Supporting Information:

Toxicology and Safety Research of Poly (N-Isopropylacrylamide) Based Thermosensitive Nanogels

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Author Contributions

Han Li, Haixia Sun, Xiangliang Yang, Shaobin Wang and Yongsheng Zhao conceived the project and designed the experiments. Han Li, Haixia Sun, Yuyu Liu, BingHan Yuan, Jingyuan Hu, Yunzhe Jiang, Qingkai Li and Suilan Cao carried out the experiments and analyzed the data. Han Li and Haixia Sun wrote the manuscript. Han Li and Haixia Sun contributed equally to this work. All authors have given approval to the final version of the manuscript.

Materials

N-isopropylacrylamide (NIPAM, Tokyo Chemical Industry Co., Ltd.), Butyl Methacrylate (BMA, Tokyo Chemical Industry Co., Ltd.), N, N'-methylene bisacrylamide (MBA, Shanghai Macklin Biochemical Co., Ltd.) are recrystallization with n-hexane and methanol, respectively. Sodium dodecyl sulfate (SDS, Sinopharm Chemical Reagent Co., Ltd.), Potassium persulfate (KPS, Shanghai Macklin Biochemical Co., Ltd.), Rhodamine B (RhB, Sigma-Aldrich), 3-Bromo-1-propene（3-BP, Macklin Inc.）, and Nile red (Sigma-Aldrich) are used directly without further purification. Lipiodol (iodized oil) is purchased from Guerbet France, Iohexol is purchased from Zhejiang Hichipharm Co., Ltd. Deionized water (Milli-Q ultrapure water 18.2 MΩ) was used in the whole experiment.

Animals

C57BL/6 mice (19–21 g of body weight, both sexes) were provided by ZhuHai Bestest Biotechnology Co., Ltd. SD Rats (360–420 g of body weight, both sexes) and Bama Miniature pigs (37-47 kg of body weight, both sexes) were provided by KINGROCK Co., Ltd. All procedures of animal experiments were in accordance with China National Animal Law on the use of laboratory animals, approved by the Institutional Animal Care and Use Committee at Peking University Shenzhen Hospital.

The synthesis of PIB nanogels

According to a modified method,[1] p(NIPAM-BMA) (PIB) nanogels was synthesized using feed emulsion polymerization. Briefly, NIPAM (2.263 g, 20 mmol), BMA (0.168 ml, 1 mmol), SDS (0.032 g, 0.1 mmol), MBA (0.032 g, 0.2 mmol) was dissolved with 160 ml deionized water in a 250 ml flask quipped with a reflux condenser, a thermometer, a N_2 bubbling inlet and outlet. The solution was heated to 70°C, while stirring for 30 minutes under N_2 atmosphere, the polymerization was initiated by adding KPS solution (10 ml, 9.5 mg/mL). After blue opalescence appeared (ca. 30 minutes), the reaction was allowed to proceed at 70°C for 4.0 h. The resultant PIB nanogels was dialyzed (the cutoff molecular weight is 14 kDa) against deionized water for three days, lyophilized and stored in a desiccator for further use.

The synthesis of RhB-monomer

According to the chemical reaction formula (Fig s1), 3-BP, anhydrous $NaCO_3$ and RhB were mixed according to the molar ratio 10:1, and stirring at a nitrogen atmosphere at 71°C for 24 hours; the mixture was dissolved with anhydrous ethanol, the $NaCO_3$ was removed by centrifugal; the excess 3-BP was removed by rotary evaporation to obtain RhB-monomer. The fluorescence quantitative standard curve of RhB-monomer was also determined (Fig s2).

The synthesis of PIB-RhB nanogels

The material preparation process is the same as the polymerization process of PIB nanogels.

