A Novel HA-based Micelle Material as a Potent Delivery System

Tse-Min Teng, *Jean-Dean Yang, Yi-Ting Hsieh, Yu-Hsiang Tien, Yi-Man Chou, Jui-Hsiang Chen and Shian-Jy Wang.*

Material and Chemical Research Laboratories, Industrial Technology Research Institute
No. 321, Sec. 2, Kuang Fu Rd, Hsinchu, 30011, Taiwan, ROC

Statement of Purpose: Amphiphilic polymeric materials are composed with hydrophilic portions and hydrophobic chains, and easily form micelles in aqueous solution *via* self-assembling. The core of micelles provides a hydrophobic micro-environment for water-insoluble molecule storage. Consequently, amphiphilic polymers as nanocarriers for drug or bioactive compound delivery have received much attraction in recent studies.[1, 2] Hyaluronic acid (HA) is a naturally occurring polysaccharide found in living organisms, especially in cartilage, joint, skin tissue, soft connective tissues and the fluid surrounding eyes. The composition of hyaluronic acid is a disaccharide repeating unit with D-glucuronic acid linked to *N*-acetyl-glucosamine through β-(1→3) linkage and the repeating unit further link to each other through β-(1→4) linkage. Hyaluronic acid could control cell permeability and regulate cell adhesion, migration, proliferation and differentiation. These unique physiological functions of hyaluronic acid reveal its potential use for clinical purpose.[3] Poly(ε-caprolactone) (PCL) is a hydrophobic biodegradable polymer extensively applied in various biomedical devices. In this study, HA-*g*-PCL copolymer was prepared in the presence of $Sn(Oct)_2$ as catalyst. The physical property analysis indicates that this amphiphilic material is suitable for drug delivery because of its low critical micelle concentration (CMC) and appropriate particle size (PS).

Methods:

Materials. Hyaluronic acid sodium salt was purchased from Shiseido. Tetrabutylammonium hydroxide aqueous solution (TBA-OH, 40% in H_2O), $Sn(Oct)_2$ and bis(cyclohexyl)methylene diisocyanate (H_{12}MDI, 90%) were received from Sigma-Aldrich. 1-Dodecanol (98%), ε-caprolactone (99%) and 1,4-diazabicyclo[2.2.2]octane (DABCO) were purchased from Acros, Alfa Aesar and MP Biomedicals Inc., respectively. Dichloromethane, petroleum ether and dimethyl sulfoxide ('BAKER ANALYZED'® A.C.S. Reagent) were provided by J.T.Baker. Sodium and hydrogen ion-exchange resins were purchased from ROHM And HAAS Shanghai Chemical Industry Co., LTD. 1-Dodecanol and dimethyl sulfoxide were dried with molecular sieve 4A before use. Other reagents were used without further purification.

Instrumentation. Gel permeation chromatography (GPC) was used to estimate molecular weight and molecular weight distribution, M_w/M_n, of poly(ε-caprolactone) with respect to polystyrene standards (Polysciences Corporation). ^{1}H-NMR spectra of the polymers were obtained on BRUKER DRX400 spectrometer using 5 mm o.d. tubes. IR absorption spectrum was analyzed with FT-IR spectrometer THERMO Nicolet 380. Critical micelle concentration (CMC) of copolymer HA-*g*-PCL was evaluated according to its fluorescence spectra, which were recorded by a luminescence spectrometer HORIBA Fluorolog-3. The particle size of HA-*g*-PCL micelle was measured by dynamic light scattering (DLS) on COULTER N4 Plus. The morphology of micelle was observed by TEM on JEOL JEM-2100F.

