The Cytoplasmic Escape and Nuclear Accumulation of Endocytosed and Microinjected HPMA Copolymers and a Basic Kinetic Study in Hep G2 Cells

Submitted: June 27, 2001; Accepted: November 28, 2001; Published: December 15, 2001

Keith D. Jensen¹, Pavla Kopecková^{1,2}, John H.B. Bridge³ and Jindřich Kopeček^{1,2}

¹Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT, USA

²Department of Bioengineering, University of Utah, Salt Lake City, UT, USA

³Nora Eccles Harrison Cardiovascular Research and Training Institute, School of Medicine, University of Utah, Salt Lake City, UT, USA

ABSTRACT The development of macromolecules as drugs and drug carriers requires knowledge of their fate in cells. To this end, we studied the internalization and subcellular fate of N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers in Hep G2 (human hepatocellular carcinoma) cells. Semiquantitative fluorometry confirmed that galactose was an effective ligand for receptor-mediated endocytosis for Hep G2 cells. The rate of internalization of a galactose-targeted copolymer was almost 2 orders of magnitude larger than that of the nontargeted copolymer. Confocal fluorescent microscopy of both fixed and live cells revealed that the polymer entered the cells by endocytosis. After longer incubation times (typically >8 hours), polymer escaped from small vesicles and distributed throughout the cytoplasm and nuclei of the cells. Polymer that entered the cytoplasm tended to accumulate in the nucleus. Microinjection of the HPMA copolymers into cells' cytoplasm and nuclei indicated that the polymers partitioned to the nucleus. The data from fixed cells was confirmed by microscopy of live, viable cells. To examine the effect of the fluorescent dye on the intracellular fate, polymers with fluorescein, Oregon Green 488, Lissamine rhodamine B, and doxorubicin were tested; no significant differences were observed.

KEYWORDS: HPMA copolymer, subcellular trafficking, endocytosis, microinjection, confocal fluorescent microscopy

INTRODUCTION

Macromolecules are being used more frequently in pharmaceutical therapies because of their unique therapeutic effects and advantages in drug delivery. Examples of macromolecules with inherent therapeutic properties include proteins, genes, and antisense oligonucleotides. Advantages of polymeric drug delivery include increased solubility of hydrophobic drugs; passive and active targeting; altered protein binding, biodistribution, pharmacokinetics, and pharmacodynamics; increased stability; and decreased immunogenicity as well as controlled release of drugs (1-3). These advantages have led to the clinical trials of several polymer-drug conjugates as well as the approval of 3 polyethylene glycol (PEG)-protein products by the Food and Drug Administration (4-6). A suitable polymeric carrier can turn an unstable, insoluble, immunogenic drug with many toxic side effects into a highly specific and effective therapeutic agent.

Conjugating a small drug to a polymer carrier changes the path of cellular entry from diffusion to endocytosis (for reviews on endocytosis see references [7-9]). This can overcome multidrug resistance because of the efflux of drugs by membrane pumps such as P-glycoprotein (3,10,11).

The activity of many drugs depends on their location within a cell. Examples include photosensitizers, genes, and antisense oligonucleotides (12-15). Because macromolecules are restricted to fewer compartments, their internalization and subcellular fate become increasingly important for the development of active therapeutics.

In this work, we chose to study the fate of N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers because of their neutral charge; high water solubility; and ease of synthesis, modification, and incorporation of drugs and targeting moieties. These polymers have been studied and used to deliver many types of drugs. The main areas of research include the delivery of anticancer drugs (3), site-specific delivery in the gastrointestinal tract (16), antisense therapy (17,18), hydrogels (19,20), and modified surfaces as platforms for epitope display (21).

To develop second-generation HPMA copolymer-anticancer drug conjugates that would localize at a predetermined subcellular location, a detailed knowledge of their internalization and subcellular fate is needed. Consequently, this study investigated the biorecognition, internalization, and subcellular fate of galactose-containing HPMA copolymers in live and fixed Hep G2 cells. To this end, the fate of polymers internalized by the endocytic route was compared with that of polymers microinjected in the cytoplasm and nucleus. A basic kinetic study of the rate of internalization was also performed.

MATERIALS AND METHODS

Materials

Hep G2 cells (human hepatocellular carcinoma) (22) were from American Type Culture Collection (Manassas, VA). Minimum essential media alpha modification (MEM- α), fetal bovine serum (FBS), L-glutamine, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and penicillin (10 000 U/mL)-streptomycin (10 000 μ g/mL) solutions were from HyClone (Logan, UT). Dulbecco's phosphate buffered saline (DPBS); cell culture grade stock solutions of 1 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH); and MEM

*Corresponding author: Jindřich Kopeček, Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 30 S 2000 E RM 301, Salt Lake City, UT 84112, USA. Tel.: 801-581-4532, Fax: 801-581-3674, E-mail: jindrich.kopecek@m.cc.utah.edu

Eagle media with nonessential amino acids and Hanks' salts (and without L-glutamine, phenol red, or sodium bicarbonate) were from Sigma (St Louis, MO). Paraformaldehyde was obtained from Aldrich (Milwaukee, WI). A SlowFade Light Antifade Kit, 5-([5-aminopentyl]thioureidyl) fluorescein (fluorescein cadaverine), 5-([5-aminopentyl]thioureidyl) 2',7'-difluorofluorescein (Oregon Green 488 [OG] cadaverine), and Lissamine rhodamine B (LR) ethylenediamine were from Molecular Probes (Eugene, OR). Glass coverslips were from VWR (South Plainfield, NJ) or Corning (Corning, NY). CytoSeal 60 was from Stephens Scientific (Kalamazoo, MI). The Modified Lowry Protein Assay came from Pierce (Rockford, IL). Sephadex G-25 (PD-10) columns, a Superose 6 (10/30) column, and a Sephadex LH-20 column were from Pharmacia (Piscataway, NJ). Deionized water was used for the preparation of all buffers. All other chemicals were of reagent grade or better.

