Diagnostic and Therapeutic Potential of Poly(benzyl L-glutamate)

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Abstract □ Poly(benzyl L-glutamate) (PBLG) microcapsules, prepared by a solvent evaporation technique for intravenous injection, are evaluated for their potential use in diagnostic computed tomographic enhancement of liver images. The smaller microcapsules, <3 μm, loaded with a radiopaque contrast material, ethyl iopanoate (IOPAE), produced prolonged opacification of the liver when delivered intravenously. *In vivo* tissue distribution studies of PBLG-^{131}I-IOPAE (5 μCi/rat, iv) showed that liver had the highest uptake (percent of injected dose/g of tissue) among other organs 24 h postinjection. An *in vitro* estrogen receptor assay in pig uteri indicated that PBLG ocnjugated with estrone did not interfere with estrogen receptor affinity, suggesting the estrogen therapy potential of PBLG–estrone.

Introduction

Microencapsulated chemotherapeutic agents for intravenous and intra-arterial sustained release drug delivery have been employed for the management of neoplasms without the risks of chronic indwelling catheters.[1-4] Chemoembolization is the combination of chemotherapy and peripheral embolization of the arterial supply to a tumor. Intra-arterial embolization of capsules 100–200 μm in diameter exert their effect on the cancer by diminishing the flow of blood and oxygen, theoretically increasing capillary permeability and prolonging the contact time between the drug and the tumor cells.[5-7] This has been most effective in the treatment of hypervascular hepatic neoplasms.

Small particles, less than 5 μm in diameter, traverse the pulmonary vascular bed when delivered intravenously. If loaded with a radiocontrast agent, small microcapsules (1) may be useful in detecting hepatic lesions by computed tomography (CT) and (2) may be more useful than ionic or nonionic radiocontrast agents which tend to be excreted rapidly by the liver and kidneys. Since most tumor cells do not have a complete reticulendothelial system (RES), their images are not enhanced by CT contrast media. Therefore, small microcapsules should have potential applications in developing a contrast density difference between normal tissue and focal hepatic lesions. Small microcapsules that have undergone surface modification may be used to target agents to specific cells.[8,9]

A spacer molecule has been inserted between a steroid and a polymer to target drugs to tissues. Conjugation of the steroid to the polymer has not interfered with the receptor binding affinity.[10,11] Therefore, the sustained release properties of receptor-mediated agents conjugated on the polymer should have potential for tumor therapy.

This study aims at evaluating the potential usefulness of poly(benzyl L-glutamate) (PBLG) microcapsules in enhancement of liver images by computed tomography (CT) and the conjugation of PBLG to receptor-mediated agent for receptor-targeted therapy.

Iopanoic acid was chosen as an ecapsulated contrast material because it is less toxic (LD_{50} = 320 mg/kg iv in rats)[12] and has been conjugated to cholesterol to produce potential tumor- or organ-imaging agents.[13,14] The capsules were coated with PBLG because it is a biocompatible poly(amino acid). Their biodistribution and CT enhancement of the liver images were studied. PBLG also provides functional groups to which bioactive agents can be bonded,[15] which means it may be useful to sustained release the agents. In this paper, we conjugated estrone (a standard estrogen agonist) to PBLG. An *in vitro* estrogen receptor assay was then performed to evaluate the potential application for estrogen therapy.

Experimental Section

Materials—The polymer used to produce the microcapsules was poly(benzyl L-glutamate), the two average molecular weights used were MW 58 000 and 43 000 and were obtained from Sigma Chemical Co. (St. Louis, MO). Polyvinyl alcohol (MW 30 000–70 000) estrone, and iopanoic acid were also obtained from Sigma. [^{131}I]Sodium iodide (specific activity 7.75 Ci/mg, 680 mCi/mL) was obtained from DuPont New England Nuclear (Boston, MA). Female Sprague–Dawley rats (100–125 g) were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN).

