Abstact
Poly(ADP-ribose) polymerase inhibitor olaparib demonstrated therapeutic effectiveness in highly metastatic triple-negative breast cancer (TNBC). However, olaparib offers a weak therapeutic response in wild-type BRCA cancers due to the drug’s poor bioavailability. Here, a bioinspired/active-tumour targeted nanoparticles system of human serum albumin with physical entrapment of olaparib was prepared via a low-energy desolvation technique using the crosslinker glutaraldehyde. The developed OLA@HSA NPs were nanosize (~140 nm), kinetically stable with a low polydispersity (0.3), exhibited olaparib entrainment (EE 76.01±2.53%, DL 6.76±0.22%) and sustained drug release at pH 7.4 with an enhancement of drug release in acidic pH. OLA@HSA NPs decreased the half-maximal inhibitory concentrations (IC50) of olaparib by 1.6-, 1.8-fold in 24 h and 2.2-, 2.4-fold in 48 h for human (MDA-MB 231) and mouse (4T1) TNBC cells, respectively, mediated by their enhanced time-dependent cellular uptake than free olaparib. The OLA@HSA NPs induced concentration-dependent phosphatidylserine (apoptotic marker) externalisation and arrested the cell population in the G2/M phase in both the tested cell lines at a higher level than free olaparib. The NPs formulation increased DNA fragmentation, mitochondrial membrane depolarisation and ROS generation than the free olaparib. The in vivo study conducted using 4T1-Luc tumour-bearing mice demonstrated strong tumour growth inhibitory potential of OLA@HSA NPs by elevating apoptosis ROS generation and reducing the level of the antiproliferative marker, Ki-67. OLA@HSA NPs reduced the occurrence of lung metastasis (formation of metastasis nodules decreased by ~10-fold). OLA@HSA NPs could be a promising nanomedicine for the TNBC treatment.

Introduction
Triple-negative breast cancer (TNBC) is the most lethal breast cancer subtype affecting the younger women population (below 40) that lacks the expression of three significant breast cancer markers, oestrogen (ER), progesterone (PR) and human epidermal growth factor (HER-2) receptors. TNBC encounters a poor prognosis and a high relapse rate due to this cancer’s invasiveness and metastatic potential [1]. Approximately 15–20% of breast cancer patients are triple-negative, indicated by the immunohistochemical staining [2]. Moreover, approximately 5% of randomly selected breast cancer patients possess germline deleterious breast cancer gene (BRCA) mutation [3]. BRCA1 and BRCA 2, located on chromosomes 17 and 13, are tumour-suppressor genes involved in the homologous recombination (HR) repair mechanism to rectify double-stranded DNA damages in the proliferating cancer cells [4]. The gene repair re-establishes the genetic sequence for normal cell functioning. However, the mutation in BRCA1 and BRCA2 genes impairs the HR mechanism resulting in the activation of oncoproteins, which predisposes the individual to high risk for breast cancer occurrence [5]. Majorly, patients with BRCA1 mutations are inclined to be affected by TNBC [6].

Olaparib is a PARP inhibitor (PARPi), first approved by the United States Food and Drug Administration (USFDA) authority to treat patients with recurrent BRCA-mutated ovarian and breast cancers. Olaparib demonstrated therapeutic benefits in phase III clinical trials (Olympia AD study) in patients with BRCA1/2-mutated HER2 negative BCs [12–14]. Olaparib was approved by USFDA as a first-line treatment to treat BRCA1/2-mutated/HER2/neu negative
breast cancer in January 2018 (oral dose/tablet. 300 mg, twice daily). In the Olympia AD Phase III trial, a statistically significant progression-free survival rate was achieved with olaparib compared to other standard chemotherapeutic treatments [13]. Olaparib is therapeutically effective in metastatic TNBC with fewer side effects than other chemotherapeutic agents, including capecitabine, eribulin and vinorelbine [14,15]. However, sequential or combination treatment of this drug with other anticancer agents is yet to be explored. Further research unravelled that the olaparib was effective in BRCA-mutated tumours of other organs [16].