Monomers

HPMA (23), N-methacryloylglycylglycylgalactosamine (MA-Gly-Gly-GalN) (24), and N-methacryloylglycylglycine p-nitrophenyl ester (MA-GG-ONp) (25) were prepared according to previously described procedures.

Polymers

The HPMA copolymers were prepared by radical precipitation copolymerization of monomers in acetone or acetone/dimethyl sulfoxide (DMSO) mixture (12.5% wt/vol) at 50°C for 24 hours using 2,2'-azo-bis-isobutyronitrile (AIBN) (0.6% wt/vol) as the initiator. The precursor polymers were purified by precipitation from methanol (MeOH) into acetone.

After reacting the precursor polymers with the fluorescent probes as described below, the polymer product was purified by precipitation to acetone and then separated on a Sephadex LH-20 column. The sample was first dissolved in DMSO (~20% wt/vol), diluted with MeOH with 0.5% CH₃COOH (to ~10% wt/vol), then applied to the column and eluted with MeOH with 0.5% CH₃COOH. For the doxorubicin (DOX) sample (polymer P5), the polymer was purified 3 times on the Sephadex LH-20 column eluted with MeOH containing 10% DMSO and 1% CH₃COOH to ensure that no free drug was associated to the conjugate. To test for free fluorescent probe and to measure the molecular weights of the polymers, the polymers were analyzed by size exclusion chromatography on a Superose 6 (10/30) analytical column. Polymer P5 was eluted in a phosphate buffered saline (PBS) solution with 30% acetonitrile. Polymers P1–P4 were eluted with PBS, and no aggregation was observed. No detectable amounts of free dyes were found. To estimate the molecular weights of the polymers, the column was calibrated with poly(HPMA) fractions (their molecular weights were determined by

laser light scattering). Before analysis, any reactive p-nitrophenoxy (ONp) groups were aminolyzed with 1-amino-2-propanol.

Polymer P1 (P-(glycylglycine-N-galactosamide)-fluorescein (P-[Gly-Gly-GalN]-Fl)

The polymer was prepared in a 2-step procedure. First, a polymer precursor containing glycylglycine N-galactosamide (-Gly-Gly-GalN) side chains and glycylglycine side chains terminated in reactive p-nitrophenyl ester groups (-Gly-Gly-ONp) were prepared. The molar ratio of monomers in the polymerization mixture for the polymer precursor was 80:15:5 for HPMA:MA-Gly-Gly-GalN:MA-Gly-Gly-ONp. The polymer precursor was purified by precipitation from MeOH into acetone. The content of ONp groups (4.5 mol%) was determined spectrophotometrically ($\epsilon_{272} = 9500 \text{ M}^{-1}\text{cm}^{-1}$ in DMSO). The second step consisted of conjugating fluorescein cadaverine with the ONp groups. Briefly, to a solution of 0.200 g polymer precursor (0.060 mmol ONp) in 1 mL DMSO, a solution of 0.0148 g (0.030 mmol) fluorescein cadaverine in 0.2 mL DMSO was added, followed by 0.02 g (0.09 mmol) diisopropylethylamine. After 3 hours of stirring at room temperature, 10 μL of 1-amino-2-propanol was added. After purification (described above), the polymer was isolated by dialysis and freeze-drying. The yield of polymer was 0.180 g (90%). The content of sugar, 14.1 mol% (0.78 mmol/g), was determined by Dionex (Sunnyvale, CA) anion exchange chromatography on a Carbowac PA-1 column using pulsed amperometric detection and 15 mM NaOH as eluent. The content of fluorescein was 1.6 mol% (0.087 mmol/g, $\epsilon_{495} = 80\,000 \text{ M}^{-1}\text{cm}^{-1}$, 0.1 M borate buffer, pH: 9.1).

Polymer P2 (P-[Gly-Gly]-Fl)

The nontargeted polymer precursor containing only fluorescein was prepared by a procedure similar to that described above, excluding the MA-Gly-Gly-GalN monomer. The 5-([5-aminopentyl] thioureidyl) fluorescein was bound to polymer precursor containing 4.7 mol% -Gly-Gly-ONp groups. After purification (described above), the polymer was isolated by dialysis and freeze-drying. The resulting copolymer contained 1.8 mol% fluorescein.

Polymer P3 (P-[Gly-Gly-OG])

To generate polymer P3, OG cadaverine was conjugated to a polymer precursor containing 4.7 mol% -Gly-Gly-ONp groups. To a solution of 0.150 g polymer precursor (0.047 mmol ONp) in 0.8 mL DMSO, a solution of 0.010 g (0.020 mmol) OG cadaverine in 0.2 mL DMSO was added, followed by 0.016 g (0.07 mmol) diisopropylethylamine. After 3 hours of stirring at room temperature, 5 μL 1-amino-2-propanol was added. After purification (see above), the polymer was isolated by dialysis and freeze-drying. The yield of polymer was 0.130 g (87%). The polymer

contained 0.109 mmol OG per gram of polymer (82% efficiency, $\epsilon_{494} = 75\,000\text{ M}^{-1}\text{cm}^{-1}$, borate buffer, pH: 9.1).

Polymer P4 (P-[Gly-Gly-LR])

The polymer containing LR was prepared by a procedure similar to those above. Briefly, from 0.300 g of polymer precursor (P-Gly-Gly-ONp; 4.7 mol% ONp) and 0.020 g LR ethylenediamine, 0.270 g of polymer-bound LR (0.090 mmol/g) was prepared. After purification (described above), the polymer was isolated by dialysis and freeze-drying. The LR was bound with 80% efficiency ($\epsilon_{560} = 122\,000\text{ M}^{-1}\text{cm}^{-1}$, MeOH).