The difference is that 5 mg RhB-monomer is added to the reaction system to participate in polymerization. And the fluorescence quantitative standard curve of PIB-RhB nanogels was determined (Fig s3)

The characterization method of PIB and PIB-RhB nanogels

The size and zeta potential of nanogels were measured by dynamic light scattering (DLS, Zetasizer Nano-ZS 90, Malvern Instrument Limited, UK) using a He-Ne laser at the wavelength of 633 nm (scattering angle was 90°). The morphology of nanogels was observed by transmission electron microscope under 200 kV voltage (TEM, Tecnai G2 20 FEI Corp., Netherlands). The samples were diluted to 1 mg/ml using ultrapure water. After ultrasonic oscillation for 10 minutes, several drops of the dilution and phosphotungstic acid solution (0.1 wt.%) were added onto a TEM copper grid (carbon film coated, 300-mesh), and dried at 25°C for TEM characterization.

Temperature-sensitive sol-gel transition of nanogels was investigated using rheological measurements. The rheological properties, including viscosity and viscoelasticity, were measured using a rheometer (MCR302, Anton Paar, Austria) with a plate (CP60-1, Φ = 60 mm), and the gap was set at 0.5 mm. The temperature sensitive viscoelasticity of nanogels was investigated from 25 to 45°C at 0.5 Pa of shear stress, 0.5°C/minutes of heating rate and 1.0 Hz of frequency. The viscosity of nanogels was measured at 25°C under a shear rate of 300 s^{-1}.

PIB-RhB was converted into gel at 37°C and 50°C, respectively. Subsequently, the supernatant was collected daily for one week to measure the concentration of free nanogels present. This allowed for the investigation of the leakage rate of free nanogels following the phase transition.

***In-vitro* elution experiment**

In order to investigate whether the PIB thermosensitive nanogels will dissociate or escape after embolization, we have built a set of *in-vitro* elution device.[2] In brief, 500 μl of PIB-RhB nanogels (3 Sets of parallel samples) were introduced into the bottom well of the releasing cell (Φ × h = 2 cm × 4 cm), and gellated at 37°C. The fresh medium (phosphate-buffered saline solution) flowed slowly through the well with a peristaltic pump at a flowing rate of 30 ml/h and 150 ml/h, and the eluent was eluted into a collecting cell. The whole apparatus was put in the dark at 37°C. The collecting cell was replaced at various times from 1 hour to 40 hours. The eluent was measured by a fluorescence spectrophotometer, EX 546 nm, EM 585 nm (RF-6000, Shimadzu. Co. Ltd., Japan).

***In-vivo* pharmacokinetic studies**

Pharmacokinetic studies were performed using 3 SD rats (360–420 g body weight), animals received a single injection of PIB-RhB dispersion systemically into the tail vein. The injection dose was 20 mg PIB-RhB/kg, and the sample concentration was 2 mg PIB-RhB/ml. The planned blood collection points are as follows: before administration, 0 min, 10 min, 30 min, 1 h, 24 h after administration. About 0.5 ml blood was collected via the jugular vein for each point, heparin was used for anticoagulation, and separate the plasma by centrifugation (3000 rpm/min, 4°C, 10 min)

within 1 hour after collection. After separation, the PIB-RhB concentration of plasmas was detected by using a spectrofluorometer, EX 546 nm, EM 585 nm (RF-6000, Shimadzu. Co. Ltd., Japan). The fluorescence intensities were brought into the standard curve (Fig s4) drawn by using rat's serum as the dispersion of PIB-RhB nanogels to calculate the plasma concentration.

In-vivo tissue distribution experiment

Tissue Distribution studies were performed using 20 C57BL/6 mice (19–21 g body weight), animals received a single injection of PIB-RhB dispersion systemically into the tail vein. The injection dose was 12.5 mg PIB-RhB/kg, and the sample concentration was 2.5 mg PIB-RhB/ml. The mice were weighed and numbered before administration, and were divided into five groups (blank control, immediately after administration, 5 min, 1 h, 24 h after administration), 4 mice in each group, half male and half female. After fixation, the mice were injected through the tail vein according to the dose of 12.5 mg PIB-RhB/kg. After being killed at different time points, the brain, heart, liver, spleen, lung, kidney, body fat, gonad, gastrointestinal tract, and skeletal muscle (Fig s5) were taken for photos in the small animal imaging equipment (Ilumina 3, Perkin Elmer, US). The excitation light was 546 nm and the emission light was 585 nm.

