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Synthesis of novel amphiphilic poly(*N*-isopropylacrylamide)-*b*-poly(aspartic acid) nanomicelles for potential targeted chemotherapy in ovarian cancer

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ABSTRACT

The purpose of this study was to merge amino terminated poly(*N*-isopropylacrylamide) (PNIPAm-NH_2) with L-Aspartic acid-*N*-carboxyanhydride (L-Asp-NCA) using thermal ring-cleavage polymerization to synthesize a pH and thermo-responsive amphiphilic PNIPAm-b-PAsp copolymer for self-assembling nanomicelles. The stimuli-responsive nanomicelles are intended for targeted delivery of methotrexate (MTX) for potential application in ovarian cancer chemotherapy. Rheological profiles of various concentrations of the block copolymer were evaluated. The thermal ring-opening polymerization of L-Asp-NCA onto PNIPAm-NH_2 yielded a copolymer with an inherent viscosity of 494.527mPas that was confirmed by advanced rheometry with a high mean molecular weight (M_W = 2.217 × 10^6kDa) computed by the partially-proportional Mark-Houwink formula. Scanning electron microscopy (SEM) elucidated the copolymer topography and pore distribution. Vibrational Fourier Transform Infrared (FTIR), Nuclear Magnetic Resonance (NMR), Differential Scanning Calorimetry (DSC), and Thermal Gravimetric Analysis (TGA) confirmed the synthesis of the amphiphilic block copolymer. The PNIPAm-b-PAsp nanomicelles were 90 nm in size with a zeta potential value of −0.539 mV (PdI ≤0), a yield of 94% and a DEE value of >77%. The *in vitro* release profile of MTX displayed constant release of MTX over 72 h. The critical micelle concentration (CMC) was computed to be 0.09 mg/mL. Furthermore, the potential of the amphiphilic PNIPAm-b-PAsp copolymeric nanomicelles to target delivery of MTX for ovarian cancer cell destruction was evaluated following determination of cytotoxicity and internalization in an ovarian cancer cell line (NIH:OVAR-5). Overall, results demonstrated that the novel PNIPAm-b-PAsp copolymer synthesized was practical for nanomicelle formation and for the potential application as a stimuli-responsive nanocarrier system for the targeted delivery of MTX in ovarian cancer.

1. Introduction

Amphiphilic copolymers have versatile properties that make them suitable for the delivery of hydrophilic and hydrophobic chemotherapeutic drugs [1–3]. These copolymers may be synthesized from aqueous-miscible and partially soluble polymers that agglomerate to form a myriad of structures including nanomicelles, tubular cylinders or vesicles. The inner core can be equilibrated by an outer shell-like surface that is dependent on the copolymer block size, polymer ratios, vehicle composition or external stimuli such as pH, temperature or ionic strength within the aqueous medium during synthesis [4–6]. Recently, significant interest has been placed on the synthesis of stimuli-responsive polymers to form nanomicelles with modified physicochemical parameters. The most commonly used stimuli are temperature and pH that induce polymer transitions when prepared from amphiphilic surfactant copolymers for application in clinical nano-enabled chemotherapeutics [7].

Poly(*N*-isopropylacrylamide) (PNIPAm) is categorized as a

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thermo-responsive polymer with a LCST of 32 °C. Furthermore, it is aqueous-miscible at physiological temperatures lower than the LCST value. However, when the LCST is exceeded it transforms into impenetrable hydrophobic agglomerates [8–11]. Polyaspartic acid (PAsp) is another synthetic polymer that is of pharmaceutical significance to deliver partially soluble drugs and has been used for numerous biomedical applications such as dialysis membranes, artificial skin and orthopedic implants [12–16]. In particular, PAsp nanomicelles with hydrophilic segments have been explored previously as a potential drug delivery vehicle for sparingly soluble chemotherapeutic drugs commonly entrapped in hydrophobic domains [17–19]. Combination of PNIPAm and PAsp as an amphiphilic copolymeric nanomicelle structure may potentially enhance the bioavailability, stability, physicochemical parameters and targeted release of chemotherapeutic drugs [20–22].

To our knowledge, stimuli-responsive nanomicelles of PNIPAm-b-PAsp prepared via thermal ring-cleavage polymerization for potential application as a targeted form of chemotherapy in Ovarian Cancer (OC) has not yet been explored. This is despite the fact that PNIPAm has been widely used with other polyamino acids as a blend to produce inter-incisive polymeric networks [23–25]. Therefore this study provides an innovative approach to firstly synthesize PNIPAm-b-PAsp employing PNIPAAm-NH_2 for copolymerization onto PAsp. This novel copolymer was subsequently used as a framework for the preparation of drug-loaded nanomicelles as a targeted form of chemotherapy in OC. The hydrophobic methotrexate (MTX) was employed as a model chemotherapeutic drug. Special interest was placed on the nanomicelle properties as well as their drug encapsulation efficiency and pharmaceutical stability. For further evaluation of nanomicelle efficacy in OC treatment, the MTX-loaded nanomicelles were incubated with OC cells (NIH:OVAR-5), and the treated cells were analyzed for their cytotoxicity and cellular uptake.