Polymer P5 (P-[Gly-Gly-DOX])

To synthesize polymer P5, DOX HCl was bound to the polymer precursor (4.7 mol% -Gly-Gly-ONp groups) in a dimethylformamide (DMF) solution in the presence of diisopropylethylamine. After purification (see above), the polymer was isolated by dialysis and freeze-drying. The polymer contained 1.9 mol% DOX (0.116 mmol/g; 65% efficiency of binding, $\epsilon_{495} = 11\,000\text{ M}^{-1}\text{cm}^{-1}$, H₂O).

Cell Culture

Hep G2 cells (Figure 1) were cultured in MEM- α media supplemented with 10% FBS in a 37°C incubator with 5% CO₂ (vol/vol) with humidified air. Antibiotics (200 U/mL penicillin and 200 $\mu\text{g/mL}$ streptomycin) were added during the microscopy of live cells and microinjection experiments. During the microscopy of live cells, the cells were cultured in MEM Eagle media without phenol red, buffered with 0.25 mM HEPES, and supplemented with 10% FBS and 0.292 mg/mL L-glutamine in open air. For all biological solutions, the pH was adjusted to 7.4 using cell culture grade HCl or NaOH as needed.

Fluorometry

The fluorescein content of polymer-incubated cells was semiquantitatively measured on an ISS (Champaign-Urbana, IL) PC1 spectrofluorometer. Hep G2 cells were seeded in 6-well (time experiment) or 24-well (concentration experiment) plates 24 hours before incubation. After polymer incubation, the cells were washed 3 times with DPBS, fresh media were administered, and the cells were returned to the incubator for 3 hours to internalize all associated polymer. The cells were next washed an additional 3 times with DPBS and dissolved with 1 M NaOH; then the fluorescence was measured (excitation: 495 nm, emission: 520 nm, 3-second integration). The protein content was measured using the Modified Lowry Protein Assay following the manufacture's procedure and the relative fluorescence was normalized to the protein content and to account for the small difference in fluorescein content of the polymers.

Figure 1. A transmitted (brightfield) image of ~13 Hep G2 cells. The nuclei of most of the cells were visible as rounded objects in the cell cluster, and 3 nuclei are indicated by arrows. The cell membranes between cells were not readily distinguishable. The scale bar is 10 μm .

Confocal Fluorescent Microscopy

Hep G2 cells were seeded on coverslips (No. 1 or 1½) 24 hours before incubation. After incubation in fresh media, the cell were washed 4 times (DPBS), fixed with 3% paraformaldehyde (in DPBS) for 20 minutes at room temperature, washed 4 times (DPBS), mounted with SlowFade Light antifade medium, and sealed with Cytoseal 60. Cells were analyzed on 1 of 3 confocal systems <Endnote 1>. System 1 consisted of a Bio-Rad (Hercules, CA) MRC-600 laser scanning confocal imaging system with a Nikon (Melville, NY) Optiphot microscope (60x plan-apo objective, numerical aperture, NA = 1.4, oil) and a krypton-argon laser (for fluorescein, excitation: 488 nm, emission: 515 nm long-pass filter). System 2 was based on a Zeiss (Thornwood, NY) LSM 510 confocal imaging system with a Zeiss Axioplan 2 microscope (100x plan-apo objective, NA = 1.4, oil) and an argon laser (for fluorescein, OG, and DOX, excitation: 488 nm, emission: 505 nm long-pass filter; for LR, excitation: 543 nm, emission: 560 nm long-pass filter). System 3 was used for the microscopy of live cells; the system was composed of a BioRad MRC 1024 confocal system equipped with a krypton-argon laser and a Nikon Diaphot microscope (100x plan-apo objective, NA = 1.3, oil; for fluorescein, excitation: 488 nm, emission: 515 nm long-pass filter). The custom-built microscope stage permitted cell perfusion with preheated media and temperature control via a water bath. The cells were incubated either on the microscope stage or preincubated in an incubator and transferred to the perfused heated stage for imaging.

The settings for all the confocal systems were adjusted so that control cells always yielded dark images (ie, no background fluorescence was visible) <Endnote 2>. The 8-bit fluorescent images were initially scaled to 256 gray levels, and colored look-up tables were applied. The software packages used to process and view the images consisted of the Confocal Assistant (26) (for images from both Bio-Rad confocal systems), the Zeiss LSM 510 Image Browser program (version 2.30.011), and Photoshop (version 6.0, Adobe, San Jose, CA) .

Microscopy of Live and Fixed Cells

The internalization and fate of the polymer could be monitored by microscopy of live cells, but imaging live cells presents several challenges not present when imaging fixed cells. The greatest challenge is keeping the cells alive and healthy for the duration of the experiment. This requires careful control of the temperature, pH, and light and the provision of fresh media. The temperature must be maintained at an acceptable level to maintain cell viability and activity. Temperature control is also vital to maintain a constant focus. Small fluctuations in the temperature change the refractive index of the immersion oil, resulting in changes in the focal plane (27). Elevated temperatures increase the evaporation rate, which can quickly result in toxic salt concentrations. To overcome these problems, we used a water bath to heat the stage, minimizing temperature fluctuation. The cell chamber was continuously perfused with fresh, preheated media.

Maintaining the pH is essential for the health of the cells. During the microscopy of live cells, a HEPES buffer system was used. The growth of the Hep G2 cells in media buffered with HEPES was slower, but it did not readily decrease the cells' metabolism for short-term experiments. Hence, the cells were cultured in a carbonate buffered system and only transferred to the HEPES buffered media during imaging.

