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Supplementary Materials for

Clinical translation of an ultrasmall inorganic optical-PET imaging nanoparticle probe

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Materials and Methods

Synthesis and characterization of cRGDY–PEG–C dots

The synthesis and characterization of poly(ethylene glycol) (PEG) and cRGDY surface-functionalized fluorescent core-shell silica nanoparticles (cRGDY–PEG–C dots) have been described previously, and the reader is referred to our earlier publication and references therein (*16*). In brief, particles were prepared by a modified Stöber-type silica condensation (*42–44*). C dots covalently sequester the organic dye molecule, Cy5 (emission maxima ~650 nm, 2 dye equivalents) in a core encapsulated by a silica shell to markedly enhance brightness and photostability (*20, 45*) over that observed with the native dye in aqueous solution. Bifunctional PEGs were derivatized with silanes for attachment to the silica surface and maleimides were used for peptide conjugation, respectively. cRGDY peptides (~6 ligands per particle (*16*)), containing the sequence cyclo-(Arg-Gly-Asp-D-Tyr-Cys) and bearing cysteine residues (Peptide International), were attached to surface-bound functionalized PEG chains via cysteine-maleimide linkages. Hydrodynamic radius, brightness, and concentrations of cRGDY–PEG–C dots, as against free Cy5 dye, were analyzed via a Zeiss LSM 510 Confocor 2 FCS using HeNe 633-nm excitation. These data were obtained in conjunction with absorption and emission spectral profiles for the encapsulated and native Cy5 dye using a Varian Cary 5000 spectrophotometer (Varian) and a fluorescence spectrofluorometer (Photon Technology International, Inc).

Radioiodination of cRGDY–PEG–C dots

Radiolabeling of cRGDY–PEG–C dots was performed by attaching radioiodine (^{124}I, ^{131}I) to the tyrosine residues (Y) of peptide moieties using the IODOGEN method (Pierce) (*20*). The radiolabeled product was eluted from PD-10 columns and assayed using a dose calibrator

(Capintec) and radioTLC. The specific activity, purity, and radiochemical yield of the ^{124}I-bound particle fractions were measured for each patient run.

Radioiodination of peptide ligands

The cRGDY free peptide, which contains the sequence cyclo-(Arg-Gly-Asp-D-Tyr-Cys), was radioiodinated with ^{124}I or ^{131}I, on the tyrosine residue using the IODOGEN method (Pierce) (*20*). The reaction mixture was transferred onto a PD-10 column and eluted with saline. Fractions were collected and radioassayed with a dose calibrator. Desired fractions were further analyzed by radioTLC, pooled and then concentrated.

Pharmacokinetics and metabolic analyses

For PK analyses, ROIs were drawn on PET imaging data (AW Workstation, GE Healthcare) to extract mean and maximum standard uptake values (SUVs) for all major normal organs and tissues, including brain, lung, left ventricle, liver, spleen, intestine, kidneys, bladder, muscle, breast, and tumor(s). For PK evaluation, SUVs were converted to %ID/g values (i.e., SUV =% ID/g tissue × patient body mass/100). Organ/tissue uptake data were supplemented by time-activity data from the blood and urine.

Venous blood and urine specimens were collected at approximately 30 min, 3 h, 24 h, 72 h, and 2 weeks after injection of ^{124}I-cRGDY–PEG–C dots. Following centrifugation of whole-blood specimens (4000 rpm, 10 min), plasma supernatant, along with urine specimens, were assayed in duplicate in a scintillation well counter (1480 Automatic Gamma Counter, Perkin Elmer, Shelton CT) calibrated for ^{124}I. RadioTLC analyses were additionally performed on biological specimens. RadioTLC analyses of the particle tracer, native peptide (cRGDY) labeled

with ^{131}I, and free iodine (^{131}I) served as standards to facilitate interpretation. For all biological samples and standards, a single drop of each radioactive fraction was placed at a starting point of 0.5 inch from the bottom cutoff of a pre-cut silica TLC plate (EMD, 5554-7, 3 inch long × 1 inch wide). The plates were developed with a mobile phase of acetic acid (1.6 mL) and methanol (0.4 ml), and read by a Bioscan AR-2000 radio-TLC imaging scanner. Activities (cpm) were converted to microCuries (μCi) and decay-corrected. Final values were expressed as %ID/g. Retention factor (Rf) values for the tracer were obtained and used for identification of the parent compound and possible metabolites.

