Evaluation of a Novel Platinum(II) Based AIE Compound-Encapsulated Mesoporous Silica Nanoparticles for Cancer Theranostic Application

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Abstract

Advanced biomedical research has established that cancer is a multifactorial disorder which is highly heterogeneous in nature and responds differently to different treatment modalities, due to which constant monitoring of the therapy response is becoming extremely important. To accomplish this, different theranostic formulations has been evaluated, however, most of them was found to suffer from several limitations extending from poor resolution, radiation damage, to high costs. In order to develop a better theranostic modality, we have designed and synthesized a novel platinum(II)-based 'aggregation induced emission' (AIE) molecule (named BMPP-Pt) which showed strong intra-cellular fluorescence and also simultaneously exhibited potent cytotoxic activity. Due to this duel functionality, we wanted to explore the possibility of using this compound as a single molecule based theranostic modality. The compound was characterized using elemental analysis, NMR and IR spectroscopy, mass spectrometry and single crystal X-ray structure determination. The compound was found to exhibit high AIE property with emission maxima at 497 nm. For more efficient cancer cell targeting, BMPP-Pt was encapsulated into mesoporous silica nanoparticles (Pt-MSNPs) and the MSNPs were further surface modified with anti-EpCAM aptamar (Pt-MSNP-E). Pt-MSNP exhibited higher intracellular fluorescence compared to free BMPP-Pt, though both of them induced similar degree of cell death via apoptosis pathway, possibly via cell cycle arrest at G1 phase. Anti-EpCAM aptamar modification was found to increase both cytotoxicity and intracellular fluorescence compared to the unmodified MSNPs. Our study showed that the EpCAM functionalized BMPP-Pt loaded MSNPs can efficiently internalize and induce apoptosis of cancer cells as well as show strong intracellular fluorescence. This study provides cues towards development of a potential single compound based theranostic modality in future.

Introduction

Due to advancement in many key technologies and development of novel treatment modalities, diagnosis and treatment of cancer has improved significantly over the past few decades. Nevertheless, it is still a formidable task to follow the tumour growth and treatment responsiveness in real time for an improved understanding of the disease and to take a better clinical decision for the choice of therapy. The idea of cancer theranostics is a relatively recent development and was introduced around 2010,[1] mainly to address the requirement for personalized medicine. Personalized onco-treatment depends heavily on real time monitoring of the treatment response, so that treatment modulation can be done at appropriate time. The integration of therapeutic and diagnostic agents to produce a theranostic technology provides a powerful means for simultaneous and real time monitoring of therapeutic responses. As of current clinical scenario, the imaging part of any theranostic approach is restricted to MRI, PET and CT technologies. Although these technologies have wide spread applications, it is sometime difficult to distinguish between normal tissue or malignant tissue using these techniques.[2] There are several classes of luminescent materials that have been tested for diagnostic purpose, especially, organic dyes and fluorescent proteins. However, organic dyes suffer from poor water solubility which restricts them from usages in bio-imaging applications. Also, organic fluorophores suffer from photo bleaching, low fluorescence intensity and intrinsically small Stokes shift which produce scattered light interferences.[3] Quantum dots (QDs) have also been widely used in bio-imaging due to their high photo-stability owing to the inorganic nature, high quantum yield and broad absorption band.[3-5] Nonetheless, QDs suffer from few disadvantages - surface defects can affect the recombination of holes and electrons and create temporary traps, which results in blinking and is undesired in bio-imaging [6]. QDs also suffer from high amount of nonspecific adsorption via electrostatic interactions, even this was found to be true for PEG (Poly ethylene glycol)-containing nanoparticles making them undesirable for selective targeting.[6, 7] Also, most luminescent materials exhibit strong luminescence in their diluted solutions, but this tends to be weakened or quenched at high concentrations, a phenomenon known as 'Aggregation-Caused Quenching (ACQ)'.[8] This quenching effect limit their application in targeted imaging as with better targeting, their concentration in the targeted tissue will increase, reducing their luminescence, and

hampering the purpose of diagnosis. In 2001, Tang and co-workers [9] discovered an exactly opposite phenomenon to ACQ which was observed in hexaphenylsilole and the phenomenon is known as 'Aggregation Induced Emission' (AIE). The aggregate formation increased their fluorescence quantum yields by more than 300 fold, transforming them into strong emitters in the solid state. Often the mechanism behind the AIE molecule is found as the 'Restriction of Intra-molecular Rotation' (RIR).[9, 10] When fully solvated and excited AIE molecule undergoes non-radiative decay by rotation of aromatic bonds, allowing the energy to decay without any emission. In contrast, in the solid state the intramolecular rotations are restricted causing the molecules to decay *via* radiative channels and thus showing strong luminescence upon excitation. Since 2001, AIE-based materials have been widely investigated for various biological applications including biological probe, immunoassay markers, PAGE visualization agents, bio-imaging and so on. As AIE complexes exhibit strongly enhanced fluorescence emission with increase in their concentration, it can be a perfect choice for tissue targeted imaging.

Nanoparticle delivery systems have demonstrable advantages, including extension of circulating half-life, passive accumulation at tumour sites due to the enhanced permeability and retention (EPR) effect, active targeting of cancer cells, reduced toxicity, and integration of multiple functionalities in a unified entity.[1, 11, 12] Among various nano-delivery systems used, mesoporous silica nanoparticles (MSNPs) have several advantages over others including well dispersity and tailorable size and structure, which ensures controllable *in vivo* pharmacokinetics and predictable outcome.[13] MSNPs are more flexible, versatile, and robust than conventional nano-delivery systems such as polymer nanoparticles, liposomes, etc. The manufacturing process of MSNPs is also relatively simple and economic, which is important to fulfil the future clinical demand and the commercialization. Although nanoparticles have been extensively explored for targeted delivery of many different drugs and other bioactives, however, till date very few reports have been published on AIE encapsulated nanoparticles for bio applications.