In-vivo embolization experiment

The renal artery embolization of PIB nanogels (mixed with iohexol, the concentration of iodine is greater than 200 mg I/ml) was performed on the right kidney normal Bama miniature pigs, the pigs were fixed in the supine position. Their groin skins were dissected, and the femoral arteries were separated using ophthalmic forceps. After the ligation of the distal arteries, a 4 F coaxial microcatheter (Terumo, Tokyo, Japan) was introduced into the proximal arteries using an 18 G puncture needle. The angiography of the pig's renal arteries was firstly performed by injecting a contrast agent (Omnipaque, 300 mg I/ml, 0.5 ml/s). Afterwards, embolic agents were slowly injected into right kidneys. Pathological examination, digital subtraction angiography (DSA, Siemens BICOR T.O.P., Germany) were used for the evaluation of renal embolization at 7 days, 28 days (medium-term embolization), and 90 days (long-term embolization) after operation.

Bioelectricity photographs of different tissue samples after embolization were operated according to the following steps: After the tissue sample is fixed, slice it with a Leica ultramicrotome (model: UC-7) with a thickness of 50-70 nm; stain with 2% uranyl acetate for 30 minutes, rinse the slice with double distilled water, the flushing volume is about 300 mL, absorb water with filter paper and bake it under the oven lamp for 15 minutes to dry the slices thoroughly; 3% lead citrate staining (in 100 mL freshly boiled double distilled water, add 0.04 g of lead citrate, then add 0.1 mL 10 mol/L NaOH, shake until the precipitate is dissolved and the solution is clear), stain for 15 minutes, rinse the section with double distilled water, the flushing volume is about 300 mL, absorb water with filter paper and bake it under the oven lamp for 15 minutes to dry the section thoroughly. Observation by Electron Transmission Electron Microscope (Japan Electron Optics Laboratory Co., Ltd., JEM-1400 PLUS), voltage: 100 KV.

Biological evaluation

In accordance with the requirements of FDA for medical devices and with reference to ISO 10993,[3] we carried out the biological evaluation of PIB-2240, including blood compatibility, *in-vitro* cytotoxicity, skin sensitization, intradermal reaction, acute toxicity, pyrogen, genetic toxicity, subchronic toxicity and implantation experiments.

Haemocompatibility:

Thrombosis

Experimental sample groups (PIB nanogels), positive control groups (glass sheet, 0.5 cm × 0.5 cm× 0.15 cm), and blank control groups (blood was not in contact with any material) were established. Each group of materials was exposed to partially anticoagulated rabbit blood for 15 minutes. Sodium citrate was then added to each tube to achieve complete anticoagulation. Plasma was separated and the remaining fibrinogen level in the plasma was measured. Statistical analysis was performed to compare the experimental sample group, positive control group, and blank control group, in order to assess the risk of thrombus formation in the experimental sample *in-vitro*.

Compared with the blank control group, the average mass concentration of fibrinogen in the positive control group was significantly reduced ($P<0.05$), indicating that the experiment was effective; The average mass concentration of fibrinogen in the experimental sample group was significantly higher than that in the positive control group ($P<0.05$); There was no significant difference in the average mass concentration of fibrinogen between the experimental sample group and the blank control group ($P>0.05$).

Coagulation

To evaluate the effect of the test sample on the coagulation time of platelet deficient plasma, we set up two control groups: a positive control group using natural latex and a blank control group where blood was not in contact with the material. Each group of materials was then exposed to fresh anticoagulant rabbit plasma for a duration of 15 minutes. The coagulation time of each tube was measured using a coagulation meter. Statistical analysis was performed to compare the coagulation time between the test sample group, positive control group, and blank control group.

The coagulation time of the blank group was 103.6 seconds: the average coagulation time of the experimental group was 103.2 seconds, accounting for 99.61% of the blank control; The coagulation time of the positive control group was 66.3 seconds, accounting for 64.00% of the blank control group. Statistical analysis showed that compared with the blank control group, the positive control group had a significant decrease in coagulation time ($P<0.05$), indicating that the experiment was effective; The coagulation time of the experimental sample group was significantly higher than that of the positive group ($P<0.05$); There was no significant difference in coagulation time between the experimental sample group and the blank control group ($P>0.05$).