The percentages of the total activity in the form of free iodide and intact particle, were derived from radioTLC results of plasma and urine, body weight–based total plasma volume (5600 ml), and PET-based estimates of total activity in the urinary bladder, respectively. Total activity in the urinary bladder was obtained by analyzing the PET-derived bladder-content activities (the SUV-based %ID/g) and bladder volumes (cm^3) as well as the measured relative peak areas (%) for particle and free iodide on thin-layer chromatograms.

PET image acquisition and processing

Low-dose whole-body spiral CT scans were obtained prior to the acquisition of each whole-body PET scan on a GE Discovery STE PET/CT scanner. Images were recorded at 4, 24, and 72 hours post-injection of the particle tracer. Positron emission data was reconstructed using the ordered subsets expectation maximization (OSEM) algorithm. Images were corrected for attenuation using the CT transmission data. All acquisitions were performed in 2D mode (septa-in) to minimize the contributions from prompt gamma emissions that accompany the positron annihilation in the decay scheme of ^{124}I. All PET image reconstructions were performed as per

the standard GE ordered subset expectation maximization (OSEM algorithm consisting of 2 iterations and 24 sub-sets with CT attenuation correction. No further image manipulation was performed.

Radiation dosimetry

The radiation dosimetry method used is an adaptation of that promulgated by the MIRD (Medical Internal Radionuclide Dosimetry) Committee, accounting for the physical properties of the administered radionuclides (^{124}I) as well as the biological properties (PK and biodistribution) of the radiopharmaceutical in individual patients. Serial whole-body PET scans enabled derivation of normal-organ absorbed dose (mGy and mGy/mBq) estimates using ROI-derived time-activity data. PET scans were acquired with all parameters identical, including the scan time. Using the patient's total-body mass (in kg) and the 70-kg Standard Man organ masses, the total-body and organ ROI data [mean standard uptake values (SUVs)] were converted to activities (fraction of the injected dose). The foregoing image-derived time-activity data were fit to exponential functions using a least-squares fitting algorithm and the resulting time-activity functions analytically integrated, incorporating the effect of physical decay of ^{124}I to yield the cumulated activities in μCi-hr/μCi in the organs and total body. Cumulated activities were used to calculate ^{124}I-labeled particle mean absorbed doses to the organs (mGy/MBq) and effective dose (mSv/MBq) for the 70-kg Standard Man anatomic model by employing the OLINDA EXM program (Vanderbilt University) (*46*).