Exposure to light results in photobleaching of the fluorescent dyes and cell death. Photobleaching is a

bigger problem with live cells as little can be done to prevent oxidation and active proteins from destroying fluorescent molecules (28). The simplest solution is reduction of the light intensity, which can be achieved by using highly sensitive detectors and by imaging intermittently and turning the light source off between image acquisition. We employed both using sensitive photomultiplier tube detectors, allowing for lower illumination levels as well as intermittent imaging.

Fixing the cells after incubation had the advantage of being able to look at samples over a wider time frame, but it did not provide the ability to monitor 1 set of cells or observe cell activity (a marker for cell viability). Antifade reagents can be added to fixed cells to reduce photobleaching as well as raise the pH, increasing the fluorescence of pH-sensitive dyes such as fluorescein. Care must also be taken when choosing and testing a fixing agent to prevent fixing artifacts. We used the data obtained from live cells to corroborate the data from fixed cells.

Microinjection

Hep G2 cells were cultured on coverslips as described above and allowed to grow until ~70% confluent. The cells were microinjected with polymer solutions either in the cytoplasm or nucleus using an Eppendorf (Madison, WI) Micromanipulator 1571 with a Transjector 5246.

RESULTS

To determine the effect of fluorescent dyes on the intracellular fate, 5 HPMA copolymers were synthesized. Their chemical structures are shown in Figure 2 and their properties are listed in Table 1. Polymers P1 and P2 were labeled with fluorescein, whereas polymers P3, P4, and P5 were labeled with OG, LR, and DOX, respectively. Only polymer P1 contained the targeting moiety galactose <Endnote 3>. All of the polymers used had similar molecular weights, low polydispersities, and low fluorescent probe amounts

Table 1. Properties of the HPMA Copolymers

Polymer Name	Functional Group	Amount (Mol%)	Molecular Weight	
			M _w	M _w /M _n
P1	Galactosamine Fluorescein	14.1 1.6	25,000	1.4
P2	Fluorescein	1.8	23,000	1.3
P3	Oregon Green 488	1.6	25,000	1.3
P4	Lissamine Rhodamine B	1.4	25,000	1.3
P5	Doxorubicin	1.9	23,000	1.3

M_w = weight average molecular weight, M_n = number average molecular weight.

Figure 2. Chemical structures of the HPMA copolymers used in this work. Polymer P1 contained galactosamine used to target it to hepatocytes and was labeled with fluorescein. Polymers P2, P3, P4, and P5 did not contain galactosamine but were labeled with fluorescein, Oregon Green 488, Lissamine rhodamine B, and doxorubicin, respectively. The number next to each monomer is its respective mole percent.

Effect of Galactose on the Rate of Polymer Internalization

The first objective was to determine the kinetic effect of incorporating galactose into the HPMA copolymers on their internalization into Hep G2 cells. Duncan et al (29) previously found that HPMA copolymers could be targeted to hepatocytes in the liver by incorporating at least 4 mol% of galactose-containing monomers. Increasing the galactose content of the polymer was recently reported to increase the internalization of HPMA copolymer in Hep G2 cells (30). The targeted polymer P1 tested in this work contained 14 mol% of galactose-containing monomers.

To determine the concentration dependence on internalization of galactose-targeted polymer P1 and nontargeted polymer P2, the cell-associated fluorescence of polymer-incubated Hep G2 cells were measured. A comparison of the relative amount of cell-associated targeted (P1) and nontargeted (P2) polymer with increasing polymer concentration is displayed in Figure 3. For the nontargeted polymer, P2, an increase in the concentration resulted in a steady increase in the amount of internalized polymer. At similar polymer concentrations, more of the targeted polymer P1 was internalized compared with the nontargeted polymer P2. The difference was particularly striking at polymer concentrations above 0.4 μM . Galactose targeting appeared to saturate above 10 μM , consistent with internalization via a membrane-bound receptor, although no competitive inhibition studies were performed.

Figure 3. Concentration-dependent internalization of galactose-targeted polymer P1 and nontargeted polymer P2 in Hep G2 cells. The cells were incubated with various concentrations of the polymers for 24 hours, and the relative cell-associated fluorescence was measured by fluorometry ($n = 1$).

The time dependence of internalization of the targeted polymer P1 and the nontargeted polymer P2 in Hep G2 cells was also evaluated. The Hep G2 cells were incubated with 7 μM of polymer P1 and P2 for 1 hour 24 minutes to 49 hours, and their relative fluorescence was measured (Figure 4). The targeted polymer P1 showed a more rapid and steady increase in the amount of polymer associated with the cells over time compared to the nontargeted polymer P2 (corresponding equations for a linear regression of the 2 data sets are displayed in the caption for Figure 4). The slope of the targeted polymer was almost 2 orders of magnitude larger than that of the nontargeted polymer.

Figure 4. Time-dependent internalization of galactose-targeted polymer P1 and nontargeted polymer P2 in Hep G2 cells. The cells were incubated with 7 μM of each polymer from 1 hour 24 minutes to 49 hours, and the relative cell-associated fluorescence was measured. Each concentration was tested twice, and all data points are shown. The equation for the linear regression of galactose-targeted polymer P1 data was as follows: $y = 5.9463x + 58.692$. The equation for the nontargeted polymer P2 was as follows: $y = 0.0602x + 54.159$.

Short-Term Internalization

The short-term fate of HPMA copolymers in live and fixed cells was monitored by fluorescent confocal microscopy. At short incubation times, polymer tended to remain in endosomes and lysosomes. A 13-image "z-series" (see <Endnote 1> for a brief explanation of confocal microscopy and references) of ~15 fixed Hep G2 cells incubated with 7 μM of targeted polymer P1 for 24 hours is seen in Figure 5. Only the polymer P1 was visible, and it was limited to small endosomal and lysosomal vesicles. Image 1/13 was focused at the bottom of the cells where they attached to the glass coverslip. Image 2/13 was acquired 1 μm above image 1/13 and revealed a collection of small fluorescent vesicles distributed throughout the cytoplasm. Each successive image was acquired 1 μm farther away from

the coverslip. Image 6/13 was taken in the perinuclear region; because no endosomal or lysosomal vesicles enter the nuclei, the nuclei appear as dark voids surrounded by fluorescent vesicles (see Figure 1 to gain a perspective of approximate size and distribution of the cells and their nuclei).

