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A ^{224}Ra-labeled polyoxopalladate as putative radiopharmaceutical

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Content

1. Experimental Materials and Methods

1.1 Materials

All reagents and solvents were purchased from Fisher Scientific (Schwerte, Germany). Barium nitrate, sodium hydroxide, sodium acetate and anion exchanger Dowex 50x8 were purchased from Sigma Aldrich (Taufkirchen, Germany). Phenylarsonic acid was purchased from Alfa Aesar (Karlsruhe, Germany). Palladium Acetate was purchased from Riedel-de Haen (Seelze, Germany). Rat serum, TLC plates, Silica Gel 60 F_{254} and RP-18 F_{254}s, were purchased from Merck (Darmstadt, Germany). Size exclusion chromatographic material, Sephadex G-15, was purchased from GE Healthcare (Uppsala, Sweden). All deionized water used was purified on-site (Millipore, deionized > 18 MΩcm).

Caution! Barium-133 and radium-224 are radioactive and all work involving these radionuclides was carried out in approved laboratories following appropriate radiation safety procedures. Barium-133 was purchased from POLATOM (Otwock, Poland) and received as [^{133}Ba]$BaCl_2$ in a 0.1 M HCl solution (specific activity: 10 MBq/mg). This material was evaporated to dryness and dissolved in deionized water. Radium-224 was isolated on-site from a ^{228}Th source purchased from Eckert and Ziegler (Braunschweig, Germany) as [^{224}Ra]$Ra(NO_3)_2$ in 1.0 M HNO_3 using ion exchange and extractive chromatography methods.

1.2 Instrumentation

^{1}H and ^{13}C NMR spectra were obtained using an Agilent DD2-400 (ProbeOne NMR probe) spectrometer at 400 and 101 MHz, respectively. Chemical shifts are reported in ppm with tetramethylsilane as the internal standard. Analytical TLC were performed on pre-coated Silica Gel 60 F_{254} and RP-18 F_{254}s and the results visualized under UV-light (λ = 254 nm). Radio-TLC were performed on pre-coated Silica Gel 60 F_{254} and RP-18 F_{254}s; the TLC plates were then used to expose phosphor imaging plates and the imaging plates were read using a FujiFilm BAS-1800II plate reader. Alpha spectroscopy measurements were performed using an Ortec Alpha Duo spectrometer with silicon surface barrier detectors (450mm^2 active area, 20 keV FWHM at 5.488 MeV α energy). Radioactivity count rates were measured using the ISOMED 2160 (MED) sodium iodide detector. pH measurements were performed using a Mettler Toledo FiveGo F2 meter with a LE438 IP67 probe.

1.3 Synthesis of Na_xBa_y-$BaPd_{15}$

This simple one-pot synthesis of $[BaPd_{15}O_{10}(PhAsO_3)_{10}]^{8-}$ (**Na_xBa_y-$BaPd_{15}$**) is based on a previously published method by Kortz and colleagues. Briefly, $Pd(OAc)_2$ (23 mg, 0.1 mmol), $PhAsO_3H_2$ (20 mg, 0.1 mmol), and $Ba(NO_3)_2$ (6 mg, 0.02 mmol) were combined in a small vial with a stir bar and 2 mL of a pH 7, 0.5 M NaOAc buffer solution. The solution was heated to 80° C for 30 min with rapid stirring. For the first few minutes, the sample was shaken to ensure that all of the $Pd(OAc)_2$ was incorporated. The solution slowly turned to an orange-brown color. After 30 min, the vial was allowed to cool to room temperature. The pH was then adjusted to 8.5-8.8 using NaOH. Prior to adjustment, the pH was approximately 5.3. Once the pH was properly adjusted, the vial was returned to the hot plate and allowed to react for an additional 60 min. Afterwards, the solution was cooled to room temperature with a final pH of the solution of approximately 8.0.