The major reason for olaparib's less sensitivity to wild-type BRCA solid tumours is the lack of PARP-BRCA-synthetic lethality. The activated HR mechanism for DNA repair by BRCA reduces the effect of PARP-inhibition. Other reasons for olaparib's less sensitivity towards the growth inhibition of wild-type BRCA tumours could be its poor bioavailability [17]. Being a BCS (Biopharmaceutical Classification System) class IV drug, olaparib suffers from poor aqueous solubility and absorption through membrane barriers. Moreover, olaparib treatment is associated with several side effects, including anaemia, nausea, myelodysplastic syndrome, acute myeloid leukaemia and pneumonitis [14,18]. The existing marketed formulations are tablets and capsules which require more dosing to overcome the poor bioavailability and has severe adverse effect of hematological toxicity. The olaparib oral formulation (Lynparza) has limited success against TNBC, which could primarily be due to its insufficient localisation to the tumour. Due to all these drawbacks, there is a need to develop nanoformulations that can maintain adequate drug concentration by releasing the drugs in a sustained manner. Various Olaparib-nanoparticles systems have been developed recently to improve the pharmacokinetic properties of the drug, tumour-targeting and improve radiosensitization [16,19–21]. Nanoformulation of olaparib, a lipid-based injectable system, enhanced the PARP inhibition and radiosensitivity of specific prostate cancer cells [19]. A PEG-poly(caprolactone) NPs system loaded with olaparib improved the therapeutic effect of radiotherapy compared to the free drug in lung cancer [20]. Several combination therapies using olaparib and other chemotherapeutic agents were studied recently and have sensitised the cancer cells towards treatment in various tested cancers, including the brain, pancreatic (BRCA-mutated) and breast cancers [16,22,23].

In our study, olaparib had been physically entrapped in human serum albumin (HSA) NPs to prepare OLA@HSA NPs via desolvation technique using ethanol (desolvating agent) and glutaraldehyde (cross linker). Albumin NPs possess unique features to be used as a drug delivery system in cancer [24]. They are nanosize, biocompatible, non-immunogenic and could carry hydrophobic drugs. Albumin NPs display tumour targetability via passive, commonly known as Enhanced Permeability and Retention (EPR) effect and active targeting mediated by the binding with secreted protein acidic and rich in cysteine (SPARC) and gp60 receptors, over-expressed on the cancer cell/cancer cell linked endothelial cells surface [25]. The study, for the first time, fabricated albumin-bound nano-olaparib and investigated its therapeutic efficacy and lung metastasis-inhibitory potential in TNBC. The NPs were characterised thoroughly for particle size, zeta potential, drug loading (DL), entrapment efficiency (EE), stability and biocompatibility. The anticancer activity was judged in vitro using murine and human TNBC cells in monolayers and human TNBC cell spheres. Further, the therapeutic efficacy and anti-metastatic ability were evaluated in vivo.

Materials and methods

Materials

Human serum albumin was purchased from Baxter Healthcare Corporation (USA). Olaparib was received as a gift sample from Dr. Reddy’s Laboratory, Hyderabad, India. 2,7-dichloro-di-hydro-fluorescein diacetate (DCFH-DA) procure from Sigma Aldrich, Mumbai, India. Methylthiazolyldiphenyl-tetrazolium bromide (MTT), Trypan blue solution and Fluromount-G were obtained from Himedia Labs (India). Spectra/Por dialysis membranes were purchased from Spectrum Laboratories, Inc. (USA).

Cell lines

Human TNBC cell line MDA-MB-231 was procured from National Centre for Cell Sciences (NCCS, Pune, India). Murine breast cancer cell lines, 4T1 and 4T1-Luc, were procured from the American Type Culture Collection (ATCC, USA). Himedia Labs (India) supplied the Penicillin Streptomycin, Dulbecco’s Modified Eagle Medium (DMEM), Minimum Essential Medium Eagle (MEM), Trypsin-EDTA, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fetal bovine serum (FBS).

