PREPARATION OF GOLD LOADED MICELLES BASED ON POLY (ETHYLENE GLYCOL) - b - POLY (4-VINYL PYRIDINE) COPOLYMERS AS A TOOL FOR BIOMEDICAL APPLICATIONS

Stefany Vircheva¹, Rayna Bryaskova¹, Stoyan Miloshev¹, Rumiana Tzoneva², Nikolay Dishovsky¹

¹ University of Chemical Technology and Metallurgy
Department of Polymer Engineering
8 Kl. Ohridski, 1756 Sofia, Bulgaria
E-mail: rbryaskova@uctm.edu

² Institute of Biophysics and Biomedical Engineering
Bulgarian Academy of Science
Acad. G. Bonchev Str., Bl.21, 1113 Sofia, Bulgaria

Received 09 November 2016
Accepted 28 February 2017

ABSTRACT

Well-defined block copolymers based on poly (ethylene glycol) monomethyl ether and poly (4-vinyl pyridine) (PEG-b-P4VP) were prepared by atom transfer radical polymerization (ATRP). PEG-b-P4VP block copolymer self assembled in an aqueous medium with formation of nanosized spherical micelles was thus prepared. Gold nanoparticles were successfully loaded into PEG-b-P4VP based micelles. This was confirmed by UV-vis, TEM and DLS analysis. The cytotoxicity of gold loaded micelles against MDA-MB-231 breast cancer cells and L929 fibroblasts was determined.

Keywords: block copolymers, micelles, gold nanoparticles, cytotoxicity.

INTRODUCTION

The design and synthesis of well-defined amphiphilic block copolymers via controlled radical polymerizations as nitroxide-mediated polymerization, atom transfer radical polymerization and reversible addition-fragmentation chain transfer polymerization attract considerable attention in the recent years due to their ability to self assemble in different nanostructures depending on their architecture, molecular mass and chain composition determining their application in various fields [1 - 2]. The polymeric micelles among them present definite interest due to their usage as nanoreactors for drug and gene delivery, metal and semiconducting nanoparticles [3]. Currently, micelles are under deep investigation as carriers for hydrophobic drugs in anticancer therapy [4]. In this regard, pH-responsive micelles attract attention as they can be used for controlled release and in vivo encapsulation. This is determined by the wide range of pH gradients present in physiological and biological systems. pH-sensitive block copolymers allow controlled micelle dissociation and triggered drug release. Once inside the cancer cells, the relatively low pH of ca 5.0 will provide the dissociation of the responsive micelles and drugs release [5]. Destabilization of these structures occurs when the protonable groups become charged below the pKa, thus leading to repulsion between the polymer chains, which in turn results in micelles dissociation [6]. This can be used as an internal stimulus for triggered drug release due to the difference
of pH of healthy and tumor cells as well as the acidic environment in endosomal and lysosomal compartments [7]. Polypyridines like poly (2-vinylpyridine) and poly (4-vynilpyridine) (P4VP) are water-insoluble at neutral or alkali pH, but become protonated and thus soluble at pH <5 [8]. Therefore, block copolymers based on polypyridines would be able to response to pH changes in the medium. Moreover, they are able to complexe with different metal ions as gold, silver, etc. [9]. Poly(ethylene glycol) is an appropriate choice for the second building block. It is a hydrophilic polymer, water soluble, biocompatible and low toxic [5].

The use of gold nanoparticles in medical, biomedical and biological field is very wide. They can be applied in diagnostics, therapy, and hygiene, immunoassay, clinical chemistry, detection and photothermalysis of microorganisms and cancer cells and tissues, targeted delivery of drug and so on [10]. Gold-based plasmonic nanostructures have been widely utilized for cancer diagnostics and therapy [11]. They have been of great interest for photothermal therapy due to their strong and tunable linear absorption in the near-infrared region, where tissue penetration can be maximized [12 - 13].

Therefore, the aim of the present work is to investigate the self assembling behavior of well-defined PEG-b-P4VP block copolymer obtained via atom transfer radical polymerization and to prepare gold loaded micelles in order to determine their cytotoxicity against normal and cancer cells.

