Supporting Information

Copper Prussian blue analogue: Investigation for multifunctional activities in biomedical applications

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1. EXPERIMENTAL PROCEDURES:

1.1. Materials

Potassium ferricyanide $K_3[Fe(CN)_{6)}]$, copper sulfate($CuSO_4$), manganese chloride ($MnCl_2$), nickel acetate ($Ni(OAc)_2$), cobalt acetate ($Co(OAc)_2$), zinc nitrate ($Zn(NO_3)_2$), silver nitrate ($AgNO_3$), ferric nitrate ($Fe(NO_3)_3$), cadmium nitrate ($Cd(NO_3)_2$), RNase, propidium iodide (PI), Triton-X, penicillin, streptomycin, kanamycin, fetal bovine serum (FBS), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide), Dulbecco phosphate buffer saline (DPBS), NBT/BCIP, sodium hydroxide (NaOH), hydrochloric acid (HCl), dimethyl sulfoxide (DMSO) and methanol (MeOH) were purchased from Sigma-Aldrich chemicals St. Louis MO, USA. Doxorubicin (Dox) was purchased from Alfa Aesar and used without further purification. A stock of solution of 5 μg/mL doxorubicin was prepared with Milli-Q water and used for the fabrication of nanoconjugates and cell culture experiments. All antibodies were purchased from Cell Signaling Technologies, USA.

1.2. Cell lines

CHO: Hamster ovary cell line, A549: human lung cancer cells; SKOV3: human ovarian cancer cells and B16F10: mouse melanoma cell lines have been purchased from ATCC. HUVEC: human umbilical vein endothelial cells were purchased from Lonza. ECV-304 cells were a kind gift from Dr. V. Shah, Chair, Gastroenterology and Hepatology Department, Mayo Clinic, Rochester, MN, USA.

1.3. Synthesis of **M-PB$_{(x-y)}$***NPs nanoparticles*

Similarly, we have synthesized the different analogues of Prussian blue such as $[Cd_3\{Fe(CN)_6\}_2]$,[1] $Mn_3[Fe(CN)_6]_2$,[2] $Zn_3[Fe(CN)_6]_2$,[3] $Fe_4[Fe(CN)_6]_2$,[4] $Ag_3[Fe(CN)_6]$,[5] $Co_3[Fe(CN)_6]_2$,[6] $Ni_3[Fe(CN)_6]_2$ [7] from 10^{-3} (M) of potassium ferricyanide $K_3[Fe(CN)_{6)}]$ with 10^{-3}(M) of cadmium nitrate ($Cd(NO_3)_2$), manganese chloride ($MnCl_2$), zinc nitrate ($Zn(NO_3)_2$), ferric nitrate ($Fe(NO_3)_3$), silver nitrate ($AgNO_3$), cobalt acetate ($Co(OAc)_2$), nickel acetate ($Ni(OAc)_2$, respectively according to published literatures.

1.4. Stability study of CPB$_{(1-1)}$ NPs at different pH solutions

Among three nanocomplexes [$CPB_{(1-1)}$NPs, $CPB_{(2-1)}$NPs and $CPB_{(1-2)}$NPs], $CPB_{(1-1)}$NPs has been considered as optimized nanoparticles based on stability and release kinetics study. Therefore, detailed characterization study including biological application have been carried out with $CPB_{(1-1)}$NPs. The as-synthesized $CPB_{(1-1)}$NPs were incubated with phosphate buffers having pH = 5, 7.4 and 10 by adjusting the volume of NaOH/HCl and the incubation was allowed to keep upto 2 weeks. The size and charge of the nanoparticles in phosphate buffers were measured by DLS instrument.

1.5. Conjugation of Doxorubicin with **CPB$_{(1-1)}$***NPs*

The $CPB_{(1-1)}$NPs based drug delivery system ($CPB_{(1-1)}$-Dox) containing Dox was fabricated by incubation of $CPB_{(1-1)}$NPs with Dox (5µg/ mL w.r.t 1 mL of $CPB_{(1-1)}$ NPs solutions) at RT under vigorous stirring. The as-synthesized nanoconjugates ($CPB_{(1-1)}$-Dox) were purified by ultra-centrifugation under 17,600 rpm.at 20 °C for 40 minutes. The intense brownish yellow colored loose pellet of $CPB_{(1-1)}$-Dox was collected and used for further characterizations and *in vitro* experiments.