Animals

Female Blab/C mice (18–20 g, 5- to 7-week old) were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India to evaluate the antitumor effectiveness. The pharmacological studies were approved by the Institutional Animal Ethics Committee of BITs-Pilani, Hyderabad. The animals were group-housed at room temperature (RT) 25 °C ± 2 °C relative humidity (50–70%) and 12:12 h light: dark cycle. They had free access to a standard chaw diet and water ad libitum. Female Wister rats used in the haemolysis study for blood collection were obtained from Sainath Agency, Hyderabad, India.

Methods

Preparation of OLA@HSA NPs

The NPs were prepared following the formula derived from the Design of Experiment (Design-Expert® version 13). The central composite design (CCD) was applied to optimise the preparation procedure of OLA@HSA NPs formulation concerning suitable particle size (PS), DL and EE. The selected independent variables, namely the polymer ratio and glutaraldehyde concentration, were considered to be the critical parameters in the preparation process that impact PS, DL and EE. These two experimental factors were varied in the design at 3 levels in 13 runs.

The optimised procedure was as follows: Into the solution of HSA (50 mg) in 2 mL of deionised (DI) water, OLA (2 mg) in 2 mL ethanol was added dropwise (1 mL/min) under stirring condition (1000 rpm) followed by the addition of glutaraldehyde volume (25% w/v, 5 μL) dropwise. After 24 h, the solution was centrifuged at 12,000 rpm, 15 min, at 4 °C. The pellet was washed twice and resuspended in DI water (2 mL) (Scheme 1). The NPs solution was lyophilised by adding mannitol (5% w/v, 1 mL) into the solution. Briefly, the mannitol-added NPs solution was kept at −80 °C for 6 h, and the frozen sample was kept for lyophilisation at −99 °C until the free-flowing powder was obtained. The blank NPs were prepared following the same protocol without the drug.
Physicochemical characterisation of OLA@HSA NPs: size, surface charge, EE, DL and morphology

The particle size and zeta potential of the HSA NPs and OLA@HSA NPs were determined by dynamic light scattering technique using Zetasizer 3600 (Malvern Instruments Ltd. UK). After lyophilising the samples, the particle size, PDI and zeta potential were measured. Olaparib content in the OLA@HSA NPs was checked by UV–Vis spectrophotometer (Jasco UV-670, Japan) and HPLC technique (Shimadzu, Japan). The chromatographic specifications were as follows: reverse phase C18 column of 150 mm × 4.6 mm, (Phenomenex, USA), mobile phase by mixing deionised water and methanol at the ratio of (36:64, v/v), the mobile phase flow rate of 1 mL/min, column temperature, 40 °C, acquisition time 10 min and injection volume 20 µL. The detection wavelength in the photo-diode array detector was set to 207 nm. The OLA@HSA NPs were dissolved in ethanol (HPLC grade), and the olaparib content was measured by HPLC. The HPLC method to evaluate the concentration of Olaparib has been detailed in the Supporting Information. The OLA encapsulation efficiency (EE) and loading (DL) were assessed in OLA@HSA NPs as per the equation [26]:

\[
\text{EE \%} = \frac{\text{Weight of drug entrapped in the nanoparticle}}{\text{Weight of total drug added initially to prepare the nanoparticles}} \times 100
\]

\[
\text{DL \%} = \frac{\text{Weight of drug entrapped in the nanoparticle}}{\text{Weight of the polymer and drug}} \times 100
\]

The surface morphology of OLA@HSA NPs was determined using the field emission scanning electron microscopy (NOVA NANOSEM 450). Samples were prepared by uniformly distributing a thin layer of nanoparticles over an adhesive carbon tape attached to aluminium stubs. The stubs were then sputter-coated with the gold of desired thicknesses and analysed at 20 kV. The morphology of the nanoparticles was visualised by transmission electron microscopy (TEM, JEM-1200EX, JEOL and Tokyo, Japan). Briefly, OLA@HSA NPs were stained with uranyl acetate (2% w/v) and later destained using distilled water. Further, the samples were dried for 10 min by placing them on copper grids and then visualised [27].

