**Supporting Information**

# Biodegradable nanoemulsion-based bioorthogonal nanocatalysts for intracellular generation of anticancer therapeutics

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## 1. Materials

All chemicals and materials for experiments were obtained from Sigma Aldrich (USA) or Fisher Scientific (USA). Further purification was not performed unless otherwise indicated. HeLa cells (ATCC CCL-2), NIH-3T3 cells (ATCC CRL-1658) and RAW 264.7 cells (ATCC-TIB-71) were purchased from ATCC. Dulbecco's Modified Eagle's Medium (DMEM) (ATCC 30-2002) and fetal bovine serum (Fisher Scientific, SH3007103) were used in cell culture.

## 2. Synthesis of PONI-GMT polymer, DTDS crosslinker, Pro-Rho and Pro-Dox

The polymer and crosslinker were synthesized according to the previous protocol.[1] Pro-Rho[2] and Pro-Dox[2,3] synthesis were done by following previous protocols.

## 3. Preparation of Nanocatalysts

Stock nanocatalysts solutions were prepared in 0.6 mL Eppendorf tubes through emulsification of a mixture of palladium (Pd) catalyst, (1,1′ bis(diphenylphosphino) ferrocene) palladium (II) dichloride and DTDS in carvacrol into an aqueous PONI-GMT (54kDa MW) solution. Briefly, Pd catalyst (3 mg/mL) and DTDS (3 wt%) were solubilized in carvacrol. Next, 3 μL of the resulting oil suspension was added to PONI-GMT aqueous solution (497 μL, 6 μM). This solution was then emulsified for 50 seconds using an amalgamator. The concentration of this nanocatalyst stock solution was defined with respect to polymer concentration (~6 μM). The formulated nanocatalyst contains Pd catalyst, crosslinker, Carvacrol oil, and PONI-GMT polymer at a 0.06:0.6:18:1 weight ratio.

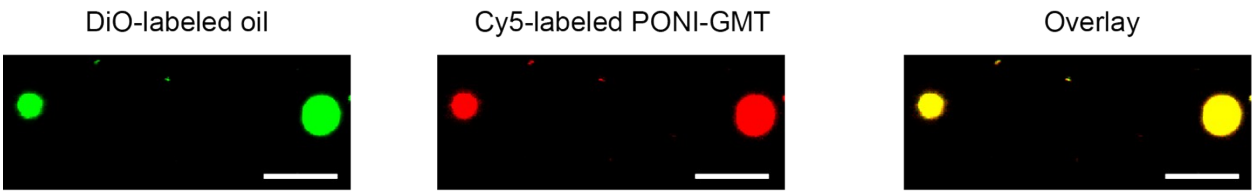
## 4. Zeta potential characterization

Zeta potential was measured in deionized (DI) water using a Malvern Zetasizer Nano ZS instrument.

## 5. DLS and TEM characterization

Dynamic light scattering (DLS) was used to measure hydrodynamic diameters of **BNE** and **Pd-BNC** in DI water using a Malvern Zetasizer Nano ZS instrument at a measurement angle of 173° (backscatter). "Multiple narrow modes" (high resolution) method was used for data analysis based on non-negative-least-squares (NNLS).

Samples were prepared for Transmission Electron Microscopy (TEM) imaging by placing a droplet of 1 μM **Pd-BNC** solution onto a 300-mesh Cu-grid coated with carbon film. The samples were analyzed using a JEOL CX-100 electron microscope. TEM images suggested no aggregation before or after catalyst encapsulation.