**EXPERIMENTAL**

**Materials**

Poly (ethylene glycol) monomethyl ether (PEG, Merck, Mn ~ 1900 g/mol) was dried under vacuum at 40°C for 24 h prior to use. Toluene (Sigma Aldrich, 99.8 %) was distilled. 4-vinlypyridine (Acros Organics, 95 %) was passed though a neutral alumina (Al₂O₃) column and distilled. Thionyl chloride (Acros Organics, 99 %), CuCl (Aldrich, 98 %), Pyridine (Sigma Aldrich, 99.8 %) and 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA, Acros Organics, 98 %) were used as received.

**Synthesis of PEG-b-P4VP block copolymer**

The synthesis and characterization of PEG-b-P4VP copolymer was reported in details in ref. [14]. In brief, the polymerization was performed in a 50 ml flask equipped with a magnetic stir bar and septum. MeOPEG-Cl macroinitiator (3g, Mn = 2050 g mol⁻¹) was added to 17 ml i-propanol and heated at 60°C in order to dissolve the macroinitiator. The mixture was deoxygenated by passing N₂. Then, the ligand (1,1,4,7,7-PMDETA, 0.330 ml) was added and the solution was stirred and deoxegenated with N₂ for 30 min. Then CuCl (0.155 g) was added. The reaction mixture was stirred until it became homogenous and green. Finally, deoxygenated 4-VP (17 ml, preheated at 50°C) was added. The polymerization was performed for 48 h at 60°C. The diblock copolymer PEG-b-P4VP was purified by passing through Al₂O₃ column to remove the copper catalyst, precipitated in cold diethyl ether and filtrated. The product was dried at 40°C under vacuum. The molar mass (Mn) of diblock PEG-b-P4VP copolymer was 6470 g mol⁻¹ as determined by SEC analysis.

**Micellization of PEG-b-P4VP micelles and preparation of gold loaded micelles**

The micelles were prepared through introduction of PEG-b-P4VP copolymer solution in methanol (c = 10 mg mL⁻¹) into 18 mL milli Q water at a controlled rate under stirring. The solution was stirred for 24 h. Dialysis against milli Q water for 48h was performed in order to eliminate methanol. HAuCl₄ aqueous solution (5 mg in 200 mL water) was added to the filtrated micellar solution aiming to prepare gold loaded micelles. The mixture was stirred for 1 day, followed by dialysis against milli Q water in order to remove the unreacted gold salt. Thermal reduction was then performed. The micelles gold concentration was determined by ICP analysis (24 mg L⁻¹ Au).

**Methods**

Dynamic light scattering (DLS). DLS measurements were performed on a Malvern ZS90 at a detection angle of 90°C. The average diameter and the polydispersity (PDI) were automatically analyzed in the cumulating mode using Malvern software.

Transmission electron microscopy (TEM). TEM pictures were recorded on a STEM JEOL JEM 2100 microscope equipped with a GATAN Orius 832 SC 1000 camera. Samples were prepared by deposition of a drop of micellar solution on carbon coated grids. The latter were subsequently dried in air.
MTT viability test. To analyze the cytotoxic effect of gold loaded micelles on L929 cells and MDA-MB-231 breast cancer cells, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (Invitrogen, USA) was performed as described in Bryaskova et al. [15]. Briefly, the adherent L929 or MDA-MB-231 cells seeded in 96-well plates were treated with 0.5 µg mL\(^{-1}\), 1 µg mL\(^{-1}\), 2 µg mL\(^{-1}\), 4 µg mL\(^{-1}\) and 8 µg mL\(^{-1}\) solutions of gold loaded micelles and incubated for 24 hours. Control cells treated with non gold loaded micelles were used. After the incubation period, MTT solution was added (5 mg mL\(^{-1}\) in PBS) and the plates were further incubated for 4 hours at 37°C. The formed formazan crystals were dissolved by addition of a solvent (5 % formic acid in 2-propanol). The absorbance was recorded at 570 nm on a plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). Six reading were carried out for each concentration. A medium containing 5 % formic acid in 2-propanol was used as a blank solution.