Here we report the design and synthesis of a platinum(II) based AIE molecule [Bis(diphenylphosphino)methane phenyl pyridine platinum(II) chloride, depicted henceforth as BMPP-Pt]. In the initial experiments, it has been proved that this molecule

exhibited strong fluorescence intensity as well as potent cytotoxic activity. To improve its cellular delivery, we encapsulated the compound into MSNPs (named as Pt-MSNP) and modified the surface of the MSNPs with an aptamar against Ephithelial Cellular Adhesion Molecule (EpCAM) for cancer cell targeting (named as Pt-MSNP-E). We evaluated its cytotoxic potency, mode of action, cellular bio-imaging, and potential for application as a single compound based theranostic modality.

Materials and methods

Materials: Potassium tetra-chloropalatinate(II), 1,2-Bis(diphenylphosphino)methane, 2-phenylpyridine, 2-ethoxyethanol, Tetraethoxysilane (TEOS), 3′-glycidoxypropyltrimethoxysilane (GOPS) and CetylTrimethyl Ammonium Bromide (CTAB) were purchased from Sigma Aldrich Chemical Company Ltd. N-Hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) have been procured from TCI. The toluene, dichloromethane, ethylacetate and hexane have been procured from Merck. Anti-Ephithelial Cellular Adhesion Molecule (aEpCAM) DNA aptamer was procured from IDT (Integrated DNA Technologies). The sequence for the same is 5'-CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA TGG GGG GTT GGC CTG-3'. The aptamer sequence was selected following previous studies by Li *et al* 2017. [14]

Characterizations: ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded in a 400 MHz Bruckner NMR spectroscope. UV-VIS absorption spectra were recorded in a Simadzu Spectrophotometer (model UV-1800 and 2550). Steady state photoluminescence (PL) spectra were recorded on Horiba Jobin Yvon Spectrofluorometer (FluoroMax-4). The solid state quantum yield of the thin film sample was measured using a calibrated integrating sphere in a Gemini Spectrophotometer (model Gemini 180).

Synthesis: Synthesis of [Pt(ppy)(ppyH)(Cl)] (ppyCl-Pt) (ppy = 2-phenylpyridine) (Scheme 1): The intermediate complex, ppyCl-Pt was synthesized by following our previous published work.[15] K_2PtCl_4 (0.30 g, 7.2mmol) and 2-phenyl pyridine (0.280g, 18.1mmol) were dissolved in 4 ml of water which was kept in a microwave vial for 10 min under microwave irradiation at 100°C. After 10 min, a green colored precipitate was

obtained and it was separated from water, dried under vacuum oven for 15 min. The crude product was recrystallized from ethanol resulting a green coloured solid product, Yield, (0.360 g, 92%). ^{1}HNMR spectra of ppyCl-Pt, (400 MHz, Chloroform-d): δ 9.63 (d, J = 6.0 Hz, 1H), 9.26 (d, J= 5.7 Hz, 1H), 8.10 (d, J = 5.2 Hz, 2H), 7.96 (t, J = 7.8 Hz, 1H), 7.74 (t, J = 7.6 Hz, 1H), δ7.64 (s, 1H), δ 7.52 (d, J = 7.8 Hz, 2H), 7.36 (dt, J = 20.8, 6.9 Hz, 4H), 7.08 (t, J = 6.7 Hz, 1H), 7.03 – 6.97 (m, 1H), 6.89 (t, J = 7.4 Hz, 1H), 6.22 (t, 1H), 6.20 (d, 1H). ^{13}C NMR spectra of ppyCl-Pt, (101 MHz, $CDCl_3$): δ 167.2, 162.3, 154.3, 151.2, 144.2, 141.0, 139.9, 138.4, 137.7, 130.8, 129.7, 129.6, 129.2, 129.0, 127.9, 127.4, 127.3, 123.9, 123.2, 123.0, 121.7, 118.0.

Synthesis of BMPP-Pt (Scheme 1): It was prepared by following the technique as reported by our group earlier.[15] To a stirred solution of ppyClPt (0.1g, 0.00019mmol ,1equivalent) in DCM (6 mL), chelate phosphine [bis(diphenylphosphino)methane] (0.071g, 1 equivalent) was added and the reaction mixture was stirred for 2 minutes to complete the reaction. The crude product was purified by column chromatography using 60-120 silica mesh. Green solid, Yield(0.135g, 95%) 1H NMR (400 MHz, CDCl3) δ 8.36 (s, 1H), 8.15 (td, J = 8.1, 1.4 Hz, 1H), 8.01 (d, J = 8.1 Hz, 1H), 7.98 – 7.87 (m, 4H), 7.84 – 7.65 (m, 5H), 7.60 – 7.40 (m, 12H), 7.28 – 7.22 (m, 1H), 7.19 (t, J = 7.8 Hz, 1H), 7.06 – 6.99 (m, 1H), 6.95 (t, J = 7.2 Hz, 1H), 4.92 (t, J = 10.5 Hz, 2H).13C NMR (101 MHz, Chloroform-d) δ 167.0, 152.3, 147.5, 141.7, 138.4, 133.9, 133.8, 133.4, 133.3, 132.8, 132.5, 131.3, 130.0, 129.9, 129.6, 129.5, 126.6, 125.0, 124.6, 120.5. 31P NMR (162 MHz, CDCl3) δ -25.7, -25.9.

Synthesis of Pt-MSNPs: 0.150 g (0.00027mM) of CTAB was dissolved in 75ml of milli Q water. Then, sodium hydroxide (2M in milli Q water, 0.5 ml) and BMPP-Pt in THF (0.030 g, 0.000039mM) was introduced to the CTAB solution dropwise sequentially. The mixture was stirred at room temperature for five hours and then the temperature of it was adjusted to 80°C. Tetraethoxysilane (TEOS, 1 mL) was added dropwise to the reaction mixture containing surfactant with vigorous stirring. The mixture was allowed to react for 12h which produced a white precipitate. This solid crude product was filtered off, washed with de-ionised water and UV grade methanol for several times and dried it in vacuum drying oven at 35°C for 2h and obtained a pure product, Pt-MSNPs.