Stability

The kinetic stability of the formulation was analysed using UV–Vis spectrophotometer (JASCO, JAPAN) for 30 days. The OLA@HSA NPs were stored at 4 °C and room temperature for 30 days. The absorbance was recorded by dissolving OLA and OLA@HSA NPs in methanol. The drug absorbance and particle size were checked daily using a UV–Vis spectrophotometer (Jasco UV 670) at a wavelength of 207 nm and Malvern Zetasizer (Malvern Instruments Ltd, UK), respectively.

X-ray diffraction analysis

OLA and OLA@HSA NPs were subjected to powder X-ray diffraction using an X-ray diffractometer (Rigaku, Ultima-IV). The lyophilised powder scanned at a range from 0 to 100° 2θ at a scan speed of 10°/min using the voltage of 40kV and an electrical current of 30 mA. The spectrum was collected and analysed using the origin software (version 9.2.0) by plotting 2θ and intensity on X and Y axis, respectively.

Differential scanning calorimetry

Thermal analysis of OLA, OLA@HSA NPs was performed using DSC (DSC 60, Shimadzu, Japan). Briefly, 5 mg of each sample was weighed and heated from 30 to 300 °C at a rate of 5 °C/min. The nitrogen environment was maintained by passing dry nitrogen at a 20 mL/min rate through the samples kept in the aluminium pans.

FTIR analysis

FTIR analysis was performed using the KBr pellet method following the previously reported protocol using an FTIR spectrometer (Jasco-4200, USA) [28]. The scanning range was kept from 4000 to 400 cm⁻¹.

X-ray photon electron spectroscopy analysis

The chemical analysis of the NPs was performed by K-Alpha X-Ray Photoelectron Spectroscopy (Thermo-Fisher, USA). The samples
were analysed bypassing the X-ray source (1375 eV) at 10-6torr vacuum pressure and the energy of the X-ray was passed at 50 and 20 eV, respectively, for wide and narrow scanning with the electron take-off angle at 56°. The obtained data were analysed using the advantage software package. Briefly, the survey of the X-ray photon electron spectroscopy (XPS) and narrow spectrum was done utilising the XPS knowledge view configuration and carbon correction process was performed. After the carbon correction all the spectra were analysed.

**Protein conformation studies**

**SDS-PAGE.** SDS-PAGE was performed for the HSA and OLA@HSA NPs, following the previously reported procedure [16]. The lyophilised NPs were resuspended in PBS, pH 7.4. Then, 15 µL of 1 mg/mL of each sample was mixed with 5 µL of loading dye. The samples were denatured by boiling at 95°C for 5 min and loaded into the 10% polyacrylamide gel. The gel was run alongside the standard ladder using electrophoresis equipment (Bio-Rad, USA) at a constant voltage of 120 Volts for 3 h. After completion of the run, the gel was washed 5 times with 100 mL of deionised water to remove SDS and buffer salts, stained using coomassie blue for 1 h, and then, kept for destaining overnight. The protein bands were observed on the gel, and the images were captured.

**Circular dichroism spectroscopy.** The secondary structure of HSA in OLA@HSA NPs was analysed using a circular dichroism (CD) spectrometer (JASCO-1500, USA). Briefly, protein solutions (0.1 µM HSA) were passed through a polyvinylidene difluoride (PVDF) filter. The solutions, placed in quartz cells of path length. 0.1 cm, were run for a scanning range of 190–260 nm under the nitrogen filter. The solutions, placed in quartz cells of path length. 0.1 cm, were run for a scanning range of 190–260 nm under the nitrogen filter. The helical content in the free HSA and OLA@HSA NPs was analysed from the plot of mean residue ellipticity (MRE) in deg cm$^2$/dmol$^{-1}$ versus wavelength (nm) in Y and X axes, respectively, according to the formula reported previously [29].

**In vitro drug release study**

The *in vitro* drug release of olaparib from OLA@HSA NPs formulation was assessed using the dialysis method [30]. In brief, OLA@HSA NPs were resuspended in PBS 7.4 (100 µg/mL OLA concentration, total volume 1 mL) and placed in a dialysis bag of MWCO 12–14 kDa bag (Spectrum Laboratories, USA). The bags were placed in the dissolution media and maintained at 37°C under continuous stirring. Media (1 mL) was collected and refilled with fresh media at predetermined time points. The samples were analysed by UV–Vis Spectrophotometer to determine the olaparib content.

