Journal of Nuclear Medicine, published on April 13, 2017 as doi:10.2967/jnumed.117.189993

Brachytherapy with Intratumoral Injections of Radiometal-labeled Polymers that Thermo-responsively Self-aggregate in Tumor Tissues

Kohei Sano[1,2,3,¶,*], Yuko Kanada[1,¶], Kengo Kanazaki[1,4], Ning Ding[1], Masahiro Ono[1], Hideo Saji[1,*]

¶ These authors contributed equally to this work.

[1]Department of Patho-Functional Bioanalysis Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto, Japan, 606-8501

[2]Kyoto University Hospital, 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto, Japan, 606-8507

[3]Department of Biophysical Chemistry, Kobe Pharmaceutical University, 4-19-1 Motoyama Kitamachi, Higashinada-ku, Kobe, Japan, 658-8558

[4]Medical Imaging Project, Corporate R&D Headquarters, Canon Inc., 3-30-2 Shimomaruko, Ohta-ku, Tokyo, Japan, 146-8501

*** Corresponding author**:

Kohei Sano, PhD, Lecture

Department of Biophysical Chemistry, Kobe Pharmaceutical University, 4-19-1 Motoyama

Kitamachi, Higashinada-ku, Kobe, Japan, 658-8558

Tel: +81-78-441-7540 Fax: +81-78-441-7541

E-mail: ksano@kobepharma-u.ac.jp

First author:

Kohei Sano, PhD, Lecture

Department of Biophysical Chemistry, Kobe Pharmaceutical University, 4-19-1 Motoyama Kitamachi, Higashinada-ku, Kobe, Japan, 658-8558

Tel: +81-78-441-7540 Fax: +81-78-441-7541

E-mail: ksano@kobepharma-u.ac.jp

Word count: 4540

Financial support: This research was supported by JSPS KAKENHI Grant Number (16H06256 and 26860991, K. S.), Shimazu Science Foundation (K. S.), and Takeda Science Foundation (K. S.). This work was partly supported by the Innovative Techno-Hub for Integrated Medical Bio-imaging Project of the Special Coordination Funds for Promoting Science and Technology, from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

Short running title: Brachytherapy with ^{90}Y-polyoxazoline

Abstract (350 words)

Brachytherapy is a type of radiotherapy wherein titanium capsules containing therapeutic radioisotopes are implanted within tumor tissues, enabling high-dose radio-irradiation to tumor tissues around the seeds. Although marked therapeutic effects have been demonstrated, brachytherapy needs complicated implantation technique under general anaesthesia and the seeds could migrate to other organs.

The aim of this study is to establish a novel brachytherapy using biocompatible, injectable thermo-responsive polymers (polyoxazoline; POZ) labeled with yttrium-90 (^{90}Y), which can self-aggregate above a specific transition temperature, resulting in long-term intratumoral retention of radioactivity and therapeutic effect. Therefore, we evaluated the tumor retention of radiolabeled POZ derivatives and their therapeutic effects.

METHODS: Using oxazoline derivatives with ethyl (Et), isopropyl (Isp), and propyl (Pr) side chains, EtPOZ, IspPOZ, Isp-PrPOZ (heteropolymer), and PrPOZ were synthesized, and their characteristic transition temperature (Tt) was measured. The intratumoral retention of ^{111}In-labeled POZ was evaluated until 7 days post-injection in nude mice bearing PC-3 human prostate cancer. The intratumoral localization of ^{111}In-labeled POZ derivatives was investigated by an autoradiographic study. Furthermore, a therapeutic study using ^{90}Y-labeled Isp-PrPOZ was performed, and tumor growth and survival rate were evaluated.

RESULTS: The Tts of EtPOZ, IspPOZ, Isp-PrPOZ, and PrPOZ (approximately 20 kDa) were >70°C, 34°C, 25°C, and 19°C, respectively. In the intratumoral injection study, Isp-PrPOZ and PrPOZ (2000

μM) with Tts lower than tumor temperature (33.5°C under anaesthesia) showed a significantly higher retention of radioactivity at 1 day post-injection (73.6% and 73.9%, respectively) than EtPOZ (5.6%) and IspPOZ (15.8%). Even at low injected dose (100 μM), Isp-PrPOZ exhibited high retention (68.3% at 1 day). The high level of radioactivity of Isp-PrPOZ was retained in the tumor 7 days post-injection (69.5%). The autoradiographic study demonstrated that the radioactivity of ^{111}In-labeled Isp-PrPOZ and PrPOZ was localized in a small area. In the therapeutic study using ^{90}Y-labeled Isp-PrPOZ, significant suppression of tumor growth and prolonged survival rate were achieved in an injection dose-dependent manner compared to that observed for the vehicle-injected group and non-radioactive Isp-PrPOZ-injected group.

CONCLUSION: The injectable ^{90}Y-labeled Isp-PrPOZ was retained for a prolonged period within tumor tissues *via* self-aggregation and exhibited marked therapeutic effect, suggesting its usefulness for brachytherapy.

Key Words: brachytherapy; polyoxazoline; thermo-responsive polymer; radiotherapy

Brachytherapy is a form of radiotherapy with 'seeds,' (therapeutic radioisotopes) that are implanted within tumor tissues. These tissues can then receive high-dose radio-irradiation. Compared with chemotherapy, hormone therapy, and external beam radiation, brachytherapy can provide a long-term anti-tumor effect with decreased damage to normal tissues; marked therapeutic effects have been demonstrated in particular to prostate, breast, and brain cancers (*1,2*). The global market for brachytherapy is expected to reach over US$2.4 billion in 2030. However, brachytherapy requires a complicated implantation technique under general anaesthesia. Seed migration is also a well-recognized phenomenon after implantation. Furthermore, seed removal can be required, although rarely (*3*). Therefore, the development of injectable radiopharmaceuticals that are rigidly retained in the tumor tissues, instead of capsule-type seeds, are desired to allow for more simple and harmless procedures (*4-7*).

