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Pharmacokinetics and biodistribution of *N*-isopropylacrylamide copolymers for the design of pH-sensitive liposomes

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ABSTRACT

The purpose of this work was to characterize the pharmacokinetics (PK) and biodistribution of pH-responsive *N*-isopropylacrylamide (NIPAAm) copolymers, and to determine the impact of some physicochemical parameters on their biological profiles. Radiolabeled copolymers of NIPAAm and methacrylic acid (MAA) of different molecular weight, amphiphilicity and lower critical solution temperature (LCST) were synthesized and injected intravenously to rats. The PK and excretion profiles were monitored over 48 h. It was found that elimination occurred mainly through urinary excretion, which was principally governed by molecular weight. Above a threshold of 32,000, the polymer chains avoided glomerular filtration and presented prolonged circulation times. Moreover, the presence of alkyl moieties at the chain extremity influenced circulation time and tissue distribution of polymer chains, hypothetically through formation of micellar structures. The polymers with an LCST situated below the physiological temperature did not circulate for prolonged periods in the bloodstream and were highly captured by the organs of the mononuclear phagocyte system. Finally, the complexation of an alkylated pH-sensitive polymer with a molecular weight of 10,000 to the bilayer of PEGylated liposomes produced a drastic change in the PK parameters, indicating that the polymer remained anchored in the phospholipid bilayer in the bloodstream. These data indicate that stable pH-sensitive liposomes can be produced using excretable NIPAAm copolymers.

1. Introduction

Over the past 20 years, there has been a growing interest in the design and use of materials that are capable of reacting adaptively and reversibly to changing environmental stimuli [1]. Poly(*N*-isopropylacrylamide) (PNIPAAm) is a polymer endowed with such properties. Since its first report in 1968 [2], it has been the object of a tremendous number publications and is still today among the most investigated stimuli-responsive polymers in the fields of pharmaceutical sciences [1] and biomedical engineering [3,4]. PNIPAAm is characterized by a lower critical solution temperature (LCST), which is around 32 °C in water [2]. This phase transition temperature can be modulated by copolymerization with more hydrophilic or hydrophobic monomers, and has been exploited to construct a variety of temperature-responsive delivery systems such as micelles [5–7], liposomes [8,9], and hydrogels [10,11]. Moreover, the introduction of ionisable moieties within the PNIPAAm structure has been shown to confer pH sensitivity to the phase transition and been used to design pH-sensitive formulations [10–15]. Among these, pH-responsive liposomes appear as promising delivery systems for the targeting of drugs to the endosomal compartment. Indeed, our group [16–19] and others [20–22] have demonstrated that terminally-alkylated copolymers of NIPAAm and methacrylic acid (MAA) can insert in liposomal bilayers and destabilize the latter at pH values typically found in the endosomes, thereby triggering the release of the encapsulated content in the endosomal compartment. Such formulations have been shown to be stable in plasma [17,18] and could be functionalized with monoclonal antibodies to specifically target tumoral cells [23]. Despite the amount of literature on PNIPAAm, very little is known about its biological fate *in vivo*. Indeed, to the best of our knowledge, no animal studies have been carried out so far to specifically characterize its pharmacokinetics (PK) and biodistribution patterns following intravenous (i.v.) injection. Such information is of utmost

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importance for scientists working with this polymer since PNIPAAm is *a priori* not biodegradable and therefore should be carefully designed to allow its clearance from the body. Elimination studies conducted with other synthetic macromolecules [24–32] have shown that excretion from the body is highly dependent on physicochemical properties, and that renal excretion is mainly controlled by the hydrodynamic volume of the polymer chains. The principal objective of the present work was to study the impact of molecular weight, amphiphilicity and LCST on the pharmacokinetics, elimination and biodistribution of NIPAAm copolymers. The information gathered from these experiments allowed the selection of a pH-responsive polymer which was then complexed to liposomes and studied with respect to its retention in the lipid bilayer under *in vivo* conditions. It was indeed found that stable pH-sensitive liposomes could be formulated with excretable pH-responsive NIPAAm copolymers. The data of this paper emphasize the importance of selecting NIPAAm copolymers with the appropriate physicochemical parameters in order to construct a clinically viable stimuli-sensitive colloidal drug carrier.