Platelet/white blood cell/red blood cell count

We set up four groups: an experimental sample group, a positive control group (natural latex), a negative control group (high-density polyethylene), and a blank control group (blood was not in contact with any material). Each group of materials is exposed to fresh anticoagulant rabbit blood in proportion. The test tubes containing the samples are then incubated in a 37 ± 1°C water bath for 60 minutes with 60 rpm oscillation. After incubation, the platelet, white blood cell, and red blood cell count of each tube are measured using a blood cell analyzer. By comparing the differences between the test group and the control groups, we can analyze the impact of the materials on the platelet, white blood cell, and red blood cell count.

The average platelet count in the blank control group was 512 × 10^9/L; The average platelet count in the positive control group was 180 × 10^9/L, accounting for 21.09% of the blank control group. The average platelet count in the negative control group was 488 × 10^9/L, accounting for 95.31% of the blank control; The average platelet count in the experimental group was 459 × 10^9/L, accounting for 89.65% of the blank control. Statistical analysis showed that there was no significant difference in platelet count between the experimental sample and the negative control group ($P>0.05$).

The average white blood cell count in the blank control group was 7.42× 10^9/L; The average white blood cell count in the positive control group was 3.37× 10^9/L, accounting for 45.42% of the blank control group. The average white blood cell count in the negative control group was 7.49 × 10^9/L, accounting for 100.04% of the blank control; The average white blood cell count in the experimental group was 7.10 × 10^9/L, accounting for 95.69% of the blank control. Statistical analysis showed that there was no significant difference in white blood cell count between the experimental sample and the negative control group ($P>0.05$).

The average red blood cell count in the blank control group was 4.24× 10^{12}/L; The average red blood cell count in the positive control group was 2.04× 10^{12}/L, accounting for 48.11% of the blank control group. The average red blood cell count in the negative control group was 4.24 × 10^{12}/L, accounting for 100.00% of the blank control; The average red blood cell count in the experimental group was 4.05 × 10^{12}/L, accounting for 95.52% of the blank control. Statistical analysis showed that there was no significant difference in red blood cell count between the experimental sample and the negative control group ($P>0.05$).

Hemolysis

We set up three groups: an experimental sample group (PIB nanogels), a positive control group (pure water), and a negative control group (physiological saline). Each group of materials is exposed to fresh anticoagulant rabbit whole blood in proportion. The test tubes containing the samples are then incubated in a 37 ± 1 °C water bath for 60 minutes. After centrifuging at 800 g for 5 minutes, the absorbance of the supernatant was measured at 545 nm. The average absorbance of the experimental sample was 0.012, the average absorbance of the positive control group was 0.855, and the average absorbance of the negative control group was 0.014. The hemolysis rate was calculated to be 0.24%, and the hemolysis rate of the experimental sample was found to be less than

5%.

***In-vitro* cytotoxicity (MTT method):**

PIB nanogels were preheated at 37°C and allowed to solidify. Then, the extraction medium (containing serum) was preheated to 37°C and added to the PIB nanogels at a ratio of 0.2 g/mL. The samples were shaken and extracted at 37°C and 60 rpm for 24 hours to obtain a 100% extract, dilute to 50%, 25%, and 12.5% with the extraction medium. The negative control and positive control groups were extracted using the same method.

The fibroblasts of mice were exposed to experimental samples at different concentrations (100%, 50%, 25%, 12.5% extract of PIB nanogels), a positive control (polyurethane membrane extract), and a negative control (high-density polyethylene membrane extract) for a duration of 24 hours. The absorbance value of each group was measured using a microplate reader, and the cell survival rate was calculated for each group.

Under the conditions of this experiment, the cell survival rates of the test sample group with 100%, 50%, 25%, and 12.5% extract were 84.76%, 78.56%, 83.71%, and 83.05%, respectively. The cell survival rates of the positive control group and the negative control group were 8.91% and 81.13%, respectively. The 100% extract of the test sample has no cytotoxicity.