2. Materials and methods

2.1. Materials

Poly(*N*-isopropylacrylamide) (PNIPAm) was purchased from Sigma Aldrich (St. Louis, MO, USA) that was hexane recrystallized, vacuum dried at 20 °C and the 2,2'-azoisobutyronitrile (AIBN) initiator agent was ethanol-recrystallized prior to use. Aspartic acid, 2-amino ethanethiol hydrochloride (AET-HCl) and triphosgene were procured from Sigma Aldrich (St. Louis, MO, USA) and vacuum dried at 20 °C. N,N'-dimethylformamide (DMF) (98%), tetrahydrofuran (THF), ethyl ether and petroleum ether (30–60 °C) was purchased from Merck Chemicals Co. (Pty) Ltd. (Darmstadt, Germany) and were used as received. Methotrexate (MTX), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 100 IU/mL penicillin/100 mg/mL streptomycin, RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS) and 0.25% $^{w}/_{v}$ trypsine 0.03%$^{w}/_{v}$ EDTA solution were purchased from Sigma Aldrich (St. Louis, MO, USA). NIH:OVCAR-5 cells were purchased from Dr. Tom Hamilton (Fox Chase Cancer Institute, PA, USA). Cell culture supernatants and urine (96-wells) were purchased from Biocom Biotech (Pty) Ltd. (Centurion, Pretoria, RSA). All other reagents and organic solvents were of analytical grade and vacuum dried prior to use. All OC cells were grown in an incubator from RS Biotechnological Galaxy (Irvine, UK) maintained at 37 °C in a fully humidified atmosphere of 5% CO_2. All cell experiments were performed in the logarithmic phase of growth.

2.2. Synthesis of amino poly(N-isopropylacrylamide (PNIPAm-NH_2)

PNIPAm-NH_2 was synthesized by radical polymerization employing a chain transfer agent 2-amino ethanethiol HCl (AET-HCl) and an initiating agent AIBN. *N*-isopropylacrylamide (4×10^{-3} mol), AIBN (0.8×10^{-5} mol) and AET-HCl (0.6×10^{-4} mol) were dissolved in 10 mL DMF. The mixture was degassed by purging with N_2 for 1 h and then refluxed at 70 °C for 10 h. Following the polymerization reaction, the solution was concentrated by condensed pressure distillation to evaporate the DMF. The yield was precipitated by introduction of diethyl ether followed by vacuum drying. Excess triethanolamine (TEA) in THF was added drop-wise to this polymer mixture at 20 °C to convert PNIPAm-NH_2-HCl into PNIPAm-NH_2. The resultant polymer was further purified by precipitation in excess diethyl ether followed by filtration with a 0.22 μm filter membrane and finally the yield was vacuum dried at 30 °C.

2.3. Synthesis of L-aspartic acid-N-carboxyanhydride using a triphosgene approach

A triphosgene approach was employed to synthesize L-Asp-NCA. Briefly, 11 g of excess triphosgene was introduced into a 7% aspartic acid tetrahydrofuran solution at 50 °C. In order to remove the phosgene gas, the solution was bubbled with N_2 for 30 min until the solution increased in clarity. L-Asp-NCA precipitated after the introduction of the solution into surfeit petroleum ether (30–60 °C) and finally the yield was vacuum dried at 30 °C.

2.4. Synthesis of the amphiphilic poly-N-isopropylacrylamide-b-polyaspartic acid copolymer

The PNIPAm-b-PAsp copolymer was synthesized by ring-cleavage polymerization of L-Asp-NCA. The reaction scheme is shown in Scheme 1(A–D). PNIPAm-NH_2 was suspended in DMF and the reaction mixture was degassed by purging with N_2 for 30 min followed by introduction of L-Asp-NCA. All reactions were conducted at 20 °C for 72 h. The resultant copolymer was purified by precipitation of surfeit diethyl and finally the yield was vacuum dried at 30 °C.

2.5. Establishment of the copolymer molecular mass

The mean molecular mass of the synthesized copolymer in aqueous solution was determined utilizing the partially-proportional Mark-Houwink relationship (Equation (1)) that correlated the inherent/intrinsic viscosity $[\eta]$ with the molar mass (M).

$$[\eta] = KM^{a} \quad (1)$$

where, a is a specific solvent-polymer interaction parameter and K is an empirical proportionality constant. Solvents with value of $a = 0.5$ is suggestive of a theta solvent. A value of $a = 0.8$ represents an ideal solvent. Flexible polymers have values between $0.5 \leq a \leq 0.8$ and partially-flexible polymers the value of $a \geq 0.8$. In this study, water was utilized as the preferred solvent which interacted with the biopolymer sequence hence displaying an elastically expandable molecular arrangement with an a value of 0.8 and an empirical proportionality K value of 6.31×10^{-5}. Viscosity evaluation of 0.01%, 0.001% and 0.0001% concentrations of aqueous suspensions of the synthesized copolymer were undertaken on a Haake Modular Advanced Rheometer System (MARS) with the mean viscosity of the dilutions evaluated and used to compute the mean molecular mass of the copolymer.