Synthesis of Pt-MSNP-GOPS: A suspension of 0.3g of Pt-MSNPs (mesoporous silica nanoparticles) in toluene (25 ml) was mixed with 0.4 ml of 3-glycidoxypropyltrimethoxysilane (GOPS) and the mixture was refluxed at 70°C for 24h, washed it with toluene and methanol for several times followed by drying at 60°C.

Synthesis of Pt-MSNP-GOPS-E[16]: The mixture of 1.5mg of Pt-MSNP-GOPS, 20µl of anti-EpCAM aptamer (1M) and 250µl of KOH (1M) was dissolved in 1ml of milliQ water was incubated for 1h. Then, the reaction mixture was washed with phosphate buffer resulting Pt-MSNP-GOPS-E, abbreviated as Pt-MSNP-E henceforth.

Preparation of solutions of BMPP-Pt to prove AIE activity: 10^{-5}M stock solution of BMPP-Pt was prepared in DCM. Four 5 ml glass tubes were taken and labelled them as 0%, 30%, 50%, 60%, 70%, 80% and 90%. 0.5 ml stock solution was added to each of the labelled tubes. Then, the hexane with the following amounts, 0ml, 1.5ml, 2.5ml, 3.0ml, 3.5ml, 4ml and 4.5ml were added to 0%, 30%, 50%, 60%, 70%, 80% and 90%, labelled tubes, respectively so that the total volume of each glass tube turned into the same total volume (5 ml).

Single crystal X-ray structure

A single crystal X-ray diffraction data set was collected on a Bruker AXS Kappa Apex II diffractometer equipped with an Oxford Cryosystem 700 Plus liquid nitrogen based cooling device. The data set was recorded at 100K using a combination of φ and ω scans to obtain a data set complete up to 78.4° in 2θ. Data reduction and corrections were done using APEX II software suite (Bruker AXS). The crystal structure was solved using direct methods (SHELXS97) available in the SHELXTL suite and the structure was refined by full matrix least squares refinement process using SHELXL97.

Computational details

The Density Functional Theory (DFT) based computations for BMPP-Pt was carried out using Gaussian09 program suit. The required structures were generated with Gauss View visualization program. All the calculations were performed at B3LYP (the Becke three parameter hybrid exchange and the Lee-Yang-Parr correlation functional)

level of theory. The particular choice for B3LYP method was based on the accuracy of calculations at this level reviewed through literature. The initial structures were optimized at same level of theory (*i.e.*, B3LYP), where Pt(II) atom was defined at LanL2DZ (with effective core potential, ECP) basis sets and all electron 6-31G (d,p) basis sets were used for lighter atoms. Use of LanL2DZ basis set with ECP was a common practice for systems containing atoms of higher atomic numbers (in case of present calculations it is Pt(II)) because the formalism approximated chemically inert core electrons to be frozen and significantly reduced the computational robustness of the Ab initio calculations without compromising the accuracy. Subsequently, frequency analysis was performed on the respective structure and absence of any imaginary frequency confirmed the global energy minima in the potential energy surface (*i.e.*, most stable structure) for the complex. The solvent parameters are incorporated through SCRF calculations via IEFPCM model for dichloromethane.

Cell culture

Human hepatocellular carcinoma cell line, Huh7 (kind gift from Dr Soma Banerjee) were cultured at 37°C, 5% CO_2, in Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100UmL^{-1} penicillin, 100µgmL^{-1} streptomycin (Invitrogen) was added to the culture medium. Cells were typically grown to 60–70% confluency, rinsed in phosphate-buffered saline (PBS; Invitrogen) and placed into fresh medium prior to treatments.

***In vitro* cytotoxicity assay**

In-vitro cytotoxicity was performed as described previously by Chowdhury *et al*.[17] Briefly, cells were cultured in 96 well plates. After 24h, cells were treated with different treatments for specific period of time. Thereafter, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) (SRL) was added to each treated and control well and incubated for 4h. Formazan crystals were solubilized in DMSO (dimethyl sulfoxide) and readings were obtained at 570nm with a differential filter of 630nm using Multiscan Microplate Spectrophotometer (Thermo Scientific). Percentage of viable cells was calculated using the following formula: Viability (%) = (mean absorbance value of drug-

treated cells) / (mean absorbance value of control) ×100. A concentration of 0.2% DMSO was found to be non-toxic and was used for dissolving BMPP-Pt, and used as control in cytotoxicity experiments.

Microscopic imaging and internalization

For microscopic imaging, cells were cultured overnight on coverslips in 6cm culture dishes, and were treated for 24h. Coverslip cultured cells were washed with 0.1 M PBS and fixed in methanol at -20°C for 10min. The coverslips were mounted with antifade mountant containing DAPI (4'-6-diamidino-2-phenylindole) (Thermoscientific) on glass slide, which were visualized by confocal fluorescence microscope using FITC ($\lambda_{ex/em}$ 490/520) and DAPI ($\lambda_{ex/em}$ 372/456) filters. Cellular uptake was further confirmed by flow cytometer; in brief, the Huh7 cells after MSNPs exposure were trypsinized, centrifuged and resuspended in PBS. The light side scatter intensity and shift in green fluorescent peak intensity was measured using flow cytometry (CytoFLEX, Beckman Coulter). Analysis of acquired data was performed using CytExpert software. For cell receptor blocking experiment, cells were treated with anti-EpCAM aptamer for 6h before nanoparticle treatment and then internalization of the aptamer tagged nanoparticles were quantitated through flow cytometry.