Sensitization:

The experiment used the extract prepared by sodium chloride injection as the polar test solution, the extract prepared by cottonseed oil as the non-polar test solution (the extraction method is identical to cytotoxicity testing), sodium chloride injection and cottonseed oil as the negative control solution, 0.5% concentration of 1-chloro-2,4-dinitrobenzene solution as the positive control solution. Guinea pigs were injected with the test solution or control solution into the skin for intradermal induction, and closed patches were used for local induction to generate sensitization. The control group animals underwent the same operation using blank extraction medium. After induction, the corresponding sample extract and control solution were applied to stimulate the experimental and control group animals. The skin reactions at the stimulation sites were graded at (24 ± 2) hours and (48+2) hours after removing the application to determine the potential risk of sample sensitization.

It was observed that both polar and non-polar extracts of PIB nanogels did not induce any skin sensitization reactions in guinea pigs.

Intradermal reaction:

The experiment used the extract prepared by sodium chloride injection as the polar test solution, the extract prepared by cottonseed oil as the non-polar test solution (the extraction method is identical to cytotoxicity testing). 0.2 mL of polar extract was injected into each rabbit's back spine at 5 points on the left side. The corresponding blank control was injected into 5 points on the other side of each rabbit. Similarly, non-polar extract and blank control were injected into the posterior

spine of each rabbit using the same method. Observe the erythema and edema at the injection site of the animal at 24 ± 2 hours, 48 ± 2 hours, and 72 ± 2 hours. The results showed that the intradermal reaction of the experimental sample and the negative control group is the same.

Acute toxicity:

The experiment used the extract prepared by sodium chloride injection as the polar test solution, the extract prepared by cottonseed oil as the non-polar test solution (the extraction method is identical to cytotoxicity testing), sodium chloride injection and cottonseed oil as the negative control solution. Using a dose of 50 mL/kg, we administered a single intravenous injection of polar extract and sodium chloride injection to different mice. Additionally, we administered a single intraperitoneal injection of non-polar extract and cottonseed oil to different mice. We observed the acute systemic toxicity of the animals at 4 hours, 24 hours, 48 hours, and 72 hours after the administration. Furthermore, we weighed the animals before and 24 hours, 48 hours, and 72 hours after the injection. In cases where clinical symptoms appeared, we conducted a gross pathological evaluation. If any abnormalities were found during the autopsy, we conducted further histological examination of the animal organs. Based on the conditions of this experiment, neither the sodium chloride injection nor the cottonseed oil extract of the test substance resulted in acute systemic toxicity in animals. The test substance did not exhibit acute systemic toxicity.

Genetic toxicity:

Bacterial reverse mutation assay

The PIB nanogels were extracted using serum-free and serum-containing media (the extraction method is identical to cytotoxicity testing). The blank extraction medium control solution was also prepared using the same method. The sample extract, negative control solution, and positive control solution were added to histidine deficient bacteria. These bacteria were then cultured in an activation system (with S9 mixture) and a non-activation system (without S9 mixture). The bacterial colonies were observed and the number of revertant colonies in each dish was counted. The number of bacterial revertant colonies for TA97a, TA98, TA100, TA102, and TA1535 in each group was calculated. The relationship between the number of bacterial revertant colonies in the negative control group and the positive control group, as well as the negative control group and the experimental group, were compared for result analysis. The bacterial reverse mutation assays of both extracts were negative.

***In-vitro* mouse lymphoma thymidine kinase assay:**

The PIB nanogels were extracted using physiological saline, serum-free and serum-containing media (the extraction method is identical to cytotoxicity testing). The blank extraction medium control solution was also prepared using the same method. The sample extract, negative control solution, and positive control solution were added to well-growing cells. These cells were then

cultured in an activation system (with S9 mixture) and a non-activation system (without S9 mixture). Each group was exposed to an activation system for 4 hours, while a non-activation system was exposed for 24 hours. Cells were inoculated with PE_0 plates 0 days after the end of exposure. Cells cultured for another 2 days after contact were inoculated with PE_2 and TFT plates. After 14 days of cultivation on all plates, observe the number of wells without colony growth on each plate under a microscope. Calculate the efficiency, relative survival rate, and TFT resistance mutation frequency of PE_0 and PE_2 plates. The total mutation frequency of each extract group of PIB nanogels is less than 126×10^{-6}. The gene mutation test for the three extracts was negative.