1.6. Preparation of standard curve of Dox and quantification of Dox in $CPB_{(1-1)}$*-Dox*

A series of doxorubicin solutions (1µg/ mL to 25 µg/ mL) were prepared in the supernatant of $CPB_{(1-1)}$NPs, obtained after ultracentrifugation (discussed before). A standard curve of doxorubicin was prepared by taking the UV absorbance of Dox at λ_{max} = 492 nm vs the concentration of Dox (µg/ mL). The absorbance of Dox (considered as unknown solution) in the supernatant of $CPB_{(1-1)}$-Dox, can be measured by UV visible spectroscopy. The concentration of Dox in unknown solution i.e. $CPB_{(1-1)}$-Dox supernatant can be measured by comparing the absorbance of unknown solution with the standard curve of Dox. Finally, % attachment of Dox in loose pellet of $CPB_{(1-1)}$-Dox was calculated.

1.7. Release of Dox in PBS

The loose pellet (around 2 mL), obtained after ultracentrifugation of 200 mL of as synthesized $CPB_{(1-1)}$-Dox and other Prussian blue nanoanalogues conjugated with Dox under 17,600 r.p.m. at 15 °C for 40 minutes was divided into several parts containing 100 µL. Each 100 µL of loose pellet ($CPB_{(1-1)}$-Dox) was mixed with 900 µL of DPBS (pH = 7.4) and incubated for different time points from 0 min to 14 days. The resultant solution was incubated for different time points. Later, the incubated solution with different time points was centrifuged using a spin-win centrifuge (LABOCENE, Scan speed: 1730R) at 10,000 rpm for 10 min at RT. The absorbance of each supernatant obtained after centrifugation was measured at 492 nm using UV visible spectrophotometer and plotted against the corresponding time points.

1.8. Cell culture experiments

Normal cells (CHO & ECV) and cancer cell lines (MCF-7, B16F10 and SKOV3) were cultured in DMEM (Dulbecco's Modified Eagle Medium) media supplemented with 10% fetal bovine

serum (FBS), 1% antibiotics (penicillin-streptomycin) and 5% L-glutamine in a humidified 5% CO_2 incubator at 37 °C for all *in vitro* experiments. HUVEC cells were cultured in EBM complete media at with 5% CO_2 at 37 °C. The nanoconjugates used for *in vitro* cell culture experiments after UV irradiation inside the hood for 10-15 minutes.

1.9. Cell viability assay using MTT reagent

Initially (10 x 10^3 cells/well) were seeded in 96 well plate and kept for 24 hours in the incubator. Cell viability assays of HUVEC, ECV-304, CHO, MCF-7, B16F10 and SKOV3 cells were performed with (i) $CPB_{(1-1)}$NPs, (ii) Dox and (iii) $CPB_{(1-1)}$-Dox for 24-48 hours in a dose dependent manner (1 to 5 μM with respect to Dox) using MTT reagents according to our published protocol.[8] Results were expressed as % viability = {[A570 (treated cells) - background]/ [A570 (untreated cells) - background]} x 100. The detailed procedure has been discussed in the ***Supporting Information***.

1.10. Cell cycle assay using Fluorescence activated cell sorting (FACS)

B16F10 cells (5 x 10^5 cells/well) were seeded in 60 mm petridish before the FACS experiment. The cells were incubated with (i) $CPB_{(1-1)}$NPs, (ii) pristine Dox, and (iii) $CPB_{(1-1)}$-Dox (2.5μM w.r.t. Dox) for 48 hour on next day at 70% confluency of cells. FACS analysis of untreated B16F10 cells and cells treated with Dox and $CPB_{(1-1)}$-Dox were performed using PI staining in a flow cytometer (FACS Canto II, Becton Dickinson, San Jose, CA, U.S.). The data were analyzed with FCS Express V3 software.[8]

1.11. Western Blot Analysis

B16F10 cells were lysed by using RIPA buffer (containing 1X protease inhibitor cocktail) after treatment with (i) $CPB_{(1-1)}$NPs, (ii) pristine Dox, and (iii) $CPB_{(1-1)}$-Dox (2.5 μM w.r.t. Dox) for 48 hour and total protein concentration was estimated by Bradford assay. Equal amount of protein (30 μg) of all samples were loaded in 15% denaturing SDS-PAGE and transferred to the nitrocellulose membrane (Thermo scientific). The membranes were then blocked 5% non-fat dry milk for 1-2 hours, and subsequently washed and incubated with primary antibodies (anti-caspase-3 at 1:500, anti-Bax at 1:1000, anti-p21 at 1:1000 and GAPDH 1:1000 dilutions) in TBST with at room temperature for 2-3 hours. Unbound primary antibodies were washed by TBST for 3 times and the membrane was incubated with secondary antibodies (alkaline phosphatase conjugated goat antibodies) for 1 hour. Protein bands were developed in the membrane by colorimetric method using BCIP/NBT substrate as according to our standard protocol.[9]