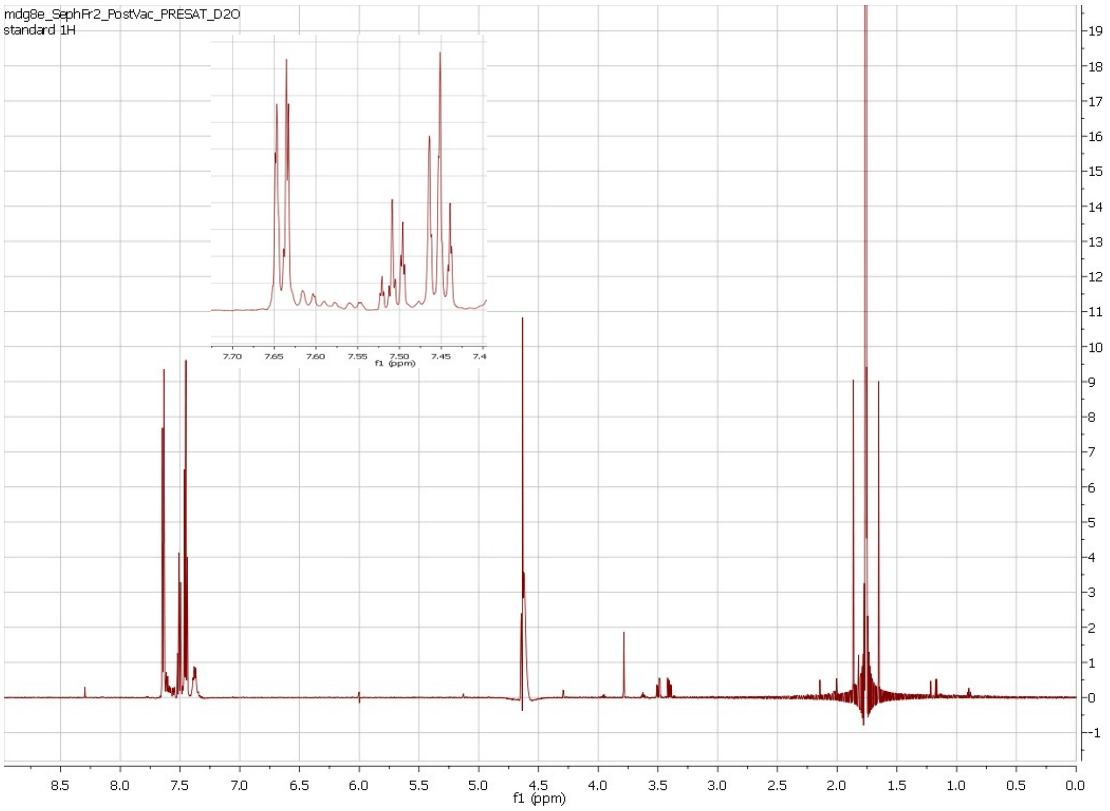

Figure S1. ^{1}H NMR spectrum of $\mathbf{Na_xBa_y\text{-}BaPd_{15}}$.

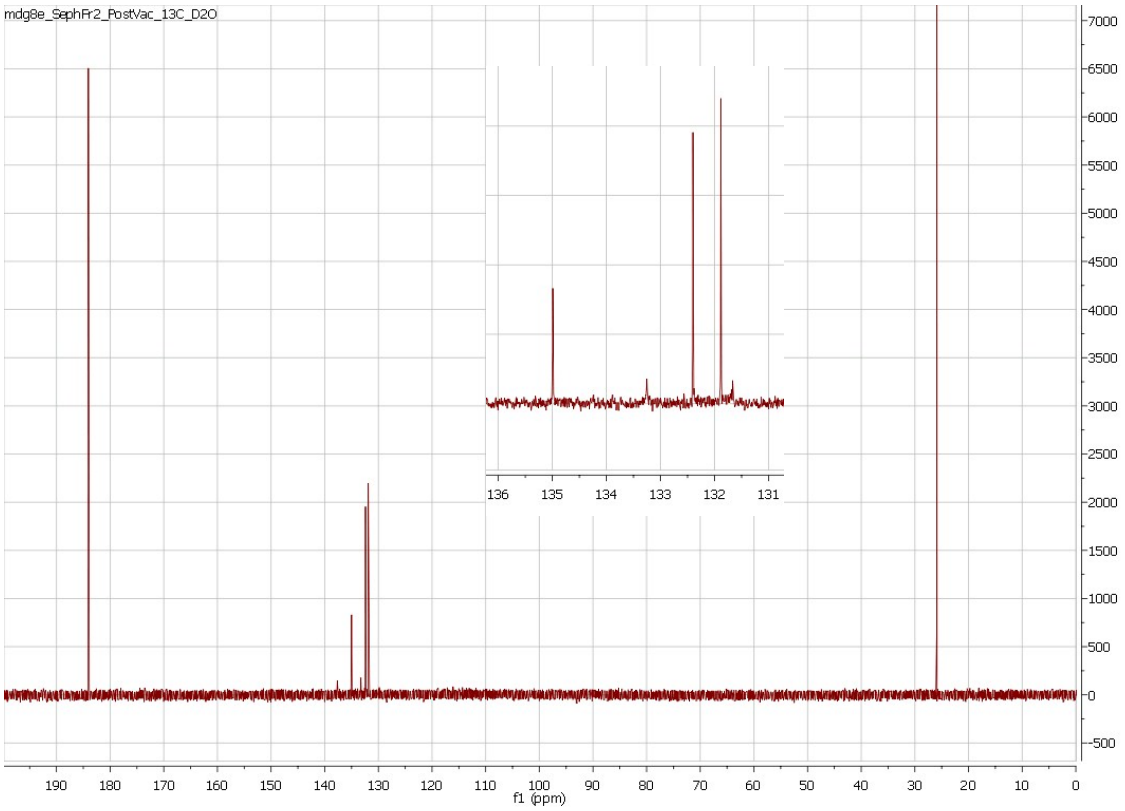

Figure S2. ^{13}C NMR spectrum of $\mathbf{Na_xBa_y\text{-}BaPd_{15}}$.