Cell cycle analysis

For detection of cells at different phases of the cell cycle, they were seeded at a density of 1×10^6, grown overnight and exposed to different treatments for 24h. Following incubation, cells were harvested and fixed in 70% ethanol for 24h at -20°C. Thereafter, the cells were centrifuged at 1500rpm and the cell pellet obtained was re-suspended in PBS. Then propidium iodide (PI, 20µg ml^{-1}) was added and the dye added mixture was incubated in dark for 30min before events were acquired in a flow cytometer (CytoFLEX, Beckman Coulter). Percentage of cells in each phase of cell cycle was calculated and plotted in bar diagram.

Flow cytometric analysis of apoptotic cells

For determination of apoptosis, Huh7 cells were seeded in 6cm dishes at a density of 1×10^6 cells. The cells were then exposed to various treatments. Thereafter, the cells were harvested, washed with PBS and re-suspended in 500µL of 1X binding buffer (BD BioSciences). Thereafter, 4µl of Annexin-V-FITC and 10µl of Propidium Iodide (PI) were added to the cells in binding buffer, followed by incubation in dark for 20min. The samples were then acquired using flow cytometer (Cytoflex, Beckmann Coulter) and analysis of acquired data was performed using CytExpert software. To detect both early and late apoptotic cells, the percentage of cells in lower and upper right (LR and UR) quadrant representative of only Annexin-V and both Annexin-V/PI positive cells respectively, were counted; and the cells in upper left quadrant representing necrotic cells was also added. A fold increase in dead cells was represented through bar diagram.

Statistical analysis

The obtained data were analyzed using the Prism® software (Version 5.01; GraphPad Software Inc., USA). The effect of various treatments was statistically analyzed using one-way ANOVA test and the level of $p < 0.05$ was considered as statistically significant. All data points represent the mean of independent measurements. Uncertainties were represented as standard deviations in the form of bars.

Results and Discussion

Till date, several theranostic formulations have been prepared and tested at various level of pre-clinical and clinical development stage. However, the expected clinical outcome has not been achieved due to various factors. As of current clinical scenario, the imaging part of any theranostic approach is heavily dependent on MRI, PET and CT technologies. These technologies involve using magnetic resonance, radioactive tracer or X-ray. However, the spatial sensitivity (such as the margin of a tumour) of these techniques is low, leading to difficulty in distinguishing between normal or malignant tissue.[2] Use of fluorescence molecule, on the other hand, can specifically differentiate between normal and fluorescent-tagged tissue. Tumour targeted delivery of a highly fluorescent molecule can specifically denote the tumour tissue and they can help monitoring the tumour size and location, leading to a better understanding of the treatment response. Among different

fluorescent molecules available, AIE molecules have obvious advantages over others with respect to higher fluorescence intensity and less fluorescence decay. Although many different AIE molecules have been developed, AIE has not been explored extensively for tumour theranostic application. Few reports are found where AIE compounds have been used for tumour detection. Huang et al.[18] has developed a tetraphenylethene based AIE molecule, which showed cell-labeling property with low toxicity. However, no tissue specificity was shown in that report. Yuan et al. [19] prepared a bio-orthogonal fluorescence turn-on probes based on AIE. It exhibited targeted cancer cell imaging, however, they need a metabolic glycol-engineered cancer cells for the binding of the dye with the cell, making practical applications challenging. Also the different strategies that have been tested before for tumour theranostics, all of them depend on two different molecular entities for therapy and diagnosis. However, two different molecules can have disparate physicochemical and bio-pharmaceutical properties, which may lead to uneven pharmacokinetics and tissue distribution, jeopardizing the sole purpose of theranostics. Single molecule based theranostic modality can be a significant advantage over the existing strategies.

Based on this hypothesis, we wanted to develop a single compound based theranostic modality, where the same molecule can be utilized for anti-cancer treatment as well as for diagnosis. We prepared a Pt(II) based AIE molecule in a two step reaction-firstly, ppyCl-Pt (Scheme 1) was prepared by reacting K_2PtCl_4 (0.30 g, 7.2 mmol) and 2-phenyl pyridine (0.280 g, 18.1 mmol) in a microwave stimulated reaction. The crude product was purified by re-crystallization in ethanol and characterized by NMR spectroscopy (^1H and ^{13}C). The yield was found to be 92%. In the second step, ppyCl-Pt was reacted with chelate phosphine [bis(diphenylphosphino)methane] (Scheme 1) to obtain BMPP-Pt. Purification of BMPP-Pt was performed using column chromatography with 60-120 silica mesh. The final product was characterized by NMR spectroscopy. The final yield was 95%. In the final compound (BMPP-Pt), all the aromatic proton signals are observed at δ= 6.8 - 8.5 ppm and two protons of P-CH_2-P are appeared at δ=4.8 ppm (**Fig. S1**†). All the aromatic carbons signals are observed at δ= 124-169 ppm and one carbon of P-CH_2-P is appeared at δ= 120.49 ppm for BMPP-Pt. The two phosphorous signals are observed at -25.6 and -25.9ppm (**Fig. S2**†). From HRMS data, the major fragmented peak appeared as $[M-Cl]^+$ at m/z 733.1502 (**Fig. S3†).** The suitable single crystals of BMPP-Pt