Poly (2-alkyl-2-oxazoline) (POZ), an isomeric polypeptide material that can be synthesized *via* living cationic ring-opening polymerization (*8*), is garnering attention as a biocompatible and hydrophilic biomaterial for drug delivery (*9,10*), in addition to the more traditional polyethylene glycol. Recently, we have demonstrated that chemically modified POZ accumulates in tumor tissue *via* an enhanced permeability and retention effect, and is rapidly cleared from the blood after intravenous injection of the probe (*11*), indicating its usefulness for biomedical applications. Moreover, POZ has a lower critical solution temperature (LCST), and can self-aggregate above a specific transition temperature (Tt) that is dependent on oxazoline composition and molecular weight of its polymers (*12,13*), which can be

controlled in a range of 10-90°C. This thermo-responsive characteristic of POZ motivated us to develop an injectable POZ radiopharmaceutical suitable for brachytherapy; ultimately, we designed therapeutic, radiolabeled POZ derivatives that are soluble at room temperature, but rapidly self-aggregate and are rigidly retained in the tumor upon intratumoral injection (Fig. 1).

In the present study, we synthesized POZ derivatives with different Tts, and determined their physicochemical properties. A metal chelator, 1,4,7,10- tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) derivative (*14,15*), was then introduced to the POZ derivatives before labelling with either indium-111 (^{111}In, γ-ray emitting radiometal, 245 and 171 keV, $t_{1/2}$=67.9 h) or yttrium-90 (^{90}Y, β^--ray emitting radiometal, 2.28 MeV, $t_{1/2}$=64.0 h). We first evaluated the tumor retention rate of ^{111}In-labeled POZ derivatives to estimate their therapeutic effect using prostate cancer-implanted mice. We also performed a therapeutic study of ^{90}Y-labeled POZ derivatives, and evaluated whether POZ-based biocompatible radiopharmaceuticals could provide long-term tumor retention and anti-tumor effect.

MATERIALS AND METHODS

Reagents

Methyl *p*-toluenesulfonate, ethylenediamine, and super dehydrated acetonitrile were purchased from Wako Pure Chemical Industries, Ltd. 2-Ethyl-2-oxazoline, 2-isopropyl-2-oxazoline, and 2-propyl-2-oxazoline were purchased from Tokyo Chemical Industry Co., Ltd. S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (*p*-SCN-Bn-DOTA) was purchased from Macrocyclics Inc. ^{111}In chloride ($^{111}InCl_3$) was kindly supplied by Nihon Medi-Physics. ^{90}Y chloride ($^{90}YCl_3$) was purchased from Eckert & Zieglr Radiopharma GmbH. Other reagents were of reagent grade and were used without further purification unless otherwise indicated.

Synthesis of amine-terminated POZ derivatives

Thermo-responsive POZ derivatives including EtPOZ composed of 2-ethyl-2-oxazoline, IspPOZ composed of 2-isopropyl-2-oxazoline, Isp-PrPOZ composed of 2-isopropyl-2-oxazoline and 2-propyl-2-oxazoline, and PrPOZ composed of 2-propyl-2-oxazoline were synthesized according to previous reports (*11*,*16*). The reaction scheme was shown in Scheme 1. In order to prepare EtPOZ, IspPOZ, Isp-PrPOZ, and PrPOZ, methyl *p*-toluene sulphonate (1 eq) was mixed with 2-ethyl-2-oxazoline (400 eq), 2-isopropyl-2-oxazoline (500 eq), a mixture of 2-isopropyl-2-oxazoline (200 eq) and 2-propyl-2-oxazoline (300 eq), or 2-propyl-2-oxazoline (500 eq) in acetonitrile, respectively, and then stirred for 14 min at 140°C under microwave irradiation (Discover, CEM Co.). Molecular

weight was determined by gel permeation chromatography with KD804 column (Showa Denko) using polyethylene glycol as a calibration standard. M.W. was 19712, 18204, 17757, and 18235 for EtPOZ, IspPOZ, Isp-PrPOZ, and PrPOZ, respectively.

Subsequently, ethylenediamine (EDA) was added to the reaction solution and stirred for an additional 7 min at 140 °C under microwave irradiation. After the reaction, the mixture was cooled to room temperature (r.t.) and the solvent was evaporated. The mixture was dissolved in methanol and dialyzed against methanol with pre-treated regenerated cellulose membrane Spectra/Por 7 dialysis tubing (molecular weight cut-off: 3.5 kDa, Spectrum Laboratories, Inc.). Each amino-terminated POZ derivative is hereinafter referred to as EtPOZ-EDA, IspPOZ-EDA, Isp-PrPOZ-EDA, or PrPOZ-EDA, according to the composition of oxazoline. M.W. is 19898, 17892, 17494, and 17676 for EtPOZ-EDA, IspPOZ-EDA, Isp-PrPOZ-EDA, and PrPOZ-EDA, respectively.

Measurement of phase transition temperature (Tt)

POZ derivatives were dissolved in phosphate buffered saline (PBS) (2000 μM) and their Tts were measured by a Zetasizer Nano (Malvern Instruments Ltd.) with heating at a rate of 1°C/min. In order to measure phase transition time, a solution of EtPOZ or Isp-PrPOZ (2000 μM, 5 μL) was dropped onto hot plate heated at 36.5°C (close to body temperature).

Preparation of ^{111}In-labeled POZ derivatives

For radiolabeling with ^{111}In or ^{90}Y, POZ-EDA derivatives prepared as mentioned above were conjugated with *p*-SCN-Bn-DOTA, a metal chelator (Scheme 1). Each POZ-EDA derivative was reacted with *p*-SCN-Bn-DOTA (5-10 eq) in methanol with K_2CO_3 for 36 h at r.t. After the reaction, unconjugated *p*-SCN-Bn-DOTA was purified by ultrafiltration with Amicon Ultra centrifugal filter units (molecular weight cut-off: 3 kDa) (Merck Millipore, Co.) and by gel filtration (PD-10 Columns, GE Healthcare). ^{111}In-labelling of POZ-EDA-DOTA derivatives was performed by mixing and reacting with $^{111}InCl_3$ (9.3-13.3 MBq) in 0.1 M acetate buffer (pH 6.0) for 10 min at r.t. After incubation, ethylenediaminetetraacetic acid (10 eq) was added and incubated for 5 min at r.t., and then the buffer was exchanged with PBS (pH 7.4) by ultrafiltration (Amicon Ultra-4, 3 kDa) to remove the un-chelated ^{111}In. Radiochemical purity was assessed by gel filtration (PD-10 Columns) eluted with PBS.

Animal model

Animal experiments were conducted in accordance with the institutional guidelines of Kyoto University, and were approved by the Kyoto University Animal Care Committee. Human prostate cancer PC-3 cells (2×10^6 cells suspended in the mixture of matrigel [50 μL, Corning] and 50 μL PBS) were inoculated subcutaneously into the right thigh of 5-week-old male Balb/c-*nu/nu* mice (Japan SLC, Inc.). When the tumor size was reached to approximately 0.7-1.0 cm in diameter, the animals were used for *in vivo* experiments. The rectal and tumor temperature were measured by thermometer or thermography.

