Supporting Information

Methods

Preparation of NanoPALs

NanoPALs were prepared by the freeze-thaw method. The lipid and PS cocktail, DPPC/DOTAP /Chol/mPEG-2000 (2:0.3:1:0.15) and verteportfin (50 to 100 nmoles) were used to prepare nanoPALs. To achieve maximum accumulation of payload in cells within 1 hour of incubation/injection, we minimized the molar ratio of PEG (0.15 mole %), as decreasing the PEG component will aid in improved cellular uptake, leading to more efficient PDT. A thin lipid film of the above cocktail was generated using the thin film evaporation method. Briefly, using a rotary evaporator, the mixture was heated to 41°C under constant pressure until the chloroform was evaporated. Any residual amount of the solvent was removed by drying the film under high vacuum for at least 2 hours. The lipid film was hydrated with bevacizumab-AF488 (labeling with AlexaFlour-488 dye conjugated as per the protocol provided by Invitrogen) or bevacizumab in PBS (6-9 μM) and the final volume was made up to 1 mL with PBS (without Ca and Mg) at 41°C. The lipid film was then subjected to 5 freeze-thaw cycles (the number of freeze-thaw cycles were optimized to minimize stress on the antibody while ensuring maximum association with the carrier). The hydrated multilamellar liposomes were then extruded through a 100 nm pore size polycarbonate extrusion membrane (prewetted in the PBS) at 41°C to yield homogenously dispersed unilamellar vesicles. The extrusion set up was maintained at 41°C to promote the liquid phase of the liposomes. The extruded sample was allowed to incubate at room temperature for 1 hour before the next step. The unassociated bevacizumab-AF488 from the crude sample was removed from the associated bulk using a Float-A-Lyzer®G2 dialysis tube, MWCO: 300kD (Molecular wt of bevacizumab-152 kD). The dialysis tubes were prewetted in DI water overnight and stabilized in PBS (without Ca and Mg) before loading the samples. The samples were dialyzed overnight at 4°C.

Physicochemical characterization of nanoPALs

A 200x dilution of the crude and dialyzed samples were analyzed for particle size and zetapotential using dynamic light scattering with a Zetasizer (Malvern instruments, Worcestershire, UK) in isotonic NaCl solvent (Table-1). The loading efficiencies and concentration of encapsulated BPD (excited at 405nm) and AF488 (excited at 488nm) were measured using a FloroMax-3 (JobeinYvon, Horiba) after the lysis of liposomes in 1% Triton X-100 in PBS. The AUC's obtained were interpolated into the standard curve to calculate the final concentrations. Each measurement was taken in triplicates and the mean of at least four formulations is reported. The concentration of unlabeled bevacizumab used for the therapeutic efficacy studies *in vitro* and *in vivo* was determined by standard BCA assay. Malachite Green Phosphate Colorimetric Assay (POMG-25H) was employed to determine the lipid content before and after column purification (data not shown).

TEM Analysis of NanoPALs

The NanoPALs were examined in a Philips CM10 transmission electron microscope (TEM) (Eindothoven, The Netherlands) and images were captured with the AMT-XR41M 4 Mpixel, cooled CCD camera and digital imaging system (Advanced Microscopy Techniques, Danvers, MA). Briefly, the NanoPALs were formulated and the samples were diluted 50 times with PBS. For those samples which required the PDT administration, 1 $J \cdot cm^{-2}$ light dose (similar to *in vitro* dose) was administered and immediately processed so that there is minimal reconstitution of the lipid bilayer after the insult. Silicon monoxide formvar-coated 200 mesh grid was used for imaging. Briefly the grids were washed with drop of filtered water for 1 min and blotted dry with filter paper. A 10 µL aliquot of the sample was pipetted onto the grid for 1 min and blotted dry followed by 1% phosphotungstic acid staining and the grid was allowed to dry at room temperature. A drop of filtered water was pipetted again and allowed to dry at room temperature

prior to imaging the nanoPALs. The TEM micrographs were captured using AMT camera system (100 kV) using a magnification of 52,000x with further digital magnification of 343,000x.

