Preparation of Novel Shell Cross-linked Thermoresponsive Hybrid Micelles with Anti-tumor Efficacy

H. Wei * and F. Dehghani **

School of Chemical and Biomolecular Engineering, the University of Sydney, Sydney 2006, Australia
* hua.wei@sydney.edu.au
** fariba.dehghani@sydney.edu.au

ABSTRACT

The shell cross-linked (SCL) thermoresponsive hybrid poly(*N*-isopropylacrylamide-*co*-aminoethyl methacrylate)-*b*-polymethyl methacrylate (P(NIPAAm-*co*-AMA)-*b*-PMMA) micelle consisting of a cross-linked thermoresponsive hybrid shell and a hydrophobic core domain was fabricated via a two-step process: micellisation of P(NIPAAm-*co*-AMA)-*b*-PMMA in aqueous solution followed by cross-linking of the hydrophilic shell layer using a novel inorganic cross-linker. The SCL micelle showed reversible dispersion/aggregation in response to the temperature cycles through the lower critical solution temperature (LCST) of the thermoresponsive hybrid shell at 36 ^{o}C, observed by turbidity measurements. The inorganic compound used as a cross-linker has anti-tumor efficacy; and the resultant SCL micelle exhibited a remarkable cytotoxic effect against HeLa cells with a very low IC_{50}.

Keywords: P(NIPAAm-*co*-AMA)-*b*-PMMA; micelle; shell cross-linked; thermoresponsive; anti-tumor efficacy.

1 INTRODUCTION

During the past decade, micelles formed by self-assembly of amphiphilic copolymers combining hydrophilic and hydrophobic segments have been developed for drug delivery applications[1,2], The strategies of covalent stabilisation via cross-linking of the micellar core or shell were explored to enhance the structural stability and integrity of the supramolecular nanostructures [3,4], which is a crucial issue in the practical applications of traditional micelles. The dissociation of non-cross-linked micelle into individual polymer chains under shear forces, dilution by gastric, blood or other body fluids and salinity fluctuations after administration may lead to rapid release of the loaded drug prior to targeted delivery, resulting in side effects *in vivo*.

Organic "small-molecule" cross-linking strategies have been developed to prepare shell cross-linked (SCL) micelles.[5,6] Wooley's group developed the use of carbodiimide coupling chemistry to link carboxylic acid groups through diamines.[7] Armes and co-workers presented the use of 1,2-bis(2-iodoethoxy)ethane as a bifunctional cross-linker for the covalent stabilization of SCL micelles.[8] However, most organic "small-molecule" cross-linking strategies suffer from expensive reagents and require purification to remove small molecule by-products. It is obvious that there is considerable scope for the development of new, improved cross-linking strategies, such as an inorganic cross-linking strategy.

There has been growing concern about organic/inorganic hybrid nanomaterials during the past decade due to the intriguing properties of the organic/inorganic hybrid micelles associated with both inorganic segments (optical, magnetic, mechanical and electronic properties, etc.) and organic polymer moieties (processability, compatibility, and stimulus-responsiveness, etc.).[9]

SCL hybrid micelles bearing characteristics of organic polymers and inorganic structures were prepared using a "silica-based" cross-linking strategy. In principle, an inorganic "silica-based" cross-linking strategy offers three advantages over conventional organic "small-molecule" cross-linking strategies: (1) inorganic "silica-based" cross-linking means that inorganic silica network structures were introduced into the organic polymer chains, which increased the rigid nature of the SCL micelles, and its stability; (2) the cross-linkers used in the inorganic "silica-based" cross-linking strategy, such as 3-(trimethoxysilyl) propyl methacrylate (MPMA), are generally regarded as low cost and having low toxicity, in comparison with organic "small-molecule" cross-linking agents; (3) the inorganic "silica-based" cross-linking resulting from an acid- or base-catalyzed sol-gel process is less labour intensive than organic "small-molecule" cross-linking reagents, and purification is more straightforward.