2. Experimental

2.1. Materials

4,4′-Azobis(4-cyanovaleric acid), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-3[3-(dimethylamino)-propyl]carbodiimide HCl (EDC), anhydrous 1,4-dioxane, NIPAAm, MAA, acrylamide (AAm), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), Sepharose® CL-4B, Sephadex® G-15, 30% hydrogen peroxide, isopropanol reagent grade, Spectra/Por® dialysis bags (molecular *cut-off* 3500 and 6–8000) and pyrene were purchased from Sigma–Aldrich (St Louis, MO). ^{14}C-acrylamide (6 mCi/mmol) and ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) (50–60 mCi/mmol) were obtained from American Radiolabeled Chemicals (St Louis, MO). Hionic Fluor® scintillation cocktail and Solvable® digestion solution were purchased from Perkin Elmer (Waltham, MA). Coatsome NC-50® egg phosphatidylcholine (96.5% purity, EPC), Sunbright DSPE-020CN® 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[poly(ethylene glycol)] 2000 (98.6% purity, DSPE–PEG) and cholesterol HP® (99.6% purity, Chol) were purchased from NOF (Tokyo, Japan). Unstabilized HPLC grade THF was purchased from JT Baker (Phillipsburg, NJ). NIPAAm and MAA were purified as described elsewhere [18]. Water was deionized and purified with a MilliQ purification system (Millipore, Bedford, MA). All other products were used without further purification.

2.2. Polymer synthesis

The alkylated initiator, DODA-501, was synthesized as previously described [33], by reacting the activated precursor disuccinimidyl 4,4′-azobis(cyanovalerate) with dioctadecylamine. Unsubstituted 4,4′-azobis(cyanovaleric acid) was used as initiator for synthesis of non-alkylated polymer. Radiolabeled random copolymers were synthesized by modification of a method previously described [14,18]. Briefly, polymers of different molecular weights were prepared by free radical copolymerization of NIPAAm, MAA, ^{14}C-AAm ± AAm in anhydrous 1,4-dioxane and in the presence of various amounts of initiator (Table 1). Prior to polymerization, ethanol from the ^{14}C-AAm solution was evaporated from the monomer blend by a flow of argon gas. The dioxane solution, containing initiator and monomers, was degassed for 10 min by bubbling argon. The copolymerization was then initiated by heating at 70 °C under inert atmosphere and constant stirring for 6–60 h. The polymer was recovered by precipitation in diethylether after solubilisation in THF. It was further dialyzed against water for 7 days and finally freeze-dried.

Table 1
Physicochemical characteristics of NIPAAm copolymers.

Polymer	Initiator (mol%)	MAA (mol%)	^{14}C-AAm (mol%)	M_n ($\times 10^3$)	M_w/M_n	Solubility at 37 °C	CAC (mg/L)
P-40k[a]	0.03	5	1	38.4	1.4	+	260
P-10k	1	5	1	11.5	1.7	+	42
P-5k	8	5	1	6.1	1.4	+	13
P-5k-NA[b]	15	5	1	5.0	1.7	+	330
P-40k-L	0.03	1.5	1	37.3	1.3	−	N.D.

[a] Also contains 5 mol% of cold acrylamide.
[b] Synthesized using non-alkylated initiator.

2.3. Polymer characterization

Number- (M_n) and weight- (M_w) average molecular weights were determined by gel permeation chromatography with a Waters Alliance GPCV2000 system (Waters, Milford, MA) operating in THF at 1 mL/min at 40 °C, and mounted with Styragel HT 5, 3, and 2 columns (Waters, Milford, MA). Monodisperse polystyrene standards were used for relative analysis calibration. The LCST was measured by turbidimetry on a Series 2 Aminco Bowman Fluorimeter (Spectronics Instruments Inc, Rochester, NY) in phosphate buffered saline (PBS, Na_2HPO_4 53 mM, NaH_2PO_4 13 mM, NaCl 76 mM, pH 7.4) at a polymer concentration of 0.05 mg/mL. The solution was heated from 25 to 60 °C with 1 °C increments under stirring, and the intensity of scattered light measured at 480 nm. An equilibration time of at least 2 min was allowed after each temperature increment. The specific radioactivity of the labelled polymers was determined by scintillation counting (Liquid Scintillation Analyser Tri-Carb 2100TR, Packard, Meriden, CT).

