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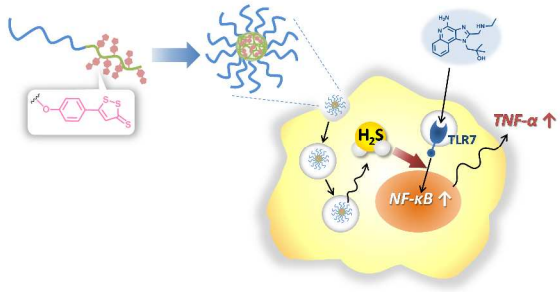

Hydrogen sulfide-releasing polymeric micelles enhanced pro-inflammatory responses induced by gardiquimod, a Toll-like receptor 7 ligand, showing the potential in immunotherapy and vaccine development.

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Polymeric Micelles for Hydrogen Sulfide Delivery

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Urara Hasegawa*[,a,b] and André J. van der Vlies[b,c]

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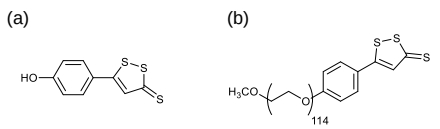
Polymeric micelles for therapeutic delivery of hydrogen sulfide (H_2S) were developed. The micelles released H_2S in murine macrophages and enhanced proinflammatory responses induced by a Toll-like receptor 7 ligand, gardiquimod. This micellar H_2S delivery system may have potential in immunotherapy and vaccine development.

Hydrogen sulfide (H_2S) has recently emerged as a gaseous signaling molecule in mammals besides nitric oxide (NO) and carbon monoxide (CO)[1]. Currently, accumulating evidence shows the physiological significance of this gas in many biological systems, especially in the cardiovascular, nervous, and immune systems[2-5].

With the discovery of the interesting physiological roles of H_2S, its therapeutic use has attracted growing attention. To administer a known amount of H_2S, the majority of studies have used inorganic sulfide salts like NaHS and Na_2S[2]. These H_2S donors spontaneously produce H_2S in aqueous media and are rapidly oxidized under aerobic condition[6]. To achieve controlled release of H_2S, several organosulfur compounds have been reported[2]. Among them, compounds having the 3*H*-1,2-dithiole-3-thione structure (dithiolethiones), such as anethole dithiolethione (ADT-OCH_3) and its desmethyl form (ADT-OH, Scheme 1a), [7] have been widely used to explore their therapeutic potential. However, these compounds are poorly water-soluble[8] and show toxic side effects[9, 10], which can be a bottleneck for therapeutic applications. In addition, the use of these low molecular weight drugs is often associated with pharmacokinetic problems such as rapid diffusion throughout the body and elimination from the kidney resulting in a low therapeutic efficacy[11].



Scheme 1. Chemical structures of (a) ADT-OH and (b) PEG-ADT.

We recently reported that the conjugation of ADT-OH with hydrophilic poly(ethylene glycol) (PEG) via a non-cleavable ether bond minimized toxic side effects of ADT-OH in murine macrophages without impairing its H_2S releasing property[12]. It was shown that this reduced toxicity may be due to the altered intracellular trafficking; the conjugate of PEG and ADT-OH (PEG-ADT, Scheme 1b) was taken up by cells via the endocytic pathway while ADT-OH entered the cytoplasm by passive diffusion through cellular membranes and thereby interacting with intracellular components to exert side effects.

The result of the PEG-ADT conjugate prompted us to develop a H_2S delivery system based on polymeric micelles. The use of nanoscale drug carriers such as polymeric micelles and vesicles have been extensively studied in the field of drug delivery in order to prolong circulation time, reduce side effects and ameliorate pharmacokinetics[13]. Here, we report H_2S-releasing polymeric micelles (ADT micelles) having a core containing dithiolethione moieties. The effects of these micellar H_2S donors on inflammation and cytotoxicity in murine macrophages were evaluated.