In our previous study[10], SCL thermo-sensitive hybrid micelles with substantial improvement in stability were prepared from poly(NIPAAm-*co*-3-(trimethoxysilyl)propyl methacrylate)-*b*-PMMA (P(NIPAAm-*co*-MPMA)-*b*-PMMA) amphiphilic block copolymers via an inorganic "silica-based" cross-linking strategy. The resulting SCL micelles had a high stability due to the presence of the cross-linked silica network, which resulted in improved entrapment efficiency (EE) as well as a rather retarded drug release as compared with PNIPAAm-*b*-PMMA micelles[11].

In this study, a novel SCL intelligent hybrid micelle was developed using an inorganic cross-linking reagent. The SCL intelligent hybrid micelle system was prepared via a

two-step procedure (Figure 1): self-assembly of poly(*N*-isopropylacrylamide-*co*-aminoethyl methacrylate)-*b*-polymethyl methacrylate (P(NIPAAm-*co*-AMA)-*b*-PMMA) copolymer into polymeric micelle in aqueous solution followed by cross-linking of the hydrophilic shell layer via the addition of a new inorganic compound as the cross-linker. The *in vitro* anti-tumor effect of the resulting SCL hybrid micelles was investigated.

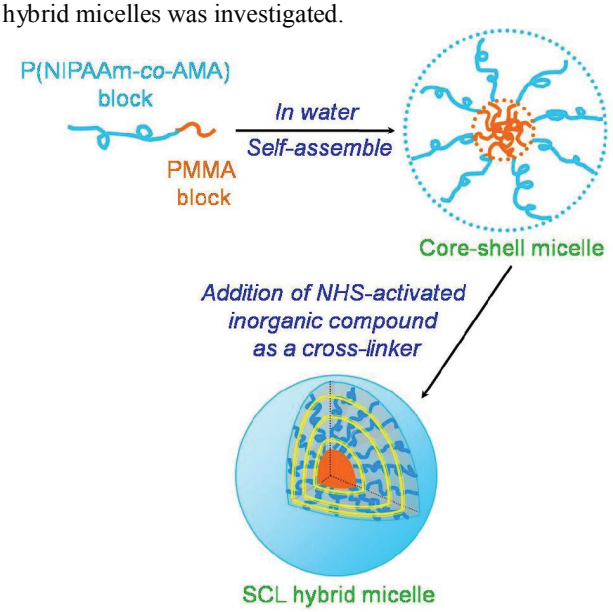

Figure 1. Schematic representation of the basic approach for the formation of the SCL hybrid micelle.

2 EXPERIMENTAL METHODS

2.1 Preparation of poly(*N*-isopropylacrylamide-*co*-aminoethyl methacrylate)-*b*-polymethyl methacrylate block copolymer (P(NIPAAm-*co*-AMA)-*b*-PMMA)

Amino-terminated PMMA (PMMA-NH_2) was prepared by free radical polymerisation using 2-amino ethanethiol hydrochloride (AET·HCl) as a chain transfer agent according to our previous report [11].

The resultant PMMA-NH_2 was reacted with dithiobis (succinimidylpropionate) (DSP) to obtain the PMMA dimmer and the PMMA were connected by disulfide bonds. The dimer was reduced by adding dithiothreitol (DTT) to the reaction mixture to obtain PMMA-SH. The product was precipitated out by the addition of water and was further dried in vacuum. NIPAAm and AMA·HCl was then copolymerized using *N*,*N*'-Azobisisobutyronitrile (AIBN) as an initiator and PMMA-SH as a chain transfer reagent in *N*,*N*'-dimethylformamide (DMF).

2.2 Preparation of SCL hybrid micelle

The inorganic cross-linker was pre-activated with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS). After the reaction, the by-product dicyclohexyurea was removed by filtration. Then cross-linking of AMA units in the micellar shell was achieved by adding the NHS-activated cross-linking agent dropwise to the P(NIPAAm-*co*-AMA)-*b*-PMMA aqueous micellar solution under constant stirring. After the addition, the mixed solution was further allowed to stir for 24 h at room temperature, then transferred to a dialysis tube and subjected to dialysis. A solid sample of SCL micelles was collected by freeze-drying for further examinations.

2.3 Measurements

The molecular weights of PMMA-NH_2 and P(NIPAAm-*co*-AMA)-*b*-PMMA were determined by a gel permeation chromatographic (GPC) equipped with a Waters 2690D separations module, and a Waters 2410 refractive index detector. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 0.3 ml/min.

