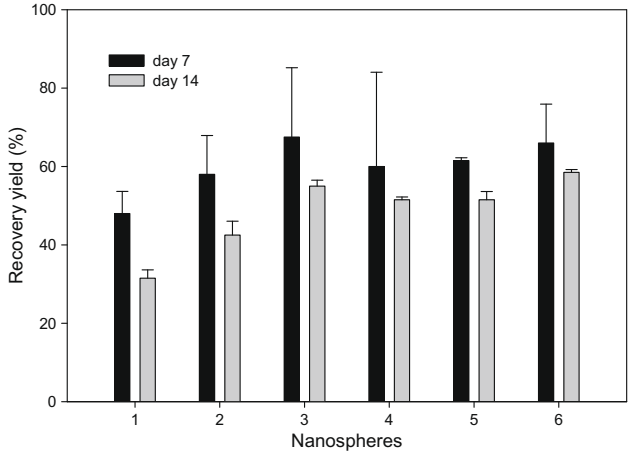

Figure 2. Recovery yields of the mALAs after incubation of the nanospheres in PBS at 37 °C. Hundred percent refers to the amount of the compounds determined before incubation. Error bar represents ±SD of the recovery yield determined in duplicate.

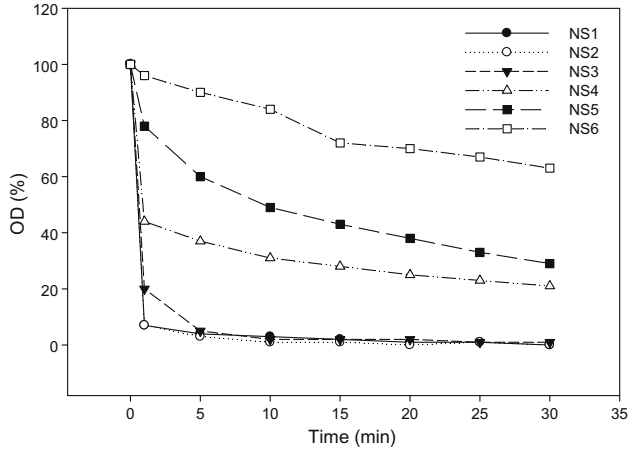

Figure 3. Oxidative destabilization of the nanospheres (NS) (500 μM ALA unit) in the presence of 1 mM HOCl. The results were calculated as the percentage of OD with 100% equal to the OD of the nanosuspensions before the addition of HOCl.

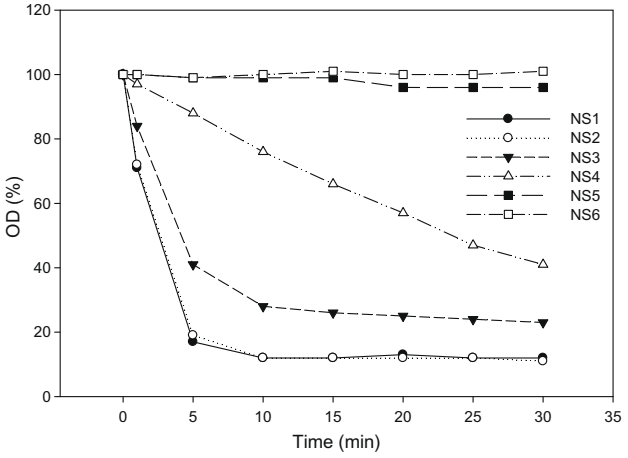

Figure 4. Enzymatic destabilization of the nanospheres (500 μM ALA unit) in the presence of esterase (5 U/mL). The results were calculated as the percentage of OD with 100% equal to the OD of the nanosuspensions before the addition of esterase.

demonstrate the oxidative destabilization in the presence of ROS, the decrease in optical density (OD) of the nanosphere suspension (nanosuspension) was measured in the presence of HOCl. In the absence of HOCl, no reduction in turbidity was observed during the incubation for 24 h (data not shown). In the presence of HOCl, the decrease in OD was immediately observed (Fig. 3). The nanospheres formed from the less hydrophobic mALAs destabilized at a faster rate and continued to a clear solution after approximately 5 min (NS1–NS3), whereas the other nanospheres did not degrade completely within the first 30 min (NS4–NS6).

According to the molecular design, the ester bonds in the mALAs were expected to be degraded by enzymatic hydrolysis. Because of water-insolubility of the compounds, the enzymatic hydrolysis was negligible in an aqueous solution (data not shown). We postulated that the hydrophobic molecules could be made more accessible to enzymes by forming them into nanospheres. This transformation would create a sufficient surface area on which the enzymatic interaction may take place.

As with HOCl, we expected the enzymatic hydrolysis to erode the nanospheres gradually and ultimately cause destabilization. Furthermore, because of the different structural complexity and hydrophobicity of the mALAs, a different rate of enzymatic hydrolysis would be expected for each nanosphere. A reduction in turbidity was immediately observed in the NS1 and NS2 and continued to an almost clear solution after approximately 30 min. NS5 and NS6 showed no appreciable decreases in OD during a similar time period (Fig. 4). The concentration of the released ALA was determined by RP-HPLC to be 468, 395, 329, 253, and 35 μM for NS1–NS5, respectively. No released ALA was detected for NS6.