***In-vitro* mammalian chromosomal aberration:**

The PIB nanogels were extracted using serum-free and serum-containing media (the extraction method is identical to cytotoxicity testing). The blank extraction medium control solution was also prepared using the same method. The sample extract, negative control solution, and positive control solution were added to CFL cells. These cells were then cultured in an activation system (with S9 mixture) and a non-activation system (without S9 mixture). After harvesting cells, cell counting, routine smearing, staining, and observation of chromosomal aberrations were performed on 300 well-dispersed metaphase cells from each experimental group under a microscope. The number and type of aberrations were recorded for each experimental group, and statistical analysis was performed using the chi-square test. The results of the chromosomal aberration test for the two extracts of PIB nanogels were negative.

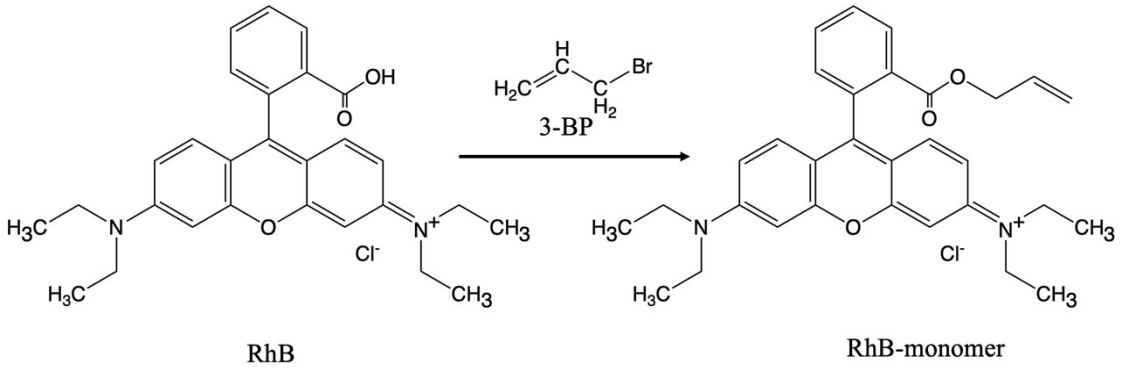
Material mediated pyrogenicity:

The PIB nanogels were extracted using sodium chloride injection (the extraction method is identical to cytotoxicity testing). Rabbits with stable body temperature were selected, and the experimental solution was injected into the ear vein at a dose of 10 mL/kg. Body temperature was measured once every 30 minutes for a total of 6 times after injection. The highest body temperature recorded was subtracted from the normal body temperature to determine the elevated temperature of the rabbit. The pyrogen test results were determined based on the temperature rise of the rabbit. The temperature rise of all three rabbits was below 0.6°C, and the total temperature rise was below 1.3°C. Therefore, the pyrogen test of the test sample extraction solution meets the requirements.

Implantation:

0.5 mL of PIB nanogels were implanted into the hip muscle of rats on one side, while the other side of the muscle was implanted with stainless steel wire tie as a negative control. After 1, 4, 12, and 26 weeks of observation, samples of the implanted tissue were taken separately. A histopathological examination was conducted after a general observation to assess the degree of inflammatory response and the formation of fibrous cystic cavities around the samples and the control. The results indicate that at 1 week and 4 weeks, the PIB nanogels showed no irritation, while at 12 weeks and 26 weeks, the PIB nanogels produced slight irritation.

Supplemental figures and tables



RhB RhB-monomer

Fig. s1 Chemical reaction formula of rhodamine monomer (RhB-monomer).