## 6. Visualization of Pd-BNC using confocal microscopy

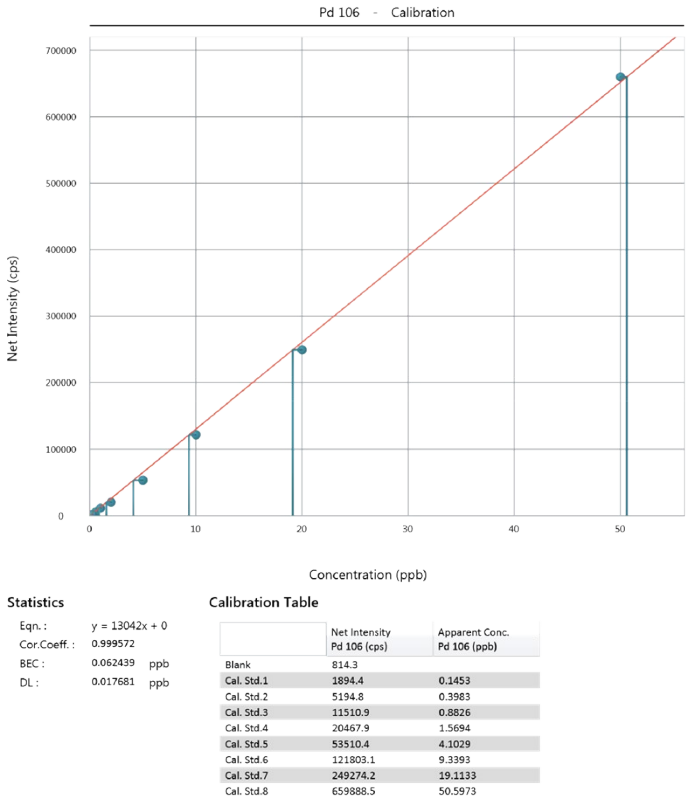
**Supplementary Figure S1.** Confocal Microscopy images of crosslinked micron-sized **Pd-BNC**. PONI-GMT-labeled with Cy5 (red fluorescence), and the oil core is loaded with DiO (green fluorescence). scale bars are 10 μm.

## 7. Quantification of Pd/Pd-BNC using ICP-MS

Pd catalysts per polymer were quantified by first adding 30 μL of **Pd-BNC** solution (6 μM) to a clean 15mL Falcon tube to which 0.5 mL of fresh aqua regia was added to the container for efficient sample digestion. The resulting solution was then diluted to 10 mL with de-ionized water. The composition of **Pd-BNC** was then analyzed on a PerkinElmer NexION 300X ICP mass spectrometer. $^{106}$Pd was measured under the standard mode with operating conditions as follows: nebulizer flow rate: 0.95 L/min; rf power: 1600 W; plasma Ar flow rate: 18 L/min; dwell time: 50 ms.

Standard ME4 solutions (0, 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40, 45 and 50 ppb) were prepared *via* serial dilutions for the calibration curve.

a)




**Statistics**

| | | |
|---|---|---|
| Eqn. : | y = 13042x + 0 | |
| Cor.Coeff. : | 0.999572 | |
| BEC : | 0.062439 | ppb |
| DL : | 0.017681 | ppb |

**Calibration Table**

| | Net Intensity Pd 106 (cps) | Apparent Conc. Pd 106 (ppb) |
|---|---|---|
| Blank | 814.3 | |
| Cal. Std.1 | 1894.4 | 0.1453 |
| Cal. Std.2 | 5194.8 | 0.3983 |
| Cal. Std.3 | 11510.9 | 0.8826 |
| Cal. Std.4 | 20467.9 | 1.5694 |
| Cal. Std.5 | 53510.4 | 4.1029 |
| Cal. Std.6 | 121803.1 | 9.3393 |
| Cal. Std.7 | 249274.2 | 19.1133 |
| Cal. Std.8 | 659888.5 | 50.5973 |

b)

| | Pd 106 (cps) | Pd (ppb) | Pd (mg) | Pd/Polymer |
|---|---|---|---|---|
| Pd-BNC (before wash) | 413047.632 | 31.671±0.716 | 3.19E-04 | 0.0591 |
| Pd-BNC (after 3 washes) | 333765.581 | 25.592±0.361 | 2.57E-04 | 0.0476 |
| Wash 1 supernatant | 4448.965 | 0.341 | | |
| Wash 2 supernatant | 2752.282 | 0.211 | | |
| Wash 3 supernatant | 1297.90 | 0.100 | | |