SUPPLEMENTARY FIGURES

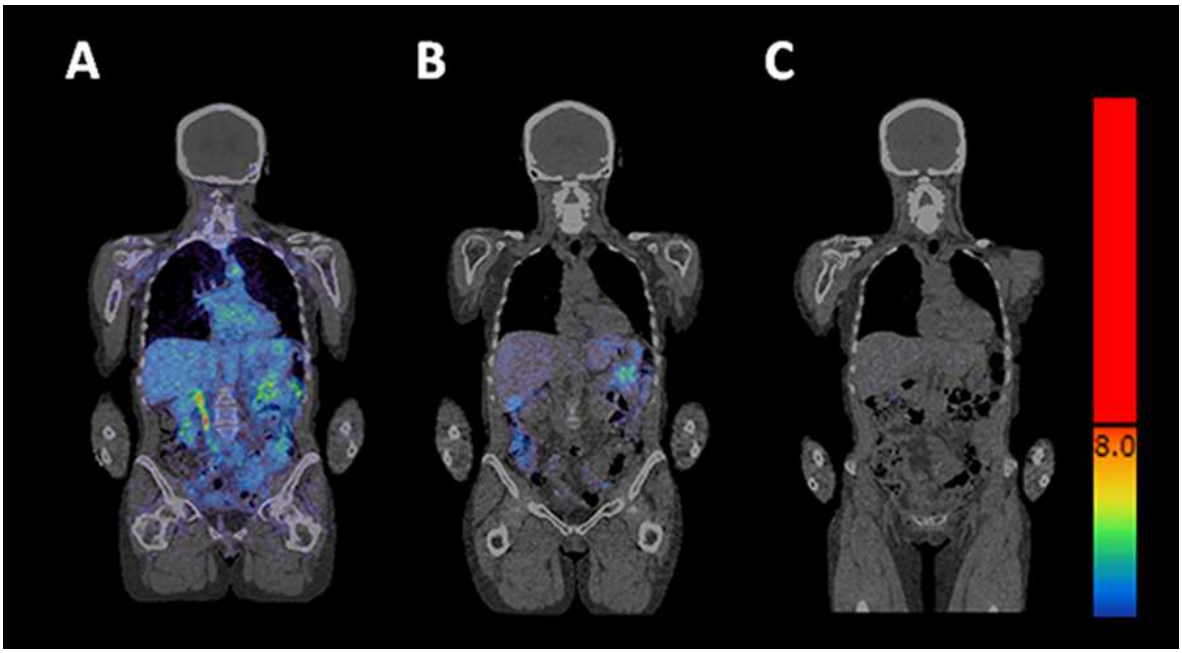

Fig. S1. Whole-body PET-CT imaging of the biodistributions of ^{124}I-cRGDY–PEG–C dots with time. (**A** to **C**) PET-CT fusion images for Patient #5, displayed as SUV values, acquired at 2 (A), 24 (B), and 72 (C) hours after i.v. injection of ^{124}I-cRGDY–PEG–C dots.

