A Supramolecular Crosslinker to Give Salt-Resistant Polyion Complex Micelles and Improved MRI Contrast Agents

Jiahua Wang, Junyou Wang,* Peng Ding, Wenjuan Zhou, Yuehua Li, Markus Drechsler, Xuhong Guo,* and Martien A. Cohen Stuart*

anie_201805707_sm_miscellaneous_information.pdf
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Experimental Section

Materials

The diblock copolymer, poly(N-methyl-2-vinyl-pyridinium iodide)-b-poly(ethyleneoxide) (P2MVP41-b-PEO205), was obtained by quaternization of poly(2-vinylpyridine)-b-poly(ethylene oxide) (P2VP41-b-PEO205) (Polymer Source, Mw/Mn= 1.03, Mw= 13.3 k) following a procedure described elsewhere.[1] The degree of quaternization is about 90%. The bis-ligand compound 1,11-bis(2,6-dicarboxypyridin-4-yloxy)-3,6,9-trioxaundecane (L2) was prepared according to literature.[2] 4-hydroxypyridine-2,6-dicarboxylic acid (chelidamic acid), Iron(III) chloride hexahydrate FeCl3·6H2O, Manganese(II) nitrate tetrahydrate Mn(NO3)2·4H2O and sodium chloride NaNO3 (analytical grade) were purchased from Sigma Aldrich and used without further purification. Fe-L3/L2 micelles solutions were made in acetate buffer at pH 5 and Mn-L3/L2 micelles solutions were made in tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4.

Characterizations

1H-NMR spectra and 13C-NMR spectra were recorded on a BRUKER AVANCE 500 spectrometer operating at 500 MHz. UV spectra for all the samples were recorded on an SHIMADZU 1800 spectrophotometer. The MRI testing and T1 relaxation time measurements were tested at a 0.47 T NMRi20-Analyst NMR Analyzing and Imaging system (Niumag Corporation, Shanghai, China).

Synthesis of 1,3,5-tris(2,6-dicarboxypyridin-4-yloxy)methyl)benzene, L3.

4-hydroxypyridine-2,6-dicarboxylic acid (chelidamic acid) 1 (2.5g, 13.3mmol) was added to a solution of thionyl chloride (7.5ml) in ethanol (25ml) at -10 °C. The mixture was stirred at room temperature for 24 h and then refluxed for an additional 2 h. After cooling, the mixture was concentrated under reduced pressure to give a residue, which was treated with water (20ml). The resultant suspension, cooled to 0 °C, was neutralized with 10% aq. Na2CO3, and filtered. The solid material was dried and recrystallized from ethanol-water (1:2) and dried under reduced pressure at 50 °C to give the diethylester 2 as white crystals. [3] A mixture of compound 2 (2.5g, 10.45mmol) and anhydrous potassium carbonate (1.31g, 9.5mmol) in dry N,N-dimethyformamide (40ml) was stirred at 25 °C in vacuo for 30 minutes, then 1,3,5-tris(bromomethyl)benzene 3 (1.13g, 3.17mmol) was added and the reaction mixture was allowed to react at 70-80 °C for 24 hours under an atmosphere of argon. The mixture was evaporated in vacuo and the residue was partitioned between dichloromethane and water. The organic layer was washed with 1% aqueous acetic acid, water and dried (sodium sulfate). The residue obtained, after removal of the solvent, was recrystallized from ethanol and dried in vacuo at 50 °C to give 4 as a white solid. [4] Hydrolysis of 2.8 g of this ester was performed by heating it in a mixture of 30 ml deionized water, 30 ml ethanol and KOH (3.37g, 60mmol) for 12 h at 60 °C. The product could be precipitated in a mixture of ethanol and water. The precipitate was washed with ice water and ethanol and freeze-dried to give the desired product 5. Yield: 57% (2.3 g, 2.58 mmol), mp > 300 °C. 1H NMR (D2O): δ 5.30 (s, 6H, CH2), 7.59 (s, 6H, aromatic H), 7.66 (s, 3H, aromatic H). 13C NMR (D2O): δ 69.74 (CH2), 111.50 (CH), 127.51 (CH), 136.73 (C), 154.83 (C), 166.30 (CO), 172.56 (CO2).
**Scheme SI 1.** Synthesis of L₃ ligand.

**Figure SI 1.** 'H-NMR spectrum of the tris-ligand L₃. L₃ was dissolved in D₂O and measured at 298 K on BRUKER AVANCE III 500 spectrometer (500 MHz).
Preparing micelles – experimental protocol

Micellar solutions were prepared by first mixing the diblock copolymer with the ligands at the relevant salt concentration. Then, a solution of the transition metal (as nitrate) was added under stirring.

Light Scattering

The Dynamic light scattering (DLS) measurement was performed with an ALV light scattering apparatus, equipped with a 400 mW argon ion laser operating at a wavelength of 532 nm. Measurements were done at a detection angle of 90°, unless stated otherwise. All measurements were performed at room temperature.

The light scattering intensity is expressed as the excess Rayleigh ratio \( R_0 \) divided by the polymer concentration. \( R_0 \) is obtained as (1) where \( I_{\text{sample}} \) is the scattering intensity of the complex solution and \( I_{\text{solvent}} \) is the intensity of the solvent. \( I_{\text{toluene}} \) is the scattering intensity of toluene (the reference), and \( R_{\text{toluene}} \) is the known Rayleigh ratio of toluene (2.1 \times 10^{-2} \text{ m}^{-1}).

\[
R_0 = \frac{I_{\text{sample}} - I_{\text{solvent}}}{I_{\text{toluene}} - I_{\text{solvent}}} \times R_{\text{toluene}} \times \frac{n_{\text{solvent}}}{n_{\text{toluene}}} \quad (1)
\]

Salt stability of the micelles was tested by titrating concentrated NaNO\textsubscript{3} solution into the micelles solution. The scattering intensity and hydrodynamic radius was recorded for each titration step.