Tumor retention analysis

In order to investigate the effect of phase Tts of POZ derivatives on tumor retention, ^{111}In-labeled DOTA-EDA-EtPOZ (^{111}In-EtPOZ), DOTA-EDA-IspPOZ (^{111}In-IspPOZ), DOTA-EDA-Isp-PrPOZ (^{111}In-Isp-PrPOZ), and DOTA-EDA-PrPOZ (^{111}In-PrPOZ) (2000 μM, 37 kBq, 5 μL PBS) were intratumorally injected into PC-3 tumors under isoflurane anaesthesia using a nano syringe (Altair Technologies Ltd.). At one, three, or seven days after injection, the tumor was excised and the radioactivity in the tumor was calculated using the injection dose as a standard. Data was presented as a percentage of tumor retention against 0 min after injection. Biodistribution of ^{111}In-labeled POZ derivative to other organs was also evaluated at one day after intratumoral injection of probes. Data was calculated as percentage injected dose (%ID). Furthermore, *in vivo* biodistribution of probes after intravenous injection was also investigated, and the data was calculated as %ID/g tissue.

In order to evaluate the localization of ^{111}In-labeled POZ derivatives in tumor tissues, the tumor tissues were excised at one day post-injection. The excised tumors were frozen, and cut into 20-μm-thick sections with a cryomicrotome. The sections were thaw-mounted in silane-coated slides, and were then placed on a phosphor image plate (BAS-SR 2040, FUJIFILM) for 1 h to obtain ^{111}In autoradiograms. The autoradiographic images were analysed using a computerized imaging analysis system (BAS2500, FUJIFILM). The same slides used in the autoradiographic study were subjected to haematoxylin and eosin staining.

In order to investigate the influence of molecular weight of POZ derivatives on tumor retention,

^{111}In-Isp-PrPOZ (5 and 10 kDa, synthesized elsewhere) (2000 μM, 37 kBq, 5 μL PBS) were intratumorally injected to PC-3 tumors. Next, to evaluate the relation of POZ concentration with tumor retention, ^{111}In-Isp-PrPOZ (20 kDa) (100, 500, 2000, and 4000 μM) (37 kBq, 5 μL PBS) were intratumorally injected into PC-3 tumors. As mentioned above, tumor retention was evaluated as a percentage of tumor retention 1 day after injection against that 0 min after injection for each condition.

Therapeutic studies using ^{90}Y-labeled Isp-PrPOZ

For therapeutic studies using Isp-PrPOZ derivatives, Isp-PrPOZ was radiolabeled with ^{90}Y as follows. Isp-PrPOZ-EDA-DOTA (2.5 mg) was incubated with $^{90}YCl_3$ in acetate buffer (0.1 M, pH 6.0) for 90 min at r.t. After incubation, ethylenediaminetetraacetic acid (10 eq) was added and incubated for 5 min at r.t., and then un-chelated ^{90}Y was eliminated by ultrafiltration.

Therapeutic studies were performed for five groups (n=12 for each group): (i) PBS-injected group (control), (ii) non-radioactive Y-labeled Isp-PrPOZ-injected group (non-radioactive), (iii) ^{90}Y-Isp-PrPOZ-injected group (0.74 MBq), (iv) ^{90}Y-Isp-PrPOZ-injected group (1.85 MBq), and (v) ^{90}Y-Isp-PrPOZ-injected group (3.70 MBq). These solutions (5 μL) were intratumorally injected, and tumor size and body weight was measured twice or thrice a week. Tumor volumes were calculated by the formula; $[\text{length}\times(\text{width})^2]/2$. From ethical point of view, mice were sacrificed when the tumor volume reached 750 mm^3 or when the body weight dropped below 80% of the body weight.

Furthermore, apart from the therapeutic studies, we prepared tumor-bearing mice (five groups, n=3

for each group). The tumor, liver, kidneys, and spleen were excised seven days after injection of each preparation, and haematoxylin and eosin staining was conducted for histological analysis.

Statistical analysis

Data are expressed as means ± standard deviation or standard error of mean from a minimum three experiments. For multiple comparisons, a one-way analysis of variance with post-test (Tukey-Kramer test) was used. The cumulative probability of survival was estimated in each group with the use of the Kaplan-Meier survival curve analysis, and the results were compared with use of the log-rank test with Bonferroni's correction for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Characterization of ^{111}In-labeled POZ derivatives

Thermo-responsive POZ derivatives including EtPOZ, IspPOZ, Isp-PrPOZ, and PrPOZ were prepared according to Scheme 1. The physicochemical properties of the synthesized POZ derivatives are characterized as follows. The Tts of EtPOZ, IspPOZ, Isp-PrPOZ(5 kDa), Isp-PrPOZ(10 kDa), Isp-PrPOZ(20 kDa), and PrPOZ were >70°C, 34°C, 26°C, 25°C, 25°C, and 19°C, respectively. Solutions of Isp-PrPOZ (Tt 25°C) and EtPOZ (Tt >70°C) were dropped on a hot plate heated to 36.5°C. Isp-PrPOZ was observed to aggregate within a second, whereas EtPOZ did not aggregate (Supplemental Video 1). The radiochemical purity of ^{111}In-labeled POZ was more than 98% for all POZ derivatives and radiochemical yield was 40-70%.

Tumor retention analysis

Intratumoral retention of ^{111}In-labeled POZ derivatives was evaluated after intratumoral injection of probes into PC-3 tumors inoculated in the flanks of Balb/c-*nu*/*nu* mice using a nano syringe. Intratumoral retention at one day of ^{111}In-labeled EtPOZ, IspPOZ, Isp-PrPOZ, and PrPOZ (M.W. approximately 20 kDa) were 5.6%, 15.8% 73.6%, and 73.9%, respectively (Fig. 2A). Isp-PrPOZ and PrPOZ, with Tts lower than tumor temperature (around 33.5°C under isoflurane anaesthesia, Fig. 2B), had a significantly higher retention rate compared to EtPOZ and IspPOZ, which had Tts higher than body temperature. Intratumoral localization of ^{111}In-labeled POZ derivatives was evaluated by autoradiography one day

after injection (Fig. 2C). EtPOZ and IspPOZ were not retained in the tumor, while Isp-PrPOZ and PrPOZ showed localization of radioactivity, suggesting rapid intratumoral self-aggregation. Handling of PrPOZ at room temperature was difficult due to its low Tt (19°C); therefore, we used Isp-PrPOZ (Tt 25°C) for further experiments. The high level of retention of radioactivity of ^{111}In-Isp-PrPOZ in the tumor was retained three and seven days post-injection (70.5% and 69.5%, respectively) (Fig. 2D). A low level of ^{111}In-Isp-PrPOZ (less than 5.4 %ID at 1 day post-injection) distributed in other normal tissues was observed (Supplemental Table 1).