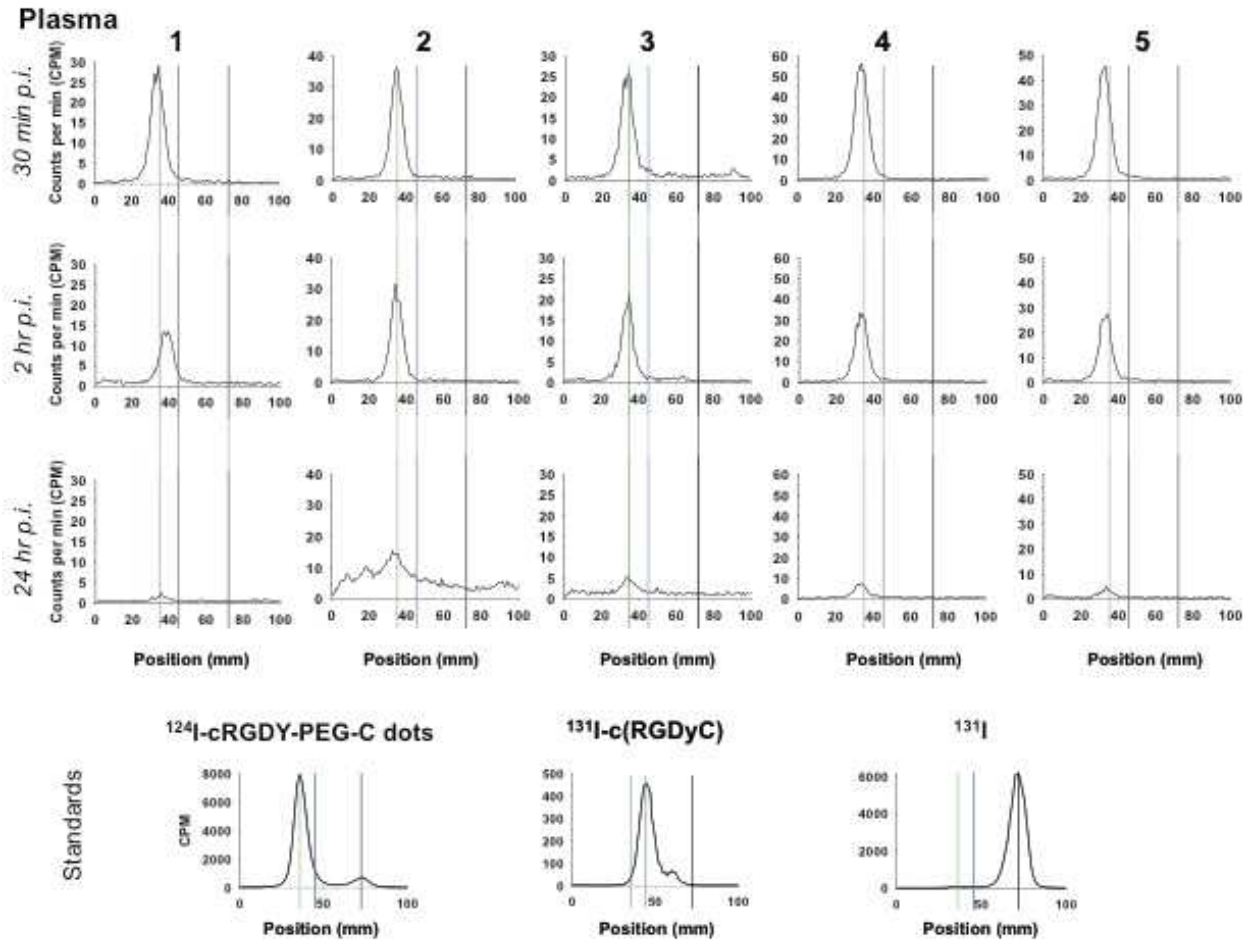

Fig. S2. RadioTLC chromatograms of plasma specimens and standards. RadioTLC (4:1 acetic acid:methanol as mobile phase) of plasma specimens (decay-corrected counts per minute, cpm, relative to collection time) for individual patients (n = 5). Chromatograms of specimens show a single peak near the origin at 0.5, 2, and 24 hours post-injection (p.i.). Vertical lines discriminate standard peaks corresponding to the particle tracer (long dashes; R_f = 0.04), ^{131}I-cRGDY (short dashes; R_f = 0.2), and ^{131}I (dotted; R_f = 0.7).