have been grown from the slow evaporation of methanolic solution. The obtained single crystal based structure exactly matches with the chemical structure of BMPP-Pt (**Scheme 1**). Interestingly, Ph_2P-CH_2-PPh_2 ligand chelating with Pt(II) forming a four membered ring. Important bond distances and bond angles of BMPP-Pt are shown in **Table 1**. We have optimized the geometry of BMPP-Pt using Gaussian 09 software. The optimized structure of HOMO is mainly located on chloride ion and LUMO located on phenylpyridine and platinum(II) (**Fig. 1**). The energy gap between HOMO to LUMO is 3.39 eV which is well matching with the observed value, 3.14 eV (394 nm) (Fig. **1**). BMPP-Pt was found to be well soluble in dichloromethane (DCM), insoluble in hexane and these are chosen to investigate the AIE property of BMPP-Pt. The photoluminescence (PL) spectra of the series of solutions (0-90%) in DCM/hexane have been recorded and observed gradual enhancement of emission intensity with increasing concentration of DCM (**Fig. 2**). In the range of 0% to 70%, no detectable emission intensity change was found presumably because of low aggregate formation, whereas from 70% to 90% the emission intensity is very high because of higher extent of aggregate formation. Six short contacts has been identified where two of the phenyl substituents are involved short contact interactions with the phenylpyridine of neighboring molecule [(**Fig. S4†)**, C28(A)-H10(B) (~2.876Å), C28(B)-H10(A) (~2.876Å), C35(A)-H29(B) (`~2.704Å), C35(B)-H29(A) (~2.704Å), C10(A)-H36(B) (~2.747Å)]. As this compound is highly emissive in solid state and having amphiphilic nature, hence it can be considered for applications in bio-imaging.

As the water solubility of BMPP-Pt was very poor, it is challenging to develop a delivery system of this compound. We have prepared BMPP-Pt encapsulated mesoporus silica nanoparticles (Pt-MSNPs) to eliminate the solubility problem as well as to make a tumour-targeted delivery system of BMPP-Pt. Different nano-formulations have been developed to provide increased safety and efficacy for cancer therapy. Among them, mesoporous silica nanoparticles (MSNPs) have attracted substantial attention due to their advantageous structural properties, which have made them applicable for diverse biomedical applications including bio-imaging for diagnostics, biosensing, biocatalysis, scaffold engineering, drug delivery etc. as well as easy functionalization with targeting ligand [20, 21]. For encapsulation of BMPP-Pt in MSNPs, first a miceller solution of BMPP-Pt was prepared using CTAB as the surfactant (**Scheme 2**). In the miceller solution of

CTAB−BMPP-Pt, tetraethoxysilane (TEOS) was then introduced to polymerize into mesoporous silica. The solid crude product was filtered, washed with water and methanol for several times and vacuum dried to yield Pt−MSNPs (**Scheme 2**). This BMPP-Pt encapsulated MSNPs was further modified by conjugating with anti- EpCAM aptamer to the surface. To conjugate with the aptamer, initially the surface of Pt-MSNPs was modified with glycidoxypropyl trimethoxy silane (GOPS) that results in Pt-MSNP-GOPS and then anti-EpCAM aptamer was added to the aqueous solution of Pt-MSNP-GOPS, incubated for one hour and washed with phosphate buffer to produce the anti-EpCAM aptamer conjugated BMPP-Pt loaded MSNPs (named as Pt-MSNP-E; **Scheme 2**). After the synthesis of Pt-MSNPs, Pt-MSNP-GOPS and Pt-MSNP-E, these have been characterized by FTIR, DLS, Zeta potential and TEM.

The FTIR spectra of Pt-MSNPs and Pt-MSNP-GOPS**,** as shown in **Fig. S5** have common peaks, which are characteristic to mesoporous silica at 1032 cm^{-1} (Si-O-Si stretching), aliphatic C-H stretching peaks at 2925 cm^{-1} and 2856 cm^{-1} due to CTAB. The peak observed at 721 cm^{-1} due to the epoxy group present in Pt-MSNP-GOPS. Furthermore, the absence of peak such as the broad absorption peak due to –OH (3485 cm^{-1}) group in Pt-MSNP-GOPS when compared with Pt-MSNPs show successful modification of surface with GOPS. Further Pt-MSNP-E shows in the IR spectrum two peaks at 3336cm^{-1} and 1635cm^{-1} which are attributed to -OH and C=N stretching frequencies respectively. These two new peaks and disappearance of epoxy peak at 721cm^{-1} strongly supporting the conjugation of anti-EpCAM aptamer on the surface of mesoporous silica. We further studied the size and shape of the different MSNPs with transmission electron microscopy (TEM). All the particles exhibited perfect spherical shape. Both blank MSNP and BMPP-Pt loaded MSNP showed a diameter in the range of ~100nm while after anti-EpCAM aptamer conjugation diameter of the particles increased to ~200nm. (**Fig. 3**). The increase in size supports the occurrence of sequential addition of GOPS and aptamer on Pt-MSNPs. The EDX results show the total nitrogen content in Pt-MSNP-GOPS and Pt-MSNP-E are 0.84 and 2.04 (in wt%), respectively. The increased nitrogen concentration further supported the successful attachment of anti- EpCAM on the surface of Pt-MSNP-GOPS **(Fig. S9, Fig. S10, Table. 3 and Table. 4)**. Also, in the TEM image, the core of the blank

MSNPs appeared to be less dense than that of the Pt-MSNP or Pt-MSNP-E. This may be due to the fact that the core of the blank MSNPs were made up of the empty micelle, whereas the core of the BMPP-Pt loaded MSNPs had significant quantity of the Pt compound in the core. We also quantified the surface charge of different MSNPs by measuring the zeta potential of the particles. The results obtained from Zeta potential show the obtained surface charge on the MSNPS, MSNPs-Pt, Pt-MSNP-GOPS and Pt-MSNP-E are -1.90, -1.99, +3.03 and +0.105, respectively (**Fig. 3d**). This observation is in well support by the presence of surface groups in blank MSNPs, MSNPs-Pt, Pt-MSNP-GOPS and Pt-MSNP-E are hydroxyl, hydroxyl, epoxy and then aptamer, respectively.