An *in vivo* biodistribution study was also performed after intravenous injection of ^{111}In-labeled POZ derivatives in normal Balb/c-*nu*/*nu* mice (Supplemental Tables 2-5). EtPOZ was cleared relatively rapidly from the blood, with no apparent retention in normal tissues, whereas Isp-PrPOZ and PrPOZ rapidly accumulated in the liver and spleen, where colloidal compounds usually accumulate, suggesting *in vivo* self-aggregation of Isp-PrPOZ and PrPOZ. IspPOZ showed a relatively high accumulation in the kidneys.

Next, the relationship between tumor retention and molecular weight of Isp-PrPOZ was investigated one and seven days after intratumoral injection of ^{111}In-labeled Isp-PrPOZ (5 kDa), Isp-PrPOZ (10 kDa), and Isp-PrPOZ (20 kDa) (Fig. 3A). Tumor retention of radioactivity was 39.4%, 54.9%, and 73.0% one day post-injection, and 14.7%, 45.2%, and 69.5% seven days post-injection, for ^{111}In-labeled Isp-PrPOZ (5 kDa), Isp-PrPOZ (10 kDa), and Isp-PrPOZ (20 kDa), respectively. Furthermore, retention of radioactivity of Isp-PrPOZ (20 kDa) in the tumor one day post-injection was independent of the

concentration of Isp-PrPOZ, in the range of 100-4000 μM (68.3%, 71.6%, 73.1%, and 62.0% for 100, 500, 2000, and 4000 μM, respectively) (Fig. 3B). There was no significant difference in intratumoral retention rate between the probe concentrations examined. Based on these results, we chose 20 kDa and 2000 μM as an effective molecular weight and probe concentration, respectively, for further *in vivo* therapeutic experiments.

Therapeutic studies using ^{90}Y-labeled Isp-PrPOZ

^{90}Y-labeled Isp-PrPOZ was obtained with a radiochemical yield of 31.1% and radiochemical purity of 96.4%. We studied five groups of PC-3 tumor-bearing mice: (i) PBS-injected group (control), (ii) non-radioactive Y-labeled Isp-PrPOZ-injected group (non-radioactive), (iii) ^{90}Y-Isp-PrPOZ-injected group (0.74 MBq), (iv) ^{90}Y-Isp-PrPOZ-injected group (1.85 MBq), (v) ^{90}Y-Isp-PrPOZ-injected group (3.70 MBq). ^{90}Y-Isp-PrPOZ significantly suppressed tumor growth in a dose-dependent manner compared to PBS and non-radioactive Y-Isp-PrPOZ (Figs. 4A and 4B). Because tumor size decreased at seven days as well as later time points after injection of ^{90}Y-Isp-PrPOZ (0.74, 1.85, and 3.70 MBq), histological analysis was performed seven days post-injection of probes. Compared to the control group and non-radioactive Y-Isp-PrPOZ group, cytotoxicity, including nuclear fragmentation, was observed in the ^{90}Y-Isp-PrPOZ-injected groups (Fig. 4C). Cellular damage was most severe in the tumors injected with 3.70 MBq ^{90}Y-Isp-PrPOZ. Survival was prolonged in mice treated with ^{90}Y-Isp-PrPOZ compared to the control and non-radioactive Y-Isp-PrPOZ-injected groups. Fifty percent of mice survived at 90 days

post-injection in the ^{90}Y-Isp-PrPOZ (3.70 MBq)-injected group (Fig. 4D). No obvious loss of body weight or systemic side effects were observed in the ^{90}Y-Isp-PrPOZ-injected group (Fig. 4E). Furthermore, histological analysis of normal tissues seven days after intratumoral injection revealed no *in vivo* cytotoxicity in the liver, kidneys, and spleen (Fig. 4F).

DISCUSSION

In this study, we developed novel injectable radiopharmaceuticals for brachytherapy that were water-soluble at room temperature and quickly self-aggregated in tumor tissue after intratumoral injection by regulating the Tt of POZ. We observed that a solution of Isp-PrPOZ with a Tt of 25°C aggregated within a second after placement on a plate heated to body temperature, indicating probable rapid seed formation in tumor tissues after injection into the body. In fact, Isp-PrPOZ was determined to have a 13.1 and 4.7-fold higher retention rate in the tumor compared with EtPOZ (>70°C) and IspPOZ (34°C), respectively, one day post-injection. Intratumoral temperature of mice under isoflurane anaesthesia was around 33.5°C; thus, IspPOZ (34°C) actually unexpectedly showed low tumor retention. *In vivo* biodistribution study by intravenous injection of probes demonstrated that POZ derivatives with Tts lower than body temperature were mainly taken up by the liver and spleen (Supplemental Tables 4 and 5), indicating self-aggregation in circulation.

The intratumoral retention of radioactivity was dependent on POZ molecular weight, but independent of probe concentration. The intratumoral retention of radioactivity improved as molecular weight increased; thus, we hypothesize that a POZ formulation greater than 20 kDa might augment the formation of POZ depots in tumor tissues. A similar injectable radiopharmaceutical, radio-iodinated elastin-like polypeptide, has been studied for brachytherapy (*6*). Liu et al. have demonstrated excellent treatment of tumors with ^{131}I-labeled polypeptides, but elastin-like polypeptide had low tumor retention when low concentrations were injected. This characteristic might be problematic as probe concentration

is diluted immediately after intratumoral injection. However, the tumor retention of ^{111}In-Isp-PrPOZ was retained in the concentration range of 100-4000 μM, which allows for the use of a lower dose of the polymer and results in the improvement of biosafety for clinical use. In a previous paper, toxicity studies in rats demonstrated that EtPOZ was safe after intravenous injection and the maximum tolerated dose was greater than 2 g/kg (*8*). Although we speculate the low toxicity of Isp-PrPOZ, the formal long-term toxicity studies would be required for clinical translation. Isp-PrPOZ was reasonably localized to a small area (4 × 5 mm as determined from autoradiography data, Fig. 2C), but localization would conversely require a multiple probe injection regimen for large tumors. By controlling the Tts of POZ used for brachytherapy, a high therapeutic effect for larger tumors *via* single probe injection would be expected.