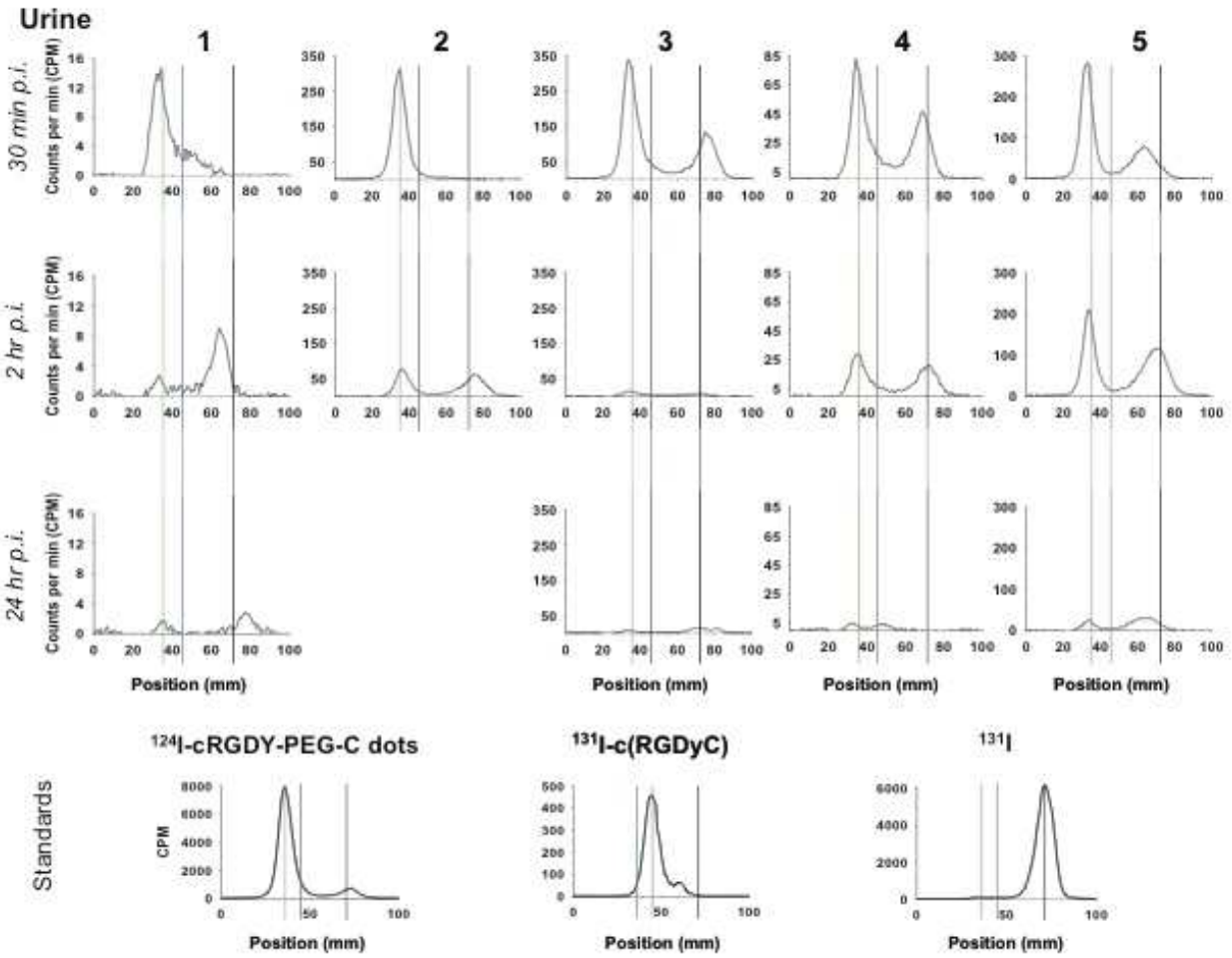

Fig. S3. RadioTLC chromatograms of urinary specimens and standards. RadioTLC (4:1 acetic acid:methanol as mobile phase) of urine specimens (background- and decay-corrected counts per minute, cpm, relative to collection time) for individual patients (n = 5). Chromatograms of specimens reveal two peaks at 0.5, 2, and 24 hours post-injection (p.i.). Vertical lines discriminate standard peaks corresponding to the particle tracer (long dashes; R_f = 0.04), ^{131}I-cRGDY (short dashes; R_f = 0.2), and ^{131}I (dotted; R_f = 0.7).

Table S1. Photophysical characterization of cRGDY–PEG–C dots by fluorescence correlation spectroscopy. The hydrodynamic size, relative brightness, and corresponding concentration of cRGDY–PEG–C dots, along with free Cy5 dye, were measured using fluorescence correlation spectroscopy (FCS). Data are means ± SD (n = 15 replicates).

	Hydrodynamic radius (nm)	**Brightness/particle (kHz)**	**Concentration (μM)**
Free Cy5 dye	0.83 ± 0.01	2.5 ± 0.04	0.025
cRGDY–PEG–C dots	2.97 ± 0.05	~6.0 ± 0.09	9.7

Table S2. Post-release product criteria for cRGDY–PEG–C dots. Criteria, as specified by the Investigational Products and Radiochemistry and Molecular Imaging Probes Core Facilities, were approved by the FDA (IND #110375). As appropriate for first-in-human trial of this size, and according to current good manufacturing practices (cGMP) for a phase I clinical trial, the criteria specified are proposed acceptance criteria supported by analytical data from validation studies and clinical trial material. TLC, thin layer chromatography.