Shelf-life stability studies

The stability of nanoPAL formulations was evaluated over a period of 60 days. After extrusion and initial dialysis overnight, the aliquots were stored under nitrogen at 4°C for 60 days. The stability here is defined in terms particle size, zeta potential and retaining capacity of both BPD-MA (verteporfin) and bevacizumab-AF488 by nanoPALs. The physicochemical characteristics were measured as mentioned above after storing the samples for 1, 30 and 60 days under similar conditions. The incorporation efficiency was measured after 1 day, 30 days and 60 days following the procedure mentioned above

Determination of release profile of NanoPAL

The release studies were carried out based on dialysis, modified for nanoPALs. Briefly, a volume of 0.5 mL of nanoPALs diluted with equal volumes of either PBS or human serum and loaded into a Float-A-Lyzer® G2 (3.8 cm in length and MWCO of 300 kD) with a cellulose ester membrane. The dialysis bag was suspended in 60 mL of PBS without Ca and Mg, maintained at 37 ± 0.5°C and was constantly rotated at 200 r.p.m using suitably sized, Teflon-coated magnetic stir bar in the beaker. At predetermined time intervals of 0.5, 1, 2, 3, 4, 18, 24, 48 and 72 hrs, 200 µL aliquots from the release media (in beaker) were sampled and replaced with 200uL of fresh PBS kept at 37°C. The BPD and bevacizumab-AF488 concentrations were quantified using fluorescence measurements, and all the experiments were done in duplicates.

Measuring the monomeric state of BPD

Aggregation of BPD, which is hydrophobic, decreases the concentration of monomeric BPD and compromises photophysical activity due to quenched fluorescence, intersystem crossing and population of the triplet state, which impedes the generation of photo-generated cytotoxic species. To measure the proclivity of BPD aggregation in nanoPAL as compared to Visudyne, an FDA approved liposomal BPD formulation used to treat macular degeneration, the fluorescence intensities of both formulations in serum were tested before and after dissolution in methanol (in order to rule out differences in BPD fluorescence signal due to discrepancies in loading efficiencies, the absolute concentration of BPD in both samples ranged from 50–80 μM). The BPD quenching factor was established after overnight incubation of formulations in 10% serum. The quenching factor was determined as follows.

$$\text{BPD quenching factor in serum} = \frac{\text{AUC in methanol-AUC in PBS}}{\text{AUC in PBS}}$$

Western blot studies

Western blots of bevacizumab treated at varying temperatures and an anti-rabbit IgG antibody was used to detect bevacizumab on the membrane. The bevazicumab binding capacity to hVEGF-A (45kDa, cell signaling technology, cat #8065) at 1:80,000 was determined at different exposure times. The bevacizumab preparations incubated at varying temperatures were used as primary antibody to detect hVEGF-A protein on the gel. Each lane with sample is followed by pre-stained protein ladder to help identify the molecular weight of VEGF on the gel.

In vitro cell association studies

Briefly, 10^5cells per well were seeded in a 24 well plate on sterile coverslips for 24 hours before adding the nanoPALs. The cells were incubated with the nanoPALs for either 1 hr or 24 hrs after which the excess unassociated nanoPALs were removed by two washes with fresh PBS. The remaining cells were fixed with 1% formalin (200 µL) and kept at 37°C for 15 min. The coverslips were given three PBS washings (3 min each) until the cells were free of fixing agent. The coverslips were mounted onto the glass slide using Gold fade-antifade solution with DAPI and the edges were sealed using nail polish. The images were taken using an Olympus FV1000 confocal microscope (Olympus America, Inc., Central Valley, CA). The lasers lines used for imaging Avastin-AF488 (green) was 488 nm, and a 405 nm line was used for BPD (red) and DAPI (blue). The emission monochromators and filters were centered at 530, 690 and 458 nm for AF488, BPD and DAPI, respectively, with a 10-20 nm band pass window. All the images were captured with a 40x water objective. The merged images are reported in the following sections.

