Biomaterials 31 (2010) 923–933

Contents lists available at ScienceDirect

Biomaterials

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

The exploitation of differential endocytic pathways in normal and tumor cells in the selective targeting of nanoparticulate chemotherapeutic agents

Gaurav Sahay [a,1], Jong Oh Kim [a,1], Alexander V. Kabanov [a,b,*,2], Tatiana K. Bronich [a,**,2]

[a] Department of Pharmaceutical Sciences and Center for Drug Delivery and Nanomedicine, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68198-5830, USA
[b] Faculty of Chemistry, M.V. Lomonosov Moscow State University, 119899 Moscow, Russia

ARTICLE INFO

Article history:
Received 5 August 2009
Accepted 29 September 2009
Available online 22 October 2009

Keywords:
Nanomaterials
Endocytosis
Drug delivery
Tight junctions
Caveolae
Nanoparticulate materials
Nanoparticles

ABSTRACT

Polymeric micelles with cross-linked ionic cores of poly(methacrylic acid) and nonionic shell of poly(ethylene oxide) (*cl*-micelles) are shown here to readily internalize in epithelial cancer cells but not in normal epithelial cells that form tight junctions (TJ). The internalization of such *cl*-micelles in the cancer cells proceeded mainly through caveolae-mediated endocytosis. In confluent normal epithelial cells this endocytosis route was absent at the apical side and the *cl*-micelles sequestered in TJ regions of the cell membrane without entering the cells for at least 24 h. Disruption of the TJ by calcium deprivation resulted in redistribution of *cl*-micelles inside the cells. In cancer cells following initial cellular entry the *cl*-micelles bypassed the early endosomes and reached the lysosomes within 30 min. This allowed designing *cl*-micelles with cytotoxic drug, doxorubicin, linked via pH-sensitive hydrazone bonds, which were cleaved in the acidic environment of lysosomes resulting in accumulation of the drug in the nucleus after 5 h. Such pH-sensitive *cl*-micelles displayed selective toxicity to cancer cells but were non-toxic to normal epithelial cells. In conclusion, we describe major difference in interactions of *cl*-micelles with cancer and normal cells that can lead to development of novel drug delivery system with reduced side effects and higher efficacy in cancer chemotherapy.

Published by Elsevier Ltd.

1. Introduction

Recent years saw rapid emergence of polymeric nanoparticulate materials (NMs) for delivery of drugs, genes and imaging agents [1–5]. Examples of materials under development include liposomes, polymer-drug conjugates, nanogels, polymeric micelles, and the like. The precise delivery of these materials to the sites of the disease, such as tumor cells, is central for successful therapies. For example, passive targeting of nanoparticles, also termed as "Enhanced Permeation and Retention (EPR)" effect, has attracted great attention in delivery of drugs and imaging agents [6,7]. EPR stems from an intrinsic property of certain tumors to accumulate NMs due to leaky vasculature and poor lymphatic drainage. The mechanisms of subsequent entry of NMs into target cells have also attracted great attention. The size, shape, charge and aggregation state of NMs were most recently revealed as critical determinants for their cellular entry and sub-cellular targeting [8,9]. Specifically, different NMs employ different endocytosis mechanisms to gain cellular entry. These mechanisms are classified as clathrin dependent (clathrin-mediated endocytosis) and clathrin independent pathways (caveolae-mediated endocytosis, clathrin- and caveolae-independent pathways and macropinocytosis) [9]. After exploitation of these entry pathways, NMs are processed through complex sorting mechanisms and are driven to specific intracellular compartments. In the field of drug delivery the mechanisms of cellular entry of NMs and their final sub-cellular distribution, could greatly affect the performance of the drugs. Therefore, understanding these mechanisms is of significance.

The purpose of this work was to evaluate cellular entry of core-cross linked polymeric micelles (*cl*-micelles) of poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) copolymer, that were recently proposed for delivery of anticancer drugs [10]. In an aqueous environment such micelles behave as nanoscale ionic gels, capable of swelling and changing charge in response to environmental changes (pH or ionic strength). Unexpectedly we found here

* Corresponding author. Department of Pharmaceutical Sciences and Center for Drug Delivery and Nanomedicine, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68198-5830, USA. Tel.: +1 402 559 9364; fax: +1 402 559 9365.
** Corresponding author. Tel.: +1 402 559 9364; fax: +1 402 559 9365.
E-mail addresses: akabanov@unmc.edu (A.V. Kabanov), tbronich@unmc.edu (T.K. Bronich).
[1] JOK and GS contributed equally to this work.
[2] AVK and TKB designed research; all authors contributed to data analysis, discussions and writing the paper.

0142-9612/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.biomaterials.2009.09.101

that such micelles in confluent epithelial cells selectively bind with the tight junctions (TJ) and do not enter the cells. However, disruption of the TJ abolishes such localization and promotes entry of the micelles into the cells. In cancer cells with absent or dysfunctional TJ the cellular entry of such micelles is not restricted by the level of cell confluency. The micelles enter the cells selectively through caveolae-mediated endocytosis, bypass early endosomes and reach lysosomes. This suggests that observed control of the delivery of the synthetic NM through formation of TJ in normal epithelial cells, and enhanced entry into cancer cells and can be exploited for design of cancer-specific drug carriers.