In general, the nanospheres prepared from less hydrophobic compounds destabilize at a faster rate. For the NS5 and NS6, the structural complexity of the molecules with three and four ALA and the resulting larger steric hindrance seem to be the additional factor in reducing the rate of enzymatic hydrolysis.[33]

To elucidate the antioxidant capability of the nanospheres, we measured their ability to protect α_1-antiproteinase (α_1-AP) from oxidation by HOCl. HOCl is a powerful oxidizing agent that can react with many biological molecules. In the presence of physiological concentrations of chloride ions, H_2O_2 is efficiently halogenated by the heme enzyme myeloperoxidase (MPO) to yield HOCl which is by far the most abundant oxidant generated by activated phagocyte cells.[34,35] In the brain, HOCl is also believed to be converted into the most damaging hydroxyl radical.[36] HOCl can chlorinate cytosolic proteins and nuclear DNA bases as well as induce lipid peroxidation in phospholipids and lipoproteins.[37,38] Furthermore, the damage caused by HOCl to the intracellular glutathione and protein thiols are irreversible and can be replaced only by the synthesis of new molecules.[39]

α_1-AP is an important biological target for HOCl, which makes it ideal for this experiment. α_1-AP is a known inhibitor of elastase, but this effect is lost when HOCl oxidizes a critical methionine residue and converts it into a sulfoxide derivative. The inactivation of α_1-AP leads to higher elastase activity which may cause enzymatic destruction of the elastic fiber in the lung.[40]

To demonstrate the different antioxidant efficacy of the six nanospheres, we compared their ability to protect α_1-AP from HOCl-induced inactivation by varying the length of incubation time (Supplementary Data: Section 1.6). We hypothesized that the observed different rates of oxidative destabilization would represent the different HOCl scavenging reactivities of the six nanospheres. In Figure 5, 100% of elastase activity represents a complete inactivation of α_1-AP by HOCl, and 0% elastase activity represents complete HOCl scavenging by ALA or by the nanospheres. We found that it took approximately 2 min of incubation for the nanospheres NS1 and NS2, 5 min for NS3 and NS4, and 30 min for NS5 and NS6 to completely scavenge HOCl and protect α_1-AP. These observa-

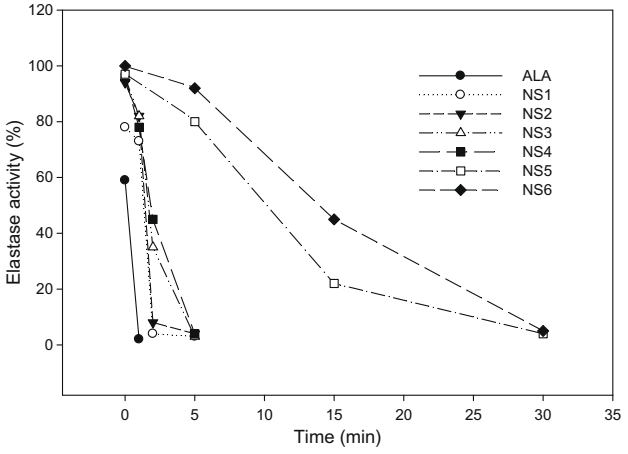

Figure 5. Incubation time-dependent protection of α_1-AP by antioxidant nanospheres and ALA against the inactivation by HOCl.

tions correlate well with the turbidity measurements (Fig. 3), which show a rapid decrease in OD for NS1 and NS2 and a much slower decrease for NS5 and NS6. Apparently, nanospheres from the more hydrophobic mALAs are more compact and stable, resulting in the reduced HOCl scavenging activity and thus reduced antioxidant protecting efficacy.

Notably, nanospheres scavenged HOCl at a slower rate in the first measured time period progressing to a much faster rate in the second measured time period. This can be explained by an induction phase[27] in which the molecules on the surface scavenge HOCl and become less hydrophobic,[32] but the amount of oxidized mALAs and the decrease in hydrophobicity may be not sufficient to overcome the stabilizing force which can be attributed to the hydrophobic interaction between the mALAs. After the induction phase, the surface molecules scavenge further HOCl and become more and more hydrophilic, which may cause a disintegration of the oxidized molecules, consequently disrupting the surface structure of the nanospheres. The increased hydrophilicity on the surface area and disrupted surface structure may allow more permeation of the aqueous HOCl solution into the nanospheres. This may accelerate the scavenging process and further disintegration of the oxidized mALAs, accounting for the rapid HOCl scavenging and thus stronger antioxidant protecting effect observed in the second measured period of this experiment.

In conclusion, a simple and versatile method for the preparation of antioxidant nanospheres from the hydrophobic mALAs has been described. The nanospheres showed remarkable physical and chemical stability, HOCl scavenging antioxidant activity and oxidative and enzymatic destabilization. Based on the results of this study, the nanospheres may be used as an antioxidant therapeutic for the prevention and treatment of oxidative stress-related diseases. Moreover, the nanospheres may be used as a drug delivery vehicle for a multitude of drugs and their antioxidant properties can offset the side effects of chemotherapeutics and other cytotoxic agents on normal cells.

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Supplementary data

Synthesis and characterization of the mALAs, preparation and characterization of the nanospheres, details of the experiments conducted, ^{1}H NMR and ^{13}C NMR spectra of the mALAs **1a**–**6a**.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.102.

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