1.4 Synthesis of the ^{133}Ba-labeled [^{133}Ba]Na-BaPd$_{15}$

Radiolabeled [^{133}Ba]**Na-BaPd$_{15}$** was prepared following the literature preparation method with the addition of tracer quantity of [^{133}Ba]$BaCl_2$. The solution was spiked with 772 kBq of [^{133}Ba]$BaCl_2$ in water prior to heating the sample.

Radio-TLC method for the radiolabeled POMs

To analyze the separation of free Ba^{2+} and [^{133}Ba]**Na-BaPd$_{15}$**, a TLC method was developed to separate both species. It was decided to follow the visibly colored POM and once a method for the POM was developed, test with ^{133}Ba to follow the POM and free [^{133}Ba]Ba^{2+}. Reverse phase TLC (RP-18) was used due to the high charge of the radiolabeled POM. Initially, several pure solvents were tested for ability to move the POM and then several mixtures of acetonitrile and water or methanol. The results are illustrated in Table S1. The best result was observed with 1/2 water/acetonitrile. Once the solvent system was developed, the method was transferred to radiolabeling with ^{224}Ra.

Table S1. TLC method development for **Na-BaPd$_{15}$** and [^{133}Ba]**Na-BaPd$_{15}$**

Solvent System	Result
methanol	Colored product streaks from origin
acetonitrile	No movement from origin
water	Colored product slightly moves
petroleum ether	No movement from origin
1:1 acetonitrile : methanol	Colored product at front but spread
4:1 Acetonitrile : methanol	No movement from origin
1:1 water : acetonitrile	Colored product at front but wide
1:2 water : acetonitrile	Colored product at front in tighter
1:3 water : acetonitrile	Streaking occurs and it seems like
1:5 water : acetonitrile	Streaking occurs and it seems like

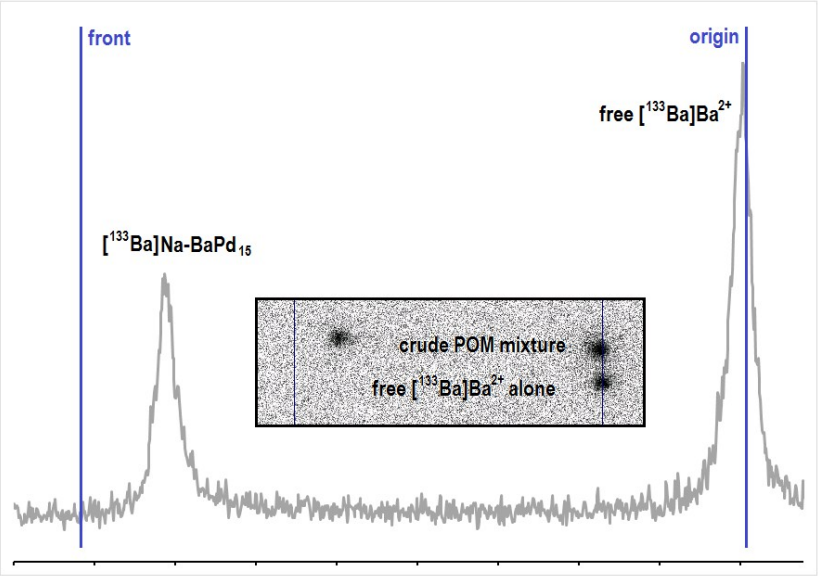

Figure S3. Radiographic image of TLC plates containing the crude [^{133}Ba]**Na-BaPd$_{15}$** mixture (top) and free [^{133}Ba]Ba^{2+} alone (bottom) using RP-TLC plates with 1:2 water: acetonitrile.

1.5 Synthesis of the ^{224}Ra-labeled [^{224}Ra]Na-Ba(Ra)Pd$_{15}$

Radiolabeled [^{224}Ra]**Na-Ba(Ra)Pd$_{15}$** was prepared as described for [^{133}Ba]**Na-BaPd$_{15}$**. Incorporation of tracer ^{224}Ra was tested by following the literature preparation method with the addition of tracer quantity of [^{224}Ra]Ra(NO$_3$)$_2$. The solution was spiked with 20 µL of [^{224}Ra]Ra(NO$_3$)$_2$ in weak nitric acid. Ion exchange was performed to remove remaining free radiometal ions from the solution. Alpha spectroscopy was performed on the crude and separated [^{224}Ra]**Na-Ba(Ra)Pd$_{15}$** to determine the radionuclides from decay found in the product. Radio-TLCs were performed to ensure that the radioactivity moves with the POMs (Figure S5). The same radio-TLCs were measured again one week later to determine the placement of the radioactivity which relates to the (decayed) radium-224.