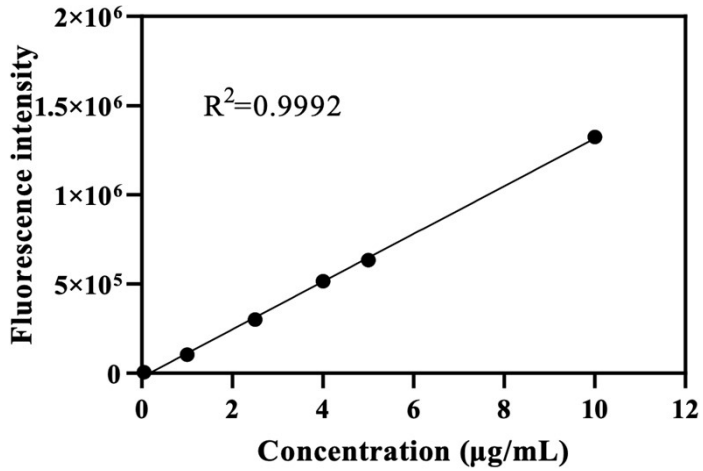


Fig. s2 The fluorescence quantitative standard curve of RhB-monomer.

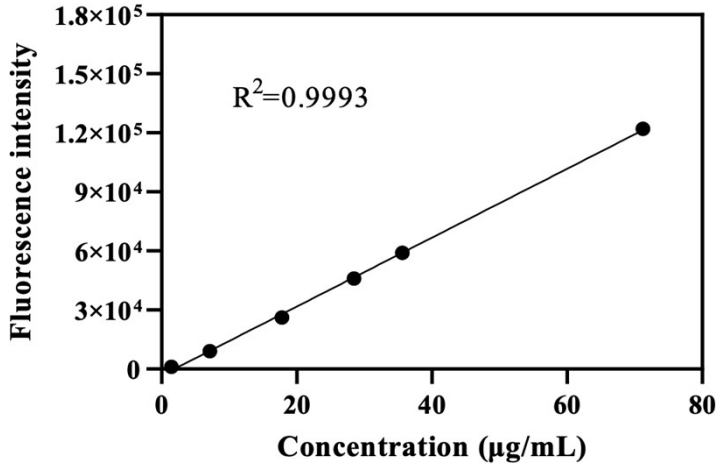


Fig. s3 The fluorescence quantitative standard curve of PIB-RhB, the linearity is good in the range of 1.4 ～71.2 μg/ml.

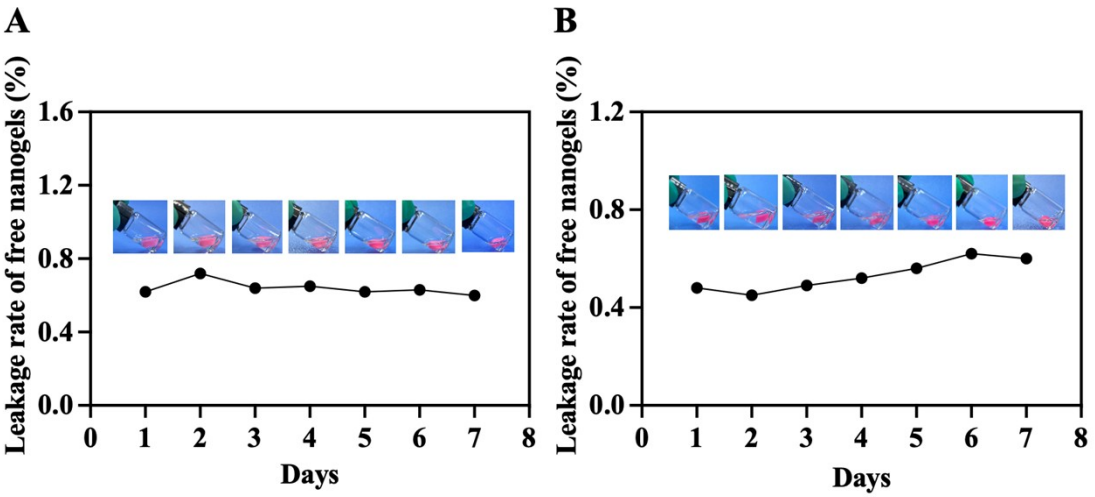


Fig. s4 The study on the leakage rate of free nanogels after phase transition at 37℃ (A) and 50℃ (B).