A 3-dimensional representation of the cells was generated from the confocal images in Figure 5 using the Confocal Assistant imaging software (26). Figure 6 shows the resulting moving image oscillating around the y-axis (print version contains a stereo-image). Most fluorescent vesicles were well distributed in the cytoplasm, although some clustering was apparent at the center and upper and lower left section of the image.

Figure 5. Confocal fluorescent slice of cells with fluorescein-labeled polymer localized in small vesicles in the cytoplasm. The Hep G2 cells were incubated with 7 μ m of the targeted polymer P1 for 24 hours. The cells were fixed and analyzed by confocal microscopy (System 1). Thirteen images were acquired at 1 μ m intervals (approximate optical slice thickness). The image shown was acquired in the perinuclear region. The dark voids of the nuclei (arrows indicate 4) were surrounded by bright fluorescent vesicles. The scale bar are 10 μ m. Movies are available in the HTML version.

Figure 6. A stereo-image from the 13 confocal slices from Figure 5 generated using the program Confocal Assistant (26). Movies are available in the HTML version.

To visualize the time-dependent internalization of targeted polymer P1, to corroborate the data from the fixed cell experiments, and to provide an indication of cell viability, live confocal microscopy of the cells was employed. To clearly display the time-dependent internalization of the targeted polymer P1 in Hep G2 cells, the cells were imaged for 65 minutes during live incubation (the cells were imaged at 10-minute intervals to reduce photobleaching and cell exposure to the laser). The average of 18 images obtained after 5 minutes of incubation is seen in Figure 7a. The average of a second series of 5 images obtained after 15 minutes of incubation is seen in Figure 7b. To more clearly visualize the change in fluorescence between $t = 5$ minutes and $t = 15$ minutes, a ratioed image was generated by dividing the image in Figure 7b by the image in Figure 7a to give the image in Figure 7c. Regions of fluorescence that did not change exhibited a pixel value of unity (purple). It was apparent that some regions of fluorescence acquired values that were as much as 3- to 4-fold greater than the background value of unity (brighter colors). In other regions, there was a decline in fluorescence (black).

To corroborate the data from the fixed cell experiments and to provide an indication of cell viability, live cells preincubated for 3 hours with polymer P1 were imaged for 47 minutes. A movie of 60 images covering the last 5-minute period of imaging is seen in Figure 8 (print version contains first image from the movie). The movie contains a group of ~ 20 Hep G2 cells imaged in the perinuclear region. High levels of vesicular motion in all the cells are clearly visible, indicating that the cells were active and hence viable. Surprisingly, several cells displayed nuclear staining after only a 3-hour incubation (1 nucleus located in the bottom left section of the image was highly stained, as were 3 or 4 additional nuclei in the upper portion of the images). All nuclear staining was visible from the beginning of imaging. The only change in nuclear fluorescence during the experiment was a small decrease in intensity, probably due to photobleaching.

Figure 7a. Confocal image of live Hep G2 cells being incubated with polymer (System 3). The cells had been incubated with 11 μ M of targeted polymer P1 for 5 minutes. The dark band is a group of ~30 Hep G2 cells surrounded by brightly stained polymer-containing media. The color bar on the right indicates the relative pixel intensity. The scale bar is in microns.

Figure 7b. Confocal image (System 3) of the live Hep G2 cells in Figure 7a after being incubated with polymer P1 for 15 minutes. The scale bar is in microns, and the color bar on the right indicates the relative pixel intensity.

Figure 7c. A ratio of the images at 5 and 15 minutes to accentuate the change in fluorescence. The averaged image in Figure 7b was divided by the averaged image in Figure 7a. Pixels with values less than 1 are black, pixels with values close to 1 are purple, pixels with values from >1 to 3 are colored red through white according to the color bar on the right of the image. The scale bar is in microns.

Figure 8. The first frame from a movie of live Hep G2 cells preincubated with polymer showing active vesicle movement and stained nuclei. The cells were incubated with 11 μ M of targeted polymer P1 for 3 hours and imaged (System 3) continuously for 47 minutes. The movie consisted of 60 confocal images that correspond to the last 5 minutes. A Kalman filter was applied to reduce the amount of noise (in images 1-31, $n = 3$, images taken every 3.6 seconds; in images 32-60, $n = 5$, images taken every 6 seconds). The movie contains a group of ~20 Hep G2 cells imaged in the perinuclear

region displaying high vesicular motion and polymer in the nucleus of 4 or 5 cells. The most prominently stained nucleus is seen as the bright circle in the middle lower left of the images. Several nuclei and vesicles are indicated in the first image. The scale bar is in microns.

Long-Term Fate

As the copolymer incubation time was increased, a subpopulation of cells began to exhibit transfer of the polymer from small vesicles into the cytoplasm and nucleus of the cells. Two groups of fixed Hep G2 cells, incubated with 12 μ M of the targeted polymer P1 for 96 hours, are seen in Figure 9. The upper group consisted of 6 cells: 3 of the nuclei in the upper group are moderately stained, 1 is significantly brighter, and 2 cells did not show significant nuclear staining. The lower group consisted of 2 cells with high levels of cytoplasmic staining; the staining of the cytoplasm of the cell on the left exceeded its nuclear staining.