**Supplementary Figure S2**. a) Calibration curve for Pd with ICP-MS, b) Quantification of Pd in **Pd-BNC**.

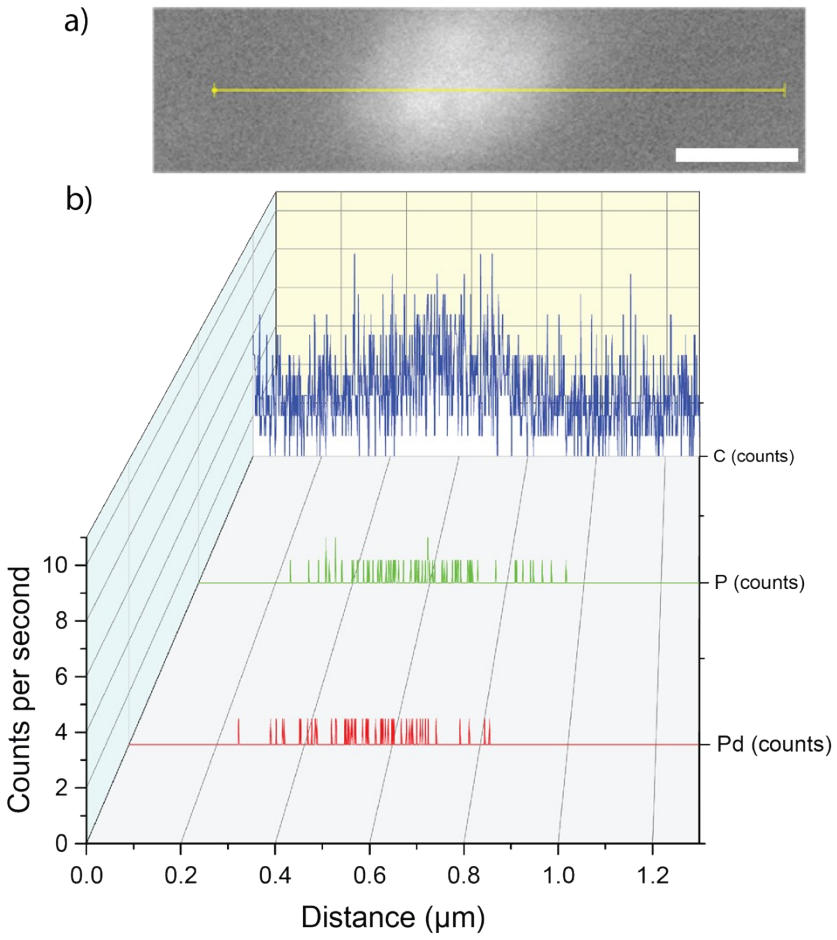
## 8. Elemental mapping analysis using EDX

In the experiment, our selected samples were carefully placed onto a 400-mesh Transmission Electron Microscope (TEM) grid. After an allocation of 30 seconds for the sample to evenly disperse, excess solution was meticulously removed using filter paper, ensuring a minimal disturbance to the sample. The samples were then subjected to a vacuum environment for 48 hours, to adequately prepare them for subsequent microscopic analysis.

For the purpose of precise micro-observation, we utilized a JEOL- JEM-2200FS Energy Filtered Transmission Electron Microscope, integrated with an Oxford 80mm^2 X-Max Energy Dispersive X-ray Spectrometer (EDS). The microscope was operated under carefully calibrated conditions:

an applied voltage of 15 kV, current fluctuation in the range of 8–10 nA, and a beam diameter confined to 6 µm. The pressure within the chamber was reduced and stabilized at 50 Pa to ensure optimal imaging conditions.

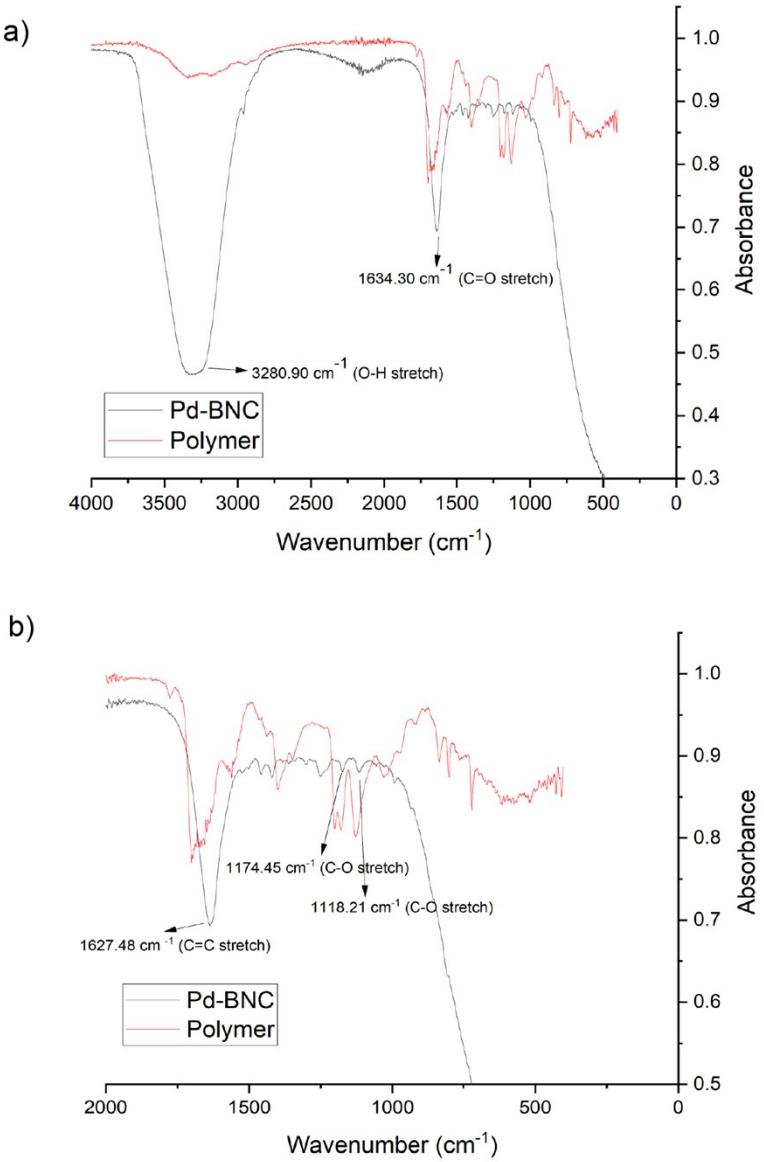
To enable a thorough characterization of the elemental composition across our sample, we employed a combination of Energy Dispersive X-ray spectroscopy (EDX) and Transmission Electron Microscopy (TEM). This powerful analytical technique allowed us to associate the elemental composition measured across a particle (line scan) with the localization of the Pd catalyst