In order to prepare ADT micelles, we designed and synthesized a block copolymer consisting of a PEG segment as a hydrophilic block and a ditholethione-bearing segment as a H_2S-releasing block (PEG-PADT, Scheme 1a) as shown in Scheme S1, ESI. Due to the hydrophobic nature of the dithiolethione structure, the PEG-PADT polymer self-assembled to form micelles. Dynamic light scattering (DLS) revealed that the hydrodynamic diameter of the micelles was 36 nm (Figure 1b). The presence of spherical micelles was further confirmed by TEM (Figure 1c). Moreover, the average molecular weight (M_w) of the micelle was determined to be 2.3×10^6 g/mol by size exclusion chromatography-low angle light scattering (SEC-LALS, Figure S2, ESI). From this data, the average aggregation number was calculated to be about 135 (M_w of PEG-PADT: 1.7×10^4 g/mol). To evaluate stability of the micelle, we determined the critical micelle concentration (CMC) by surface tension measurement. As can be seen from Figure S7 in ESI, the CMC was calculated to be 91 pM.

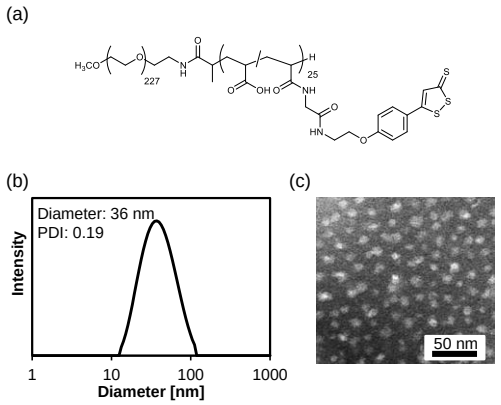


Figure 1. Characterization of ADT micelles. (a) Chemical structure of PEG-PADT. (b) Size distribution by DLS. The Z-average diameter and polydispersity index (PDI; μ_2/Γ^2) were calculated using the cumulant method. (c) TEM image of the micelles negative stained with 3wt% Preyssler-type phosphotungstate solution.

We investigated whether ADT micelles were capable of releasing H_2S. Previously, we have shown that the PEG-ADT conjugate and ADT-OH released H_2S in the presence of murine macrophages as well as their cell lysate, but not in fetal bovine serum (FBS)[12]. This observation indicates that these H_2S donors were stable in extracellular space and released H_2S by the action of intracellular enzymes after cellular uptake. As observed for these H_2S donors, the micelles released H_2S in cell lysate but not in FBS (Figure 2a). We next measured H_2S release in the presence of RAW Blue macrophages. Cells were cultured in the presence ADT micelles (100 μM) and the H_2S concentration in the culture medium was measured using a fluorescent H_2S-detection dye, WSP-1 (Scheme S2, ESI)[14]. As shown in Figure 2b, the H_2S concentration gradually increased up for 2 h. Since similar release profiles were observed for ADT-OH and the PEG-ADT conjugate[12], the micellization did not seem to impair the H_2S releasing property of dithiolethione moieties.

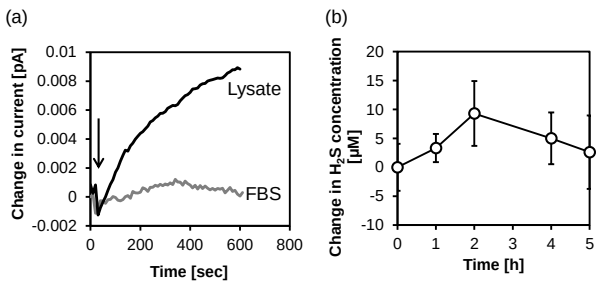


Figure 2. H_2S release from ADT micelles. (a) H_2S release in the presence of FBS and cell lysate. ADT micelles were added to 10% FBS/PBS (gray line) and cell lysate/PBS (black line) and H_2S was detected by a H_2S electrode sensor. The arrow indicates the time point when ADT micelles were added. Concentration of the dithiolethione moieties: 50 μM. (b) H_2S release in the presence of RAW Blue macrophages. ADT micelles were added to the culture medium. The H_2S concentration in the culture medium was measured using a fluorescent dye WSP-1. Concentration of the dithiolethione moieties: 100 μM.