Samples of the free [^{224}Ra]Ra^{2+} (freshly separated from column) and [^{224}Ra]**Na-Ba(Ra)Pd$_{15}$** were analyzed by alpha spectrometry to understand the relative ratios ^{224}Ra and its daughters in the product (Figure S4). The relative ratios of radium and its daughters ^{212}Pb and ^{212}Bi are not significantly changed from the starting radium-solution to the [^{224}Ra]**Na-Ba(Ra)Pd$_{15}$** product. This suggests that the radiometal uptake is not selective for radium, so lead- and bismuth-centered BaPd$_{15}$ POMs are also produced. It should be noted that the significant peak tailing in the POM spectrum results from self-shielding due to the excessive acetate.

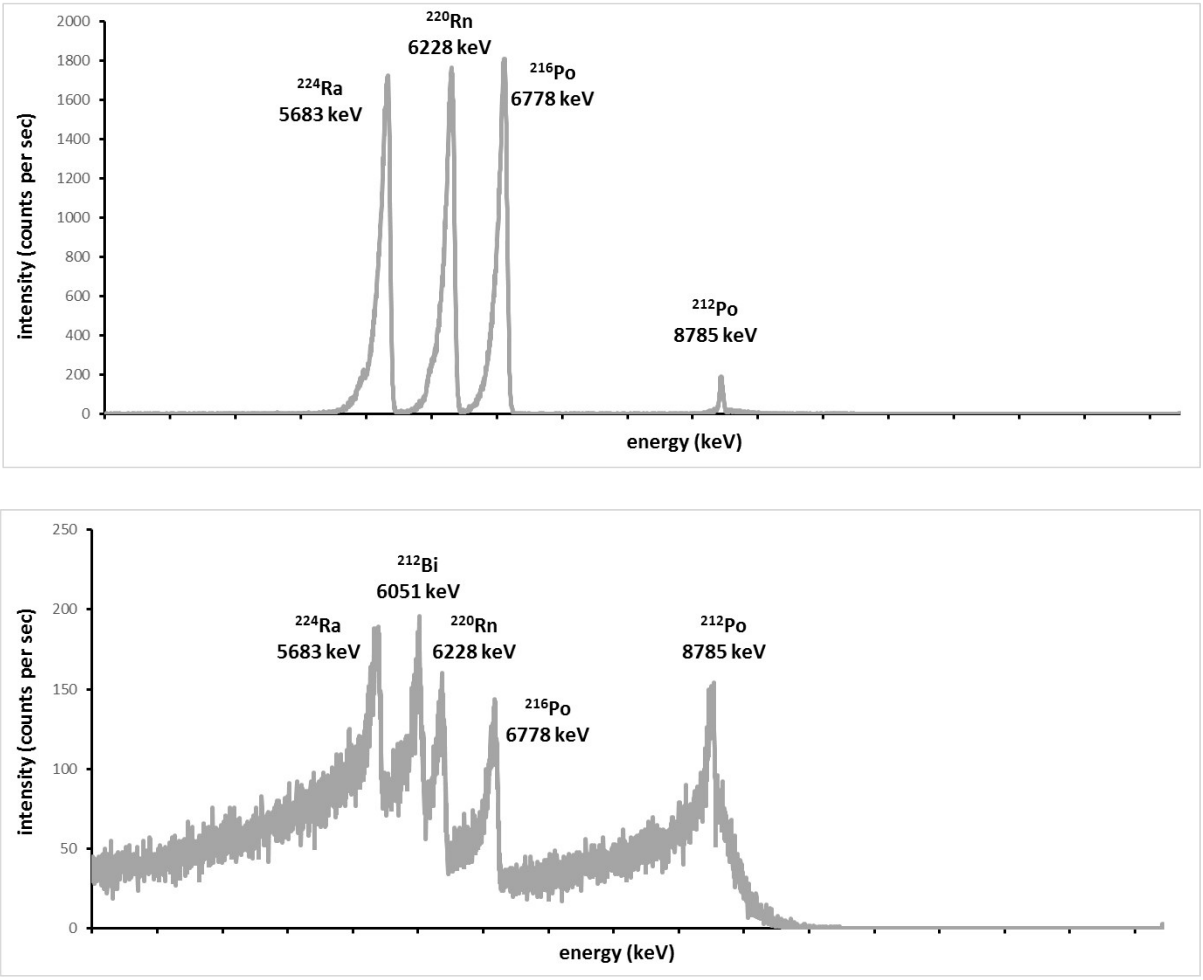

Figure S4. Alpha spectrum of freshly separated, uncontacted [^{224}Ra]Ra^{2+} solution (top) and cation-exchange separated [^{224}Ra]**Na-Ba(Ra)Pd$_{15}$** product (bottom).