Radiolabeling of Ethyl Iopanoate—Iopanoic acid (2 g, 3.5 mmol) was dissolved in absolute alcohol (50 mL), and thionyl chloride (0.4 mL, 5.25 mmol) was added. The reaction was refluxed for 3 h. After cooling, the reaction mixture was evaporated and reconstituted in methylene chloride (100 mL). The organic mixture was washed twice with NaOH (5%, 25 mL) and twice with water (25 mL). The methylene chloride layer was dried over $MgSO_4$ and evaporated to dryness, yielding 1.73 g of ethyl iopanoate (IOPAE) (82.4%). The structure was proved by ^{1}H nuclear magnetic resonance and mass spectrometry (M^+ 599) (performed at the University of Texas Medical School Analytical Chemistry Laboratory, Houston, Texas). The radioisotope exchange reaction was carried out using a known procedure with some modification.[8] Briefly, the ester (10 mg) and 0.3 mL of tetrahydrofuran were placed in a vial and treated with 1.6 mCi of [^{131}I]sodium iodide (in 100 μL of 0.1 M sodium borate buffer) and pivalic acid (25 mg).

The reaction vial was sealed and heated at 150 °C for 1.5 h. The vial was cooled and the solvent was evaporated under N_2. The IOPAE was reconstituted in methylene chloride (0.1 mL), chromatographed on a silica gel column, and eluted with methylene chloride/methanol (9:1). This yielded 0.54 mCi of ethyl iopanoate (34%). Radiochemical purity was determined by cochromatography on a silica gel plate (Bioscan System 200, Washington, DC) it was eluted with methylene chloride/methanol (9:1); unlabeled ester served as the standard, with a retardation factor of 0.80.

Small Microcapsule Preparation—PBLG (0.7 g) and unable IOPAE (0.3 g) were dissolved in methylene chloride (30 mL). To this mixture was added [^{131}I]ethyl iopanoate (320 μCi). This organic phase was emulsified in an aqueous solution (200 mL) containing polyvinyl alcohol (1% w/v). The mixture was stirred at 2000 rpm for 24 h to ensure complete evaporation of methylene chloride. The suspension was then centrifuged (12 000 rpm) for 10 min. The microcapsules were separated, washed with water to remove any excess polyvinyl alcohol, and centrifuged again. The resulting microcapsules were filtered through a nylon cloth (5-μm mesh). The final concentration was 154 μCi in 18 mL of water. Preparation of unlabeled PBLG–IOPAE microcapsules was similar to the preparation of labeled particles, except [^{131}I]IOPAE was not used. The final concentration prepared was 30 mg of I/mL of water.

Particle Size Analysis—The size distribution of the microspheres was estimated with a Coulter counter and Coulter Channelyzer (Coulter Electronics, Hialeah, FL). Microcapsules were suspended in saline solution containing Nonidet P40 (0.0001%) as dispersant. Measurements were made after sonication for 1 min (Branson Cleaning Equipment Co., B-1200R-1, Shelton, CT) in order to cause deaggregation.

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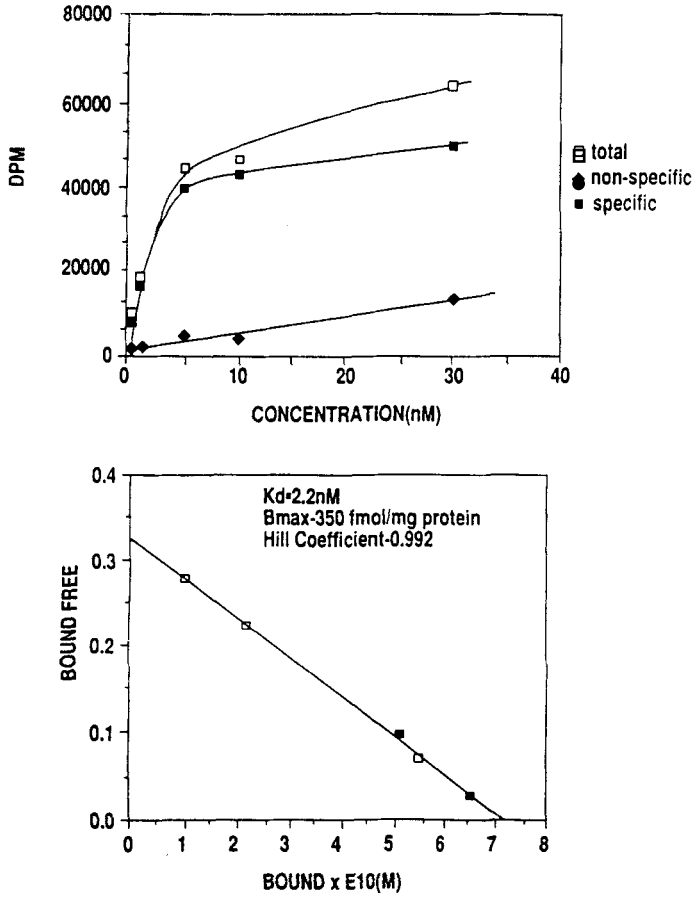