RESULTS AND DISCUSSION

The amphiphilic PEG-b-P4VP block copolymer is prepared by ATRP using a novel macroinitiator based on PEG as reported elsewhere [14]. Micellization of amphiphilic PEG-b-P4VP diblock copolymer in water is performed by dissolution of PEG-b-P4VP copolymer in methanol (a good solvent for both blocks), followed by slow addition of the dissolved copolymer into water at a controlled rate. Thus micelles consisting of P4VP core and PEG shell were prepared (Scheme 1).

The average hydrodynamic diameter (Dh) and particles size distribution of PEG-P4VP micelles are measured by DLS. Micelles of Dh value of 75 nm and PDI of 0.17 are obtained (Fig. 1a).

The morphology of the micelles is examined by TEM. TEM pictures of deposited micelles show the presence of well-defined spherical micelles with an average diameter of 70 nm (Fig. 1b).

Further, PEG-b-P4VP based micelles in water are loaded with gold nanoparticles. This is achieved using HAuCl\(_4\) as a gold precursor. Accumulation of gold salt within the micellar core is expected due to the high affinity of the nitrogen atom of P4VP pyridine rings towards gold. The loading of micelles is performed by addition of a gold salt to the micellar solution of the copolymer providing gold diffusion into the micelles.
This step proceeds within 24 h. The non-reacted gold salt is removed by dialysis against water followed by reduction and formation of gold nanoparticles in P4VP core of the micelles. The formation of gold loaded micelles is confirmed by UV-vis spectroscopy through the appearance of a peak at 530 nm. It is attributed to gold (Fig. 2a).

DLS analysis is applied to determine the average hydrodynamic diameter and particle size distribution of the gold loaded micelles prepared. It is found that the hydrodynamic diameter of the latter increases to 90 nm with PDI = 0.2 (Fig. 2b). TEM analysis confirms also the preparation of spherical gold loaded micelles with an average diameter of 80 nm (Fig. 3a). The selected area electron diffraction pattern (SAED) of gold loaded micelles indicates that the gold nanoparticles are nanocrystalline. The patterns of SAED are indexed as (111), (200), (220), and (311) reflections characteristic for the face centered cubic (fcc) structure of gold (Fig. 3b).

Finally, the cytotoxicity of gold nanoparticles incorporated into PEG-b-P4VP based micelles is tested. MTT test is conducted for 24h aiming this. MDA-MB-231 breast cancer cells and L929 fibroblasts are incubated varying the gold nanoparticles concentrations within the range of 0.5µg mL⁻¹ - 8µg mL⁻¹. Micelles of no gold nanoparticles presence are used as control samples. The latter are used for reference in cell viability determination (Fig. 4).
The results show that after 24h of incubation the viability of both cell types decreases gradually with increase of gold nanoparticles concentration. Fig. 4 reveals that the gold loaded micelles prepared are more toxic to MDA-MB-231 breast cancer cells in comparison to the normal L929 fibroblasts cells and reach 44.5% and 54% cell viability, respectively, in case of 8µg mL⁻¹ gold content (p < 0.01) (Fig. 4). For micelles containing lower gold concentrations (0.5µg mL⁻¹ - 4 µg mL⁻¹) the tendency observed is preserved. It is worth adding that the gold loaded micelles are more active towards MDA-MB-231 than L929 cells at relatively high cell viability (Fig. 4).

CONCLUSIONS

PEG-b-P4VP block copolymers were successfully prepared by atom transfer radical polymerization. The self-assembling behavior of PEG-b-P4VP diblock copolymer in an aqueous medium with formation of well-defined spherical micelles was investigated. TEM and DLS analysis were applied. The micelles prepared were successfully loaded with gold nanoparticles. The latter were introduced to P4VP core of the micelles. Appropriate analytical techniques were applied. The cytotoxicity test of gold loaded micelles performed in respect to normal and cancer cells clearly demonstrated that the gold loaded micelles showed high activity against cancer cells. It increased with gold concentration increase. These results demonstrated that the gold loaded micelles prepared can find a potential application in biomedicine.

Acknowledgements

This study was supported by the Scientific Research Center of the University of Chemical Technology and Metallurgy, Sofia, Bulgaria, through project No 11513.

REFERENCES