In vitro cytotoxicity assay

All percent cell viability studies were carried out in a 24 well plate (10^5 cells/mL). Cells were exposed to free drug or liposomal drug for 1 hr after which PDT was administered. The equivalent dose of BPD and bevacizumab used for all the *in vitro* studies was 250 nM and 25 nM, respectively. The light dose was 1 $J \cdot cm^{-2}$ with a fluence of 50 $mW \cdot cm^{-2}$. Wells were washed with PBS to remove unbound liposomes and cellular debris, and the cells were fixed to the plate by adding 500 µl of 1 mg/mL MTT into each well followed by incubation at 37°C for 1 hour where MTT reagent was reduced to formazan. This was measured by dissolving bound formazan with equal volumes of DMSO in all wells before measuring the absorption at a

wavelength of 570 nm with a SpectraMax M5 (Molecular devices, CA). Percent of cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \frac{\text{Flourescence intensity of treated cell population}}{\text{Fluorescence intensity of untreated cell population}} X\ 100$$

The percent of cell viability was determined and plotted as a function of concentration of drug.

Cellular association studies – Multi-color flow cytometery analysis

After addition of the nanoPALs and other controls, the cells were incubated under standard cell culture conditions for a further 1 hr or 24 hrs in the dark. The culture media was removed and cells washed three times with PBS and 200 µL/well of 0.25% EDTA-trypsin was added to the cells for 10 minutes in the incubator. The resulting cell suspension was then centrifuged at 1,000 r.p.m for 5 min at 4°C in a bench-top centrifuge. The cell pellet was then resuspended in 500 µL of PBS and the resulting suspension was strained to remove any aggregates using 5 mL polystyrene round-bottom tube with cell-strainer cap bought from Becton Dickinson Labware (Le Pont De Claix, France). The flow cytometry analysis was performed using a FACSAria™ cytometer. For each sample, at least 10^4 events were acquired and the gating was determined from the unstained cell population and heat maps show the cell population by analysis[33] of AF488 (representing Bevacizumab uptake) and side scatter (representing BPD uptake) pseudo dot plots using FlowJo™ software (Tree Star, Inc; Ashland, OR). BPD fluorescence intensity was measured using the instrument's "AlexaFlour 430 channel" modified to collect red emission (Ex 430nm, Em 690 nm) whereas AlexaFlour 488-labeled bevacizumab fluorescence intensity was measured using the instrument's "FITC channel" (Ex 488nm, Em 530 nm).

Four-to-six-week-old male Swiss nude mice weighing approximately 20 grams (Cox Breeding Laboratories, Cambridge, MA) were anesthetized using a cocktail of 84 mg/kg ketamine and 12 mg/kg xylazine. AsPC-1 cells of low passage number (<20 passages) in monolayer were collected and re-suspended in 50% Matrigel and 50 µL containing 10^6 cells was injected subcutaneously into the right scapula region for the subcutaneous model. The tumors were allowed to grow until the volume reached 100 mm^3 (within 14 days), after which experiments and treatments began: including tumor response, H&E analysis and tumor distribution profiles of NanoPALs pre- and post-treatment. To develop an orthotopic tumor model, a minor surgery under aseptic conditions was performed on the nude mice and the cell suspension prepared as above was injected near the pancreas. The tumor was allowed to grow for 10 days after which treatments and imaging experiments were peformed.

Tumor response study

The tumor bearing mice were allocated into 7 different groups randomly before starting the treatment (Table 3). The formulations were injected and allowed an incubation period of 1 hr. Here, we chose to administer 0.5 mg/kg BPD and ~15 mg/kg bevacizumab via an *i.v* route, and administered a light dose of 75 $J \cdot cm^{-2}$ at (690 nm). The PDT doses were administered to various groups as shown in Table-2. The tumor was then irradiated with a fluence rate of 100 $mW \cdot cm^{-2}$. The mice were monitored on a daily basis to record changes in the body weight and tumor volumes. The length and width of s.c. tumors were measured with a digital caliper, and the tumor volume was calculated using the formula, ½ ($Length \cdot Width^2$). The absolute average tumor volumes and the percent average body weight changes of each group are reported here. For the orthotopic tumor response studies, mice were sacrificed on day 4 and the tumors were harvested to record tumor weights.