The therapeutic effect of ^{90}Y-Isp-PrPOZ was enhanced in a dose-dependent manner, with 50% of mice surviving 90 days after injection of ^{90}Y-Isp-PrPOZ (3.70 MBq). Cytotoxicity such as nuclear fragmentation in the tumor was observed within a week post-injection of ^{90}Y-Isp-PrPOZ. The injected radioactivity was 5-17% of radioactivity that was administered in other reports on injectable radiopharmaceuticals for brachytherapy (*5,6*) leading to decreasing exposure dose for clinical trials. However, there might be still be room for improvement in the chemical modification of POZ derivatives. The radiochemical yield of ^{90}Y-labeled Isp-PrPOZ was not high (approximately 30%), which might be improved by changing the chelator from DOTA to diethylenetriamine pentaacetic acid (DTPA), the clinical chelator for ^{90}Y-labeled ibritumomab tiuxetan (*17*).

Although this therapeutic study was conducted using the PC-3 (human prostate cancer)-bearing

mouse model, we hypothesize that this probe could be applied to other cancers including breast, brain, and gynaecologic that are currently treated by capsule seed-type brachytherapy (*18-21*). Brachytherapy is also conducted to prevent tumor recurrence after surgical treatment. Recently, breast-conserving surgery with whole breast radiation therapy has become the standard treatment of early-stage breast cancer (*22*). In these cases, injectable radiopharmaceuticals, which do not require seed removal and have decreased difficulty of handling, would be extremely useful.

In conclusion, we demonstrated the potential of the novel injectable radiopharmaceutical, ^{90}Y-labeled POZ, for brachytherapy. The Tt of Isp-PrPOZ was determined to be 25°C, *i.e.* Isp-PrPOZ is soluble at room temperature and self-aggregates at body temperature. The optimized ^{111}In-Isp-PrPOZ (20 kDa, 2000 μM) was highly retained seven days after injection, and ^{90}Y-Isp-PrPOZ exhibited a high therapeutic effect *via* intratumoral aggregation with no damage to normal tissue. Therefore, we conclude that ^{90}Y-Isp-PrPOZ would be used for treating cancer with currently marketed capsule-type seeds.

DISCLOSURE

The costs of publication of this article were defrayed in part by the payment of page charges. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734. This research was supported by JSPS KAKENHI Grant Number (16H06256 and 26860991, K. S.), Shimazu Science Foundation (K. S.), and Takeda Science Foundation (K. S.). This work was partly supported by the Innovative Techno-Hub for Integrated Medical Bio-imaging Project of

the Special Coordination Funds for Promoting Science and Technology, from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. No potential conflict of interest relevant to this article was reported.

REFERENCES

1. Tanderup K, Ménard C, Polgar C, Lindegaard JC, Kirisits C, Pötter R. Advancements in brachytherapy. *Adv Drug Deliv Rev.* 2017;109:15-25.
2. Perera M, Krishnananthan N, Lindner U, Lawrentschuk N. An update on focal therapy for prostate cancer. *Nat Rev Urol.* 2016;13:641-53.
3. Older RA, Synder B, Krupski TL, Glembocki DJ, Gillenwater JY. Radioactive implant migration in patients treated for localized prostate cancer with interstitial brachytherapy. *J Urol.* 2001;165:1590-2.
4. Hruby M, Subr V, Kucka J, Kozempel J, Lebeda O, Sikora A. Thermoresponsive polymers as promising new materials for local radiotherapy. *Appl Radiat Isot.* 2005;63:423-31.
5. Hruby M, Pouckova P, Zadinova M, Kucka J, Lebeda O. Thermoresponsive polymeric radionuclide delivery system-an injectable brachytherapy. *Eur J Pharm Sci.* 2011;42:484-8.
6. Liu W, McDaniel J, Li X, et al. Brachytherapy using injectable seeds that are self-assembled from genetically encoded polypeptides in situ. *Cancer Res.* 2012;72:5956-65.
7. de la Puente P, Azab AK. Delivery systems for brachytherapy. *J Control Release.* 2014;192:19-28.
8. Viegas TX, Bentley MD, Harris JM, et al. Polyoxazoline, chemistry, properties, and application in drug delivery. *Bioconjug Chem.* 2011;22:976-86.
9. Hruby M, Filippov SK, Panek J, et al. Polyoxazoline thermoresponsive micelles as radionuclide delivery systems. *Macromol Biosci.* 2010;10:916-24.
10. Adams N, Schubert US. Poly(2-oxazolines) in biological and biomedical application contexts. *Adv*

Drug Deliv Rev. 2007;59:1504-20.

11. Kanazaki K, Sano K, Makino A, Homma T, Ono M, Saji H. Polyoxazoline multivalently conjugated with indocyanine green for sensitive in vivo photoacoustic imaging of tumors. *Sci Rep.* 2016;6:33798.
12. Luxenhofer R, Han Y, Schulz A, et al. Poly(2-oxazoline)s as polymer therapeutics. *Macromol Rapid Commun.* 2012;33:1613-31.
13. Roy D, Brooks WL, Sumerlin BS. New directions in thermoresponsive polymers. *Chem Soc Rev.* 2013;42:7214-43.
14. Koukouraki S, Strauss LG, Georgoulias V, et al. Evaluation of the pharmacokinetics of ^{68}Ga-DOTATOC in patients with metastatic neuroendocrine tumors scheduled for ^{90}Y-DOTATOC therapy. *Eur J Nucl Med Mol Imaging.* 2006;33:460-6.
15. Gaertner FC, Luxenhofer R, Blechert B, Jordan R, Essler M. Synthesis, biodistribution and excretion of radiolabeled poly(2-alkyl-2-oxazoline)s. *J Control Release.* 2007;119:291-300.
16. Huang H, Hoogenboom R, Leenen MA, et al. Solvent-induced morphological transition in core-cross-linked block copolymer micelles. *J Am Chem Soc.* 2006;128:3784-8.
17. Wagner HN Jr, Wiseman GA, Marcus CS, et al. Administration guidelines for radioimmunotherapy of non-Hodgkin's lymphoma with ^{90}Y-labeled anti-CD20 monoclonal antibody. *J Nucl Med.* 2002;43:267-72.
18. Keller BM, Pignol JP, Rakovitch E, Sankreacha R, O'Brien P. A radiation badge survey for family

members living with patients treated with a ^{103}Pd permanent breast seed implant. *Int J Radiat Oncol Biol Phys.* 2012;70:1374-80.