2. Material and methods

2.1. Materials

PEO-*b*-PMA diblock copolymer ($M_w/M_n = 1.45$) was purchased from Polymer Source Inc., Canada. The block lengths were 170 and 180 repeating units for PEO and PMA, respectively. The concentration of carboxylate groups in the copolymer samples was determined by potentiometric titration. Dox hydrochloride is a kind gift from Dong-A Pharmaceutical Company, South Korea. Calcium chloride, 1,2-ethylenediamine (ED), cystamine (Cys), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), triethylamine (TEA), methanol and dimethylformamide (DMF) were obtained from Sigma–Aldrich (St Louis, MO). DOXIL™ was purchased from Ortho Biotech (Horsham, PA) and SP1049C was a kind gift from Supratek Pharma Inc. (Montreal, Canada). Alexa 488-labeled cholera toxin subunit B (Alexa 488-CTB), Alexa 488-labeled transferrin (Alexa 488-Tf), WGA (Wheat Germ agglutinin), DiD (Vybrant® DiD cell-labeling solution), Lysotracker™ (red or green), rab-5 GFP (Organelle lights™ Endosome-GFP), Polystyrene beads (FluoSpheres® carboxylate modified microspheres, blue fluorescent 365/415 and FluoSpheres® amine modified microspheres, red fluorescent 580/605), fetal bovine serum (FBS) (both dialyzed and heat inactivated), Dulbecco's Modified Eagle's Medium (DMEM), were purchased from Invitrogen Inc (Carlsbad, CA). Bovine serum albumin (BSA) and NUNC™ chambered glass coverslips for live cell imaging was purchased from (Fisher Scientific, Waltham, MA). MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Research Products International (Prospect, IL). All other chemicals were of reagent grade and used without further purification.

2.2. Cell lines

Madin-Darby Canine Kidney epithelial (MDCK) cells, Caco-2 (human epithelial colorectal adenocarcinoma) cells, MCF-7/ADR (Dox resistant human breast adenocarcinoma) cells, 3T3 mouse embryonic fibroblasts (MEFs), KO (knock out) cell line (homozygous for a disruption of the caveolin-1 gene Cav-1 −/−, ATCC CRL-2753) and 3T3 MEFs WT (wild type, Cav-1 +/+) cell line (ATCC CRL-2752) were maintained in DMEM, containing 10% heat inactivated FBS and 0.01% penicillin/streptomycin. Primary mouse micro-vessel lung microvasculature endothelial cells (MVEC) were kind gift from Dr. Joseph Vetro (University of Nebraska Medical Center). MVEC cells were incubated in RPMI-media with 0.1% penicillin/streptomycin in the presence of MEM vitamins and sodium pyruvate. Primary BBMEC were maintained as previously described [11].

2.3. Synthesis of Dox-conjugated PEO-b-PMA

NHS (2.34 mg, 0.02 mmol) and EDC (6.0 mg, 0.03 mmol) in CH_2Cl_2 (1.0 ml) were added to a solution of 100 mg PEO-*b*-PMA in 20 ml DMF/methanol (1:1 v/v) and stirred for 2 h at R.T. The Dox (9.1 mg, 0.016 mmol) and TEA (4.5 μl, 0.032 mmol) in methanol were added to this solution and the reaction mixture was stirred continuously for additional 24 h. Organic solvents were evaporated in vacuum, and resulting mixture was dialyzed against distilled water for 2 days using a dialysis membrane (MW cutoff 3500 Da). Dox-conjugated PEO-*b*-PMA was further purified using size exclusion chromatography and lyophilized. The degree of conjugation was 2.7 Dox per copolymer chain as determined by ^{1}H-NMR spectroscopy.

2.4. Synthesis of fluoresceinthiocarbamyl ethylenediamine (FITC-ED)

FITC-ED was synthesized using previously reported procedure [12]. Briefly, ED (200 mg, 1.5 mM) was dissolved in the mixture of 50 ml methanol and 0.5 ml TEA. Solution of FITC (117 mg, 0.3 mmol) in 10 ml methanol containing 100 μl TEA was added dropwise to ED solution over a 30 min period followed by stirring for additional 1 h. The resulting solution was filtered and ED-FITC was recovered by precipitation in 10 ml of methanol. The precipitate was dried in air, and used without further purification.

2.5. Synthesis of FITC-labeled PEO-b-PMA

NHS (2 mg, 0.0174 mmol) and EDC (4.8 mg, 0.025 mmol) were dissolved in 1 ml CH_2Cl_2 and added to a solution of 100 mg PEO-*b*-PMA (0.78 mmol carboxylic groups) in 2.5 ml DMF. The reaction mixture was stirred overnight at R.T. After that, 4 mg of FITC-ED in 200 μl DMF and 20 μl TEA were added to the mixture and stirred for additional 12 h. Organic solvents were evaporated in vacuum, and resulting mixture was dialyzed against distilled water for 2 days using a dialysis membrane (MW cutoff 3500 Da). FITC-labeled PEO-*b*-PMA was further purified by size exclusion chromatography and lyophilized.

