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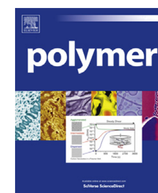

Hepatocyte-targeted fluorescent nanoparticles based on a polyaspartamide for potential theranostic applications

Emanuela Fabiola Craparo [a], Mariano Licciardi [a], Alice Conigliaro [b, c], Fabio Salvatore Palumbo [a], Gaetano Giammona [a], Riccardo Alessandro [b], Giacomo De Leo [b], Gennara Cavallaro [a, *]

[a] *Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF), Università di Palermo, Palermo, Italy*
[b] *Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi (Di.Bi.Me.F.), Università di Palermo, Palermo, Italy*
[c] *Dipartimento di Biotecnologie Cellulari ed Ematologia, Sapienza Università di Roma, Rome, Italy*

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Here, the synthesis of a galactosylated amphiphilic copolymer bearing rhodamine (RhB) moieties and its use for the preparation of polymeric fluorescent nanoparticles for potential applications in therapy and diagnosis are described.

To do this, firstly, a fluorescent derivative of α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was synthesized by chemical reaction with RhB, and with polylactic acid (PLA), to obtain PHEA-RhB-PLA. Then, the derivatization of PHEA-RhB-PLA with GAL-PEG-NH_2 allows obtaining PHEA-RhB-PLA-PEG-GAL copolymer, with derivatization degrees in -PLA and -PEG-GAL equal to 1.9 mol% and 4.5 mol%, respectively. Starting from this copolymer, liver-targeted fluorescent nanoparticles were prepared by high pressure homogenization–solvent evaporation method, and showed nanoscaled size, slightly negative zeta potential and spherical shape. Chemical and enzymatic stability of fluorescent dye covalently linked to the copolymer backbone by ester linkage was demonstrated until 4 days of incubation. Finally, thanks to the covalently-linked fluorescent RhB, it was demonstrated that these galactosylated nanoparticles interact with HepG2 cells that are positive for the asialoglycoprotein receptor (ASGPR), while these do not interact with HeLa cells that are negative for the same receptor, demonstrating the contributor of ASGPR to the internalization process.

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1. Introduction

In the field of nanomedicine, the use of polymeric nanoparticles bearing a fluorescent probe for imaging is a promising application to evaluate intracellular trafficking and/or events, and body distribution of therapeutic systems, in a non-invasive way [1]. Moreover, the potential of these systems to enable the detection of many processes inside the cells, could allow the understanding of disfunctionalities and the subsequent occurrence of diseases. Some of these fluorescent polymer-based systems are currently proposed for simultaneous applications combining both therapy and diagnosis [2,3].

To realize fluorescent polymer-based nanoparticles, the polymeric materials used as the matrix should offer excellent biocompatibility, multiple functional groups for further conjugation with dyes, drugs, and/or targeting ligands, and the ability to form stable particles that persist over a long time also in biological environments. Moreover, by varying the polymer, the preparation method, and the kind of surface functionalization, it is possible to precisely engineer these particles to a specific application.

Complementary to the passive targeting to an organ or a tissue, which is mainly obtained with nanoparticles with high circulation time depending on their size, shape, surface charge and chemistry, active targeting with ligands can also be exploited to reach cell specificity [1]. A targeting moiety can be conjugated to the fluorescent particle to improve the localization and binding of the dye in the area to image, and to modify its pharmacokinetics. The targeting moiety can be for instance an antibody, protein or peptide, an oligonucleotide, a saccharide, or any other molecular template known for its specific affinity for a cellular compartment, cellular receptor, biological fluid or tissue [4].

* Corresponding author. Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF), Università di Palermo, via Archirafi 32, 90123 Palermo, Italy.
E-mail address: gennara.cavallaro@unipa.it (G. Cavallaro).

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Galactosylation is a well-established strategy to obtain a hepatocyte active targeting because targeting via galactosylated carriers exploits highly specific interactions of galactose ligands with asialoglycoprotein receptor (ASGPR) that is specifically and abundantly found on hepatocytes [5,6].

In this paper, the realization of fluorescent polymeric nanoparticles targeted to hepatocytes by following easily scaling up processes and by using starting materials with suitable structural and functional properties, is reported. In particular, the synthesis of a fluorescent amphiphilic polyaspartamide-based copolymer starting from α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) is reported. PHEA is a biocompatible, synthetic protein-like polymer, already used for obtaining polymeric carriers with potential application in controlled and targeted drug delivery [6–10]. PHEA was functionalised with rhodamine B, with acid terminated PLA and with a galactosylated PEG derivative in order to obtain PHEA-RhB-PLA-PEG-GAL graft copolymer. The latter was used for the preparation of liver-targeted fluorescent polymeric nanoparticles by using the high pressure homogenization-solvent evaporation technique [11], without the use of surfactants or stabilizing agents. Obtained nanoparticles showed nanometric size, spherical shape, low surface charge, and were stable after dispersion in physiological-mimicking condition until 24 h. Moreover, stability of linkage of RhB on these polymeric nanoparticles until 4 days was also demonstrated. Preliminary in vitro studies demonstrated the absence of cell toxicity, and the contributor of ASGPR to the internalization process of galactosylated nanoparticles in HepG2 cells.