19. Petr MJ, McPherson CM, Breneman JC, Warnick RE. Management of newly diagnosed single brain metastasis with surgical resection and permanent I-125 seeds without upfront whole brain radiotherapy. *J Neurooncol.* 2009;92:393-400.
20. Keys HM, Roberts JA, Brunetto VL, et al. A phase III trial of surgery with or without adjunctive external pelvic radiation therapy in intermediate risk endometrial adenocarcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol.* 2004;92:744-51.
21. Ashamalla H, Rafla S, Zaki B, Ikoro NC, Ross P. Radioactive gold grain implants in recurrent and locally advanced head-and-neck cancers. *Brachytherapy.* 2001;1:161-6.
22. Veronesi U, Cascinelli N, Mariani L, et al. Twenty-year follow-up of a randomized study comparing breast-conserving surgery with radical mastectomy for early breast cancer. *N Engl J Med.* 2002;347:1227-32.

Figures

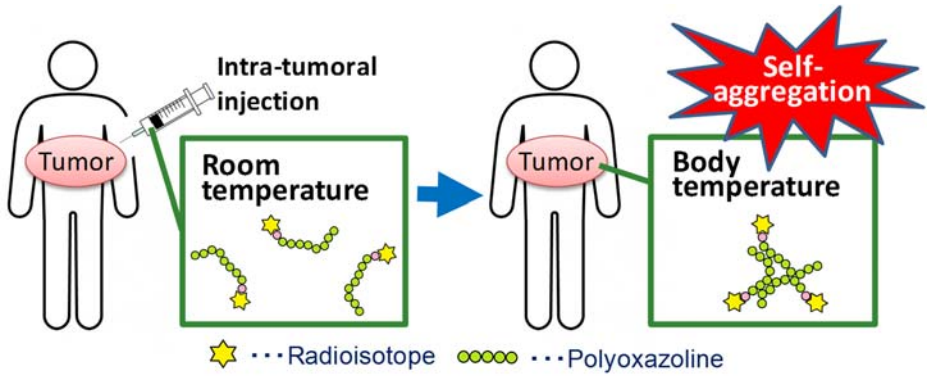


FIGURE 1. Concept of injectable polymer (polyoxazoline; POZ) labeled with radiometal that are thermo-responsively self-aggregated in tumor tissues for brachytherapy

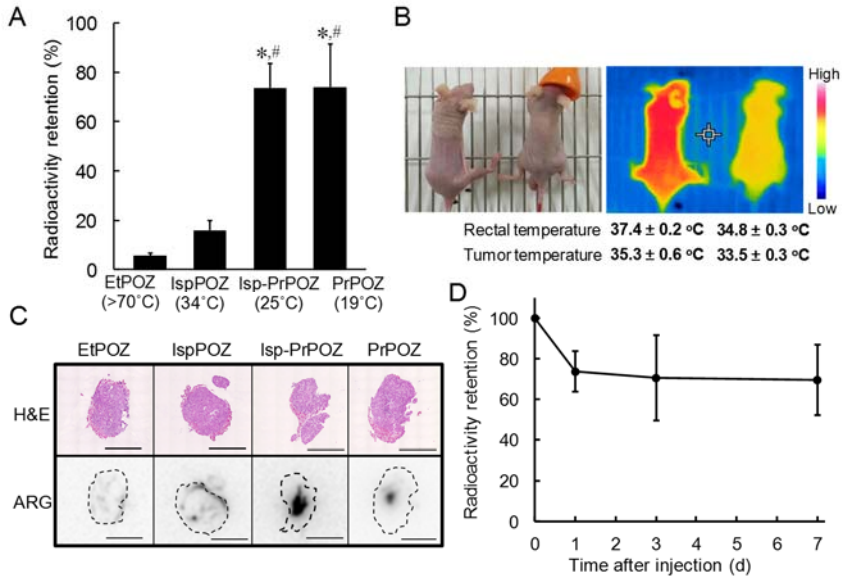


FIGURE 2. Evaluation of tumor retention of ^{111}In-labeled polyoxazoline (POZ) derivatives in PC-3 tumors. (A) Radioactivity retention (%) in the tumor 1 day after intratumoral injection of ^{111}In-labeled POZ derivatives (*$P < 0.01$ vs. EtPOZ and $^{\#}P < 0.01$ vs. IspPOZ). Data are means ± standard deviation. (B) Rectal and tumor temperature with (right) or without (left) isoflurane anaesthesia (2%). (C) Localization of radioactivity in the tumor determined by ARG. Scale bar, 10 mm. (D) Long-term retention of radioactivity in the tumor after injection of ^{111}In-labeled Isp-PrPOZ(20 kDa). Data are means ± standard deviation.

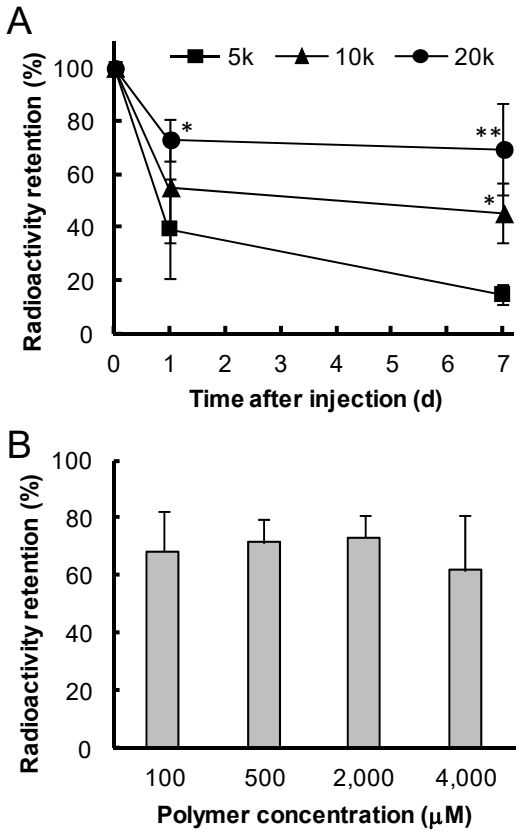


FIGURE 3. Effect of POZ molecular weight and probe concentration on intratumoral radioactivity retention after injection of ^{111}In-Isp-PrPOZ. (A) Radioactivity retention (%) of ^{111}In-Isp-PrPOZ with different molecular weight (5, 10, and 20 kDa) 1 and 7 days post-injection (**$P < 0.01$, *$P < 0.05$ vs. 5 kDa). Data are means ± standard deviation. (B) Radioactivity retention (%) of ^{111}In-Isp-PrPOZ(20 kDa) with different concentration (100, 500, 2000, and 4000 μM) 1 day post-injection. Data are means ± standard deviation.