Figure 1—Saturation curve and Scatchard analysis of estrogen receptor assay.

In Vivo Tissue Distribution—PBLG microcapsules loaded with [^{131}I]ethyl iopanoate (5.7 μCi in 0.6 mL of water) were administered to rats via the tail vein. The control group was given [^{131}I]IOPAE only. Rats (N = 3/group) were killed at 20 min and 2, 5, and 24 h after injection. The percentage of injected dose in a given organ or weight of tissue was determined by γ counter.

Preparation of 3-(Aminoethyl)estrone—Estrone (5.0 g, 18.5 mmol) was dissolved in 80 mL of anhydrous N,N-dimethylformamide, and sodium hydride (4.4 g, 185 mmol) was slowly added to the solution to generate reactive phenoxide in situ. Care was taken to avoid rapid evolution of hydrogen gas. Chloroethylamine (4.3 g, 55 mmol) was finally added into the solution, and the mixture was allowed to react at 60 °C for 4 h. The product was precipitated with a large volume of water and the precipitate collected. For purification, the crude product was dissolved in methylene chloride and then washed with water. Evaporation of methylene chloride yielded 3-(aminoethyl)estrone, which was further washed with ethyl ether to give 3.0 g (52%) of product (mp 140–142 °C) 3-aminoethyl estrone hydrochloride mp 180 °C dec. ^{1}H NMR (ppm) 2.78 (2, t, $CH_2CH_2NH_2$), 3.01 (2, t, $CH_2CH_2NH_2$), 4.00 (2, t, $COCH_2$).

Coupling of 3-(Aminoethyl)estrone to PBLG—Conjugation of (aminoethyl)estrone to PBLG was accomplished via an imide linkage. Briefly, 3-(aminoethyl)estrone (1.25 g, 4 mmol) was added to a 7-mL dioxane solution containing PBLG (0.88 g, 4 mmol). The mixture was allowed to react at 60 °C for 2 days. The conjugate formed was collected by precipitating the dioxane solution with water and then filtering. For purification, the solid was dissolved in methylene chloride. Insoluble impurities were removed by filtration. The methylene chloride solution was washed with cold aqueous hydrochloric acid solution (0.2 N), water, and saturated NaCl. Evaporation of methylene chloride yielded 0.4 g of conjugated product. Anal. calcd: C, 70.73; H, 7.60; N, 6.60. Found: C, 66.70; H, 6.45; N, 6.00. The degree of substitution was 12% based on the elemental analysis data. ^{1}H NMR (ppm): 2.86 (2, t, CH_2CH_2-NHCO), 3.70 (2, t, CH_2CH_2NHCO), 4.04 (2, t, $COCH_2$).

Estrogen Receptor Assay for Estrone–PBLG Conjugate—The estrogen receptor assay was performed as previously described.[16,17]

Table 1—Tissue Distribution of [^{131}I]Ethyl Iopanoate-Loaded Poly(benzyl L-glutamate) Microcapsules after Intravenous Injection to Rats[a] (n = 3/group)

Organ	20 min	2 h	5 h	24 h
Blood	1.53 ± 0.71	1.46 ± 0.21	1.30 ± 0.26	0.29 ± 0.06
Lung	1.86 ± 0.40*	1.06 ± 0.16*	1.02 ± 0.19	0.28 ± 0.03*
Liver	1.80 ± 0.78	1.20 ± 0.15*	1.16 ± 0.14*	0.64 ± 0.06*
Kidney	0.74 ± 0.28	0.58 ± 0.09	0.58 ± 0.05	0.24 ± 0.08

[a] Data represent the average (mean ± SD) of percentage of injected dose/g of tissue. *Significant difference ($p < 0.05$; t-test) of corresponding organs between microcapsule and control groups (data shown in Table 2).