Once it was established that ^{224}Ra was actually incorporated into the POM and not just the daughters, a radioactive TLC was performed to demonstrate that the radioactivity moves with the POMs. In Figure 11, it is shown that a large activity moves with the front as expected with the POMs. Streaking of the POM is noted in this chromatogram; this could be related to the different metals incorporated into the POM. The TLC was left for 1 week to decay and reimaged, which ensures that the only remaining activity results from ^{224}Ra. Though the signal is significantly weakened, the chromatogram confirms that ^{224}Ra was incorporated into POM.

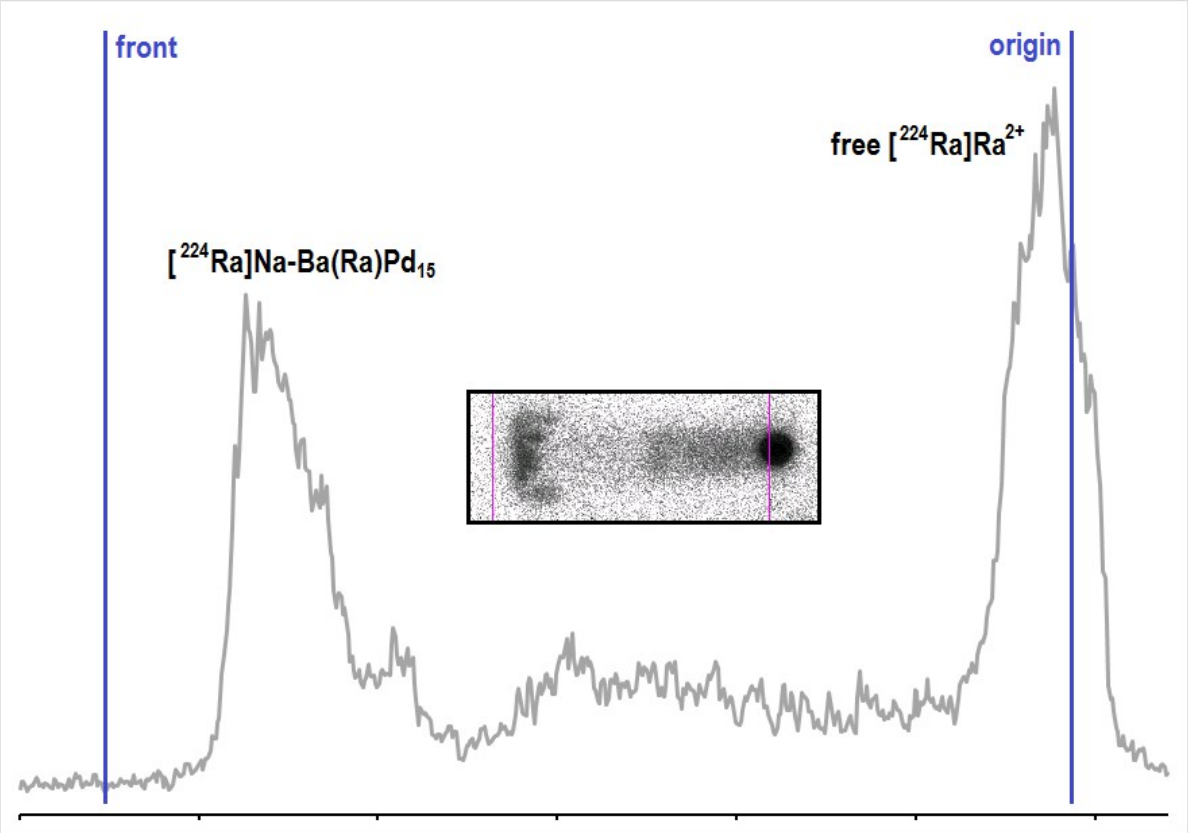

Figure S5. Radio-TLC (small image) and quantification of the crude [^{224}Ra]**Na-Ba(Ra)Pd$_{15}$** immediately after preparation.

1.6 POM-Purification Evaluations

Several methods were tested to purify the **Na_xBa_y-$BaPd_{15}$** product. Various counter-ions were tested to precipitate the product as a salt to leave behind the free Ba^{2+} and excess acetate ions in solution. Two chromatographic methods were tested to isolate the $BaPd_{15}$ complex from the free Ba^{2+}. First, free barium was simply extracted from the solution by cation exchange chromatography. Second, a size exclusion column was tested to remove the free barium and excess sodium acetate from the solution.