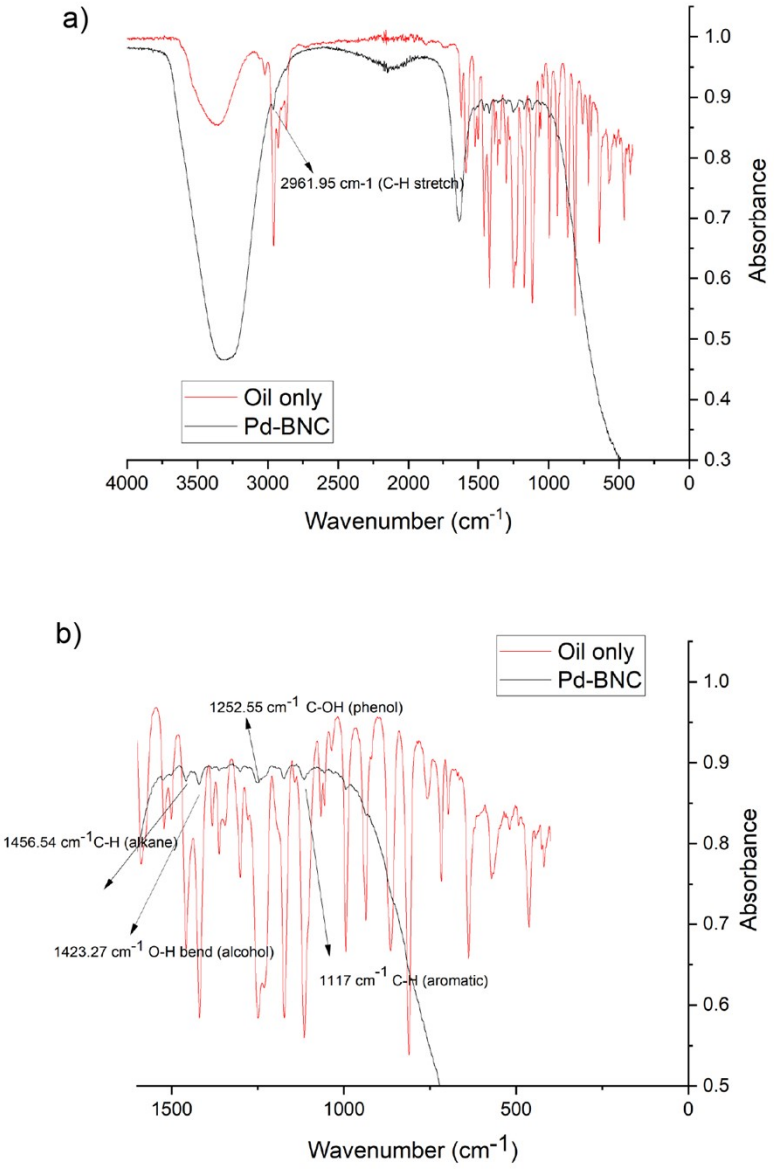

within the polymer matrix.