The metabolic activity of RAW blue macrophages was evaluated after 1 d culture in the presence of ADT micelles by the MTT assay. As can be seen in Figure 3a, while ADT-OH significantly reduced metabolic activity at 100 μM, ADT micelles did not show obvious toxicity. Furthermore, to test the toxicity of H_2S, we used Na_2S, a sodium salt of H_2S, which immediately forms H_2S (and HS^-) in physiological buffers. Na_2S did not affect cell viability up for 1 mM showing that H_2S itself is not toxic in this concentration range.

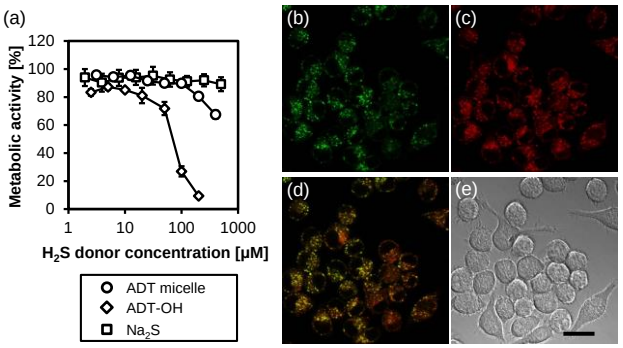


Figure 3. (a) Cytotoxicity of ADT micelles, ADT-OH and Na_2S in RAW Blue macrophages as determined by the MTT assay. (b-e) Intracellular distribution of the coumarin-labeled ADT micelles in RAW Blue macrophages. (b) ADT micelles. (c) Endosomes and lysosomes (rhodamine-labeled dextran). (d) Merged image. (e) Phase contrast image. Scale bar: 20 μm

To confirm cellular uptake of ADT micelles in RAW Blue macrophages, we prepared coumarin-labeled ADT micelles (for details, see Scheme S3 and Figures S3-S5, ESI). Rhodamine-labelled dextran was used as a marker for endosomes and lysosomes. As shown in Figure 3 (b-e), ADT micelles were internalized by RAW blue macrophages within 30 min and colocalized with endosomes and lysosomes showing that the intracellular internalization of the micelles occurred through the endocytic pathways.

H_2S has been implicated as a signaling mediator in various inflammatory conditions[15]. Although the disparate roles of H_2S in inflammation have been reported, one mode of its action is upregulation of proinflammatory mediators. Several reports showed that in acute inflammatory diseases such as sepsis, endotoxemia and acute pancreatitis, endogenously produced H_2S is involved in the proinflammatory signaling pathway by enhancing the translocation and activation of the transcription factor nuclear factor-κB (NF-κB) which plays key roles in the activation of innate immunity [16-18].

The proinflammatory effect of ADT micelles under inflammatory conditions was assessed using RAW Blue macrophages stimulated with qardiquimod (GDQ), an imidazoquinoline compound, which stimulates Toll-like receptor (TLR) 7 to induce proinflammatory responses. RAW blue macrophages are stably transfected with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter which can be induced by NF-κB. RAW Blue macrophages were cultured in the presence of the H_2S donors for 3 h prior to stimulation with GDQ. The production of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α), was quantified 2 h after GDQ stimulation. The activation of NF-κB was determined by measuring the SEAP level in the culture medium after 24 h. Under GDQ-induced inflammation, ADT micelles enhanced both TNF-α production and NF-κB activation as shown in Figure 4. On the other hand, the addition of ADT micelles alone, i.e., without GDQ, did not show any effects. These results suggest that ADT micelles were able to potentiate GDQ-induced inflammation. Although not significant, the addition of Na_2S also slightly increased TNF-α production, but did not

increase SEAP level in the culture medium. This weak effect may correlate with its instantaneous H_2S release which decreases H_2S concentration within a few minutes (Figure S6, ESI). In contrast, ADT-OH treatment slightly decreased the production of TNF-α in GDQ-induced inflammation. In addition, the activation of NF-κB was observed for ADT-OH alone (without GDQ treatment). Since cellular damage also can induce NF-κB activation, these conflicting results may relate to the side effect of ADT-OH.