Table 2—Tissue Distribution of [^{131}I]Ethyl Iopanoate after Intravenous Injection to Rats[a] (n = 3/group)

Organ	20 min	2 h	5 h	24 h
Blood	1.51 ± 0.10	1.41 ± 0.23	0.99 ± 0.04	0.28 ± 0.12
Lung	1.20 ± 0.16	0.64 ± 0.03	0.70 ± 0.31	0.13 ± 0.06
Liver	1.62 ± 0.18	0.97 ± 0.01	0.73 ± 0.06	0.39 ± 0.03
Kidney	1.02 ± 0.10	0.61 ± 0.02	0.60 ± 0.33	0.24 ± 0.11

[a] Data represents the average (mean ± SD) of the percentage of injected dose/g of tissue weight.

Briefly, uteri cytosol was prepared from a uterine homogenate containing 30 g of EDTA (1.5 mM) and sodium azide (3 mM) in 80 mL of Tris buffer (10 mM, pH 7.4). To investigate the nature of estradiol interaction with the estrogen receptor site, saturation curves were calculated for [^{3}H]-estradiol (10^{-5}–10^{-10} M) in the presence and absence of estradiol (10^{-5} M). The concentration of estrone–PBLG that decreased specific radioligand binding by 50% (IC_{50}) was determined. Protein concentrations were determined according to the method of Lowry et al.[18]

Computed Tomographic Evaluation of PBLG-Microencapsulated IOPAE—Computed tomography (CT) imaging was performed on two New-Zealand male rabbits (3 kg) with a High Speed Advantage CT System (GE Medical System, Milwaukee, WI). The field-of-view (FOV) was 25 cm. A no-contrast prescan was performed before intravenously injection of PBLG–IOPAE microcapsules. The scan region was started from the upper liver field to the bladder. The slices (5 mm for liver, 10 mm for kidneys) were collected at 120 kV and 220 mA. Each rabbit was administered PBLG–IOPAE microcapsules (100 mg of I/kg of body weight). The image was collected at 0, 15, 30, 60, 120- and 1440-minute intervals during a 3-min infusion. Each rabbit was anesthetized with ketamine (50 mg/kg, im), xylazine (3 mg/kg, im), and acepromazine (1 mg/kg, sc). The supplement dose used was ketamine (10 mg/kg, im) and xylazine (0.8 mg/kg, im). Blood samples were taken at 1, 7, 14, and 21 days for the analysis of blood urea nitrogen (BUN), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphate, and cell counts.

Results

In Vitro Estrogen Receptor Assay of Estrone–PBLG Conjugates—The size distribution of microcapsules determined was in the range of 1–3 μm. The Scatchard analysis of [^{3}H]-estradiol binding in pig uteri indicated a single class of binding sites with a mean binding affinity constant (K_d) of 2.2 nM and a mean receptor density (β_{max}) of 350 fmol/mg of protein. The protein concentration used was 1 mg/mL of cytosol. Hill analysis (coefficient 0.992) indicated that estradiol has competitive reversible binding ability (Figure 1). The IC_{50} for estrone was 5×10^{-8} M and for estrone–PBLG was 5×10^{-7} M (based 12% conjugation).

***In Vivo* Tissue Distribution of Small Microcapsules**—The results of tissue distribution studies for [^{131}I]IOPAE and PBLG–[^{131}I]IOPAE microcapsule groups are shown in Tables 1 and 2. The liver radioactivity–uptake ratio in the PBLG group was higher than that of the control group.