1.12. Transmission electron microscopy for cellular internalization

B16F10 cells were grown in a T75 flask, and for each treatment two flasks were used. When the cells were 60-70 % confluent the cells were treated (i) $CPB_{(1-1)}$NPs, and (ii) $CPB_{(1-1)}$-Dox (2.5 μM w.r.t. Dox) for 4 hours. The dose of $CPB_{(1-1)}$NPs and $CPB_{(1-1)}$-Dox with respect to pellet volume is 10 μL/mL: where, the concentrations of copper and iron in the loose pellet were Cu: 4.37 μg/μL & Fe: 2.82 μg/μL. Cells were trypsinized, washed two times with PBS. The cells were fixed using 2.5% glutaraldehyde and washed with PBS followed by post-fixation with 1% osmium tetroxide. Then, the samples were dehydrated through a varied % of acetone and embedded in epoxy resin.[9] Finally, they were polymerized at over 60 °C. Thin sections were incised on an ultramicrotome (Leica, Germany), placed on a mesh of copper grids and stained

with 2 % uranyl acetate, followed by counter staining with lead citrate. TEM images were visualized on a HITACHI H7500 (Hitachi, Japan) transmission electron microscope, operating at 80 kV.[9]Also, we have carried out EDAX analysis by using high resolution transmission electron microscopy (HRTEM) (Model: FEI TECHNAI G2 200 kV S-twin) to determine the elemental analysis inside the B16F10 cancer cells.

1.13. Fluorescence quenching of Dox

25 mL of metal Prussian blue analogues [$CPB_{(x-y)}$NPs] nanoparticles (where 'M' be Mn, Cu, Co, Ni, Fe, Zn and Cd) were incubated with Dox (5 μg/mL with respect to. the volume of NPs solution) for 0-10 minutes and fluorescence spectrum of individual solution was taken by fluorescence spectroscopy. The graph for each solution was plotted against fluorescence intensity vs the wavelength. Also, Dox was mixed with $CPB_{(1-1)}$NPs in increasing doses and fluorescence intensity was measured to check the quenching profile of Dox (λex = 490 nm and λem = 500-700 nm) as a concentration dependent manner.

1.14. Fluorescence release profile of Dox in DPBS

The loose pellet (around 2 mL), obtained after ultracentrifugation of 200 mL of as synthesized $CPB_{(1-1)}$-Dox under 17,600 rpm. at 15 °C for 40 minutes was divided into several parts containing 100 μL. Each 100 μL of loose pellet ($CPB_{(1-1)}$-Dox) was mixed with 900 μL of DPBS (pH = 7.4) and incubated for different time points from 0 min to 14 days. The resultant solution was incubated for different time points. Later, the incubated solution with different time points was centrifuged using a spin-win centrifuge (LABOCENE, Scan speed: 1730R) at 10,000 rpm for 10 min at RT. The fluorescence of each supernatant obtained after centrifugation was

measured at λex = 490 nm and λem = 500-700 nm using fluorescence plate reader and plotted against the corresponding time points.

2. Characterization techniques

2.1. UV-visible spectroscopy:

The ultraviolet and visible absorption spectrum of $CPB_{(1-1)}$NPs was recorded with UV-visible spectroscopy (JASCO) dual-beam spectrophotometer (Model V-570) in a quartz cuvette from 800 nm to 200 nm with a resolution of 1 nm .In which 1ml of the reaction mass (before centrifugation) was measured.

2.2. X-Ray diffraction method:

The brownish intense loose pellets of as-synthesized $CPB_{(1-1)}$NPs samples were obtained by centrifugation at a 17,400 rpm at 15^0C for 45 minutes in Thermo scientific, Sorvall-WX ultra 100. The glass slide was coated with this loose $CPB_{(1-1)}$NPs pellet by evaporating the solvent repeatedly and submitted for XRD analysis. The structure and phase purity of the as-synthesized $CPB_{(1-1)}$NPs samples were determined by X-ray diffraction method (XRD) analysis using a Bruker AXS D8 Advance Powder X-ray diffract meter (using CuKαλ=1.5406 Å radiation).

2.3. Transmission Electron microscopy (TEM)

For the analysis of shape, size and its structural morphology of the chemically synthesized $CPB_{(1-1)}$NPs were characterized on by a FEI Tecnai F12 (Philips Electron Optics, Holland) instrument operated at 100 kV. From the pictures the particles are mono dispersive with unique morphology was observed. IMAGEJ software was used to measure the particle distribution.

2.4. Dynamic light scattering (DLS)

The radius of synthesized $CPB_{(1-1)}$NPs was calculated from a Malvern Instruments Ltd, Zetasizer Ver. 6.20 and Serial number: MAL1004428. 100 μL of as-synthesized $CPB_{(1-1)}$NPs solution was

taken in the sample chamber for the measurement of the size. Zeta potential of $CPB_{(1-1)}$NPs solution was measured in zeta potential analyzer using quartz cuvette taking 20 µL of $CPB_{(1-1)}$NPs in 1mL of deionized water.