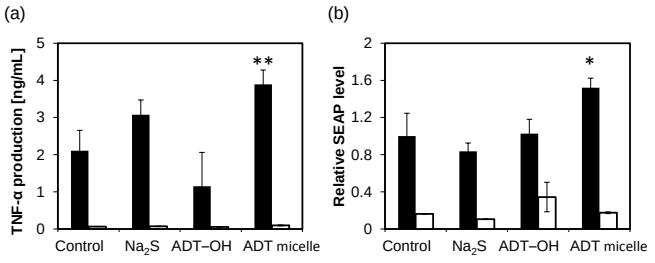


Figure 4. Effects of H_2S donors on GDQ-induced inflammation in RAW Blue macrophages. RAW Blue macrophages were treated with 50 μM Na_2S, ADT-OH and ADT micelles for 3 h and thereafter stimulated with 1 μg/mL GDQ. (a) TNF-α production as measured by ELISA. (b) NF-κB activation as determined by the level of SEAP in the culture medium. Solid column; GDQ (+), Empty column; GDQ (-). *p<0.05,**p<0.01 versus control, GDQ (+).

Recently, the therapeutic potential of TLR7 ligands has gained attention in cancer immunotherapy and vaccine development. It has been reported that they can boost both humoral and cell-mediated responses to vaccines targeting infectious diseases and cancer[19]. Among TLR7 ligands, GDQ is known as one of the most potent TLR7 ligand exerting approximately ten times higher activity than imiquimod, another extensively used TLR7 ligand[20]. In this study, we showed that ADT micelles can further enhance the proinflammatory effect of GDQ. Therefore, the micelles would be useful in the TLR-based therapies by improving the efficacy of TLR ligands.

Furthermore, it is of importance in immunotherapy to deliver immunomodulatory drugs to lymph nodes, where a substantial fraction of immature dendritic cells and macrophages exists[21]. For this purpose, the use of nanoparticles with well-controlled size has been demonstrated to be the crucial factor for lymphatic uptake from the interstitial space[22]. For instance, it has been reported that PEGylated nanoparticles in the range of 20-50 nm in diameter efficiently accumulated in the draining lymph nodes following intradermal injection. Therefore, it can be expected that ADT micelles, which are of a particulate nature, may show potential in H_2S delivery, especially when targeting the draining lymph nodes in immunotherapy.

In conclusion, we describe a nanoscale H_2S delivery system based on polymeric micelles. The micelles released H_2S in the presence of cell lysate and RAW Blue macrophages. The micelles reduced cytotoxicity compared to the small H_2S donor ADT-OH. Furthermore, the micelles successfully enhanced the GDQ-induced proinflammatory responses in a synergistic manner in murine macrophages. These results suggest that the micellar H_2S delivery system may expand the possibility of therapeutic use of H_2S.

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Notes and references

[a] Frontier Research Base for Young Researchers, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, Japan. E-mail: urara.hasegawa@chem.eng.osaka-u.ac.jp, Tel: +81-6-6879-7365, Fax: +81-6-6879-7367.
[b] Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, Japan.
[c] Frontier Research Center, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, Japan.
Electronic Supplementary Information (ESI) available: Experimental details, Synthetic schemes, GPC charts, SEC-LALS chart, TEM image and DLS data of coumarin-labelled ADT micelles. See DOI: 10.1039/c000000x/

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