TEM experiments were carried out on a JEM-100CX II instrument operating at an acceleration voltage of 80 keV.

Average SCL micelle diameters in aqueous solution with different concentrations were measured by Nano-ZS ZEN3600 (MALVERN, UK) instrument. The scattering angle was fixed at 90 °C. All data are averages of three independent determinations.

Turbidity of the uncross-linked and cross-linked micelle aqueous solution ([polymer] = 1000 mg/L) at various temperatures was measured at 542 nm with a Lambda Bio40 UV-Vis spectrometer (Perkin-Elmer), respectively. Sample cell was thermostated in a refrigerated circulator bath at different temperature from 26 to 56 °C prior to measurements. The LCST was defined as the temperature producing a half increase of the total increase in turbidity.

In vitro cytotoxicity of P(NIPAAm-*co*-AMA)-*b*-PMMA copolymer and the SCL hybrid micelle was evaluated by MTT assay, respectively. 6.0×10^3 HeLa cells were incubated in each well of a 96-well plate. After incubation for 24 h in incubator (37 °C, 5% CO_2), the culture medium was changed to 200 μL of Dulbecco's Modified Eagle Medium (DMEM) containing the material at various concentrations and the mixture was further incubated for 48 h. DMEM with the material was then replaced by fresh DMEM and 20 μl of MTT solution (5 mg/ml) was added to the HeLa cells. After incubation for 4 h, the MTT medium was removed from each well, 200 μl of DMSO was added, and the mixture was shaken at room temperature. The optical density (OD) was measured at 570 nm with a microplate reader Model550 (BIO-RAD, USA). The cell viable rate was calculated by the following equation: Viable cell (%) = ($OD_{treated}$ / $OD_{control}$) ×100, where $OD_{control}$ was obtained in the absence of the material and $OD_{treated}$ was obtained in the presence of the material.

3 RESULTS AND DISCUSSION

3.1 Synthesis of P(NIPAAm-*co*-AMA)-*b*-PMMA

P(NIPAAm-*co*-AMA)-*b*-PMMA copolymer was synthesized by copolymerisation of NIPAAm and AMA using AIBN as an initiator and sulfhydryl-terminated PMMA (PMMA-SH) as a chain transfer reagent. The successful preparation of the target block copolymer was confirmed by molecular weights determined by GPC as shown in Table 1.

Table 1. GPC data of PMMA-NH_2 and P(NIPAAm-*co*-AMA)-*b*-PMMA.

	M_n	M_w/M_n
PMMA-NH_2	6,500	1.82
P(NIPAAm-*co*-AMA)-*b*-PMMA	13,100	1.51

3.2 SCL micelle formation

Shell cross-linking was verified as follows. After cross-linking, the SCL micellar aqueous solution was diluted 3-fold. If no shell cross-linking had occurred, dissolution of the micelle into the individual block copolymer chains would be expected. However, DLS study indicated that the size distribution of the micelle solution after dilution (123.1 nm, PDI 0.294) varied slightly as compared with the size before dilution (115.6 nm, PDI 0.22), which confirms that the shell cross-linking was successful.

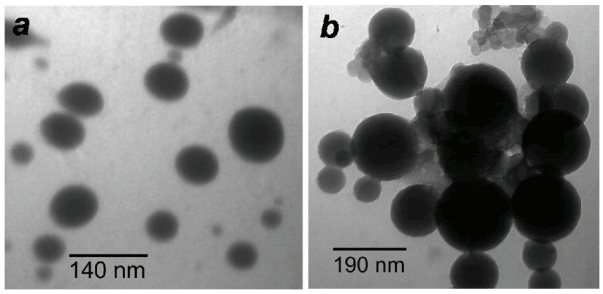

Figure 2. TEM images of SCL micelles in (a) aqueous media and (b) methanol, respectively.

The morphology of the resulting SCL micelles was further investigated by TEM. A typical photograph of the SCL micelles is shown in Figure 2a, in which the SCL micelles were well-dispersed and clearly displayed a regularly spherical shape with size around 40~130 nm in diameter. The results suggest that the diameter observed by TEM was in agreement with the results obtained by particle-size analyzer.