Synthesis of PCL-OH. Glassware and Teflon blades were dried at 50-60 °C for 1 day before use. To a 250 mL glass reactor equipped with mechanical mixing blades were added ε-caprolactone (50 g, 439 mmol) and 1-dodecanol (4.05 g, 22 mmol) under nitrogen atmosphere. The two reactants were well-mixed for 10 minutes at 100 °C before the addition of stannous octoate (44 mg, 0.1 mmol, 0.5 mol% to 1-dodecanol). The ring-opening polymerization reaction was carried out in a 110 °C oil bath for 16 h with continuous mechanical stirring at 200 rpm. The resulting mixture was cooled to room temperature and then diluted with dichloromethane (50 mL). The crude product was purified by reprecipitation twice in cold diethyl ether (2 L, 4 °C). The white precipitate was collected by filtration and dried *in vacuo* for 24 h to give pure PCL-OH (52 g, 96%). The polymer was identified by ^{1}H-NMR using *d*-chloroform as *d*-solvent and its molecular weight was estimated by GPC.

Preparation of HA-TBA Salt. Hyaluronic acid sodium salt (Mw 16000, 6.0 g) was dissolved in DIW (600 mL) and then the solution was eluted through a column filled with hydrogen ion-exchange resin (450 g). The collected hyaluronic acid solution was added tetrabutylammonium hydroxide (40% in H_2O, 9.8 mL) with magnetic stirring and the neutralization was kept at room temperature for 3 h. The solution was concentrated on rotatory evaporator under vacuum and then lyophilized to afford dry hyaluronic acid tetrabutylammonium salt (HA_{16k}TBA, 9.2 g, 99%). The product was characterized by ^{1}H-NMR and used without further purification.

Synthesis of HA-*g*-PCL. PCL-OH (Mw 2740, 2.95 g, 1.08 mmol) was dried with toluene (9 mL) *in vacuo* at 70 °C before use and then dissolved in DMSO (8 mL), followed by addition of H_{12}MDI (0.28 mL, 0.97 mmol). DABCO (6.5 mg, 2000 ppm) and $Sn(Oct)_2$ (3.3 mg, 1000 ppm) diluted with DMSO (1 mL) were added at 60 °C and the reaction was kept at 60 °C for 20 h to generate pre-polymer PCL_{2740}-NCO. HA_{16k}TBA (3.0 g, 4.84 mmol) was dried *in vacuo* overnight and then dissolved in DMSO (25 mL) at 40 °C. The pre-polymer PCL_{2740}-NCO was thoroughly transferred to HA_{16k}TBA solution at 60

^{o}C and additional DABCO (11.8 mg, 2000 ppm) and $Sn(Oct)_2$ (5.9 mg, 1000 ppm) in DMSO (1 mL) were added. The resulting mixture was kept for another 24 h. The HA_{16k}TBA-*g*-PCL copolymer in a dialysis bag (MWCO 12-14000) was dialyzed against DMSO for 1 day and then in DIW for 2 days. Water was completely evaporated to dryness and the crude product was further purified within Soxhlet extractor using dichloromethane as solvent to remove free PCL-OH fragment. The residue was dissolved in DIW and the solution was eluted through a column filled with sodium ion-exchange resin (500 g). The solution was concentrated on rotatory evaporator under vacuum and then lyophilized to provide comb-like HA_{16k}-*g*-PCL copolymer. This product was characterized by ^{1}H-NMR and FT-IR.

Results:

PCL-OH. The preparation method of PCL-OH polymer involved ring-opening polymerization (ROP) of ε-CL using 1-dodecanol as initiator in the presence of stannous octoate. PCL-OH was purified by reprecipitation twice in dichloromethane/diethyl ether system at low temperature to remove unreacted small molecules. The synthetic PCL-OH polymer was characterized by ^{1}H-NMR and GPC. According to the calculation of integral ratio, the number of repeating ε-CL was evaluated to be 22. GPC data indicated the molecular weight of PCL-OH was 2740 g/mol.

HA-TBA Salt. The sodium ions on native hyaluronic acid were replaced by hydrogen ions *via* ion-exchange process. Hyaluronic acid was then neutralized with equal mole of tetrabutylammonium hydroxide to afford HATBA, which is soluble in highly polar organic solvents, such as DMSO and DMAc. Accordingly, HA-TBA could be chemically modified through various synthetic reactions.