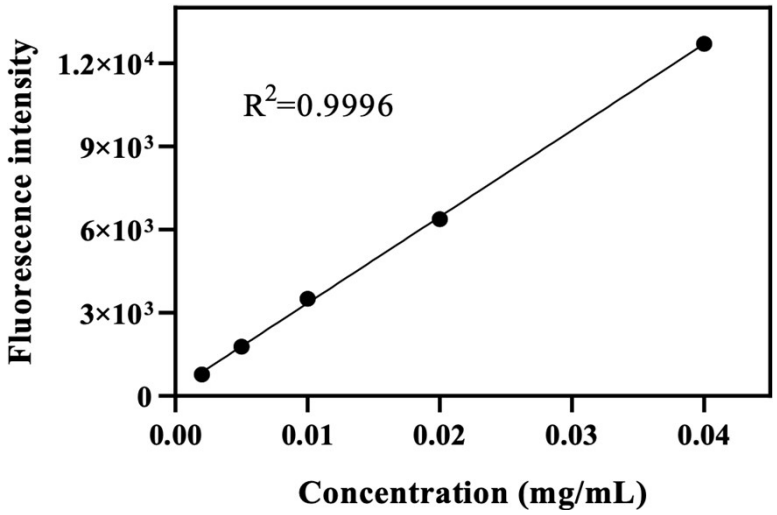


Fig. s5 The fluorescence quantitative standard curve of PIB-RhB in rat's serum as the dispersion.

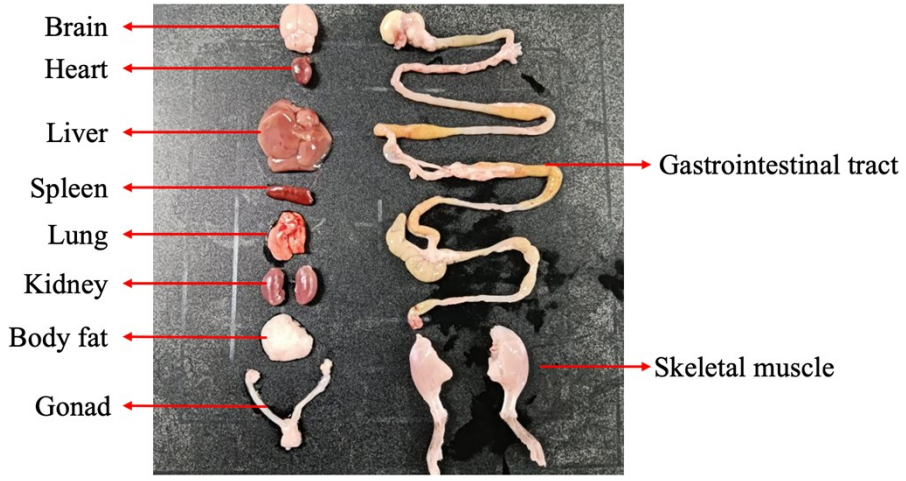


Fig. s6 The brain, heart, liver, spleen, lung, kidney, body fat, gonad, gastrointestinal tract, and skeletal muscle of mice.

Table s1 The average cumulative release amount of PIB-RhB at different flowing rates

Elution time	Flowing rate=30 mL/h				Flowing rate=150 mL/h			
	Fluorescence intensity			Average cumulative release amount	Fluorescence intensity			Average cumulative release amount
	channel 1	channel 2	channel 3		channel 1	channel 2	channel 3	
blank	99.0	111.2	134.5	/	88.6	89.9	91.3	/
1 h	73.4	62.7	64.2	0%	145.2	166.9	168.9	1.6%
3 h	116.7	112.6	117.1	0%	86.5	100.8	113.5	1.6%
6 h	132.3	132.2	126.7	0%	74.8	77.3	74.8	1.6%
18 h	173.7	167.7	222.6	0%	25.4	26.6	28.9	1.6%
30 h	119.6	120.0	112.9	0%	39.9	37.6	42.1	1.6%
40 h	56.1	27.8	30.9	0%	38.9	38.2	49.1	1.6%

Supplemental references

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