2.5. Fourier transformed infrared spectroscopy (FTIR):

By the stretching and bending frequencies in the FTIR spectra we can characterize the functional groups present in the $CPB_{(1-1)}$NPs nanoparticles. The Fourier transformed infrared (FTIR) spectra were recorded using thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets. In which the loose pellet was coated on glass plate and evaporated water completely and submitted to FT-IR analysis.

2.6. Thermo gravimetric analysis:

The thermal stability of $CPB_{(1-1)}$NPs and its Dox conjugate ($CPB_{(1-1)}$-Dox) was explained by using their thermo gravimetric analysis. TGA was recorded using on TGA/SDTA851^{c}, from METTLER TOLEDO, Switzerland at 10^{o} C/minute from 25-800^{o}C under N_2 atmosphere.

2.7. ***Inductively coupled plasma optical emission spectrometry (ICP-OES):***

An inductively coupled plasma optical emission spectrometer (ICP-OES, IRIS intrepid II XDL, Thermojarrel Ash) had used to calculate the concentration of Cu and Fe in the in pellet. The loose pellet (100ul) was dissolved in 50 ml of milli-Q water. Then finally calculated the concentration of metals (Fe, Cu, K) present in the 100 µl pellet, from this information we had determined the chemical composition present in the resultant $CPB_{(1-1)}$NPs.

2.8. Brunauer–Emmett–Teller (BET)

The BET instrument Quadra Sorb Station 3 (verson 5.06) was used to measure the surface area, pore size and pore diameter of $CPB_{(1-1)}$NPs and $CPB_{(1-1)}$-Dox. As synthesized $CPB_{(1-1)}$NPs and its doxorubicin conjugated $CPB_{(1-1)}$-Dox were dried by lyophilizer and submitted ~50 mg of each

sample to BET analysis. From this analytical data we have explained doxorubicin is conjugated to $CPB_{(1:1)}$NPs. Further to measure the surface area, pore size and pore diameter of other analogues of Prussian blue nanoparticles Micromeritics TriStar II 3020 (Version 2.00) was used.

Table S1. Reaction conditions for the synthesis of $CPB_{(x-y)}$NPs (x,y=1-2) and their comparative study.

Expt No.	**Sample name**	**10^{-3}M $K_3Fe(CN)_6$ (mL)**	**10^{-3}M $CuSO_4$ (mL)**	**Molar ratio**	**Total Volume (mL)**	**Size from TEM (nm)**	**Hydrodynamic diameter (nm)**	**Zeta Potential (mV)**
1.	$CPB_{(1-1)}$	25	25	1:1	50	30-75	87.2 ± 2.7	- 28.6 ± 10.7
2.	$CPB_{(2-1)}$	33.4	16.6	2:1	50	40-130	178.5 ± 5.6	- 12.2 ± 4.73
3.	$CPB_{(1-2)}$	16.6	33.4	1:2	50	50-140	511.6 ± 7.8	- 25.6 ± 4.32

SI-Table-2: Stability studies of $CPB_{(1-1)}$-Dox in DPBS with respect to hydrodynamic diameter measured by DLS.

Hydrodynamic diameter (0 days in DPBS)	Hydrodynamic diameter (7 days in DPBS)	Hydrodynamic diameter (14 days in DPBS)
89.2±4.7	91.5±1.3	98.2±2.4

SI-Table: 3

BET surface area, pore size and pore volume of different Prussian blue analogues:

S.NO	Complex code	Pore size (A^o)	BET surface (m^2/g)	Pore diameter distribution (Avg. width) in A^o
1	$CPB_{(1-1)}NPs$	161	315	1018-17
2	$CPB_{(1-1)}$-Dox	112	358	954-17
3	AgPB	81	22	2107-17
4	CdPB	5	22	1912-17
5	MnPB	151	59	1466-17
6	ZnPB	269	2	1901-18
7	NiPB	23	545	686-17
8	CoPB	22	448	1752-17
9	FePB	80	141	1449-17

SI-Table-4: Cell viability difference of $CPB_{(1-1)}NPs$, DOX and $CPB_{(1-1)}$-Dox treated MCF-7 cells between 24 and 48 hours.

Sample treated with	Concentration	% viability (24 hours)	% viability (48 hours)	Difference between % viability
$CPB_{(1-1)}NPs$	1 µM	107	101	N/A
	2.5 µM	93	99	N/A
	5 µM	100	100	0
DOX	1 µM	93	100	N/A
	2.5 µM	103	98	5
	5 µM	103	71	32
$CPB_{(1-1)}$-Dox	1 µM	100	91	9
	2.5 µM	94	59	35
	5 µM	79	46	33

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