**Haemolysis assay**

Blood was collected retro-orbitally from the rat eye and mixed with the ethylenediamine tetra acetic acid (EDTA) solution to prevent coagulation [18]. The collected blood was centrifuged at 3000rpm, 4°C for 10 min to separate the plasma. The RBCs isolated were washed thoroughly and resuspended in PBS 7.4 to form a 5% v/v RBC suspension. OLA@HSA NPs (12.5–250 µg/mL) were added to the RBC suspensions in tubes and incubated at 37°C for 1 h. Triton X-100 (1%) and PBS 7.4 were positive and negative controls, respectively. The samples were then centrifuged at 7000rpm, 4°C for 20 min. The supernatant was analysed by measuring the absorbance at 576 nm using a Spectramax Multiplate reader (Molecular Devices, USA). The % haemolysis was then calculated using the following formula:

$$\text{% hemolysis} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{ve}}}{\text{Abs}_{\text{ve}} - \text{Abs}_{\text{ve}}} \times 100$$

$\text{Abs}_{\text{sample}}$ the absorbance of the sample  
$\text{Abs}_{\text{ve}}$ the absorbance of the negative control  
$\text{Abs}_{\text{ve}}$ the absorbance of the positive control

**Cell viability assay.** MDA-MB-231 and 4T1 cells were seeded in 96 well plates (10,000 cells/wells) and incubated overnight. The next day, old media was replaced with fresh media containing 100 µL of olaparib and OLA@HSA NPs (0–100 µg/mL) and incubated for 24 h. Then, 50 µL of this MTT reagent solution (5 mg/mL) was added into each well and incubated for 4 h before adding the solvent, DMSO (150 µL), to dissolve the formed purple formazan crystals. After 1 h, the absorbance was measured at 570 nm and a reference wavelength of 620 nm using the Spectramax™ Multiplate Reader (Molecular Devices, USA). The equation used to calculate cell viability is given below:

$$\text{Cell viability} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{ve}}} \times 100$$

$\text{Abs}_{\text{sample}}$ the absorbance of the sample  
$\text{Abs}_{\text{ve}}$ the absorbance of control

**Cytotoxicity (apoptosis) assay.** Cancer cells were seeded in a 12 well tissue culture plate (50,000 cells/well) and incubated overnight. Rhodamine-labelled OLA@HSA-Rh NPs were added to the cells at a 25 µg/mL OLA concentration and incubated for 1, 4 and 8 h (the procedure to prepare the rhodamine-labelled HSA has been included in the Supporting Information). Next, the cells were washed thoroughly, trypsinized and centrifuged. The cell pellets were suspended in cold PBS and analysed using a flow cytometer (BD FACS ARIA, Germany). The forward scatter (FSC), detecting the scatter along the laser path and the side scatters (SSC), detecting the scatter at 90° angle relative to the laser, were considered for data acquisition or gating. FSC versus SSC plot was used to identify cells based on their size and complexity, and a total of 10,000 cells were gated, excluding the debris region. A total of 10,000 live cells were gated for the data acquisition. The data was obtained as the histogram plots (count vs. PerCP-cy5-5a) in FACS Aria III, BD Biosciences, USA) Software. Similarly, for fluorescence microscopy studies, the OLA@HSA NPs-treated cells were washed, fixed using 4% paraformaldehyde and stained using DAPI (5 min, 1 µg/mL). The images were captured using a fluorescent microscope (Leica, Germany).

