Dithiolethione-Bearing Polymeric Micelles for Hydrogen Sulfide-Based Therapy

André J. van der Vlies and Urara Hasegawa

Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
urara.hasegawa@chem.eng.osaka-u.ac.jp

ABSTRACT SUMMARY
This study aims to develop novel polymeric micelles for hydrogen sulfide (H$_2$S) delivery. Well-defined amphiphilic diblock copolymers composed of a hydrophilic poly (ethylene glycol) block and a hydrophobic H$_2$S-releasing block bearing dithiolethione moieties were synthesized. The polymers formed micelles of 36 nm in diameter with a narrow size distribution. In murine macrophages, the micelles enhanced the proinflammatory response induced by gardiquimod, a Toll-like receptor 7 ligand, without obvious toxicity.

INTRODUCTION
Hydrogen sulfide (H$_2$S) has emerged as a gaseous signaling molecule that plays important roles in immunological, cardiovascular and nervous systems.$^{1}$ Recently, several organosulfur compounds, including dithiolethiones, have been reported as H$_2$S donors and used to explore the therapeutic potential of this gas.$^{2}$ Although the discovery of these small H$_2$S donors opens up new possibilities, side effects and low solubility of these compounds remain an issue for practical applications.

Polymeric nanoparticles have been widely used as drug carriers in the field of drug delivery. This approach not only improves drug solubility and stability, but also prolongs circulation time, reduces side effects, alters pharmacokinetics and, in some cases, enhances accumulation of drugs in certain tissues (e.g. tumor and the draining lymph nodes). Among known nanoparticles, polymeric micelles, spherical self-assemblies from amphiphilic block copolymers, have been recognized as one of the most promising drug carriers due to their unique characteristics such as easy formulation and functionalization, high colloidal stability, high drug loading capacity and low toxicity.

Here we report novel polymeric micelle-based H$_2$S donors. Polymeric micelles having a core containing H$_2$S-releasing anethole dithiolethione (ADT) moieties (ADT micelles) were prepared. H$_2$S release property and cytotoxicity of the micelles were evaluated. Furthermore, the proinflammatory effects in macrophages were also examined.

EXPERIMENTAL METHODS
Amphiphilic diblock copolymers containing ADT moieties (PEG-PADT, Figure 1(a)) were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization of N-acryloyl glycine tert-butyl ester using PEG-pyrrole carbodithioate as a chain transfer agent (CTA) followed by CTA end group removal, deprotection of tert-butyl ester group and conjugation with amino group-modified ADT derivatives (ADT-NH$_2$). ADT micelles were prepared by adding a PEG-PADT/DMF solution dropwise to milliQ water followed by dialysis. The micelles were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). A fluorescent H$_2$S detection dye, WSP-1, was used to detect H$_2$S release from ADT micelles in the presence of murine macrophages, RAW Blue cells. The effects on gardiquimod (GDQ)-induced nuclear factor-$\kappa$B (NF-$\kappa$B) activation and the cytokine production in RAW Blue cells were assessed by Quanti Blue and ELISA assays. Cytotoxicity of the micelles was assessed by MTT cell viability assay.

RESULTS AND DISCUSSION
We first prepared PEG-poly(N-acryloyl glycine tert-butyl ester) block copolymers by RAFT polymerization. The polymerization proceeded quantitatively in well-controlled manner as shown by $^1$H NMR and GPC. After removing the protecting groups in TFA, the
polymers were conjugated with ADT-NH₂. The degree of modification was about 70% by 1H NMR.

PEG-PADT block copolymers were dispersed in water to form ADT micelles. DLS showed that the micelles were monodisperse with a diameter of 36 nm (Figure 1 (b)). Further characterization by TEM confirmed that the polymers formed spherical micelles (Figure 1 (c)).

![Chemical structures of PEG-PADT and ADT-OH. (b) Size distribution of ADT micelles by DLS. (c) TEM image of ADT micelles.](image)

We investigated the H₂S release property of ADT micelles. Since it has been reported that the H₂S release from ADT is induced by metabolic digestion, we incubated ADT micelles in the presence of murine macrophages, RAW Blue cells, and measured H₂S concentration in the culture medium. In the case of the rapid H₂S releaser Na₂S, H₂S concentration increased immediately and dropped to zero within 30 min while the small organosulfur compound ADT-OH gradually increased H₂S concentration up for 1 h. Furthermore, the increase of H₂S concentration was even slower for ADT micelles.

In order to assess cytotoxicity of the different H₂S donors, the cell viability was evaluated by the MTT assay. Na₂S did not show any toxic effect while ADT-OH significantly reduced cell viability at 100 μM. On the other hand, ADT micelles did not seem to affect the cell viability up to 200 μM. (Figure 2).

The effect of ADT micelles in inflammation was assessed using RAW Blue macrophages. Gardiquimod, a Toll-like receptor 7 ligand, was used to induce inflammation. ADT micelles exerted a synergistic effect with gardiquimod, and enhanced NF-κB activation and TNF-α production.

![Cytotoxicity of Na₂S, ADT-OH and ADT micelles.](image)

**CONCLUSION**

We developed a micellar form of H₂S delivery system. ADT micelles were capable of releasing H₂S in the presence of murine macrophages. The micelles significantly reduced cytotoxicity of the small H₂S donor ADT-OH, in murine macrophages. Moreover, ADT micelles exerted a synergistic effect with gardiquimod and enhanced proinflammatory responses. This polymeric micelle-based H₂S delivery system may have potential in immunotherapy and vaccine development.

**REFERENCES**


**ACKNOWLEDGMENTS**

We thank Dr. E. Mochizuki (Osaka University, Japan) for TEM experiments and Prof. M. Sadakane (Hiroshima University, Japan) for supplying Preyssler-type phosphotungstate staining agent. This work was supported by Grant-in-Aid for Young Scientists (B), No. 24700482, from the Japan Society for the Promotion of Science.