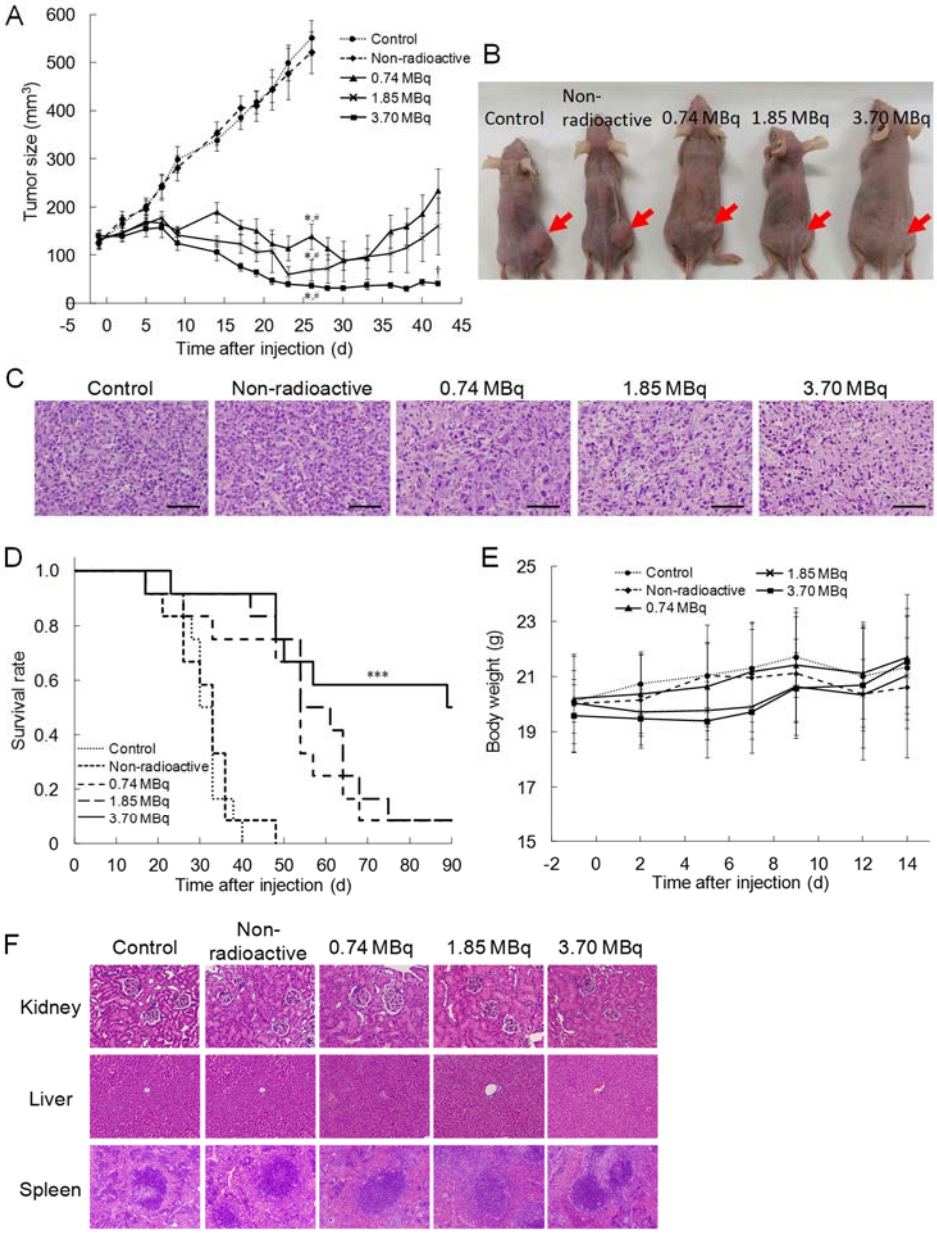
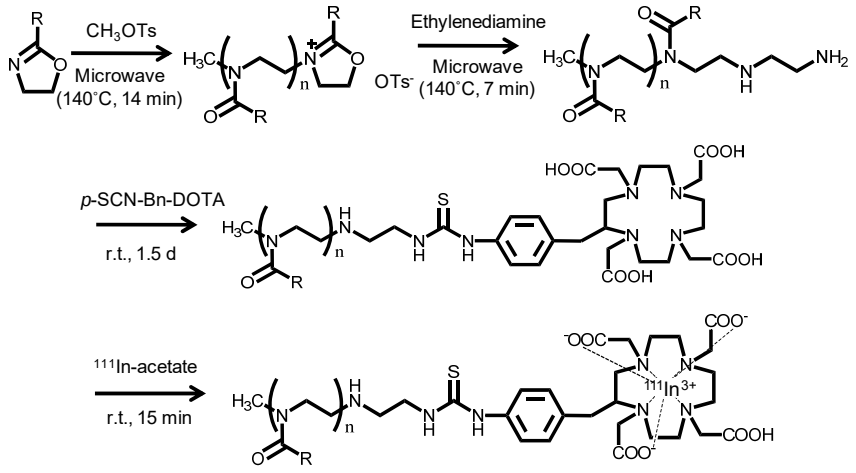


FIGURE 4. Therapeutic effects by intratumoral injection of ^{90}Y-labeled Isp-PrPOZ in PC-3 tumor bearing mice. (A) Tumor growth inhibition by ^{90}Y-labeled Isp-PrPOZ. Data are means ± standard error of mean. (n=9-12 mice in each group) (*$P < 0.01$ vs. control, $^{\#}P < 0.01$ vs. non-radioactive Y-Isp-PrPOZ, $^{\dagger}P < 0.05$ vs. 0.74 MBq). (B) Representative pictures of PC-3 tumor bearing mice 40 days after treatment with each preparations. (C) Histological analysis by haematoxylin and eosin staining. Scale bar = 100

μm (D) An analysis using Kaplan-Meier survival curve of treatment of ^{90}Y-labeled Isp-PrPOZ in PC-3 tumors (n=12 mice in each group) (***$P < 0.01$ for treatment compared to other groups.). (E) The change of body weight of tumor-bearing mice treated with each preparations. Data are means ± standard deviation. ($n = 12$). (F) Histological analyses (n = 3) of kidney, liver, and spleen at 7 days post-treatment with each preparations.