A general trend was observed that in the majority of the cells, polymer remained in small vesicles at incubation times less than 8 hours. As the incubation times were lengthened, the proportion of cells with cytoplasmic or nuclear staining increased. Nuclear staining appeared to follow cytoplasmic staining and increased with longer incubation times. The shortest incubation time that exhibited a high degree of nuclear staining was 3 hours (Figure 8). The presence of the polymer in the media was not required for cytoplasmic entry of the polymer; cells incubated with polymer for 24 hours followed by incubation in fresh media for 2 to 4 days produced results equivalent to those for continuous polymer incubation (data not shown).

Figure 9. Lysosomal escape and nuclear accumulation of polymer after long-term incubation. A confocal image (System 1) of 9 Hep G2 cells incubated with 12 μ M of targeted polymer P1 for 96 hours. Polymer had entered the cytoplasm of all the cells and, to various extents, the nuclei that are seen as bright or dim ovals inside the cells. The scale bar is 10 μ m.

Initial long-term experiments suggested that concentration also played a role in the ability of polymer to escape into the cytoplasm or nucleus. Samples incubated with higher concentrations of polymer exhibited more and brighter cytoplasmic and nuclear staining (data not shown). In subsequent experiments, cytoplasmic and nuclear staining was found even at lower polymer concentrations, but it was less prevalent and more difficult to detect. Problems affecting detection include photobleaching, fluorescence quenching, and background fluorescence <Endnote 2>.

Fate of Copolymers after Microinjection

When the copolymers escaped from their membrane-bound vesicles, they had a tendency to accumulate in the nucleus, as manifested by a higher degree of staining of the nucleus as compared to the cytosol. If the polymer's escape from lysosomes was the result of membrane rupture, all of the contents of the lysosome—including the lysosomal enzymes—would be present in the cytoplasm. This could disrupt normal cellular functions and potentially alter the fate of the polymer in the cell. To eliminate this potential problem, as well as test the polymer's fate when administered directly to the cytoplasm or nucleus, polymers were microinjected into the cells' cytoplasm and nuclei.

The intracellular fate of the microinjected polymer was determined (15 minutes to 4 days postinjection) using confocal fluorescent microscopy. Fifteen minutes after nontargeted polymer P2 was microinjected into the cytoplasm, it had already entered the nucleus, as was evident by nearly equal staining of the cytoplasm and nucleus (Figure 10a). By 60 minutes postinjection, the polymer P2 had predominantly localized in the nucleus (Figure 10b). At longer times (2 hours to 4 days postinjection), the staining of the nucleus increased relative to the cytoplasm and remained 4 to 10 times brighter (Figure 10c) than the cytoplasm, although the fluorescence of the cells at time intervals above 24 hours was noticeably dimmer. In Figure 10c, the polymer was predominantly located in the nucleus of the microinjected cells. The polymer in the cytoplasm was visible only with a higher detector gain. Transfer of polymer from the cytoplasm to the nucleus was also observed with live cells (data not shown). To determine the effect of galactose on nuclear accumulation of the polymers, the targeted polymer P1 was also tested, which yielded equivalent results (data not shown).

To determine if the polymer would remain in the nucleus, the cells were imaged after nuclear microinjection of the polymer. A typical image of cells 15 hours after nuclear injection of nontargeted polymer P2 is seen in Figure 11. The nuclei of the injected cells were significantly brighter than the respective cytosol. This preferential nuclear partitioning was found in all microinjected samples analyzed from 1 to 96 hours postinjection, with both targeted and nontargeted polymers P1 and P2 (data not shown).

Figure 10a. A confocal image (System 2) 15 minutes after cytoplasmic injection of polymer. Untargeted polymer P2 was injected into the cytoplasm of the 2 Hep G2 cells visible. The polymer had already entered the nuclei, which were equally as stained as the cytosol. The scale bar is 10 μ m.

Figure 10b. A confocal slice of several Hep G2 cells 60 minutes after cytoplasmic injection of untargeted polymer P2. The polymer had already begun to accumulate in the nuclei of the cells, which are more brightly stained than the corresponding cytosol. The dark structures inside the nuclei are probably nucleoli that excluded the polymer. The scale bar is 10 μ m.

Figure 10c. A confocal slice of 5 Hep G2 cells 24 hours after cytoplasmic injection of untargeted polymer P2. The polymer was predominantly located in the nuclei of the cells with only minor amounts staining the cytoplasm, which was barely visible at these settings. The scale bar is 10 μ m.

Figure 11. A confocal slice of 4 Hep G2 cells 15 hours after nuclear injection of targeted polymer P1. Only the nuclei of the cells were visible—the cytoplasm was nearly black. The scale bar is 10 μ m.

DISCUSSION

An understanding of the internalization and subcellular fate of macromolecules is required for the rational design of effective macromolecular therapeutics. The delivery of macromolecules to their target site is currently the limiting factor for many large compounds, including antisense oligonucleotides (15).

As macromolecules have generally been considered to be impermeable to cell membranes, it is well accepted that they enter cells via endocytosis and are often used as markers of endocytosis (eg, fluorescently labeled dextrans [31] and polyvinyl-pyrrolidone [32]). It is generally accepted that endocytosed macromolecules remain in membrane-bound vesicles (1), unless some type of permeation enhancer (eg, fusogenic peptides [33] or cell-penetrating peptides [34]) is used. An exception may be polycationic polymers as evidence of escape of polyethyleneimine from endosomes and lysosomes due to endosomal burst, as was recently reported (35).

Many studies have led to a general understanding of the fate of HPMA copolymers, especially *in vivo* (1-3). HPMA copolymers with molecular weights below the renal threshold (~45 kd [36]) are rapidly cleared from the blood (37). Higher molecular weight polymers have a longer blood residence time, and no unexplainable amounts of copolymer accumulate in organs (37). An increase in the molecular weight of macromolecules also leads to passive targeting in tumors because of the enhanced permeation and retention (EPR) effect (3).