The lyophilized SCL micelles were dissolved in an organic solvent to demonstrate their adequate cross-linking and to assess their shape and integrity in organic solvent,. As can be seen in Figure 2b, the structure of SCL micelles was maintained as spherical particles after switching the solvent from water to methanol. It is concluded that cross-linking of the micellar shell was effective and the micellar structure was locked by the cross-linked shell layer.

3.3 Thermoresponsive behavior

We examined the turbidity of the uncross-linked and cross-linked micelle aqueous solution as a function of temperature to determine the thermoresponsive properties of the uncross-linked and cross-linked micelles.

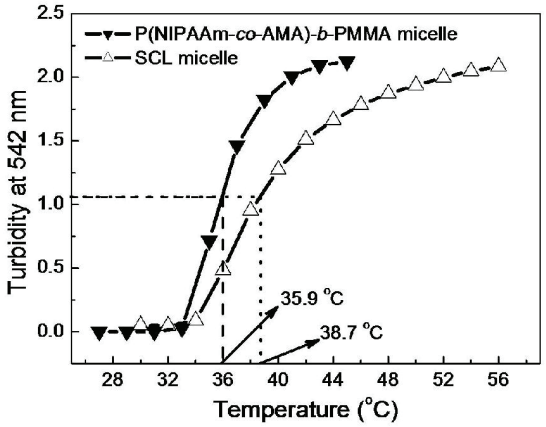

Figure 3. LCST of uncross-linked and cross-linked micelles determined by turbidity at 542 nm, [polymer] = 1000 mg/L.

As presented in Figure 3, the uncross-linked P(NIPAAm-*co*-AMA)-*b*-PMMA micelle underwent a change in the thermoresponsive P(NIPAAm-*co*-AMA) shell at a temperature close to 36 °C. Generally, in the case of a random copolymer of NIPAAm with hydrophilic co-monomer, the LCST shifts to a higher temperature because incorporation of the hydrophilic co-monomer favors chain stretch, resulting in an increase of the LCST [12,13]. Since AMA is more hydrophilic, the P(NIPAAm-*co*-AMA) copolymer exhibits a higher LCST than that (33 °C) of PNIPAAm homopolymer. Additionally, it can be seen from Figure 3 that the response rate of the LCST behavior for P(NIPAAm-*co*-AMA)-*b*-PMMA micelle remained fast, comparable to that of pure PNIPAAm, which indicates that the introduction of an appropriate amount of AMA co-monomer into the PNIPAAm chain as the cross-linking sites resulted in P(NIPAAm-*co*-AMA) copolymer with unaltered response rate, albeit a small increase in the LCST value as compared with PNIPAAm homopolymer. Similar findings were observed in our previous work [14].

However, due to the incorporation of the inorganic cross-linker into the micellar structure, the neighbouring

thermosensitive P(NIPAAm-*co*-AMA) chains in the shell were diluted and interrupted. As a result, the temperature sensitivity of the shell was slightly weakened. It is apparent from Figure 3 that the temperature response rate of the SCL micelle became slightly slower as compared with the uncross-linked micelle, and the relatively retarded response rate accounted for an elevated LCST at 38.7 ^{o}C for the SCL micelle.

3.4 Cytotoxicity study

In order to determine whether the new inorganic cross-linker based SCL hybrid micelle exhibits the anti-tumor efficacy as expected, cytotoxicity of P(NIPAAm-*co*-AMA)-*b*-PMMA copolymer and the resulting SCL micelle against HeLa cells was investigated, respectively.

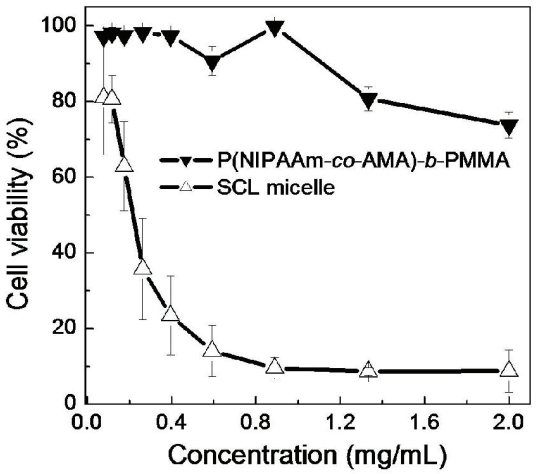

Figure 4. Cytotoxicity of P(NIPAAm-*co*-AMA)-*b*-PMMA copolymer and the resulting SCL micelles against HeLa cells.