Pt-MSNPs, Pt-MSNP-GOPS and Pt-MSNP-E were found to be showing green emission in water with emission maxima at 479, 478 and 479nm, respectively (**Fig. S6†**). After the synthesis of Pt-MSNPs**,** we investigated the total loading of BMPP-Pt into the MSNPs by the help of UV-VIS spectroscopic study. To estimate the total amount of BMPP-Pt that has been incorporated into the mesopores of mesoporous silica, the absorbance of a set of known concentration solutions of BMPP-Pt (in the range of 5μg/mL to 25μg/mL) has been recorded and then plotted the absorbance value (at λ_{max} = 233nm) vs. concentration. Thereafter, 1 mg of Pt-MSNPs and 10 μl of HCl were added into 2ml of DCM in a round-bottomed flask, stirred it for overnight and then recorded the absorbance spectra. It was expected that all the incorporated Pt(II) compounds would be expelled out from the mesopores into the solution. The concentration of this unknown solution has been determined through extrapolation into the calibration curve as stated earlier and the amount is determined to 28 μg of BMPP-Pt / mg Pt-MSNP (**Fig. S7†, Table. 2**)

Analysis of cellular uptake of Pt-MSNPs

In the last few years, cancer research has focused on the optimization of the clinical methodologies to better target the tumour through development of new therapeutic strategies. In this context, MSNPs have shown considerable promise as potential versatile drug delivery vehicles [20, 21]. However, till date, the major hindrance with MSNP-based therapy has been identifying and tagging of fluorescent molecules that can track the NPs, as well as ones that can regulate their target-specific entry. In this study, we have encapsulated the BMPP-Pt complex in MSNPs. The BMPP-Pt is an ‘aggregation induced

emission' compound and hence is a promising candidate as fluorescent tracker that can be loaded in MSNPs and monitored or used for cancer diagnosis. In our study, to confirm the internalization of AIE active BMPP-Pt complex, we exposed Huh7 liver cancer cells to free BMPP-Pt for 24h. Confocal microscopy and flow cytometric analysis revealed that these compounds are internalized, though the fluorescent signal was weak **(Fig 4a)**. Thereafter, to evaluate whether encapsulation of BMPP-Pt into MSNPs can enhance their cellular internalization property, Huh7 liver cancer cells were treated with BMPP-Pt and Pt-MSNPs and were monitored for their relative fluorescence in comparison to free BMPP-Pt compounds. Interestingly, both confocal microscopic analysis and flow cytometric detection of intra-cellular green fluorescence showed an increased fluorescence signal in Pt-MSNPs as compared to free BMPP-Pt or untreated control in Huh7 cells after exposure of 24h **(Fig 4a and b)**. Cells treated with free BMPP-Pt exhibited only weak signal, whereas BMPP-Pt encapsulated MSNPs showed strong intracellular green signal and an increased shift in peak (FL1) compared to free BMPP-Pt when analyzed through flow cytometry, signifying better internalization efficiency of the Pt-MSNP compound compared to free BMPP-Pt **(Fig 4b)**. It has been observed in multiple studies that MSNP encapsulation enhance cellular uptake of different active compounds.[22-24] The increased internalization efficiency and their diagnostic potential further prompted us to investigate and compare the cytotoxic property of Pt-MSNP with BMPP-Pt.

Pt-MSNPs induce cytotoxicity and apoptosis in Huh7 cancer cells

The cytotoxic potential of the BMPP-Pt compound was evaluated in the Huh7 cells through MTT assay **(Fig 5a)**. The compound was found to be highly cytotoxic, apart from its intra-cellular AIE property that was evident from the previously discussed results. This allowed us to consider the BMPP-Pt compound as potential theranostic agent which could be utilized for both detection and treatment of cancer cells. As the previous data showed that encapsulation of BMPP-Pt into MSNPs enhance their cellular uptake, we compared the cytotoxic potential of free BMPP-Pt to that of MSNP encapsulated BMPP-Pt. Both free BMPP-Pt and Pt-MSNPs showed considerable cytotoxic effect on Huh7 cells **(Fig 5a and b)** with IC_{50} values with both the treatment was ranging around 1μM. Blank MSNPs show only very low level of cell death, that also at a very high concentration **(Fig. S8†)**. Next we

wanted to analyze the mode of action of BMPP-Pt for the cytotoxicity. We evaluated whether the cytotoxicity observed with BMPP-Pt treatment was due to necrosis or apoptosis. Necrosis happens when the cells die due to physical or chemical assault and is characterized by inflammation and tissue damage whereas apoptosis, also known as programmed cell death, happens due to metabolic dysfunction in a cell and usually no inflammation or tissue damage occurs in this, making apoptosis the pathway of choice for eliminating cancer cells. The percentage of apoptotic and necrotic cell population was quantified by flow cytometric analysis with Annexin-V/Propidium Iodide (PI) staining after treatment with free BMPP-Pt or Pt-MSNPs at equal dose of Pt (IC_{50}) for both the cases. Interestingly, free BMPP-Pt treatment induced an augmentation in necrotic cell population (~18.5%; PI positive and Annexin-V negative) **(Fig 5c)**. This might be attributed to the high cytotoxic potential of free BMPP-Pt as also analyzed through MTT assay: sudden exposure to a highly cytotoxic compound produced chemical damage to the cell, triggering its necrosis. Importantly, treatment with MSNP encapsulated BMPP-Pt reduced necrotic cell death and induced an increase in number of cells undergoing apoptosis (~80%; PI negative and Annexin-V positive as well as PI positive and Annexin-V positive) **(Fig 5c)**. Slow release of the BMPP-Pt from the MSNPs may protect the cells from sudden damage and instead induce programmed cell death. Hence, based on the results it can be concluded that MSNP encapsulation reduces the cytotoxic potency of BMPP-Pt complex and preferentially induces apoptosis in Huh7 cancer cells which might be of potential therapeutic benefit.