**Supplementary Figure S3**. Detailed EDX and TEM Analysis of **Pd-BNC**. a) Representative TEM image and corresponding EDX line scans for **Pd-BNC**. (Scale bar = 200 nm). b) EDX line scan detailing the Carbon (C Kα1,2), Palladium (Pd Lα1), and Phosphorus (P Kα1) signals for the particle illustrated in Figure S3a. The figure demonstrated that Pd and P elements were co-localized within the nanocatalyst. The source of the Phosphorus in our experiment is our Pd catalyst, [1,1′-bis(diphenylphosphino)ferrocene palladium (II) dichloride. Remarkably, this area of co-localization also corresponded with the region that displayed an increased count of Carbon (C) elements form the PONI-GMT polymers and Carvacrol oil.

## 9. Infrared Spectroscopy of Pd-BNC



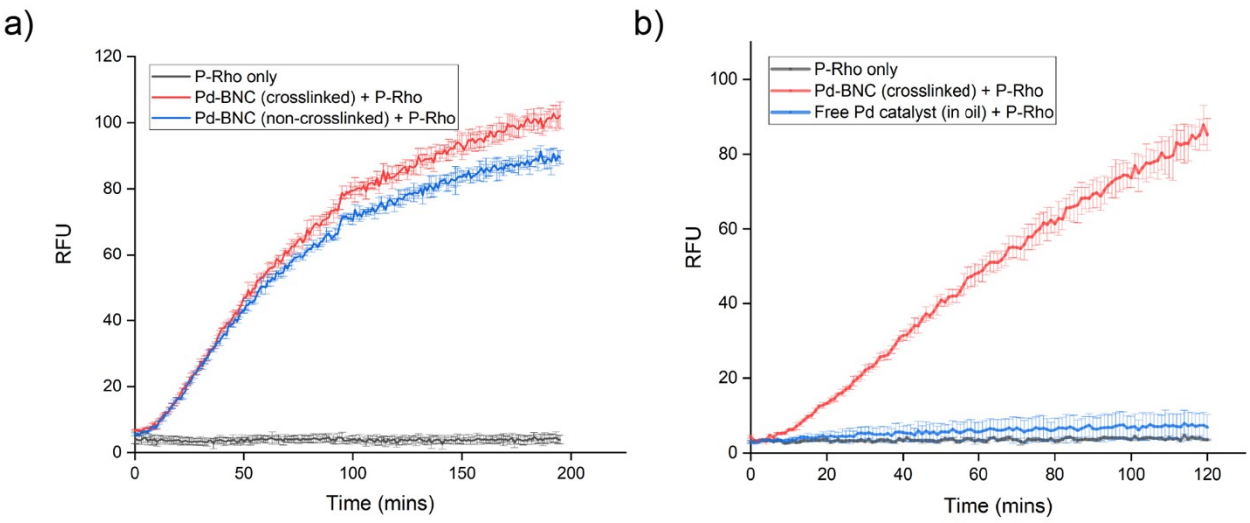

**Supplementary Figure S4.** FTIR Spectra of Pd-BNC as compared to PONI-GMT polymer. a) Full IR Spectrum of PD-BNC vs Polymer. b) Zoomed in IR Spectrum of Pd-BNC vs Polymer.




**Supplementary Figure S5.** FTIR Spectra of Pd-BNC as compared to carvacrol oil. a) Full IR Spectrum of Pd-BNC vs oil. b) Zoomed in IR Spectrum of Pd-BNC vs oil.

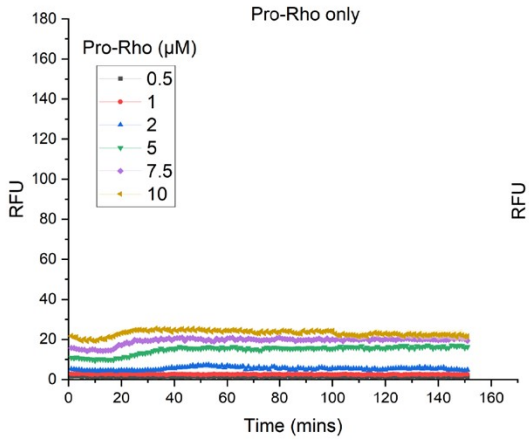
## 10. Catalytic Activity of Pd-BNC Nanocatalysts

The **Pd-BNC** activity was quantified by the activation of a rhodamine-based pro-dye. Briefly, different concentrations of pro-rhodamine solutions were prepared in phosphate-buffered saline (PBS) and then mixed with **Pd-BNC** in a black 96-well plate. Each experiment was done in triplicates. The Pd catalyst within the **Pd-BNC** mediated the cleavage of propargyl carbamate and allowed for the release of the fluorescent rhodamine molecule. The fluorescence generation was monitored using a Molecular Devices M2 microplate reader ($\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 521 nm, cutoff = 515 nm). **Pd-BNC** displayed a substrate inhibition kinetic model.