**Annexin-V assay.** Cancer cells were seeded at a seeding density of 0.5 × 10$^6$/well in 12 well plates. Following overnight incubation, the cells were treated with olaparib and OLA@HSA NPs (OLA concentration 12.5 µg/mL, 25 µg/mL) for 24 h at 37°C. The study was carried out as per the manufacturer's instructions. The cells with no treatment served as controls. The cells suspensions were analysed by flow cytometry (no of gated cells, 10,000) (FACS Aria III, BD Biosciences, USA). Fluorescence of FITC and PI was measured at 535 nm and 550 nm, respectively. PI versus Annexin V-FITC with quadrant gating was done as dot plots. Each quadrant represents the cell populations with the following characteristics: necrotic (Q1, Annexin V$^+$, PI$^+$), late apoptosis (Q2, Annexin V$^+$, PI$^+$), live cells (Q3, Annexin V$^-$, PI$^-$), early apoptosis (Q4, Annexin V$^-$, PI$^+$).
**Cell cycle analysis.** MDA MB231 and 4T1 cells were seeded in a 12 well plate (1 × 10^6 cells/well). The next day, cells were treated with olaparib, OLA@HSA NPs (OLA concentration. 12.5 µg/mL, 25 µg/mL) for 24 h. After the treatment, the cells were harvested and washed with 1 mL PBS (pH 7.4), and then, centrifuged at 1500 rpm for 5 min. The pelleted cells were fixed in 70% ice-chilled ethyl alcohol overnight at −20°C. Fixed cells were centrifuged at 1500 rpm for 5 min. Cell pellets were suspended in ice-chilled PBS (1 mL) followed by centrifugation at 1500 rpm for 5 min. Finally, the cells were resuspended in 0.5 mL of the staining solution (20 µg/mL propidium iodide, 200 µg/mL RNase and 400 µL PBS 7.4). The cell cycle arrest was then analysed using the flow cytometer.

**Nuclear staining assay.** Cancer cells were seeded in a 12 well tissue culture plate (50,000 cells/well). After 24 h, the cells were treated with olaparib, OLA@HSA NPs (OLA concentration as 10 µg/mL) and incubated at 37°C, 5% CO₂ for 24 h. The cells were washed using PBS 7.4, fixed using 4% paraformaldehyde, stained using DAPI (1 µg/mL, 5 min) and acridine orange. 6 µg/mL, 20 min) using PBS 7.4, fixed using 4% paraformaldehyde and then embedded in OCT medium, frozen and cryo-sectioned at 4 µm thickness using cryotome (Leica biosystems, Germany). The obtained data was then processed using Image J software.

**Mitochondrial membrane potential assay.** The cancer cells were seeded in a 12 well tissue culture plate (50,000 cells/well). After 24 h, the cells were treated with olaparib and OLA@HSA NPs (olaparib concentration. 10 µg/mL) (incubation conditions. 37°C, 5% CO₂ for 24 h). After incubation, the cells were washed using PBS 7.4, trypsinized and collected by centrifugation. Total DNA was isolated using GSure DNA genomic isolation kits. The isolated DNA was checked for absorbance at 260 nm and 280 nm and the ratio was obtained. A ratio of 1.8 is considered to be pure DNA. The isolated DNA was run on agarose gel (1.5%) containing 0.5 µg/mL ethidium bromide. The gel was then observed for DNA fragmentation under the microscope using the Fusion Pulse Gel Doc system (Vilber, Germany).

**DNA fragmentation assay.** MDA MB 231 and 4T1 cells were seeded in a 12 well tissue culture plate (50,000 cells/well). After 24 h, the cells were treated with olaparib, OLA@HSA NPs (OLA concentration. 10 µg/mL) and incubated at 37°C, 5% CO₂ for 24 h. After incubation, the cells were washed using PBS 7.4, fixed using 4% paraformaldehyde, stained using DAPI (1 µg/mL, 5 min) and acridine orange. 6 µg/mL, 20 min) and observed under fluorescence microscope at blue (358 nm) and green (480–490 nm) channels for DAPI and acridine orange, respectively.

**Growth inhibition/live-dead cells assay in spheroids.** Spheroids were incubated with olaparib and OLA@HSA NPs (OLA concentration. 6 µg/mL for 24 h). Next, the spheroids were visualised under an optical microscope (Leica Microsystems, Germany) at predetermined time points (0, 3 and 6 days). The images were captured at 10X magnification, and the diameter was measured in the images [32]. For live-dead cells estimation, after 24 h of the treatment with OLA and OLA@HSA NPs (6 µg/mL), the spheroids were stained with calcein blue and PI at a concentration of 2 and 4 µM, respectively, and incubated for 30 min at 37°C. The stained spheroids were observed under the fluorescence microscope using FITC (Ex/em. 495/519 nm) and the rhodamine (540/570 nm) filter.