The CUMULANT method was used to analyze the mean apparent hydrodynamic radius \( (R_h) \), which is
where $q$ is the scattering vector, $k$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the solvent, and $\Gamma$ is the measured average decay rate of the correlation function. The CONTIN method is used to analyze the distribution of particle (C3Ms) radius.

For angular-dependent DLS, ten correlation functions $g_2(t)$ were recorded at 6 angles $\theta$, from 60 to 135° in increments of 15°, to evaluate the angular dependence of the diffusion coefficient. It is known that asymmetric particles always give rise to a dependence of $D (= \Gamma/q^2)$ on $q^2$, but for spherical particles, the $D (= \Gamma/q^2)$ values should be independent of the scattering vector, because of the undetectable rotational motion.\(^\text{[6]}\)

\begin{align*}
q &= (4\pi n/\lambda) \sin(\theta/2)
\end{align*}

Cryogenic Transmission Electronic Microscopy (Cryo-TEM)

A few microliters of samples were placed on a bare copper TEM grid (Plano, 600 mesh), and the excess liquid was removed with filter paper. This sample was cryo-fixed by rapidly immersing into liquid ethane cooled to -170 to -180 °C in a cryo-box (Carl Zeiss NTS GmbH). The specimen was inserted into a cryo-transfer holder (CT3500, Gatan, Munich, Germany) and transferred to a Zeiss EM922 EFTEM (Zeiss NTS GmbH, Oberkochen, Germany). Examinations were carried out at temperatures around -180 °C. The TEM was operated at an acceleration voltage of 200 kV. Zero-loss filtered images were taken under reduced dose conditions (500-2000 e/nm²).

All images were recorded digitally by a bottom-mounted CCD camera system (UltraScan 1000, Gatan) and processed with a digital imaging processing system (Digital Micrograph 3.9 for GMS 1.4, Gatan).

Cytotoxicity Assay

Cell viability was determined by a typical 3-(4,5-dimethyl-2-yi)-2,5-diphenyltetrazolium bromide (MTT) assay. SMCC-7721 cells were seeded into a 96-well culture plate at a density of 5 x 10³ cells well⁻¹ and cultured for 24 h in a 5% CO₂ incubator at 37 °C. The cells were treated with Mn-L₃ micelles at 0–60 μM or vehicle control for 24 h; the cell viability was measured by a microplate reader at 490 nm with the MTT staining assay. Optical density (OD) was read at 490 nm and background was subtracted at 630 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, USA). Cell viability was expressed as a percentage of the corresponding control value. The data are expressed as the average of five replicates ± standard deviations (SD).
**Figure SI 3.** Angular dependence of the apparent particle radius (solid) and the self-diffusion coefficient (open) for Mn-L$_2$ and Mn-L$_3$ micelles systems.

**Figure SI 4.** The autocorrelation function for Mn-L$_2$ micelles (0% of L$_3$) at low and high salt concentration.
Figure S15. a: size and size distribution of Mn-L2-L3 micelles (20% of L3) at low and high salt concentration; b: the autocorrelation function for Mn-L2-L3 micelles at low and high salt concentration.

Figure S16. a: size and size distribution of Mn-L2-L3 micelles (40% of L3) at low and high salt concentration; b: the autocorrelation function for Mn-L2-L3 micelles at low and high salt concentration.
**Figure SI 7.** a: size and size distribution of Mn-L2-L3 micelles (60% of L3) at low and high salt concentration; b: the autocorrelation function for Mn-L2-L3 micelles at low and high salt concentration.

**Figure SI 8.** a: size and size distribution of Mn-L2-L3 micelles (80% of L3) at low and high salt concentration; b: the autocorrelation function for Mn-L2-L3 micelles at low and high salt concentration.
**Figure SI 9.** a: size and size distribution of Mn-L₃ micelles (100% of L₃) at low and high salt concentration; b: the autocorrelation function for Mn-L₂-L₃ micelles at low and high salt concentration.

**Figure SI 10.** Plots of longitudinal relaxation rate, 1/T₁ vs Mn²⁺ concentration for Mn-L₂ micelles and Mn-L₃ micelles solutions, inset figure is vitro T₁-weighted MR imaging of Mn-L₃ micelles at various Mn²⁺ concentration.
Figure S11. a: size and size distribution of Fe-L2-L3 micelles as a function of L3 ligand percentage; b: Scattering intensity (normalized by the original value I0, where no salt is added yet) for Fe-L2-L3 micelles at different L3% with increasing salt concentration.

Figure S12. The relaxivity $r_1$ of Fe-L2-L3 micelles at different L3 fractions. $r_1$ is the longitudinal relaxivity of water protons in the presence of Fe-L2-L3 micelles.
Figure S13. a: Scattering intensity of Zn-L2-L3 micelles at different L3% with increasing salt concentration; b: Rₙ and PDI of Zn(II)-L3 micelles.

Figure S14. a: Scattering intensity for Ni-L2-L3 micelles at different L3% with increasing salt concentration; b: Rₙ and PDI of Ni-L3 micelles.

References