Characteristic	**Test**	**Specification**
Physicochemical	pH	6.0–8.0
Purity	Radiochemical purity (TLC)	No less than 90%
Purity	Test for free iodine (TLC)	No greater than 10%
Radionuclide identity	Half-life test (gamma spectrum analyzer, on incoming ^{124}I)	Conforms to expected spectrum
Specific activity	TLC, absorbance, and dose calibrator	27.8–57.4 GBq/μmol

Table S3. Stability measurements of cRGDY–PEG–C dot batches (*n* = 2) by fluorescence correlation spectroscopy. To assess long-term particle stability, FCS analyses of two particle batches in water were conducted at approximately 1 month and 1–2 years following particle synthesis. Hydrodynamic size and the number of dye equivalents (equiv) per particle, a measure of the relative brightness, were monitored. The results of these batches were used to assess the expected shelf life of these particles for clinical trial studies. Data are means ± SD (*n* = 15 replicates).

	Time post-synthesis (~1 month)		**Time post-synthesis (~1–2 yrs)**	
Batch #	Radius (nm)	Dye equiv/particle	Radius (nm)	Dye equiv/particle
1	3.40 ± 0.07	2.91	3.36 ± 0.06 (~2 yrs)	2.50 (~2 yrs)
2	3.21 ± 0.05	2.24	3.36 ± 0.07 (~1 yr)	2.32 (~1 yr)

Table S4. Individual patient PK results for intravenously injected ^{124}I-cRGDY–PEG–C dots.

	%ID/g(×10^{-3})														
Patient ID	**#1**			**#2**			**#3**			**#4**			**#5**		
Time post-injection (h)	3	24	72	3	24	72	3	24	72	3	24	72	3	24	72
Brain	0.44	0.3	0.06	0.6	0.34	0.15	0.71	0.29	0.05	1.16	0.43	0.18	0.49	0.14	0.07
Parotid	4.14	3.87	0.14	6.15	4.76	5.06	2.23	1.14	0.03	4.29	3.72	1.78	17	4.68	1.16
Heart	5.26	0.94	0.2	6.1	1.91	0.49	4.54	1.06	0.13	5.51	2.22	0.47	7.58	1.18	0.23
Lung	2.5	0.46	0.07	1.94	0.65	0.25	2.1	0.33	0.06	1.45	0.57	0.18	1.75	0.53	0.79
Thyroid	0	0	0	20.28	42.79	25.28	7.64	2.99	2.86	3.08	3.25	3.99	3.44	0.75	0.11
Liver	3.59	1.06	0.94	5.31	2.24	1.28	3.81	1.68	0.66	5	3.55	1.34	7.33	2.23	1.95
Spleen	3.21	1.26	0.2	3.82	1.94	1.06	3.19	1.06	0.43	3.22	1.65	0.72	4.6	1.84	2.86
GI	8.36	4.14	0.11	16.18	2.94	2.15	3.05	0.89	0.3	1.16	1.96	0.97	3.51	0.63	0.23
Kidney	3.14	2.39	0.41	7.46	4.29	3.09	3.96	3.21	1.56	5.71	5.54	3.74	33.65	5.4	3.11
Spine	1.8	1.03	0.17	1.82	0.81	0.32	2.78	0.56	0.11	2.32	1.73	0.41	1.42	0.56	0.37
Bladder	14.54	12.9	0.66	42.38	29.82	4.01	17.7	31.49	0.88	21.88	9.16	0.67	82.46	9.7	2.26
Muscle	1.43	0.43	0.07	1.71	0.37	0.28	0.96	0.23	0.05	1.24	0.61	0.21	1.75	0.47	0.23
Plasma	6.2	0.75	0.1	8.7	2.1	0.5	7.79	1.27	0.13	8.98	2.27	0.63	9.14	1.27	0.16
Urine	7.7	5.42	0.25	64.1	29.1	6.3	15.16	14.21	0.34	25.32	9.65	5.38	86.5	19.5	2.26

Table S5. Summary of PK results for ^{124}I-cRGDY–PEG–C dots delivered by systemic injection. (**A** and **B**) %ID/g values for tissues (A) and for biological specimens (B). Data are median values and interquartile ranges (n = 5).