2. Experimental

2.1. Materials

Rhodamine B (RhB), anhydrous *N*,*N*′-dimethylformamide (*a*-DMF), α-lactose, D,L-poly(lactic acid) (PLA acid terminated, Mw = 10–18 kDa), sodium cyanoborohydride, 1,1′-carbonyldiimidazole (CDI), *N*,*N*′-disuccinimidyl carbonate (DSC), anthrone, anhydrous dimethylacetamide (*a*-DMA), poly(ethylene oxide) standards, esterase from porcine liver or lipase from porcine pancreas type II were purchased from Sigma–Aldrich (Italy). Diethylamine (DEA), triethylamine (TEA), O-(2-aminoethyl)-O′-methyl poly(ethylene glycol) 2000 (PEG_{2000}) (≤0.4 mmol NH_2/g), poly(ethylene glycol)bis(amine) 2000 (H_2N-PEG-NH_2), ethyl ether, dichloromethane were obtained from Fluka (Italy). All reagents were of analytic grade, unless otherwise stated.

α,β-Poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared by aminolysis of polysuccinimide (PSI) with ethanolamine in DMF solution [12]. Spectroscopic data were in agreement with the attributed structure [12]. Weight average molecular weight ($\overline{M}_w$) of PHEA was 38.7 kDa ($\overline{M}_w/\overline{M}_n = 1.62$), determined by SEC analysis.

The SEC system (Waters, Mildford, MA) was equipped with a pump system, two Phenogel columns from Phenomenex (5 μm particle size, 10^3 Å and 10^4 Å of pores size), and a 410 differential refractometer (DRI) as concentration detector. Analyses were performed with 0.01 M LiBr DMF solution as eluent with a flow of 0.8 ml/min and poly(ethylene oxide) standards (range 145–1.5 kDa) to obtain the calibration curve. The column temperature was set at 50 °C (±0.1 °C).

2.2. Synthesis of PHEA-RhB copolymer

Derivatization of PHEA with RhB to obtain the PHEA-RhB copolymer was carried out by using CDI as coupling agent to activate the RhB carboxyl groups. A calculated amount of CDI dissolved in a-DMF (26.0 mg/ml) under argon atmosphere, was added dropwise to a RhB solution in a-DMF (8.7 mg/ml), according to $R_1 = 1.25$ being:

$$R_1 = \frac{\text{moles of CDI}}{\text{moles of RhB}} \tag{1}$$

The clear solution was stirred at 40 °C for 4 h under argon atmosphere. Simultaneously, 33.3 mg/ml of PHEA were dissolved in a-DMF at 40 °C under argon atmosphere and then DEA, as catalyst, was added. The amounts of PHEA and DEA were calculated according to $R_2 = 0.01$ and $R_3 = 5$, being:

$$R_2 = \frac{\text{moles of RhB}}{\text{moles of hydroxyl} - \text{carrying PHEA repeating units}} \tag{2}$$

$$R_3 = \frac{\text{moles of DEA}}{\text{moles of RhB}} \tag{3}$$

After activation time, the resulting polymeric solution was added drop-wise and slowly to CDI-activated RhB (aRhB) solution. The reaction mixture was left under argon atmosphere and continuous stirring at 40 °C for 48 h, then was precipitated in diethyl ether and the suspension was centrifuged at 4 °C for 15 min, at 9800 rpm by using a Centra MP4R IEC centrifuge, equipped with a 854 rotor and temperature control. The obtained solid product was recovered, washed four times with ethanol, separating the washing mixture by centrifugation at 4 °C for 15 min, at 9800 rpm. Then, the product, dried under vacuum, was obtained with a yield of 85 wt% based on the starting PHEA weight.

2.3. Characterization of PHEA-RhB copolymer

PHEA-RhB derivative was characterized by ^{1}H NMR and SEC analyses.

^{1}H NMR spectra were obtained by a Bruker Avance II-300 spectrometer, working at 300 MHz. ^{1}H NMR (300 MHz, D_2O, 25 °C, TMS): δ 1.15 (m, $12H_{RhB}$ $\mathbf{CH_3}$–CH_2–); δ 2.71 (m, $2H_{PHEA}$ –CO–CH–$\mathbf{CH_2}$–CO–NH–); δ 3.29 (t, $2H_{PHEA}$ –NH–$\mathbf{CH_2}$–CH_2–O–); δ 3.58 (t, $2H_{PHEA}$ –NH–CH_2–$\mathbf{CH_2}$–O–); δ 4.65 (m, $1H_{PHEA}$ –NH–**CH**(CO)CH_2–); δ 8.00–8.50 (m, $10H_{RhB}$ **H**–Ar). $\overline{M}_w$ of PHEA-RhB graft copolymer, determined by SEC analysis, was found to be 35.0 kDa ($\overline{M}_w/\overline{M}_n = 1.41$).

2.4. Evaluation of molar extinction coefficient (ε) of PHEA-RhB

ε of RhB and PHEA-RhB were evaluated in bidistilled water, in concentration ranging between 10^{-7}–10^{-4} M, by recording UV spectra with a by a RF-5301PC spectrofluorometer (Shimadzu, Italy). The absorption at maxima wavelength was measured and the ε was calculated from the curve obtained by plotting absorbance versus sample solution concentrations. Each experiment was repeated in triplicate. For RhB, the maxima wavelength was 554 nm ($y = 117{,}044x$, $R^2 = 0.9996$), while for PHEA-RhB was 561 nm ($y = 53{,}200x$, $R^2 = 0.99$).

2.5. Synthesis of PHEA-RhB-PLA graft copolymer

Derivatization of PHEA-RhB with acid terminated PLA to obtain the PHEA-RhB-PLA graft copolymer was carried out by using CDI as coupling agent. In particular, a calculated amount of CDI dissolved in a-DMF (123 mg/ml) under argon atmosphere, was added dropwise to acid terminated PLA solution in a-DMF (175 mg/ml), according to $R_4 = 2$ being:

$$R_4 = \frac{\text{moles of CDI}}{\text{moles of PLA}} \tag{4}$$

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