The effect of material concentration on the proliferationof HeLa cells was studied. It can be seen from Figure 4 that the cell viability was all above 70 % in the range of the copolymer concentration recorded from 0 to 2.0 g/L, indicating that the block copolymer exhibited no apparent cytotoxicity. However, it is evident from Figure 4 that the IC_{50}, a concentration at which only 50 % cells survive was 0.2 g/L for the SCL micelle, and the cell viability was well below 10 % when the concentration of the SCL micelle exceeded 0.8 g/L. These results indicate that the cytotoxicity of the SCL hybrid micelle against HeLa cells was greater than that of the block copolymer due to the introduction of the bioactive inorganic cross-linker into the micelle structure, which further underpins that the cross-linker conjugated in the micelle structure still exhibited anti-tumor efficacy. It turns out that a novel SCL hybrid micelle system bearing anti-tumor activity was developed successfully as expected.

4 CONCLUSIONS

In summary, we have developed for the first time SCL thermoresponsive hybrid micelle by a two-step strategy: self-assembly of P(NIPAAm-*co*-AMA)-*b*-PMMA copolymer to form polymeric micelle in an aqueous solution and then cross-linking of the peripheral P(NIPAAm-*co*-AMA) block using an inorganic compound as a cross-linker. DLS measurements, TEM observation and LCST analyses confirmed the amphiphilic, shell cross-linked and thermoresponsive hybrid structure. The SCL micelles exhibited significant cytotoxicity against HeLa cells due to the presence of the inorganic structure demonstrated by cytotoxicity study. Taking advantages of its anti-tumor efficacy, it is expected that the SCL thermoresponsive hybrid micelle could be a promising candidate for cancer therapy as well as a novel carrier with much improved stability for the effective encapsulation and controlled release of various drugs.

REFERENCES

[1] C. Allen, D. Maysinger, A. Eisenberg, Colloids Surf. B: Biointerfaces 16, 1999, 3.
[2] H. Wei, S. X. Cheng, X. Z. Zhang, R. X. Zhuo, Prog. Polym. Sci. 34, 2009, 893.
[3] Y. T. Li, B. S. Lokitz, C. L. McCormick, Macromolecules 39, 2006, 81.
[4] J. Y. Zhang, X. Z. Jiang, Y. F. Zhang, Y. T. Li, S. Y. Liu, Macromolecules 40, 2007, 9125.
[5] H. Huang, T. Kowalewski, E. E. Remsen, R. Gertzmann, K. L. Wooley, J. Am. Chem. Soc. 119, 1997, 11653.
[6] J. J. Yuan, O. O. Mykhaylyk, A. J. Ryan, S. P. Armes, J. Am. Chem. Soc. 129, 2007, 1717.
[7] K. L. Wooley, J. Polym. Sci. A 38, 2000, 1397.
[8]) V. Butun, A. B. Lowe, N. C. Billingham, S. P. Armes, J. Am. Chem. Soc. 121, 1999, 4288.
[9] B. Boury, R. J. P. Corriu, V. Le Strat, P. Delord, M. Nobili, Angew. Chem. Int. Ed. 38, 1999, 3172.
[10] H. Wei, C. Cheng, C. Chang, W. Q. Chen, S. X. Cheng, X. Z. Zhang, R. X. Zhuo, Langmuir 24, 2008, 4564.
[11] H. Wei, X. Z. Zhang, Y. Zhou, S. X. Cheng, R. X. Zhuo, Biomaterials 27, 2006, 2028.
[12] A. Polozova, F. M. Winnik, Langmuir 15, 1999, 4222.
[13] S. Koga, S. Sasaki, H. Maeda, J. Phys. Chem. B 105, 2001, 4105.
[14] H. Wei, W. Q. Chen, C. Chang, C. Cheng, S. X. Cheng, X. Z. Zhang, R. X. Zhuo, J. Phys. Chem. C 112, 2008, 2888.