A homopolymer of HPMA is uncharged and has a low affinity for cell membranes, although this can be easily changed by incorporating hydrophobic, charged, or targeting moieties (3). Using galactose to target HPMA copolymers to the liver has been very successful, and a galactose-targeted anticancer HPMA copolymer is already in Phase 1 clinical trials (38).

Although there have been studies demonstrating the ability of HPMA copolymers to be targeted to hepatocytes *in vivo* (1,3) and *in vitro* (30,39) using galactose, the kinetics of internalization were not evaluated. In a concentration-dependent internalization study (Figure 3), the nontargeted polymer P2 showed a linear dose response curve expected for fluid-phase pinocytosis of macromolecules. In contrast, we found that the targeted polymer P1 was internalized in a manner consistent with receptor-mediated endocytosis, although no competitive inhibition studies were performed. A basic study of the rate of internalization revealed that the targeted polymer was internalized at a rate almost 2 orders of magnitude faster than that of the nontargeted polymer P2 (Figure 4). It should be noted that the recent data by David et al (30) indicate that even more dramatic differences in uptake should be possible using polymer with higher amounts of trivalent galactose.

Microscopic evidence of the lysosomotropic nature of HPMA copolymers was previously demonstrated (39). At shorter incubation times, we found that the staining of the cells was punctate, consistent with internalization via endocytosis (Figure 5). A computer-generated 3-dimensional reconstruction of confocal slices was used to determine the spatial distribution of polymer in the cells (Figure 6). It was found that the distribution of fluorescent vesicles was not completely random; some clusters of vesicles were visible particularly at the center and upper and lower left sections of the cells. This was not unexpected, as endosomes and lysosomes follow predefined tracks in cells, rather than randomly moving throughout the cytoplasm. The punctate staining did not depend on galactose content or on the fluorescent markers tested, as all of the polymers (P1–P5) yielded images similar to Figure 5 (data not shown) at short incubation times.

As the dyes are fairly hydrophobic, their presence can affect the permeability and intracellular fate of the polymer in an analogous way, as seen by Abdellaoui et al (40). The amount of dye on each polymer was designed to be high enough to obtain good images at reasonable concentrations, while being as low as possible to minimize the effects of the dyes. Although no obvious evidence of dye interactions was observed for any of the polymers tested, more studies would be required to preclude possible effects.

Using fixing agents and procedures always raises questions about their effect on the cells and distribution of fluorescent markers. Although microscopy of live cells has its own set of limitations and challenges, it can provide invaluable information. Corroboration of the distribution of fluorescent markers using microscopy of live cells provides a strong validation of the fixed cell data. Because only live cells have active endocytosis and vesicular movement, monitoring this movement provides a marker of the cells' viability. The rate of vesicular movement directly corresponds to the health of the cells, as it slows and eventually stops as the cells' health worsens.

Microscopy of live cells also revealed evidence of internalization via endocytosis, as well as the rapid internalization of the galactose-targeted polymer P1 into Hep G2 cells. The image in Figure 7c is a ratio of the cell after 5 and 15 minutes of incubation. The magnitude of changes in fluorescence between the 2 time points was striking. The ratioed pixel values indicated changes in the fluorescence ranging from 1/3 to 4 times as high between the 2 time points. Most of the decreases occurred at the cell membranes, which is consistent with endocytosis of the polymer.

Microscopy of live cells (Figure 8) also demonstrated the viability of cells with polymer accumulated in their nuclei, consistent with the data of the fixed cell (Figure 9). Because vesicular movement is a good marker for cell

viability, the high and constant activity demonstrates that these cells were alive and active. Of greatest interest, we found polymer in the nuclei of at least 4 cells after incubation for only 3 hours—the shortest time for nuclear accumulation observed. This demonstrated that entry of the polymer into the cytoplasm and nucleus had not compromised the cell's viability and that escape from endosomes and lysosomes could occur within hours after beginning incubation.

The escape of polymer from the endosomes and lysosomes was quite unexpected. To ensure that we were not observing an artifact, the experiment was repeated numerous times. We reproducibly monitored entry of the polymer into the cytoplasm and nucleus of live and fixed Hep G2 cells following incubation, without the aid of any membrane disruptor, pH stabilizer, or any other extraneous chemical or physical intervention. Although unexpected, such a result is not without precedence. Hovorka et al (41) reported cytoplasmic localization of HPMA copolymer in EL-4 cells in a preliminary study.

Endosomal escape mechanisms are of utmost importance in gene and antisense therapy. Problems with delivery of these and other macromolecules have resulted in a concentrated study of various types of membrane permeation enhancers. One of the main limitations is the lack of understanding of the mechanism of cytoplasmic entry. Two prominent solutions are the destabilization of the endosomal membrane by fusogenic peptides (33) and direct cytoplasmic entry of macromolecules, mediated by protein transduction domains (34).

The ability of the polymers to escape from lysosomes may have a potential for the delivery of many drugs that are active only in the cytoplasm or nucleus of the cell. Nevertheless, it should be noted that the release of polymer from the endosomes and lysosomes observed in this work was a slow process, often not being observed in a significant number in cells until after 8 hours of incubation. This would probably be too long for enzymatically sensitive molecules such as antisense oligonucleotides. However, preliminary studies revealed that conjugating a TAT peptide to HPMA copolymers allowed the copolymers to enter the cytoplasm and nucleus in less than 5 minutes (42).

