Polymer 75 (2015) 25–33

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Glutathione-bearing fluorescent polymer-curcumin conjugate enables simultaneous drug delivery and label-free cellular imaging

Soma Dey [a], Lizebona August Ambattu [b], P.R. Hari [a], M.R. Rekha [b], K. Sreenivasan [a, *]

[a] Laboratory for Polymer Analysis, BMT Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojappura, Trivandrum, 695012, Kerala, India

[b] Division of Biosurface Technology, BMT Wing, SCTIMST, Poojappura, Trivandrum, 695012, Kerala, India

ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form
3 August 2015
Accepted 12 August 2015
Available online 14 August 2015

Keywords:
Glutathione
Polymer-curcumin conjugates
Cryo-TEM
Confocal microscopy

ABSTRACT

Curcumin is a natural polyphenol with potential anticancer property. However, the medicinal assets of curcumin cannot be properly utilized due to its low aqueous solubility, instability and subsequent poor bioavailability. Conjugation of curcumin to a suitable hydrophilic polymer is a useful approach for augmenting the aqueous solubility of the drug. In addition, nano-structured materials with intrinsic fluorescence property are of prime importance in the current biomedical domain. In this study, a novel glutathione containing biodegradable, non-toxic and water soluble, fluorescent polymer was developed. Curcumin was covalently conjugated to this polymer to fabricate nano-sized micelle forming fluorescent polymer-curcumin conjugate. The ability of the entity as a safer curcumin delivery vehicle and label-free cellular imaging probe was demonstrated in C6 glioma cells.

1. Introduction

The term “nanomedicine” is now widely contemplated and has shown enormous potential to cancer management right from detection to treatment [1]. At present polymer based nano-sized therapeutics are also important candidates for the fabrication of nanomedicine for cancer treatment. Among various “polymer therapeutics”, polymer-drug conjugate is a vital vehicle for anti-cancer drug delivery [2]. For many of the anticancer drugs poor aqueous solubility is the leading limitation that can be improved by direct covalent conjugation of the drug to a hydrophilic polymer. Polymer-drug conjugates also have the capability to control the release profile of a drug. Conjugation of hydrophobic drugs to suitable polymers offers a chance to alter drug pharmacokinetics and biodistribution which are basically useful for drugs exhibiting poor bioavailability (e.g. curcumin), faster clearance and/or off target toxicities (anticancer drugs) [3].

Curcumin (Ccm), the natural polyphenol, possesses tremendous medicinal assets including potential chemopreventive and chemotherapeutic activity [4,5]. It is well known that nuclear transcription factor kappa beta (NF-κβ) is the key controller of inflammation, apoptosis, cellular proliferation and resistance in cells and most tumor cells express constitutively active NF-κβ to mediate their survival. Interestingly, Ccm can block NF-κβ and consequently can suppress NF-κβ regulated gene products to kill tumor cells [6]. Various studies proved that Ccm can induce cellular apoptosis by inhibiting multiple cell signaling pathways and because of this (i) possibility to develop resistance in cancer cells towards Ccm is very less likely and (ii) Ccm can exhibit pleiotropic therapeutic effect in cancer [6,7]. Unfortunately, Ccm is associated with limitations like extremely low aqueous solubility, instability and consequent poor bioavailability [8]. In order to redress these problems, one of the most useful approaches is the synthesis of polymer-Ccm conjugate.

Nanomaterials with intrinsic fluorescence are of extreme interest in the biomedical field for their potential use in drug delivery, cellular imaging, diagnostic and therapeutic applications [9,10]. Quantum dots, organic dye conjugated nanostructures etc. are widely used as fluorescent biomaterials. However, these materials are associated with several issues such as cumbersome synthetic steps and in many instances show severe cytotoxicity [11,12]. Photoluminescent polymeric materials are advantageous in this regard as they possess varied functionalities facilitating desired surface modification and own low intrinsic toxicity along with the competence of label-free cellular imaging [13,14].

Recently, a family of amino acid based aliphatic, biodegradable,

* Corresponding author.
E-mail address: sreeni@sctimst.ac.in (K. Sreenivasan).

http://dx.doi.org/10.1016/j.polymer.2015.08.020
0032-3861/

photoluminescent polymers (BPLPs) was developed [15] and using them various fluorescent nano materials was reported for drug delivery, imaging and tissue engineering applications [16,17]. Glutathione (GSH) is a natural tripeptide consisting of L-glutamic acid, L-cysteine and glycine. GSH is a potential antioxidant that helps in cancer regression. Herein, we report the synthesis and evaluation of a novel GSH containing water soluble fluorescent polymer (GSHBP) and the micelle forming fluorescent GSHBP-Ccm conjugate for the safer delivery of the natural anticancer drug Ccm and simultaneous label-free *in vitro* cellular imaging.

2. Materials and methods

2.1. Materials

L-Glutathione (reduced; GSH), poly(ethylene glycol) (PEG; Mw = 1000), citric acid (anhydrous; CA), 1,3-dicyclohexyl carbodiimide (DCC), 4-Dimethylaminopyridine (DMAP) and Nile Red (NR) dye were purchased from Sigma–Aldrich, Bangalore, India. Curcumin (Ccm; 95% total curcuminoid content) from rhizome of turmeric was obtained from Alfa Aesar. Dimethyl sulfoxide (DMSO) and Ethanol (EtOH) were obtained from Merck, Mumbai, India. Deionized water was used during all the reaction and purification process and ultra pure water (18.2 mΩ resistivity) was obtained from the Milli-Q water purification system.