**Spheroid study**

**Penetration in spheroids.** Spheroids were grown using the liquid overlay method reported previously [31]. Briefly, MDA-MB-231 cells (1 × 10⁴ Cells/well) were seeded in a 96-well plate, which is pre-coated with sterile agarose solution (1.5% w/v). Agarose was prepared by using serum-free DMEM media and 50 µL was added to each well of a 96-well plate. The formation of dense and spherical spheroids of similar sizes was ascertained by an optical microscope. Spheroids were treated with OLA@HSA-Rh NPs (25 µg/mL OLA) for 24 h and washed with PBS. The Z-stacked images (at fixed X and Y axis) were taken at 10 µm intervals using the confocal laser scanning microscope (TCS SP8, Leica Microsystems, Germany) at 10X magnification. The images were processed using Image J software.

**In vivo study**

**Tumour inhibition study.** 4T1-Luc cells (1.5 × 10⁶ in 100 µL) in cold PBS were injected into the left flanks of the 6-week old female balb/c mice. Once the tumour volume reached approximately 80–100 mm³, the mice were randomly divided into three groups (control, olaparib and OLA@HSA NPs; n = 5) and intravenously injected via the tail vein with olaparib and OLA@HSA NPs (OLA dose 50 mg/kg). The dosing was given every alternative day for ten days (treatment duration 21 days). Mice were administered with D-luciferin (150 mg/Kg, intraperitoneal) periodically at day 0, 5, 10, 15 and 21 to check the tumour progression. D-luciferin was injected to the mice, 10 min before capturing the image using the IVIS-Lumina in vivo imaging system (PerkinElmer, Inc., USA) (Ex/Em. 620/780 nm). The tumour volume was measured by the equation, length X width²/2, during the experiment every alternate day. The body weight was measured at the same time. On day 21, the tumour mass was surgically extracted from the mice anaesthetised using ketamine/xylazine and weighted. The animals revived after surgery and were monitored via bioluminescence imaging using luciferin-D on day 7, postsurgery. Following live-animal imaging, mice were sacrificed, the lung tissues were weighed and photographed and the tumour nodules were counted.

**ROS detection assay.** MDA MB 231 and 4T1 cells were seeded in a 12 well tissue culture plate (50,000 cells/well). After 24 h, the cells were treated with olaparib, OLA@HSA NPs (OLA concentration as 10 µg/mL) and incubated at 37°C, 5% CO₂ for 24 h. After incubation, the cells were washed thrice using PBS 7.4, fixed and treated with JC-1 dye (2 µM) for 20 min. The labelled cells were observed under the fluorescence microscope (ex/em. 504/529 nm, 20X objective). The cells were treated similarly with JC-1 dye for 30 min for flow cytometry analysis. The cells were pelleted and washed thoroughly with cold PBS, pH 7.4. The mitochondria membrane potential was analysed for 10,000 viable cells by measuring the fluorescence intensity using the flow cytometer (BD FACS ARIA, Germany). The obtained data was then processed using (FACS Aria III, BD Biosciences, USA).

**Immunohistochemistry**

**Tunel assay/detection of Ki-67.** The isolated tumour tissues were emersed in OCT medium, frozen and cryo-sectioned at 4 µm thickness using cryotome (Leica biosystems, Germany). The TUNEL assay (R&D System TM TACS TdT In Situ Apoptosis Detection Kit, Product code- 481230 K) was performed according to the manufacturers’ instructions. Briefly, the tumour tissues were incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h. The TUNEL reaction mixture composed of nucleotide mix, TdT enzyme and equilibration buffer was added to the fixed tumour tissue slides and incubated in a dark, humid atmosphere
at 37°C for 1 h. Samples were washed three times with 1× PBS to remove the unincorporated fluorescein-dUTP. Next, the tissue sections were stained with DAPI for 5 min. Slides were visualised using a fluorescence microscope (Leica, Germany). For Ki-67 analysis, the tumour sections were blocked in blocking buffer for 1 h and incubated overnight at 4°C with Ki-67 primary antibody (Rabbit mAb #9129). Next, the tissue sections were washed thrice with PBS and incubated with secondary antibody (Alexa Fluor® Plus 488) for 2 h at RT in the dark. Finally, the tissues were washed with PBS and visualised under a fluorescence microscope (Leica Microsystems, Germany).