Histology (H&E Analysis)

The tumor bearing mice were injected with nanoPALs and allowed an incubation period of 1 hr. The tumor was then irradiated to achieve a fluence of 75 $J \cdot cm^{-2}$ with an irradiance of 100 $mW \cdot cm^{-2}$. The mice were then sacrificed on day 1 or day 3 after the PDT treatment, and the tumor tissue was collected and frozen in OCT compound. 5-µm-thick tumor tissue sections were mounted onto a clean slide using a Microm HM 550 cryostat (ThermoScientific, Pittsburg, PA) and H&E staining was performed as follows. The slides were air dried for 30 min, washed with DI water twice and fixed with alcoholic formalin for 1 min. The sections were then stained with hematoxylin for 5 min, followed by two washes with acetic acid. Bluing was done in 0.2% ammonia water or saturated lithium carbonate solution for 10s followed by a water wash and stained for eosin, dehydrated in 95% alcohol, 100% alcohol and citrisolv prior to placing the coverslip. The whole slide images were captured using a NanoZoomer RS (Hamamatsu, Rockville, MD).

In vivo Fluorescence Imaging and Analysis

Hyperspectral fluorescence images of freshly excised organs from PDAC mice were acquired using the CRi Maestro system before and after tail vein injection of fixed BPD (0.5 mg/kg) and Bevacizumab-Alexa Fluor 488 (15 mg/kg) doses for each formulation. BPD images were collected using a 445-490 nm bandpass excitation filter, a 515 nm longpass emission filter and a liquid crystal tunable emission filter (LCTEF), and the excitation collected from 520 to 800 nm in 10 nm intervals. For bevacizumab-Alexa Fluor 488, we used a 480-500 nm bandpass excitation filter, a 515 nm longpass filter and the LCTEF to acquire images from 520-800 nm in 10 nm intervals. A custom MATLAB routine was written to batch process the images, which performs linear spectral deconvolution pixel-by-pixel to break each spectrum into component spectra (auto fluorescence, bevacizumab-Alexa Fluor 488 conjugates and BPD). Basis shape spectra

for spectral unmixing were acquired as follows. (1) Auto fluorescence basis spectra (including the liver, spleen, skin, muscle, lung and tumor) were measured for a mouse prior to drug injection. (2) The basis spectra for BPD were measured from BPD dissolved in phosphate buffered saline with or without 2% TritonX-100, a detergent that enhances BPD solubility. (3) The basis spectrum of Bevacizumab-Alexa Fluor 488 was acquired *in vivo* from auto fluorescence-corrected images of the tail immediately following tail-vein injection of the antibody-dye conjugate (in the absence of BPD). To verify accurate spectral unmixing, the reduced chi-squared was calculated for the spectral fit at each pixel based on the noise statistics (a measured variance-signal curve) of the CCD camera and the fit residuals. All images were background corrected (to subtract dark current) and normalized by their exposure time for global comparison.

Staining protocol for serial tumor sections

Subcutaneous PDAC tumors from above were excised at various time points following tail vein drug injection, embedded in OCT compound and frozen at -80°C. A cryotome was used to cut 20-μm-thick cryosections. Sections were then (i) fixed in a pre-cooled mixture of 1:1 acetone: methanol for 15 minutes at -20°C, (ii) air dried for 30 minutes at room temperature, and (iii) washed three times, for 5 minutes each, in PBS with gentle agitation. A Pap Pen was used to encircle the tissue specimens for mounting small-volume blocking and staining solutions. A blocking solution (Dako Protein Block Reagent) was applied for 30 minutes at room temperature, followed by application of the immunostains, at ~5 μg/mL antibody each, diluted in a background reducing buffer (Dako Antibody Diluent, Background Reducing) for 2 hours at room temperature in a humidifying chamber. Finally, the slides were washed again three times, for 5 minutes each, in PBS with gentle agitation, mounted (Invitrogen SlowFade Gold with DAPI) with a coverslip and sealed with nail polish.