Glioma cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. 3-(4, 5-Dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT reagent), fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium were purchased from Sigma–Aldrich (Bangalore, India). Trypsin/EDTA and Nutrient F-12 Ham were purchased from Invitrogen (Bangalore, India).

2.2. Synthesis of fluorescent biodegradable polymer (GSHBP)

In order to obtain GSHBP, in a three neck round bottom flask PEG and CA (1:1 by no. of mole) were allowed to react under N_2 (g) blanket at 160 °C with gradual addition of GSH (molar ratio GSH: CA = 0.5). The reactants were melted at 160 °C with constant stirring and kept at that temperature for about 20 min. Then the temperature was brought down to 135 °C and the reaction was continued for nearly 2 h. The highly viscous polymer was collected from reaction vessel in hot condition and it was purified after cooling by dialysis against deionized water (using membrane of molecular weight cut off MWCO 1000). Finally, the product was lyophilized and refrigerated in amber colored glass bottle for future use. The synthetic scheme is shown in Scheme 1A.

2.3. Synthesis of micelle forming GSHBP-Curucmin conjugate (GSHBP-Ccm)

GSHBP (500 mg) was dissolved in 10 mL DMSO and the free carboxylate groups in GSHBP were activated using DCC in presence of catalytic amount of DMAP with stirring at room temperature for 1 h. Then Ccm (4.07 mM in DMSO) was added to it and the reaction mixture was stirred at 65 °C temperature under N_2 for 8 h. The reaction mixture was cooled to room temperature and purified by dialysis against DMSO for 1 day followed by distilled H_2O for 2 days using a dialysis membrane of MWCO 1000. Finally the dialyzed intense yellow colored product was lyophilized and stored in refrigerator in an amber colored glass bottle for further use. The reaction scheme is depicted in Scheme 1B.

2.4. Physicochemical characterizations

The absorption and emission spectra of the fluorescent polymer and polymer-drug conjugate were recorded using Ultraviolet–Visible (UV–Vis) spectrophotometer (Cary model 100 Bio UV–Visible spectrophotometer, Melbourne, Australia) and spectrofluorometer (Cary Eclipse model EL 0507) respectively. Fourier Transform Infrared (FTIR) spectra were recorded using a Nicolet 5700 FTIR Spectrometer. Nuclear Magnetic Resonance (NMR) spectra (both ^1H and ^{13}C) were analyzed by 500 MHz spectrometer (Brucker Avance DPX 500). NMR spectra were recorded in DMSO-D6 solvent at room temperature. The hydrodynamic diameter and zeta potential of the fluorescent polymer-drug conjugate micelles were determined by dynamic light scattering instrument (Malvern Zetasizer Nano ZS, UK) at 25 °C and in aqueous buffer solution (pH = 7.4). Morphology of the nano-sized micelles was visualized by recording transmission electron microscopic images (TEM; JEM-2100, JEOL, Japan) under cryogenic condition. Molecular weight of the polymer and its degradation behavior was determined using gel permeation chromatography (GPC) and liquid chromatography (LC) (Waters Assoc Inc.; Milford, USA, model 600 pumps). For GPC analysis Styragel column and THF were used as the stationary phase and mobile phase respectively. Refractive index (RI) detector was used to generate the GPC chromatogram. Whereas, in LC analysis C18 Reprobond column was used as the stationary phase and 0.1% H_3PO_4 aqueous solution was employed as the mobile phase. UV detector was used in LC analysis (using λ = 214 nm for citric acid).

2.5. Estimation of fluorescence quantum yield of GSHBP

Fluorescence quantum yield (Q.Y.) of GSHBP was determined by William's method [18] using anthracene as the reference compound. An aqueous solution of GSHBP polymer was prepared to determine the excitation wavelength at which the polymer gives highest intensity of fluorescence emission and then the absorbance of the solution at that optimal excitation wavelength was measured. From this solution six solutions were prepared (by dilution with gradient concentration) such that the absorbance of each of the six solutions was in the range of 0.01–0.1. Fluorescence emission spectra for the set of solutions were recorded (keeping both the emission and excitation slit width as 5 nm) and the integrated fluorescence intensity (area under the curve) for each solution was calculated. Finally, the integrated fluorescence intensity against absorbance was plotted for all the six solutions with different concentrations. Similar plot was also done for the reference compound and the fluorescence Q.Y. was calculated based on the following equation.

$$\Phi_s = \Phi_r \cdot \frac{Slpoe_s}{Slope_r} \cdot (\eta_s/\eta_r)^2$$

Where, Φ = Q.Y., Slope = slope of integrated fluorescence intensity Vs absorbance plot, η = refractive index of the solvent used, s = sample r = reference. Here we used ethanolic solution of anthracene as the reference ($\Phi_r = 0.27$) [15].

2.6. Degradation of GSHBP in physiological condition

In order to study the degradation of GSHBP polymer in physiological condition, the polymer was dissolved in PBS (pH = 7.4; 31 mg/mL concentration) and the solution was kept in an orbital shaker at 37 °C at 120 rpm. After a desired period of time, aliquot (20 μL) of degradation medium was taken out followed by replacement with similar volume of fresh PBS. The sample (20 μL) was analyzed using LC to determine the amount of CA present in the degradation medium (by means of a calibration curve). After 15 days of degradation study, the remaining polymer solution was lyophilized followed by GPC analysis (2% w/v solution of degraded