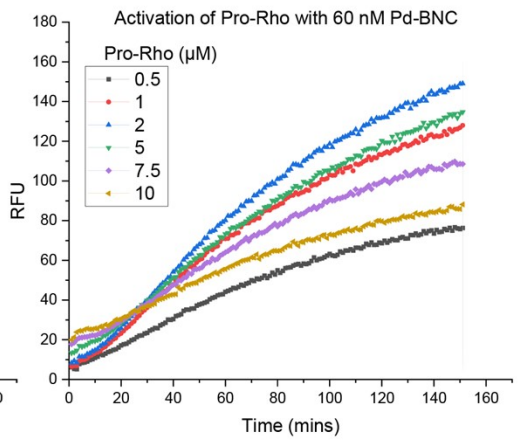



**Supplementary Figure S6.** a) Catalytic activity of **Pd-BNC** (60 nM polymer, 220 nM of Pd catalyst) in crosslinked and non-crosslinked state with P-Rho (2 μM). b) Catalytic activity of **Pd-BNC** (60 nM) compared to free catalyst dispersed in carvacrol oil with P-Rho (2 μM).

a)




b)



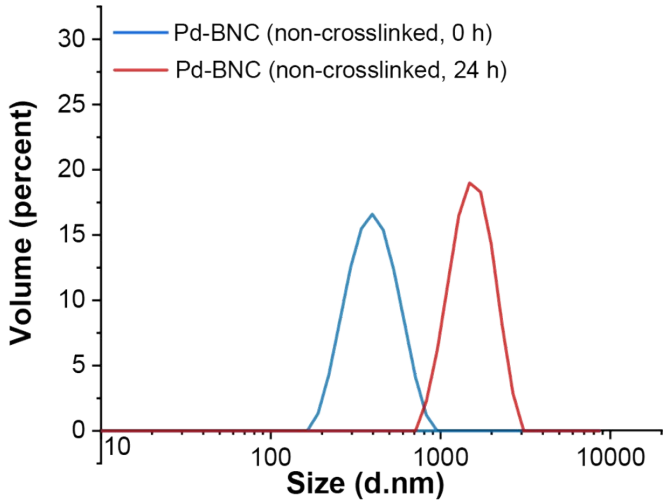

c) $K_m$ and $V_{max}$ calculations

Equation used: $Y=V_{max}*X/(K_m + X*(1+X/Ki))$

| | Velocity of reaction (μM/min) |
|---|---|
| Substrate inhibition | |
| Best-fit values | |
| **$V_{max}$** | **3.137** |
| **$K_m$** | **1.873** |
| $K_i$ | 2.214 |
| Std. Error | |
| $V_{max}$ | 0.608 |
| $K_m$ | 0.5272 |
| $K_i$ | 0.581 |
| 95% Confidence Intervals | |
| $V_{max}$ | 1.203 to 5.072 |
| $K_m$ | 0.1950 to 3.550 |
| $K_i$ | 0.3651 to 4.063 |
| | |
| Goodness of Fit | |
| Degrees of Freedom | 3 |
| $R^2$ | 0.9873 |
| Absolute Sum of Squares | 0.002896 |
| Sy.x | 0.03107 |
| Constraints | |
| $K_m$ | $K_m > 0.0$ |
| $K_i$ | $K_i > 0.0$ |
| Number of points | |
| Analyzed | 6 |

**Supplementary Figure S7**. a) Pro-Rho only at different concentrations. b) Catalytic Activation of **Pd-BNC** with Pro-Rho at different concentrations. c) $K_m$ and $V_{max}$ calculations.

## 11. DLS analysis of non-crosslinked Pd-BNC



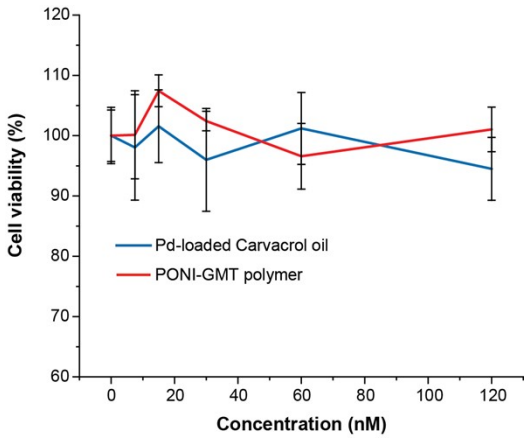

**Supplementary Figure S8.** DLS analysis of the non-crosslinked **Pd-BNC** at 37 °C. The size of **Pd-BNC** increased which indicates limited stability without the crosslinking initiated by DTDS.