Most of the apoptosis inducing drugs act by inhibition of cell cycle. After demonstrating treatment with Pt-MSNP resulted in apoptotic cell death, next we wanted to find out whether the apoptosis induced by Pt-MSNP was cell cycle dependent. Cell cycle analysis was done after treatment with free BMPP-Pt or Pt-MSNP at equal dose of Pt (IC_{50}) for both the cases followed by PI staining. With both the treatment, a significant increase in sub-G1 population (BMPP-Pt- ~6%; Pt-MSNP- ~12%) was observed compared to control (~0.45%) (**Fig. 5d**). Sub-G1 population signifies the presence of dead cells (fragmented or hypo-diploid DNA content), corroborating the cytotoxicity data. Notably, with Pt-MSNP treatment, a significant increase in G1 population (~55%) was also detected compared to BMPP-Pt (~37%) or control (~34%) **(Fig 5d)**, whereas other than increase in

sub-G1 population (dead cells), no significant difference was found between control and BMPP-Pt treated cells. This data indicates that treatment with free BMPP-Pt induced cell cycle independent necrosis whereas Pt-MSNP treatment resulted in cell cycle arrest at G1 stage followed by apoptosis. In this context, it has been demonstrated that the clinically approved highly successful anticancer drug cisplatin exerts its cytotoxic effects via similar mechanisms only.[25-27]

Anti-EpCAM aptamer functionalized Pt-MSNPs show increased internalization and cytotoxicity

The silica nanoparticles, especially those with mesopores, have attracted the interest of the scientific community due to their potential to be applied in the nanomedicine field.[28] When compared to the organic nanocarriers, MSNPs are more resistant to pH, temperature variations and also to mechanical stress, which renders them an improved capacity to protect the drug cargo when in contact with body fluids.[20, 21] Here we observed that the MSNPs can be loaded with BMPP-Pt complex which can have both cytotoxic as well as diagnostic property. Because of its well acclaimed property in the nanomedicine field, and supported by our results discussed above, we were interested in functional modification of the Pt-MSNP complex with cancer cell specific targeting molecules. The objective was to develop a single compound based theranostic agent which can be targeted specifically to cancer cells. Epithelial cell adhesion molecule (EpCAM) is known to be highly expressed in most of the solid tumours, including liver.[29-31] It has been reported as a putative cancer stem cell marker, and is regarded as a target antigen for cancer therapies using antibody and aptamers.[32, 33] Also, an aptamer based tumour targeting can rescue the inherent issues associated with antibody, such as larger size and immunogenicity. Aptamer against EpCAM was procured and used as a surface functionalization agent on Pt-MSNP complex. The cellular uptake study of anti-EpCAM aptamer functionalized Pt-MSNP complex was confirmed through flow cytometry **(Fig 6a)**. A significant shift in green fluorescence (FL1) peak was observed between MSNP encapsulated Pt and MSNP encapsulated Pt functionalized with anti-EpCAM aptamer **(Fig 6a)**. Confocal microscopic images also confirmed the internalization of the same in Huh7 cells **(Fig 6b)**. Importantly, pre-incubation of Huh7 cells with anti-EpCAM aptamer for 6h before treatment with Pt-

MSNP-E caused a negative shift in fluorescence intensity of Pt-MSNP-E nanoparticles thus proving that prior EpCAM receptor blocking limits the entry of the aptamer tagged nanoparticles **(Fig 6c)**. Also, the tagging of the aptamer to the Pt-MSNPs and hence enhanced internalization was also confirmed as we observed a shift in fluorescence when Huh7 cells were treated with aptamer-tagged Pt-MSNP-E when compared to only Pt-MSNP-GOPS **(Fig 6d)**. Furthermore, the Pt-MSNP-E appeared to impart cytotoxicity in Huh7 cells, as analyzed through MTT assay **(Fig 6e)**. Interestingly, in addition to its effective internalization when compared to Pt-MSNP complex, the Pt-MSNP-E compound showed significantly better cytotoxicity in Huh7 cells in the concentration range, 0-1μM, when exposed for 24h. However, it showed partly lower cytotoxicity beyond the concentration range of 1μM, which is rationalized as follows. Nanoparticles which are attached with anti-EpCAM aptamer (Pt-MSNP-E) would probably be internalized in the tumor cells through receptor mediated endocytosis whereas nanoparticles without aptamer (Pt-MSNP) could be internalized through different other pathways like, pinocytosis. As receptor mediated endocytosis is dependent on the number of that specific receptor on the surface of the cell, it may get saturated at high ligand concentrations[34] whereas, the non-aptamer tagged nanoparticles does not face such hindrance as its entry is independent of receptor saturation. Probably due to this reason, Pt-MSNP-E exhibited better cellular internalization and higher cytotoxicity at low concentrations compared to Pt-MSNP; whereas at high concentration, Pt-MSNP exhibited better cytotoxicity. Hence, here we observed that anti-EpCAM aptamer conjugated Pt-MSNPs were able to induce better cell death than MSNP encapsulated platinum(II), whereas free platinum(II) was causing necrosis, which indicates that anti-EpCAM aptamer conjugation of MSNPs was facilitating the NPs entry through proper channel causing maximum toxicity.

Conclusion

Nanotechnology has the prospect to impact many long-standing challenges in both biology and medicine, for example selective drug delivery and sensitive detection of a disease.[35, 36] Of late, MSNPs have attracted considerable attention as a promising component of multi-modal nanoparticle system. It has shown immense prospect for many biomedical applications primarily due to their straightforward synthesis, tunable pore size,

relatively low-toxicity in the biological environment, and also for its capability to carry various payloads within its porous core. In recent years, this potential capacity of MSNPs have attracted more and more research with the objective of development of a single competent nanoparticle system for drug delivery and treatment.[12, 20] However, currently, there are challenges not only behind the targeted delivery of drug loaded MSNPs specifically to cancer cells, but also for identification of appropriate tagging compounds that can switch this potent drug delivery vehicle to a prospective theranostic agent.