SCHEME 1. Synthesis scheme of ^{111}In-labeled POZ derivatives.

Supplemental Table 1 Biodistribution results of ^{111}In-labeled POZ derivatives in nude mice 1 day after intratumoral injection of probe. Results are expressed as means (%ID) ± SD (n=3).

Organ	**EtPOZ**	**IspPOZ**	**Isp-PrPOZ**	**PrPOZ**
Heart	0.18 ± 0.05	0.09 ± 0.07	0.02 ± 0.01	0.01 ± 0.00
Lungs	0.23 ± 0.04	0.15 ± 0.06	0.08 ± 0.11	0.05 ± 0.03
Liver	1.14 ± 0.10	1.40 ± 0.43	3.28 ± 3.88	2.36 ± 0.75
Kidneys	0.72 ± 0.06	1.36 ± 0.32	5.38 ± 0.74	1.07 ± 0.15
Stomach	0.25 ± 0.03	0.11 ± 0.05	0.27 ± 0.04	0.08 ± 0.02
Intestine	0.84 ± 0.08	1.52 ± 0.66	1.87 ± 0.62	0.50 ± 0.07
Pancreas	0.20 ± 0.05	0.09 ± 0.04	0.16 ± 0.07	0.06 ± 0.02
Spleen	0.17 ± 0.07	0.13 ± 0.08	0.30 ± 0.38	0.07 ± 0.02

Supplemental Table 2 Biodistribution results of ^{111}In-EtPOZ in nude mice after intravenous injection of probe. Results are expressed as means (%ID/g) ± SD (n=3).

	1 h	6 h	24 h
Blood	10.36 ± 0.26	5.54 ± 0.29	2.25 ± 0.31
Heart	2.49 ± 0.67	1.18 ± 0.17	0.71 ± 0.06
Lungs	3.26 ± 0.75	1.75 ± 0.24	1.18 ± 0.30
Liver	1.93 ± 0.29	1.34 ± 0.14	1.50 ± 0.33
Kidneys	3.36 ± 0.43	2.55 ± 0.27	1.97 ± 0.44
Stomach	1.49 ± 0.65	0.70 ± 0.44	0.35 ± 0.12
Intestine	1.10 ± 0.11	0.54 ± 0.17	0.43 ± 0.07
Pancreas	1.78 ± 0.32	1.02 ± 0.14	0.69 ± 0.15
Spleen	1.43 ± 0.31	1.04 ± 0.14	1.03 ± 0.25
Muscle	0.50 ± 0.19	0.33 ± 0.04	0.33 ± 0.07

Supplemental Table 3 Biodistribution results of ^{111}In-IspPOZ in nude mice after intravenous injection of probe. Results are expressed as means (%ID/g) ± SD (n=3).

	1 h	6 h	24 h
Blood	6.50 ± 3.74	0.39 ± 0.30	0.10 ± 0.10
Heart	3.02 ± 0.91	2.72 ± 0.45	2.81 ± 0.50
Lungs	2.98 ± 0.82	1.47 ± 0.27	1.57 ± 0.22
Liver	10.78 ± 5.74	14.57 ± 4.95	11.31 ± 4.28
Kidneys	29.91 ± 1.85	18.62 ± 1.20	15.97 ± 2.46
Stomach	2.79 ± 0.55	5.52 ± 1.46	4.82 ± 1.34
Intestine	2.02 ± 0.14	2.79 ± 1.35	3.52 ± 0.32
Pancreas	3.10 ± 0.27	4.07 ± 0.33	4.31 ± 0.12
Spleen	3.52 ± 0.66	4.80 ± 0.91	4.47 ± 0.86
Muscle	0.61 ± 0.14	0.62 ± 0.20	0.56 ± 0.16

Supplemental Table 4 Biodistribution results of ^{111}In-Isp-PrPOZ in nude mice after intravenous injection of probe. Results are expressed as means (%ID/g) ± SD (n=3).

	1 h	6 h	24 h
Blood	0.25±0.05	0.06±0.01	0.03±0.02
Heart	0.40±0.05	0.35±0.01	0.39±0.06
Lungs	20.50±3.45	20.03±3.15	14.05±2.84
Liver	28.20±1.81	25.72±2.34	32.02±3.26
Kidneys	17.49±1.60	17.41±1.74	12.75±1.34
Stomach	1.42±0.55	0.74±0.30	0.96±0.18
Intestine	0.85±0.06	0.67±0.21	0.67±0.34
Pancreas	0.91±0.11	1.07±0.04	1.00±0.10
Spleen	19.87±3.70	16.45±1.88	20.75±0.67
Muscle	0.21±0.08	0.21±0.07	0.20±0.16

Supplemental Table 5 Biodistribution results for ^{111}In-PrPOZ in nude mice after intravenous injection of probe. Results are expressed as means (%ID/g) ± SD (n=3).

	1 h	6 h	24 h
Blood	0.22±0.04	0.17±0.05	0.05±0.02
Heart	0.41±0.06	0.31±0.19	0.42±0.06
Lungs	3.57±0.46	3.59±1.10	3.04±0.49
Liver	56.29±4.78	58.27±6.39	52.24±8.60
Kidneys	7.02±0.60	7.33±1.27	5.10±0.49
Stomach	0.68±0.08	0.64±0.20	0.34±0.07
Intestine	0.56±0.04	0.54±0.02	0.52±0.02
Pancreas	1.09±0.10	1.32±0.14	0.99±0.04
Spleen	22.91±4.45	23.50±12.35	25.88±12.64
Muscle	0.16±0.09	0.35±0.24	0.15±0.06

Supplemental Video 1: Real-time observation of self-aggregation of POZ derivatives when heated at around body temperature (36.5°C).

Images were obtained after drop the solution of EtPOZ and Isp-PrPOZ (2000 μM) on a plate heated at 36.5°C. Rapid aggregation of Isp-PrPOZ with Tt under body temperature was shown.