Precipitation method

For the precipitation method, freshly prepared **Na_xBa_y-$BaPd_{15}$** was combined with an excess of several compounds (triphenylphosphine, guanidinium chloride, tetrabutylphosphonium chloride, tetraethylammonium bromide, tetrahexylammonium bromide, tetradodecylammonium bromide, dimethyldioctadecylammonium chloride, 15-crown-5, and 18-crown-6). The samples were shaken and allowed to react overnight if no initial reaction was noted. Further studies were performed with the guanidinium chloride salt. A sample of **Na_xBa_y-$BaPd_{15}$** was split into 4 x 400 µL aliquots and an excess of guanidinium chloride (20 mg per sample) was added to form **Gua-$BaPd_{15}$** (4726 g/mol; 6.427 mg expected per aliquot). The sample was centrifuged, the solution removed, and the **Gua-$BaPd_{15}$** dried to determine mass. A value of 6.06 ± 0.29 mg (n = 4) was found resulting in a 94% recovery of the expected product. To determine free Ba^{2+} in the decanted solution, $NaSO_4$ was added to precipitate $BaSO_4$. The samples were centrifuged, the solvent removed, and the $BaSO_4$ dried to determine the mass of 0.86 ± 0.06 mg (n = 4) (quantitative recovery of barium).

Cation exchange method

For cation exchange chromatography, Dowex-50 resin was slurried and a 1 mL bed volume of resin added to a small pipette column. To prevent unfavorable pH conditions, the resin was converted from the H^+ form to Na^+ by washing the column with 1 M NaOH and then adjusting to pH 7 using H_2O. Once the column was prepared, the $BaPd_{15}$ solution was added to the column. An aliquot of water was added to strip the product from the column. The POM was easily followed on the column due to its orange-brown color and only colored fractions were collected from the column to avoid sample dilution. For non-radioactive samples, precipitation as barium sulfate was used to check for free Ba^{2+}. For radioactive samples, a NaI detector was used to determine the activity present in the original spike and separated product to determine the percent of ^{133}Ba lost on the column. Production yields are calculated from the determined values of free Ba^{2+} (Table S2).

Table S2. The experimental activity and separation efficiency for [^{133}Ba]**Na-$BaPd_{15}$** by cation exchange.

Sample	Crude Activity (kBq)	Separated Activity (kBq)	Percent Barium Bound
$BaPd_{15}$ w/ 1 µL $^{133}BaCl_2$	16.4	4.4	27.8
$BaPd_{15}$ w/ 10 µL $^{133}BaCl_2$ (n = 4)	193.2 ± 20.8	58.0 ± 5.8	30.1 ± 0.8

Size exclusion chromatography

For size exclusion chromatography, Sephadex G-15 (MWCO = 1000 Da) was tested to isolate the large POM molecule from the significantly smaller starting materials. Initial tests using small pipette columns demonstrated the need for a larger column. A large 14 mm diameter, 85 mm tall Sephedex G-15 column was tested for the removal of free, unreacted starting materials. The column was wet with water and then 2 mL of the crude **Na_xBa_y-$BaPd_{15}$** solution was added to the column (Figure S6). The colored eluent was eluted using water and collected in a clean vial. ^{13}C NMR was measured to determine the presence of acetate in the sample.

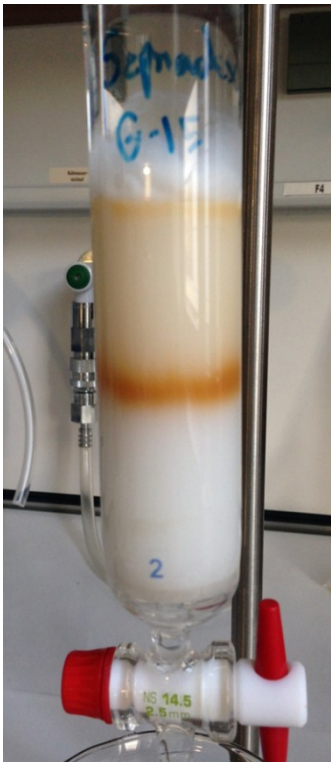

Figure S6. Elution of non-radioactive **Na_xBa_y-$BaPd_{15}$** using Sephadex G-15 size exclusion resin.

To determine the free [^{133}Ba]Ba^{2+} concentration with the POM after radiolabeling, crude [^{133}Ba]**Na-$BaPd_{15}$** was added to the column and eluted as described. The activity was determined using a NaI detector and the percentage eluted determined.

1.7 Radiochemical Stability Studies for [^{133}Ba]Na-BaPd$_{15}$

A simple dialysis study was performed to analyze the stability of [^{133}Ba]**Na-BaPd$_{15}$** in an aqueous environment. A 500 µL aliquot of crude and separated [^{133}Ba]**Na-BaPd$_{15}$** solutions were transferred to separate pre-conditioned Pur-A-Lyzer Midi 1000 dialysis tubes (MWCO = 1000). The dialysis tubes were submerged into separate beakers containing 80 mL of water with stirring. Fractions of the dialysis water were collected at various time points and the activity determined using the NaI detector. Any activity observed is assumed to be free [^{133}Ba]Ba^{2+} as barium bound to the [^{133}Ba]**Na-BaPd$_{15}$** could not pass through the dialysis membrane. The method was done using [^{133}Ba]$BaCl_2$ (10µL with 772 kBq). The results are illustrated in Table S3. A statistically relevant amount of [^{133}Ba]Ba^{2+} is observed to diffuse in both dialysis solutions using higher activity.