In this regard, it is worthwhile to mention that even today conventional cancer therapy usually suffers from poor treatment efficiency and severe adverse effects; and cancer cure heavily relies on early diagnosis and therapy monitoring *in situ*. In this context, the use of fluorescence imaging has the advantage of high sensitivity, but conventional fluorophores suffer from aggregation-caused quenching, and hence, their application in diagnosis is severely impeded. In contrast, AIE molecules have major positives in terms of excellent photo-stability and a lack of self-quenching, and therefore can be effectively utilized as theranostic platforms by conjugating them with already established therapeutic vehicles or modalities. We hence thought of utilizing and associating the imaging and cytotoxic property of AIE molecules with the existing MSNPs and develop a potent theranostic agent for effective cancer therapy.

A new Pt(II) based AIE active complex was synthesized. Platinum based compounds like, cisplatin are extensively utilized even today for cancer therapy. This compound was then successfully incorporated into the mesopores of silica. Strategies were thereafter developed to conjugate functional entities like, anti-EpCAM aptamer on the surface of MSNPs to improve their selectivity towards the cancerous cell. Cellular internalization of the free and MSNP encapsulated AIE compounds was compared and studied in cancer cells. Also the anti-EpCAM aptamer conjugated Pt-MSNPs complex were investigated for their cellular internalization potential and cytotoxic property. In summary, we for the first time report the use of novel Pt-based AIE molecules incorporated in anti-EpCAM aptamer functionalized MSNPs as future theranostic regimen for cancer cells. Our study can revolutionize both early detection and therapy of cancer cells and also monitors their treatment *in situ*.

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Title of the Schemes

Scheme 1. Reaction scheme for the synthesis of compounds of ppyCl-Pt (**1**) and BMPP-Pt (**2**).

Scheme 2. Preparation of BMPP-Pt loaded MSNPs (**3**), modification of the MSNPs with GOPS (**4**) and conjugation of anti-EpCAM aptamer with the GOPS modified MSNPs (**5**).

Figure Legends

Figure 1. (a) and (b) represent the DFT based ground state optimized HOMO and LUMO orbitals of BMPP-Pt; (c) displays absorbance spectrum of BMPP-Pt at 0.69 x 10^{-5}M dichloromethane; (d) shows ORTEP diagram of BMPP-Pt.

Figure 2. (a) Luminescent images of BMPP-Pt (λ_{ex} = 365nm) in different fractions of dichloromethane (DCM) in hexane with keeping the concentration of BMPP-Pt as 2×10^{-5}

M; (b) The corresponding photoluminescence spectra of BMPP-Pt in DCM-hexane mixed solvents (λ_{ex} = 365nm) (upto 70% PL intensity is very low, not visible PL intensity); (c) The plot of PL intensity at 497 nm with varying concentration of hexane into DCM.

Figure 3. (a, b and c)Transmission electron microscopic image of blank MSNPs, Pt-MSNPs and Pt-MSNP-E; (d) Zeta potential of blank MSNPs, Pt-MSNPs**,** Pt-MSNP-GOPS and Pt-MSNP- E.

Figure 4. Internalization studies of MSNP, BMP-Pt and Pt-MSNPs by confocal microscopy and flow cytometry. (a) Huh7 cells were seeded on cover slips and treated for 24h with either MSNP, BMP-Pt or Pt-MSNPs and were counter-stained with DAPI. Fluorescence was observed under confocal microscope and represented. For each panel, 1: DAPI (nuclear stain); 2: green fluorescence from BMPP-Pt; 3: overlay of both 1 and 2; 4: phase contrast image for cellular morphology (b) The Huh7 cells after treatment with the above compounds for 24h were harvested from 6cm dishes, were re-suspended in PBS and were acquired using flow cytometer (Cytoflex, Beckmann Coulter). The scale bar represents 100 µm. Control represents un-treated cells. The symbol (*) represents a significant difference ($p<0.05$) as compared to un-treated control cells.

Figure 5. Effect of BMP-Pt and Pt-MSNPs treatment on cyto-toxicity of Huh7 cells. (a & b) Cells were treated with different concentrations of BMP-Pt and Pt-MSNPs, respectively for 24h and cell viability was analyzed through MTT assay and is represented as bar diagram. The symbol (*) represents a significant difference ($p<0.05$) as compared to un-treated control cells. (c) Flow cytometric analysis of cell death (apoptosis and necrosis) using Annexin-V/PI was done after 24h treatment. Fold increase in total number of dead cells is represented through bar diagram. Here, (*) represents a significant difference ($p<0.05$) as compared to control (un-treated) cells. (d) Number of cells in each phase of the cell cycle was analyzed by propidium iodide (PI) staining of Huh7 cells exposed to BMPP-Pt and Pt-MSNPs for 24h through flow cytometry. Percentage of cells in each phase of cell cycle is represented by bar diagram.

Figure 6. Internalization and cytotoxic effect of Pt-MSNP-E treatment on Huh7 cells. (a) Comparative analysis of uptake of Pt-MSNPs and Pt-MSNP-E by the cells through flow cytometry is represented as bar diagram. (b) Confocal images of the internalized Pt-MSNP-E in Huh7 cells after 24h of treatment (left: untreated cells; right: Pt-MSNP-E treated cells) For each panel, 1: DAPI (nuclear stain); 2: green fluorescence from BMPP-Pt; 3: overlay of both 1 and 2; 4: phase contrast image for cellular morphology. (c) Comparison of cellular uptake of only Pt-MSNP-E with free aptamer pre-treatment followed by treatment with Pt-MSNP-E (depicted in the figure as Ap~Pt-MSNP-E) through flow cytometry. In Ap~Pt-MSNP-E group, the cells were pre-treated for 6h with free aptamer to block the receptor-mediated entry of the nanoparticles followed by Pt-MSNP-E treatment for 24h. (d) Analysis of cellular uptake Pt-MSNP-E compared to Pt-MSNP-GOPS as analyzed through flow cytometry 24h after treatment. (e) Representation of cell viability of Huh7 cells after treatment of Pt-MSNPs and Pt-MSNP-E post 24h exposure, analyzed through MTT assay. The symbol (*) represents a significant difference ($p<0.05$) as compared to control cells.