HA-*g*-PCL. The grafting ratio of PCL on HA-*g*-PCL copolymer was estimated according to the integral ratio between terminal CH_3 on PCL and amide-CH_3 on HA. The low solubility of PCL fragment in water would cause the lower signal intensity and therefore co-solvent D_2O/DMSO-d_6 = 8/2 was used to obtain practical information. ^{1}H-NMR spectra indicated that the grafting ratios of HA-*g*-PCL vary from 6~8% in most cases (**Figure 1**).

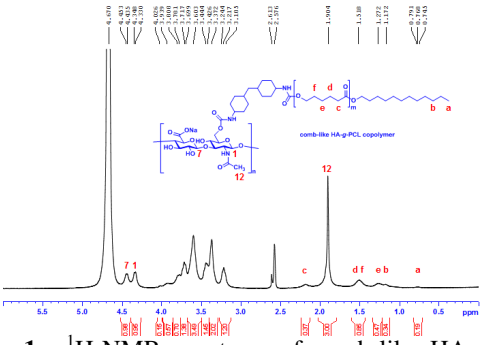

Figure 1. ^{1}H-NMR spectrum of comb-like HA-*g*-PCL copolymer and its structural assignments.

Unique functional groups, including ester and carbamate, could be characterized by FT-IR. Fluorescence spectra data revealed CMC of this HA-based material was around 1.6×10^{-3} mg/mL (**Figure 2**), an extremely low value. Consequently, it is possible to be a nanocarrier for active molecule delivery in aqueous system. The particle sizes of HA-*g*-PCL micelles ranged from 190 to 240 nm and TEM images also demonstrated their actual morphology and sizes (**Figure 3**). In addition, this copolymer is highly biocompatible even at a concentration of 300X CMC (cell viability > 90%, **Figure 4**).

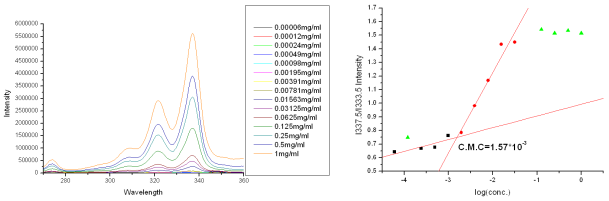

Figure 2. The graphs indicate the estimation method for CMC of comb-like HA-*g*-PCL and the value is around 1.6×10^{-3} mg/mL.

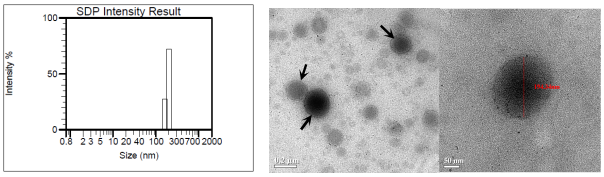

Figure 3. The particle size distribution of HA-*g*-PCL micelles was measured by DLS (left) and the micelle morphology was observed by TEM (right).

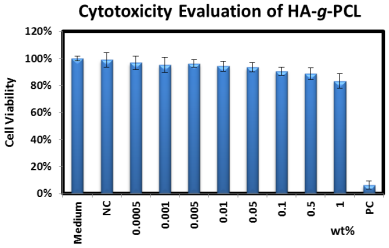

Figure 4. The cytotoxicity of HA-*g*-PCL material was evaluated using MTT method.

Conclusions: In this work, a novel HA-based copolymer was synthesized and characterized. The results of its physical properties analysis, including critical micelle concentration, particle size, TEM image and cytotoxicity, indicated that this micelle material has potential to be utilized as a nanocarrier for small molecule delivery. Further investigations about endocytosis with HA-based micelle and its metabolism pathway in cell are ongoing.

References:

(1) Tong, R.; Cheng, J. *Polym. Rev.*, **2007**, *47*, 345.
(2) Chung, T.-W.; Tyan, Y.-C.; Yang, J.-D. *J. Microencapsul.* **2010**, *27*, 514.
(3) Lee, H.; Ahn, C.-H.; Park, T.-G. *Macromol. Biosci.* **2009**, *9*, 336.