Table S3. Dialysis results examining the stability of [^{133}Ba]**Na-BaPd$_{15}$**.

Time		5 m	15 m	30 m	45 m	60 m	2 h	4 h	24 h	48 h	72 h	96 h
Percentage Ba Leaked	**Crude**	2.2	6.0	9.9	12.6	15.4	22.3	26.5	37.4	44.0	46.9	46.8
	Separated	-0.3	0.6	0.5	0.3	2.0	2.5	3.8	9.7	11.7	11.3	12.5

21.8 Biological Compatibility Study of [^{133}Ba]Na-BaPd$_{15}$ using Rat Serum

A 100 µL aliquot of the purified [^{133}Ba]**Na-BaPd$_{15}$** solution was combined with 400 µL of rat serum in an Eppendorf tube. The tube was transferred to a thermomixer, where it was heated to 37°C and shaken at 300 rpm. At various time points (1, 2, 4, 8, 24, and 48 h), a 1 µL sample of the mixture was taken and spotted onto a reverse phase TLC plate. The TLC was run as previously described with 1/2 water/ acetonitrile. Additionally, a "zero" time point was ran using the uncontacted [^{133}Ba]**Na-BaPd$_{15}$** solution.

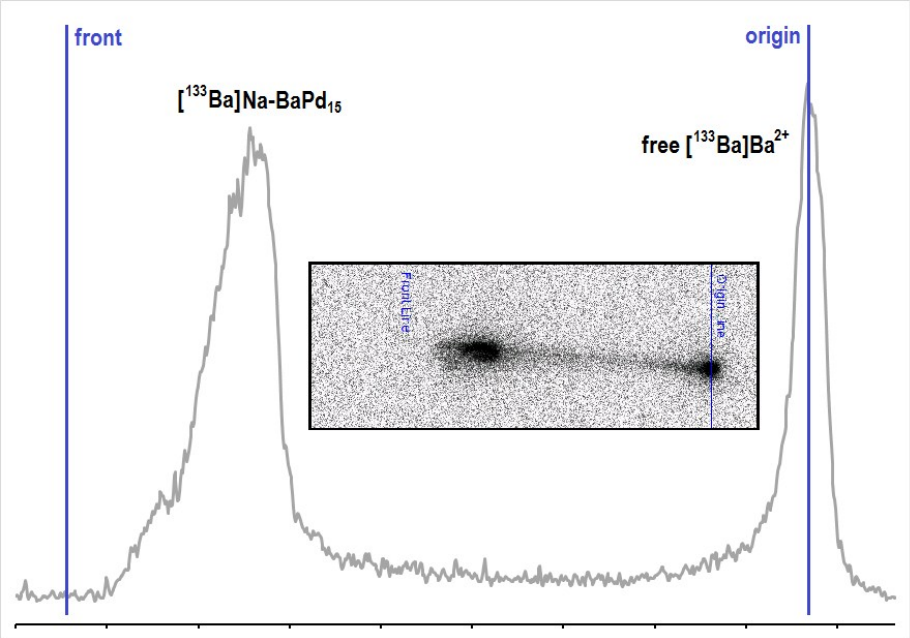

Figure S7. Radio-TLC image (small figure) and quantification of the uncontacted [^{133}Ba]**Na-BaPd$_{15}$**.