Table 3—CT Liver and Kidney Uptake in Hounsfield Units (HU) after Administration of PBLG–IOPAE Microcapsules to Rabbits[a]

Time (min)	Liver[b]		Kidney
	Region 1	Region 2	
No contrast	93.6 ± 2.6	85.9 ± 2.0	54.0 ± 3.6
0	109.8 ± 2.9	99.0 ± 3.0	74.9 ± 4.7
15	104.1 ± 2.8	89.5 ± 2.3	67.2 ± 4.6
30	100.4 ± 2.1	84.6 ± 2.6	46.7 ± 4.2
60	102.3 ± 2.5	84.1 ± 2.3	48.7 ± 3.7
120	94.5 ± 2.1	86.7 ± 2.6	50.1 ± 2.7
1440	83.4 ± 2.1	77.5 ± 2.3	46.1 ± 4.2

[a] The dose level was 100 mg of iodine/kg of body weight. [b] At each time interval two regions of the liver were selected and the HU value was determined.

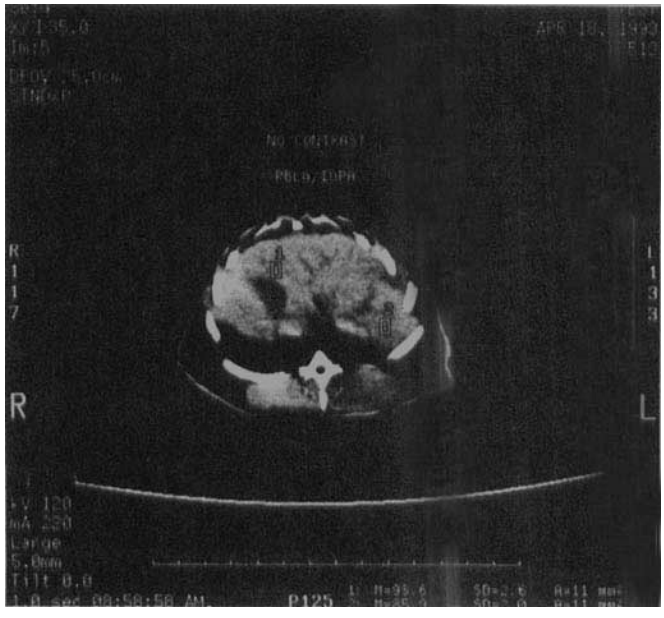

Figure 2—CT images of the liver from a rabbit at no contrast.

Computed Tomographic Imaging Studies—The liver and kidney uptake of PBLG–IOPAE microcapsule is shown in Table 3. The highest uptake appeared at 15–60 min postinjection (Figures 2–4). The microcapsules produced a prolonged contrasting effect up to 2 h postinjection. The average Hounsfield unit in the liver was higher than that of the prescan (no contrast). The radiocontrast Hounsfield units were returned to baseline at 24 h postinjection.

Blood Chemistry—On day 1 after administration of PBLG–IOPAE particles, SGOT, SGPT, and LDH levels were significantly increased. The SGOT value changed from 25–30 to 100–120 IU. The SGPT value changed from 35–40 to 70–80 IU. The LDH value changed from 65–80 to 500–560 IU (data not shown). On day 7, these values were back to baseline. There were no significant changes in these values on days 14 and 21. No significant changes in values, after injection of microcapsules, were noted for BUN, creatinine, alkaline phosphate, total protein, and bilirubin.

Discussion

Microcapsules have been prepared by many methods, including coacervation, interfacial polymerization, mechanical methods, polymer dispersion matrix encapsulation, and solvent evaporation.[19] Sustained-release microcapsules have been prepared from ethylcellulose[5,6] and poly(D-lactide).[19] There is