Detection of fluorescence in the cytoplasm and nuclei of the cells does not necessarily require that polymer escape from the endosomes or lysosomes. Some may contend that cleavage of the fluorescent tag from the polymer and its subsequent diffusion into the cytoplasm and nuclei could also result in cytoplasmic and nuclear staining. The first point to consider is that the linkage between the fluorescent tags is fairly stable. The Gly-Gly spacer is the most likely site for degradation by lysosomal enzymes, but it was previously shown to be very stable when incubated with tritosomes (harvested

lysosomal enzymes) (43). The second point is the diffusion of free dyes through membranes. We tested and found that free fluoresceinamine would diffuse out of live cells within a matter of minutes. We also found no unusual fluorescence above background levels for cells loaded with free fluoresceinamine, washed, incubated in fresh media, and fixed and analyzed by confocal microscopy (data not shown). These results indicate that any small amounts of fluorescent probes with permeabilities equal to or greater than fluoresceinamine that could be cleaved from the polymers would not accumulate in cell membranes or other compartments, but would quickly diffuse throughout the sample.

Another important point to note is that it is unknown how much of the internalized polymer was able to escape into the cytoplasm. The pH sensitivity of the dyes, as well as the huge differences in fluorescent intensity among the cytoplasmic, nuclear, and vesicular compartments, makes quantitation of this microscopic data unreliable. Although the percentage of polymer that escaped may be low, the relatively large volume of the cytoplasm and nucleus would require a significant amount in order to be detected by confocal microscopy.

The fixed cell experiments demonstrated that polymers entering the cytoplasm tended to accumulate in the nucleus. To corroborate this nuclear partitioning and eliminate the possibility of lysosomal enzymes being present as a result of lysosomal rupture, various polymers were microinjected directly into the cytoplasm or nucleus. Nuclear entry of the polymers occurred rapidly. At the shortest time after cytoplasmic injection (15 minutes), the nontargeted polymer P2 had already evenly distributed into the nucleus (Figure 10a). Within 1 hour, the polymer accumulated in the nucleus (Figure 10b), and only very small amounts of polymer were visible in the cytosol after 24 hours (Figure 10c). Polymer microinjected into the nucleus tended to remain localized there (Figure 11).

Nuclear accumulation of the polymers was another unexpected result. The polymers were almost completely neutrally charged. Nuclear localization did not depend on the presence of galactose in the polymer. All of the polymers (P1–P5) displayed nuclear localization, although the DOX conjugate (polymer P5) had less pronounced nuclear staining (data not shown). Dimmer nuclear staining of the DOX conjugate was not unexpected as binding to DNA causes fluorescence quenching of DOX. Additional studies are needed to begin to understand why the polymer partitions to the nucleus. Subcellular fractionation of radiolabeled polymers would prove useful in deducing the effects of the fluorescent probes on the polymers' fate. Regardless of the reason for nuclear accumulation, such an effect should be explored to increase the efficacy of drugs that act on nuclear components.

Of the fluorescent probes used, DOX (the anticancer drug attached to 2 HPMA copolymers in clinical trials [3]) is the most interesting. In vitro, free DOX accumulates in the nucleus (39,44) as does DOX bound to HPMA copolymers via lysosomally degradable spacers (45). DOX bound to PEG via an acid-sensitive linker was found in the cytoplasm (44). In vivo, polymer-DOX conjugates were found to accumulate in tumors via the EPR effect (3). Results from this research suggest that some of the DOX HPMA copolymer conjugate that is internalized by endocytosis will enter the cytoplasm and nucleus. This may be at least partly responsible for the toxicity of HPMA copolymers with DOX attached via nondegradable spacers. The degradable spacers used to link the DOX to the HPMA copolymer conjugates in clinical trials are efficiently cleaved by lysosomal enzymes, likely making them more efficient at delivering the drug to the cell's interior than were the slow-escaping polymers observed in this work.

CONCLUSIONS

We monitored the internalization and subcellular fate of HPMA copolymers in Hep G2 cells by fluorometry and confocal fluorescent microscopy. As expected, we found that Hep G2 cells recognized galactose-containing polymers that were internalized by endocytosis. After internalization, the polymer remained in small vesicles for several hours. In less than 3 hours, polymers began to escape into the cytoplasm, but this typically took more than 8 hours to be completed. There was considerable variability among cells in the same sample—with 1 subset exhibiting quick cytoplasmic entry, while another subset remained in endosomes and lysosomes even after 4 days. Once polymer entered the cytosol, it accumulated in the nucleus. Nuclear accumulation was confirmed using microinjection. All data of fixed cells were corroborated by microscopy of live cells. Studies relating the physiological state (eg, cell cycle) of particular cells to the endosomal escape of polymers may help elucidate the mechanism of these phenomena.

ACKNOWLEDGMENTS

The research was supported in part by National Institutes of Health (NIH) grants CA51578 and CA88047 from the National Cancer Institute. Partial support of KDJ was provided by NIH training grant GM08537 and by a fellowship from the American Foundation of Pharmaceutical Education. Doxorubicin was a kind gift from Dr A. Suarato (Pharmacia, Milan, Italy).

ENDNOTES

1. The main advantage of confocal fluorescent microscopy (28) is the reduction of unfocused light from an image. A focused light source greatly reduces fluorescence from sample sections out of the focal plane or field of view. An aperture placed in front of the detector (PMT or camera) further excludes the

unfocused fluorescence from the image. The result is a clear image of a narrow optical section in the x-y plane of the sample. Successively imaging 1 section—moving the sample in the z-axis and re-imaging the sample—results in a series of images (often termed a z-series) covering the whole volume of the sample. With imaging software, one can use the series of confocal optical sections to calculate a 3-dimensional representation of the sample. See Figures 5 and 6 for an example of confocal slices and 3-dimensional reconstruction, respectively.

2. The aperture of the confocal system limits the amount of unfocused light reaching the detector. Thus, high fluorescence and bright light sources (lasers) are typically required to image specimens. Cells also have a moderate level of autofluorescence. To account for this, control cells not incubated with polymer must always be imaged and the detector adjusted to give a black control image. The result is a further reduction in the amount of fluorescent polymer that can be detected.

3. More accurately N-acyl galactosamine.

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