2.4. Determination of the critical association concentration (CAC)

The CAC was determined by a steady-state pyrene fluorescence method [34]. The shift in pyrene excitation wavelength from 333 to 338 nm corresponding to the transfer of molecules from hydrophilic to hydrophobic environment was monitored and used to determine the apparent CAC. Polymer solutions with concentrations ranging from 4×10^{-2} to 5×10^{3} mg/L were incubated overnight at 37 °C with 2×10^{-7} M pyrene. The I_{338}/I_{333} intensity ratio of each solution was then measured at 37 °C on a Series 2 Aminco Bowman Fluorimeter, after 5 min equilibration. The CAC was determined graphically on an I_{338}/I_{333} vs. concentration plot as described elsewhere [34].

2.5. Preparation of dual-labelled, PEGylated, liposomes

Liposomes were prepared by the film-hydration/extrusion method [35]. Briefly, EPC, Chol, DSPE–PEG, ^{14}C-DODA-P(NIPAAm-*co*-MAA) and ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) (57:38:3:1.2:0.8 mol%) were dissolved in chloroform. The organic solvent was eliminated under rotary evaporation during at least 2 h and the lipid film placed under vacuum for another 20 min. The dried film was then hydrated overnight with an isotonic buffer solution of HEPES (20 mM and NaCl 140 mM, pH 7.4) and extruded through 400-, 200- and 100-nm polycarbonate membranes using a LiposoFast® manual extruder (Avestin, Ottawa, ON, Canada) to yield 120 nm vesicles (PI = 0.07, as determined by dynamic light scattering). Free polymer was removed by size exclusion chromatography on a Sepharose® CL-4B column (25 cm height, 1.5 cm width) using HEPES buffer as an eluent. Adequate separation of liposomes from free polymer was assessed by scintillation counting of each collected fraction. Fractions containing liposomes were pooled and lipid concentration was determined by the phosphorous assay [36]. Specific radioactivity of both isotopes was assessed by dual scintillation counting.

2.6. PK studies

All animal studies were conducted as approved by the Animal Welfare and Ethics Committee of the University of Montreal in accordance with Canadian Council on Animal Care guidelines. Male Sprague–Dawley rats weighing 280–320 g (Charles River, Montreal, QC, Canada) were housed individually in metabolic cages. The polymer solution/liposomal formulation was injected in the subclavian vein under isoflurane anaesthesia. The quantities injected were ~8 mg/kg of polymer (2.5–5 mCi/kg of ^{14}C) and 9.5 mg/kg of lipids for ^{3}H/^{14}C labelled liposomes (0.5 mCi/kg of ^{14}C and 4 mCi/kg of ^{3}H). On each rat, 300-μL blood samples were collected in EDTA-coated capillary tubes by the lateral saphenous vein after 15, 30 min, 1, 2, 4, 6, 12 and 24 h. The last blood sample was collected under anaesthesia by the subclavian vein 48 h post-dosing. The animals were then sacrificed, organs were flushed with saline and collected for analysis. Urine and faeces were collected at 4, 12, 24 and 48 h after injection. Five to seven animals were included in each group.

Blood samples and 200–800 mg of organ homogenate were weighed, digested at 60 °C with isopropanol and Solvable® digesting solution, and bleached with 30% hydrogen peroxide solution. Total urine and faeces collections were weighed and aliquots were digested with Solvable®. Preparations were left at 60 °C for at least 48 h and then mixed with Hionic Fluor® scintillation cocktail. Radioactivity was assessed by scintillation counting. The polymer amounts in faeces, urine, organs and blood are expressed as percentage of injected dose (%ID). For blood, the %ID remaining in the animal was calculated by multiplying the blood radioactivity per gram by 0.064 × body weight in grams [37].

2.7. Calculations of PK parameters

To simplify comparison between groups, PK data were treated by non-compartmental analysis of percentage of injected dose per gram of blood (%ID g^{-1}) vs. time profiles. Maximal concentration (C_{max}) corresponds to the maximum concentration measured. Apparent first-order terminal elimination rate (K_{el}) was estimated with a linear least-squares regression on the semi-log plot of the plasmatic concentration vs. time curve using the using the last 3–4 points of the curve.