## 12. Mammalian cell culture

T75 cell culture flasks were used for culturing all mammalian cells. Standard low-glucose growth media (DMEM supplemented with 10% FBS and 1% Pen-Strep) was used for HeLa cells while 3T3 cells and RAW 264.7 cells were cultured in high-glucose DMEM (supplemented with 10% FBS and 1% Pen-Strep) under physiological conditions (37 °C in a humidified atmosphere of 5% $CO_2$). Once the cells reached ~80-90% confluency, they were washed with phosphate-buffered saline (PBS) and trypsinized using 0.05% trypsin-EDTA solution. Further, the cells were collected, centrifuged, and resuspended in fresh culture media. Trypan blue was used to stain cells in a 1:1 ratio by volume and counted by hemocytometer for subsequent experiments.

## 13. Cytotoxicity studies

10,000 cells/well of 3T3 cells were plated in a 96-well clear plate and allowed to grow overnight. **Pd-BNC** at different concentrations were then incubated with the cells for 24h. The cells were then washed with PBS three times to remove dead cells and excess **Pd-BNC**. Cell viability was measured using Alamar blue assay which involved using 10% Alamar Blue reagent (Invitrogen) in serum containing media in each well and incubating for 3 h at 37 °C and 5% $CO_2$. The supernatant from each well was then transferred to a 96-well black plate and the fluorescence intensity was then measured (Ex/Em: 560/590 nm) using a SpectraMax M2 microplate spectrophotometer. All concentrations were studied in triplicates.




**Supplementary Figure S9.** Viability of human fibroblast NIH-3T3 cells (ATCC CRL-1658) after 24 h exposure to Pd-loaded carvacrol oil or PONI-GMT polymers.

## 14. Hemolysis Assay

Human whole blood (pooled, mixed gender) was purchased from BioIVT Elevating Science and processed as soon as received. Red blood cells were collected through centrifugation at 5000 rpm for 5 min followed by washing 4 times with PBS buffer and then diluted in PBS to a final concentration of approximately 5% (v/v). **Pd-BNC** were serially diluted using PBS and incubated in 96-well plates (200 µL/well). The blood cell suspension (20 µL/well) was added to each well and the plates were incubated at 37 ˚C for 1 h while shaking at 150 rpm PBS and Triton X-100 (0.1%) served as negative and positive controls, respectively. After incubation period, the mixture was centrifuged at 3000 rpm for 7 minutes and 120 µL of supernatant was transferred to a new 96-well plate. The absorbance was recorded at 560 nm in each well, and hemolysis was calculated using the following formula:

$$\text{Hemolysis} = \frac{OD\ 560_{Sample} - OD\ 560_{PBS}}{OD\ 560_{Triton} - OD\ 560_{PBS}} X\ 100$$

## 15. Cytokine response using qRT-PCR

Cells were plated in 24-well plates at a density of 50000 cells/well. Cells were treated with different concentrations of **Pd-BNC** for 48 h. Following treatments, approximately 1.5 µg RNA was harvested from cells using the PureLink RNA Mini Kit (Ambion) following the manufacturer's instructions. SuperScript IV Reverse Transcriptase was used for the conversion of approximately 150 ng of RNA to cDNA, along with RNaseOut, 10 mM dNTPs, and 50 µM Random Hexamers (ThermoFisher, Pittsburgh, PA), also following the manufacturer's instructions.

RT-PCR was performed on cDNA as prepared above using a CFX connect real-time system with iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA). All DNA primers were purchased from Integrated DNA Technologies (Caralville, Iowa). The following primer sequences were used:

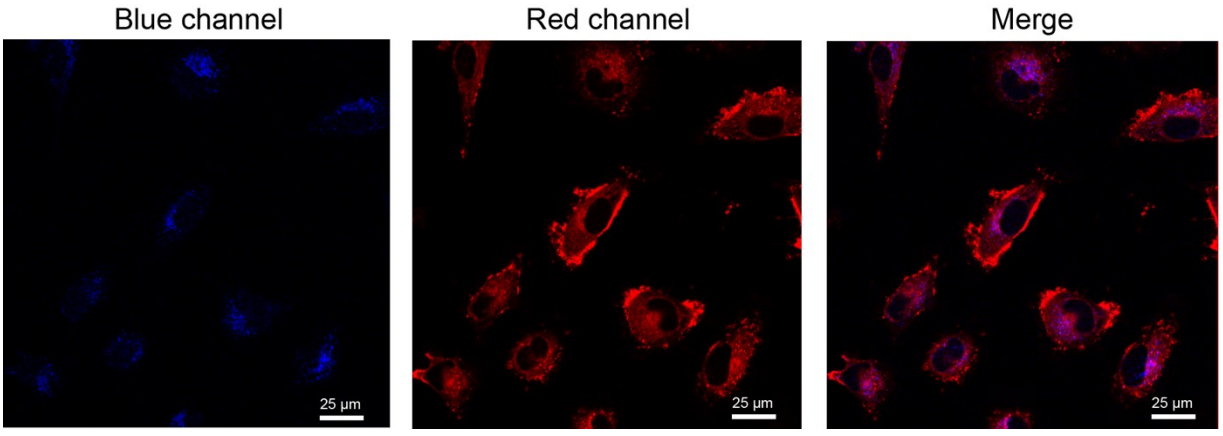
β-Actin (forward) 5'-GATCAGCAAGCAGGAGTACGA-3',
β-Actin (reverse) 5'-AAAACGCAGCGCAGTAACAGT-3',
iNOS (forward) 5'-GTTCTCAGCCCAACAATACAAGA-3',
iNOS (reverse) 5'-GTGGACGGGTCGATGTCAC-3',
TNF-α (forward) 5'-CCTGTAGCCCACGTCGTAG-3',
TNF-α (reverse) 5'-GGGAGTCAAGGTACAACCC-3'.

Analyses were performed as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min. Then denaturing occurred at 95 °C for 30 s followed by annealing at 57 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the $C_t$ value of the gene of interest to that of the β-actin housekeeping gene, by the $2^{\Delta\Delta Ct}$ method. Three biological replicates were performed for each control group and three technical replicates were used for each biological replicate.

## 16. Intracellular uptake of Pd-BNC and pro-fluorophore activation

30,000 cells/well of HeLa cells were seeded in a 4-well chamber Lab-Tek II chambered coverglass plates and were kept for 24 hrs to reach confluency. The cells were then washed with PBS and further incubated with Cy5-labelled **Pd-BNC** (60 nM) for 24 hrs. Next, the media was removed, and cells were washed with PBS three times to remove any excess **Pd-BNC**. Pro-rhodamine (50 μM) in fresh media was then added to cells and further incubated for 6 hrs. The cells were then washed once with PBS and were imaged by confocal microscopy on a Zeiss LSM 510 Meta microscope by using a 40× objective. Confocal microscope settings were as follows: green channel, $\lambda_{ex}$ = 488 nm and $\lambda_{em}$ = BP 505–530 nm; red channel, $\lambda_{ex}$ = 543 nm and $\lambda_{em}$ = LP 650 nm. Emission filters: BP, band pass; LP, high pass.

For LysoTracker studies, LysoTracker Blue DND-922 (Invitrogen) was incubated at 100 nM with cells for 30 min before microscopy experiments to confirm endocytosis.



**Supplementary Figure S10.** Confocal Microscopy images of HeLa cells incubated with Cy5-labelled **Pd-BNC** (60 nM) stained with Lysotracker Blue DND-922 (100 nM). Co-localization of Lysotracker stain with Cy5 from the polymer in **Pd-BNC** confirms endosomal uptake. (Scale bars = 25 μm).

## 17. Intracellular prodrug activation

10,000 cells/well of HeLa cells were seeded in a 96-well plate 24 h before the experiment. Next day, cells were washed with PBS once and incubated with **Pd-BNC** (60 nM polymer, 220 nM of Pd catalyst) for 24 hrs. Further, cells were washed with PBS three times and treated with Pro-Dox at different concentrations (2, 5, 7.5, 10 μM) for 24 hrs. After that cells were completely washed off with PBS and 10% Alamar blue in serum-containing media was added to each well and incubated for 3 h at 37 °C. Cell viability was then determined by measuring fluorescence at $\lambda_{ex}$ = 560 nm and $\lambda_{em}$ = 590 nm using SpectraMax M2 plate reader.

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2. M. A. Miller, B. Askevold, H. Mikula, R. H. Kohler, D. Pirovich and R. Weissleder, *Nat. Commun.*, 2017, **8**, 15906.
3. J. Li, J. Yu, J. Zhao, J. Wang, S. Zheng, S. Lin, L. Chen, M. Yang, S. Jia, X. Zhang and P. R. Chen, *Nat. Chem.*, 2014, **6**, 352–361.