Excitation of DAPI and fluorescently labeled (Alexa Fluor 488, 568 and 647) bevacizumab (administered *in vivo*), anti-human cytokeratin 8 (CK8, clone LP3K, R&D Systems) and anti-mouse PECAM-1 (CD31; clone 390, Millipore) was carried out using 405, 488, 559 and 635 nm lasers, respectively. Tissue sections were stained as explained in the staining protocol. Lasers were scanned sequentially to reduce channel crosstalk. Mosaic images of entire tumor cross-sections were collected and stitched together using the Olympus FluoView software. The CK8 antibody selectively stains human cancer cells to define the tumor boundary. An automated fill of the CK8 channel, with CD31-positive pixels removed, gives a map of the extravascular tumor space.

Image analyses

All analyses were performed using custom MATLAB routines for batch processing. For display and all analyses, the channels were autothresholded to reject background signal using an intensity threshold to reject 99.5% of background (calculated from histograms of control, unstained images) for each channel. Wavelet transforms and image correlation coefficients amongst the color channels were used to eliminate bubbles and extraneous, reflective objects from the analyses. Statistical analysis of confocal imaging data (colocalization, percent permeation and mean fluorescence signal) amongst multiple treatment groups were analyzed using a Kruskal-Wallis one-way analysis of variance, ANOVA.

Supplementary Figure Legends

Figure S1

Determination of the suitable separation method for the nanoPALs by analyzing the changes in particle size (A), PDI (B), and the percent loading of BPD (C) and bevacizmab (D) before and after the separation. DRV represent nanoPALs synthesized by dehydration rehydration method and FT represents nanoPALs synthesized by free-thaw cycles. Both column purification and dialysis have been used as separation methods here. **p<0.01 represent significant differences as determined by ANOVA.

Figure S2

The confocal images show the qualitative uptake pattern of BPD (red) and bevacizumab-AF488 (green) by AsPc-1. Each image is a mosaic stitch of (3x3) x3 = 27 random fields on a single coverslip. 40X water objective was employed for imaging at zoom 3; pmt and laser intensity was maintained constant throughout the process. Cationic NanoPAL (B, C), Neutral NanoPAL (D, E), Anionic NanoPAL (F, G) were incubated with cells at an equivalent dose of 25nM Bevacizumab-AF488 and 250nM BPD for 1 hr (B, D, F) and 24 hrs (C, E, G). Visudyne + bevacizumab-AF488 cocktail incubated for 24 hrs was used as a control (A). Magnification bar: 100µm

Figure S3

Figures A and B represent the qualitative accumulation of nanoPAL; 1hr and 24hrs uptake pattern of BPD (red) and Bevacizumab-AF488 (green) in the orthotopic PDAC tumors is marked based on the reflected light images D and E respectively, (C) *In situ* whole tumors from each group were exposed and analysis of the hyperspectral fluorescence images was performed to

quantify accumulation of BPD and bevacizumab -Alexa Fluor 488.The y- axis indicates relative florescence units (RFU's) determined after batch processing (MATLAB analysis) and subsequent image J analysis of the tumors corrected for the auto florescence. The results are mean +/- s.d, n = 2 mice per group. Asterisks denote statistically significant differences compared to the corresponding treatment at 1 hr *P<0.05 (two way ANOVA and bonferroni post tests) (F) shows the tumor weights 4 days post PDT with nanoPAL precursors in each group. The results are mean +/- s.d, $n \geq 3$ mice per group. Asterisks denote statistically significant differences compared to the corresponding non treated mice, *P<0.05, ***P<0.001 (non parametric-two tailed t-test).

Figure S4

The percent temporal release profiles of (A) BPD and (B) Bevacizumab from nanoPALs. The results are mean +/- s.d.'s n = 2 per group. Asterisks denote *p<0.05 (t-test).

Supplementary Figure Captions

Figure S1: Optimization of purification method

Figure S2: Cellular association studies of nanoPAL

Figure S3: pilot studies to determine the nanoPAL tumor uptake timeline

Figure S4: release profile of cargo from NanoPALs