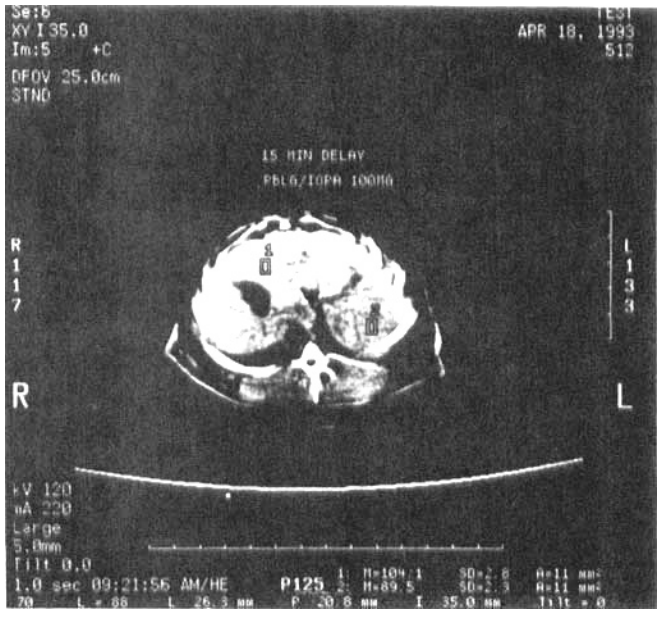

Figure 3—CT image of the liver from a rabbit at 15 min postinjection of PBLG–IOPAE (100 mg of I/kg of body weight).

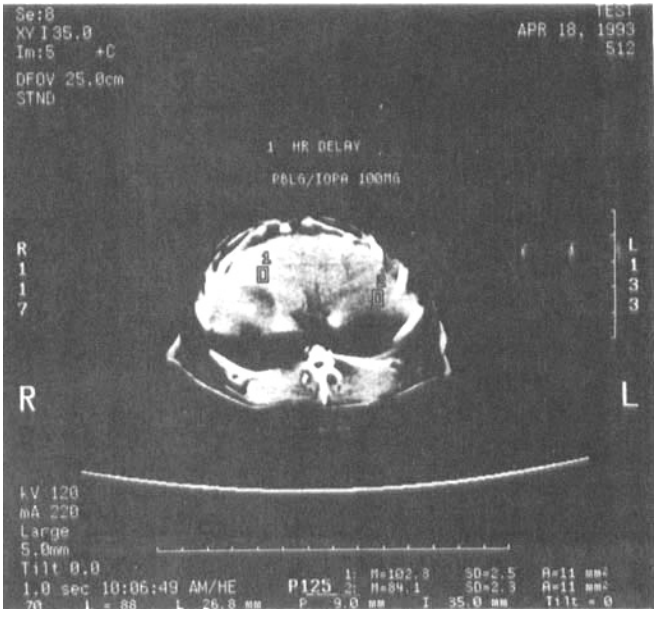

Figure 4—CT image of the liver from a rabbit at 1 h postinjection of PBLG–IOPAE (100 mg of I/kg of body weight).

voluminous literature on the preparation and use of encapsulating polymers designed for sustained drug release. Poly(benzyl L-glutamate) was chosen for this study because of its (1) biocompatability and (2) the availability of modifiable side chain functional groups.

A homogeneous nonaggregated preparation of a <3-μm microencapsulated imaging agent was prepared as previously described. The material encapsulated was a radiocontrast agent (iopanoic acid). The microencapsulated agent was administered to an animal by intravenous injection. The imaging agent was then detected by computed tomography. The highest liver imaging CT enhancement in a rabbit with PBLG–IOPAE observed was between 15 to 60 min. Similar results were observed from our *in vivo* tissue distribution data.

The liver enzymes SGOT, SGPT, AND LDH were increased when compared to values at preadministration of the micro-

capsules. These transient changes returned to baseline 1 week after injection.

Using particulate contrast media for CT enhancement of liver imaging was reported.[20-22] However, most studies show that the particles were quickly degraded into water-soluble material and eliminated from the body. By using the microencapsulation technique, (1) the microcapsules produced sustained-release properties in the liver and spleen, and (2) the administration route could be changed (e.g. oral changed to iv). Small microcapsules loaded with radiopaques may actively be taken up by Kupffer cells surrounding the neoplasm; thus, they may possibly produce a contrast density difference between normal liver tissue and focal hepatic lesions.

The receptor binding affinity of estrone–PBLG conjugation decreased 10-fold when compared that of to estrone itself. Our data suggests that estrone conjugated to PBLG polymer displayed potential as an estrogen receptor targeted therapy.

In summary, small microcapsules loaded with a radiocontrast agent produce a prolonged opacification of the liver. PBLG–estrone conjugates have the potential to be useful in estrogen therapy.

References and Notes

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