A

Tissue / **Time post-injection (h)**	**%ID/g(× 10^{-3})**		
	4	**24**	**72**
Lungs	1.94 (1.75-2.10)	0.53 (0.46-0.57)	0.18 (0.07-0.25)
Heart	5.51 (5.26-6.10)	1.18 (1.06-1.91)	0.23 (0.20-0.47)
Liver	5.00 (3.8-5.31)	2.23 (1.68-2.24)	1.28 (0.94-1.34)
Spleen	3.22 (3.21-3.82)	1.65 (1.26-1.84)	0.72 (0.43-1.06)
GI	3.51 (3.05-8.36)	1.96 (0.89-2.94)	0.30 (0.23-0.97)
Kidneys	5.71(3.96-7.46)	4.29 (3.21-5.40)	3.09 (1.56-3.11)
Bladder	21.90 (17.7-42.38)	12.90 (9.7-29.82)	0.88 (0.67-2.26)
Muscle	1.43 (1.24-1.71)	0.43 (0.37-0.47)	0.21 (0.07-0.23)
Parotid	4.29 (4.14-6.15)	3.87 (3.72-4.68)	1.16 (0.14-1.78)
Thyroid	3.44 (3.08-7.64)	2.99 (0.75-3.25)	2.86 (0.11-3.99)
Brain	0.60 (0.49-0.71)	0.30 (0.29-0.34)	0.07 (0.06-0.15)
Spine	1.82 (1.80-2.32)	0.81 (0.56-1.03)	0.32 (0.17-0.37)

B

Specimen / **Time post-injection (h)**	**%ID/g(× 10^{-3})**			
	0.5	**4**	**24**	**72**
Blood	13.9 (13.5-14.1)	8.7 (7.79-8.98)	1.27 (1.27-2.1)	0.16 (0.134-0.50)
Urine	96.8 (45.2-109.0)	25.3 (15.20-64.10)	14.20 (9.65-19.50)	2.26 (0.34-5.38)

Table S6. Percentage of total body activity of ^{124}I-labeled nanoparticle or free iodide (^{124}I). Data are based on the estimated plasma, urine, and total body extracellular fluid compartment activities as well as the radioTLC analyses of plasma and urine at 0.5 and 24 hours post-injection of ~5 mCi ^{131}I-cRGDY–PEG–C dot. Data are the percentage of the total activity representing free iodide relative to that in the form of intact particle from a representative patient.

	Percentage (%) of total activity	
Time post-injection (h)	**Particle**	**Iodide**
0.5	99	1
24	97.5	2.5

Table S7. Organ- and tissue-specific cumulated ^{124}I activities by patient. Data are medians ± SD (n = 5). The cumulative tracer activities in the GI tract, heart, and bladder were counted intact; contents were not emptied prior to performing measurements.

	Cumulative activity (μCi-h/mCi)						
Patient ID	**1**	**2**	**3**	**4**	**5**	**Median**	**SD**
GI	1.28	3.05	0.62	0.98	0.45	0.98	1.04
Kidneys	0.17	0.45	0.37	0.86	0.66	0.45	0.27
Bone (marrow)	2.97	3.01	3.49	3.09	2.51	3.01	0.35
Brain	0.07	0.13	0.09	0.16	0.08	0.09	0.04
Lungs	0.12	0.14	0.13	0.17	0.18	0.14	0.03
Heart	0.17	0.28	0.28	0.33	0.20	0.28	0.06
Liver	0.78	1.29	0.80	1.60	1.38	1.29	0.37
Bladder	0.49	1.55	1.89	0.60	1.08	1.08	0.60
Muscle	5.73	10.50	6.50	12.20	6.98	6.98	2.81
Spleen	0.07	0.12	0.09	0.13	0.06	0.09	0.03
Thyroid	0.00	0.19	0.09	0.10	0.00	0.09	0.08

Table S8. Complete metabolic profile in male subjects. The datum in each cell represents a single measurement collected at a given time post-injection. Specimens were collected at 5 time points. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Base, baseline; BUN, blood urea nitrogen; Hb, hemoglobin; LDH, lactate dehydrogenase; NR, normal range; RBC, red blood cells; WBC, white blood cells.