2.6. Synthesis of pH-sensitive Dox-conjugated PEO-b-PMA

Dox was conjugated to PEO-*b*-PMA copolymer through an acid-sensitive hydrazone bond. EDC (6.0 mg, 0.03 mmol) dissolved in CH_2Cl_2 (1.0 mL), hydrazine hydrate (1.6 mg, 0.03 mmol) were added to a solution of 100 mg PEO-*b*-PMA (0.78 mmol carboxylic groups) in 20 ml DMF/methanol (1:1 v/v) and stirred for 24 h at R.T. Organic solvents were evaporated in vacuum, resulting mixture was dialyzed against deionized water for 2 days, and hydrazine-modified PEO-*b*-PMA was isolated by lyophilization. 10 mg Dox in 2 ml of methanol containing 5 μl TEA were mixed with hydrazine-modified PEO-*b*-PMA dissolved in 20 ml DMF/Methanol (1:1 v/v) and reacted for 24 h. Organic solvents were evaporated in vacuum, and the mixture was dialyzed against distilled water for 2 days using a dialysis membrane (MW cutoff 3500 Da). The pH was adjusted and maintained at 8–9 during the dialysis. The resulting conjugate was further purified using PD-10 columns and lyophilized. The degree of conjugation was 1.3 Dox per copolymer chain as determined by ^{1}H-NMR spectroscopy.

2.7. General procedure for the synthesis of cl-micelles

cl-Micelles were prepared by the previously described method [10]. In brief, Dox-conjugated PEO-*b*-PMA/Ca^{2+} or FITC-labeled PEO-*b*-PMA/Ca^{2+} complexes were prepared by mixing an aqueous solution of corresponding of PEO-*b*-PMA with a solution of $CaCl_2$ at a molar ratio of $[Ca^{2+}]/[COO^-] = 1.3$. EDC was added into solution of PEO-*b*-PMA/Ca^{2+} complexes to create an active-ester intermediate with carboxylic groups of PMA segments followed by addition of the solution of ED or Cys as bifunctional cross-linkers. The extent of cross-linking was controlled by the ratio of the amine functional groups to carboxylic acid groups. The reaction mixture was allowed to stir overnight at R.T. After completion of the reaction, ethylenediaminetetraacetic acid (EDTA) (1.5 molar equivalent) was added followed by dialysis, first, against 0.5% aqueous ammonia, and, second, against distilled water to remove metal ions and byproducts of the cross-linking reaction.

2.8. Electrokinetic mobility and size measurements

Electrophoretic mobility measurements were performed using a "ZetaPlus" analyzer (Brookhaven Instrument Co.) with a 30 mW solid-state laser operating at a wavelength of 635 nm. ζ-potential of the particles was calculated from the electrophoretic mobility values using Smoluchowski equation [13]. Effective hydrodynamic diameters of the particles were measured by photon correlation spectroscopy (DLS) in a thermostatic cell at a scattering angle of 90° using the same instrument equipped with a Multi Angle Sizing Option (BI-MAS). All measurements were performed at 25 °C. Software provided by the manufacturer was used to calculate the size of the particles and polydispersity indices. The diameters mean values were calculated from the measurements performed at least in triplicate.

2.9. Atomic Force Microscopy (AFM)

Samples for AFM imaging were prepared by depositing 5 μl of an aqueous dispersion of *cl*-micelles (ca. 0.2 mg/ml) onto positively charged 1-(3-aminopropyl) silatrane mica surface (APS-mica) for 10 min. followed by surface washing with deionized water and drying under argon atmosphere. The AFM imaging in air was performed with regular etched silicon probes (TESP) with a spring constant of 42 N/m using a Multimode NanoScope IV system (Veeco, Santa Barbara, CA) operated in a tapping mode. The images were processed and the widths and heights of the particles were measured using Femtoscan software (Advanced Technologies Center, Moscow, Russia).

2.10. FACS analysis

MDCK or MCF-7/ADR (5×10^4 cell/well in 24-well plates) cells were plated and experiments were performed on either confluent or non-confluent cells. Cells were exposed to various concentrations of different NMs that include i) Dox-labeled PEO-*b*-PMA copolymer, ii) Dox-labeled *cl*-micelles, iii) FITC-labeled *cl*-micelles, iv) carboxylate modified polystyrene beads, v) amine modified polystyrene beads. Cells were then washed and trypsinized, centrifuged, resuspended in PBS (pH 7.4, 1% BSA) and followed by FACS analysis. In select experiments, 3T3 MEF Cav KO or WT cells were exposed to i) Dox-labeled *cl*-micelles, ii) Dox-labeled PEO-*b*-PMA copolymer, iii) DOXIL™. The cells positive for fluorescence were termed as the % gated cells and