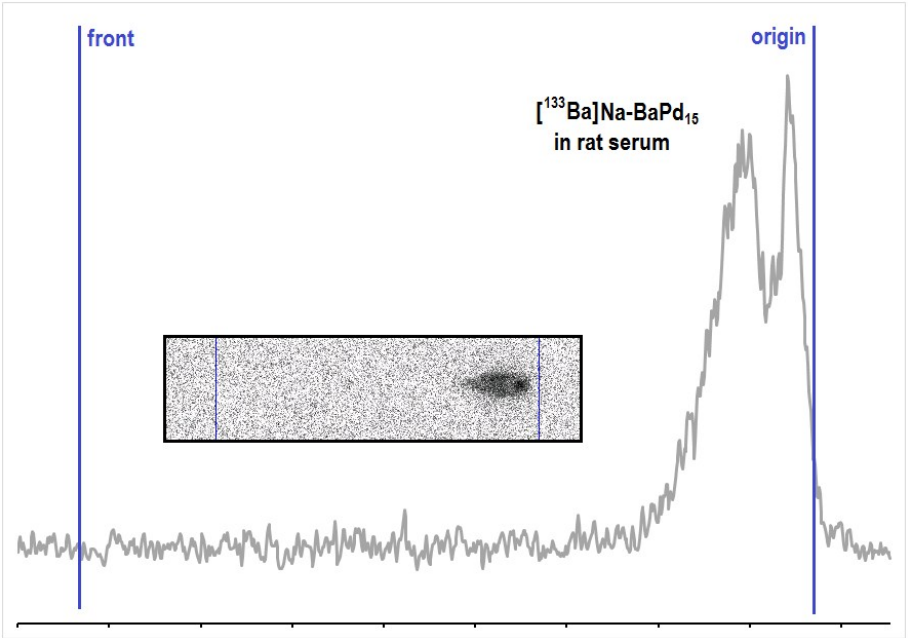

Figure S8. Radio-TLC image (small figure) and quantification of the [^{133}Ba]**Na-BaPd$_{15}$** incubated with intact serum.

Table S4. Stability of [^{133}Ba]**Na-BaPd$_{15}$** with and without serum incubation at given time points.

Sample	% at solvent front	% slightly off	% at origin
[^{133}Ba]**Na-BaPd$_{15}$** without incubation	70.8	-	29.2
[^{133}Ba]**Na-BaPd$_{15}$** incubated 1h	-	66.2	33.8
[^{133}Ba]**Na-BaPd$_{15}$** incubated 2 h	-	56.3	43.7
[^{133}Ba]**Na-BaPd$_{15}$** incubated 4 h	-	62.1	37.9

A study was performed to evaluate the difference between the POM and $BaCl_2$ in the presence of rat serum. Thus, 1 µL of **[^{133}Ba]**Na-$BaPd_{15}$ purified by cation exchange separation was added to 4 µL of rat serum and allowed to contact for 1 h at 37°C and 300 rpm using the thermomixer. The same was done with a 1 µL aliquot of the 1/100 [^{133}Ba]$BaCl_2$ sample which was added to 4 µL of rat serum and allowed to contact for 1 h at 37°C and 300 rpm using the thermomixer as well. TLCs were performed for all three samples as previously described and an imaging plate was exposed for 2 h with all three TLCs. The results are illustrated in Figure S9.

The uncontacted [^{133}Ba]**Na-BaPd$_{15}$** was observed to have 85.5% of the activity at the front line and 14.5% remaining at the origin. The [^{133}Ba]**Na-BaPd$_{15}$** incubated with the serum proteins had 54.6% slightly off the baseline and 45.4% remaining at the origin. [^{133}Ba]Ba^{2+} was observed to be 100% at the origin. The difference in retention factor for the uncontacted [^{133}Ba]**Na-BaPd$_{15}$** and the [^{133}Ba]**Na-BaPd$_{15}$** with rat serum shows the high interaction of the POM with serum protein. This is reinforced by the lack of movement from the [^{133}Ba]Ba^{2+} on its TLC as it has no affinity for serum proteins.

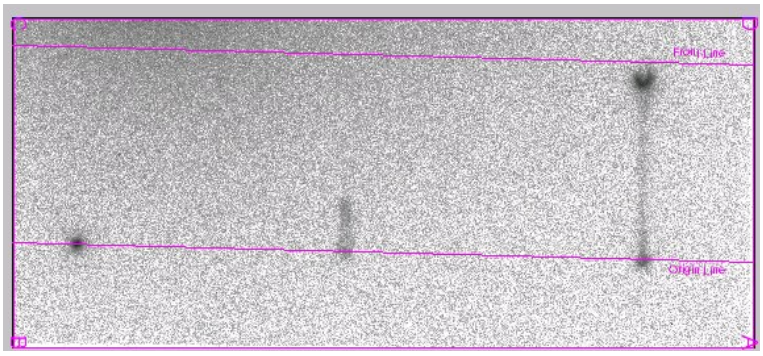

Figure S9. Radiographic image of TLC plates containing [^{133}Ba]Ba^{2+} with intact serum proteins (left), POM with intact serum proteins (middle), and uncontacted [^{133}Ba]**Na-BaPd$_{15}$** (right).

Additionally, a 5 µL sample of the [^{133}Ba]**Na-BaPd$_{15}$**-serum mixture was taken and 20 µL of methanol was added to denature the proteins. The proteins crashed out as a white precipitate. The tube was centrifuged at 14,000 rpm for 2 min. Interestingly, the protein pellet at the bottom contained the entire

colored product and the solution was clear. A likely possibly is the formation of a protein corona around the highly charged [^{133}Ba]**Na-BaPd$_{15}$**. A TLC was performed using the supernatant solution from this sample. There was no activity found in the supernatant. Although it was visibly clear that the colored product remained with the protein pellet, it was expected to detect free [^{133}Ba]Ba^{2+}. This suggests that the separation is effective and the species is fully intact prior to contact with methanol.