	Time post-injection (h)	#1						#3						#4						Male
		Base	0.5	2	24	72	2 wk	Base	0.5	2	24	72	2 wk	Base	0.5	2	24	72	2 wk	NR
Metabolic profile	Na	139	137	138	137	139	135	140	137	139	140	141	135	138	138	138	138	141	140	[136-144 mEq/l]
	K	4.2	4.1	4.6	3.9	4.6	4.3	4.1	4.6	H	4.3	4.2	4.4	4.2	4.4	3.9	4.3	4.5	4.3	[3.5-5.1 mEq/l]
	Cl	103	101	101	101	105	103	105	103	104	106	108	103	105	103	102	103	106	106	[98-109 mEq/l]
	CO_2	29	31	32	32	31	24	27	26	28	28	26	25	26	27	29	32	29	29	[24-30 mEq/l]
	Calcium	10	8.6	8.9	9.3	9.3	10.3	10.3	9.2	9.6	9.5	9.3	9.8	9.2	8.7	8.9	9.2	8.8	9.1	[8.5-10.5 mg/dl]
	Glucose	98	77	79	77	83	136	141	76	77	82	84	118	123	95	82	67	78	142	[70-99; <140 (non-fasting) mg/dl]
Renal	BUN	11	21	18	20	16	14	8	9	8	9	10	8	37	38	38	35	32	31	[6-20 mg/dl]
	Creatinine	0.9	0.9	0.8	0.8	0.9	0.9	1.1	1	1	1.1	1	0.9	1.7	1.5	1.5	1.5	1.5	1.7	[0.6-1.3 mg/dl]
Hepatic function	Total bilirubin	0.6	0.2	0.3	0.3	0.3	0.6	1.1	0.9	H	1	0.8	0.7	0.8	0.6	0.6	0.4	0.3	0.7	[0-1.0 mg/dl]
	Total protein	6.8	6.1	6.2	6.7	6.8	7.0	6.6	6.9	7.6	7.4	7.4	6.5	6	6.5	6.6	6.4	6.2	6.3	[6.3-8.1 g/dl]
	Albumin	3.8	3.9	3.9	4.2	4.3	4.2	4.2	4.5	5	4.8	4.8	4.3	3.9	4.2	4.4	4.2	4.1	4	[4.0-5.2 g/dl]
	ALP*	77	89	98	100	105	88	75	67	61	74	73	59	50	52	58	60	58	66	[33-97 U/l]
	AST	24	29	27	20	22	28	45	29	37	32	35	21	24	40	42	36	36	22	[10-37 U/l]
	ALT	21	21	24	23	28	18	57	41	46	44	42	23	18	35	36	36	36	21	[5-37 U/l]
	LDH	237	--	--	--	--	191	204	--	--	--	--	185	204	--	--	--	--	224	[12-246 U/l]
Blood counts	WBC	5.4	15.6	14.2	6.6	5.3	7.4	5.7	6.4	8	6.9	6.7	5.4	2.7	3.6	3.5	3.6	3.6	3.3	[4-11 K/mcl]
	RBC	3.96	3.37	3.50	3.65	3.70	3.96	4.81	4.7	5.2	5.18	4.93	5.28	3.02	3.04	3.00	2.97	2.94	3.02	[4.2-5.6 M/mcl]
	Hb	10.3	9.3	10.1	10.1	10.2	11.1	12.9	13	13.9	13.7	13.9	14.1	10.1	10.1	10.2	9.6	9.7	10.2	[13-17 g/dl]
	Hematocrit	32.9	29.1	29.8	30.7	31.5	34.1	39	38.8	43.4	43.1	41.2	43.3	28.2	28.6	28.3	28	28.1	28.5	[38-52 %]
	Platelets	303	191	186	182	186	282	315	301	347	369	313	355	155	128	133	130	131	140	[160-400 K/mcl]

*For patients older than age 55 (Patient #4), the ALP range changed to 45–129 U/l.