**ROS generation in tumour tissue.** The olaparib, OLA@HSA NPs were injected intravenously to mice bearing 4T1-Luc tumours (~60 mm³) with an equivalent dose of 5 mg/kg. After 24 h of postinjection, the mice were injected intratumorally with the DCFH-DA at concentration of 25 μM (50 μL). After 30 min of DCFH-DA administration mice were sacrificed, tumour tissues were collected and immersed in OCT media. The tumour tissues were cryosectioned at 5 μm of size using cryotome (Leica Biosystem, Germany). The frozen tissue sections were observed under a fluorescence microscope (Leica, Germany) using the FITC filter (ex/em 495/519 nm). The images were processed, and the fluorescence intensities were determined by Image J software. The area of the images was selected. The fluorescence intensity of the region of interest was compared with the free drug.

**H & E staining.** Lung sections of 4 μm thickness were processed with xylene, different concentration of alcohols and washed with water. Briefly, tumour tissues were dissected into multiple parts. One portion was fixed in 10% formaldehyde by dipping the tissue for 24 h. Next, the tissues were washed and processed through different concentrations of alcohols, 100–30% for 1 h each, followed by xylene (100%) incubation for 4–5 min [33]. The nuclei were stained with haematoxylin (0.5%) followed by eosin (0.5%) for staining the cytoplasm. The slides were washed thoroughly with water, followed by ethanol solutions (30–100%) and washed with mounting media (Fluoromount G) before visualisation under an optical microscope with 10 X magnification and a bright field exposure (Leica, Germany).

**Western blot**

The western blot was performed for determining the protein expression of apoptotic markers PARP1, WH2AX and p53 (cell signalling technology, USA, product code PARP1- 9542, WH2AX-2577 and p53- 9282) in tumour tissues. The tumour tissues were cut into small pieces and homogenised in RIPA buffer containing protease inhibitor and 200 μM phenylmethylsulfonyl fluoride (PMSF) using bead homogeniser MINILYS® (Bertin Technologies, France). The protein samples were centrifuged at 16,000g for 20 min at 4°C, and the supernatant were collected. The extracted proteins were quantified by a BCA protein assay kit (TaKaRa Bio Inc, USA). 30 μg of protein were loaded on the sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) for separation. The separated proteins were then transferred onto PVDF membranes (Bio-Rad, USA) using wet transfer method. Then, membranes were blocked with 5% (W/V) non-fat milk powder prepared in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for one hour. The membranes were incubated with primary antibodies for proteins, WH2AX and P53 (CST, dilution 1:7000 each) for overnight at 4°C. On next day, the membranes were washed thrice with TBST for 30 min each washing and incubated with secondary antibodies (Anti-rabbit, CST and 1:7000) for 2 h at room temperature. After completion of the incubation period the membranes were thoroughly washed. The blots were detected using enhanced chemiluminescence (ECL; Bio-Rad, USA) solution. The blots were scanned using ChemiDoc TM Gel Imaging System (Eppendorf fusion plus). The protein bands were further quantified using Image J software (Version 6.0). β-actin was used as a housekeeping gene for normalisation with other proteins.

**Statistics**

All the above experiments were performed in triplicates, and the data are shown as mean and standard deviation (mean ± SD). The significance of the difference between all the groups was evaluated by one-way ANOVA using GraphPad Prism 9 software (Graph Pad, Inc., California, USA). The p-values < .05 reflected statistically significant analysis. *, **, *** illustrates p-values < .05, .01 and .001, respectively.

**Results**

**Physicochemical characterisation of the OLA@HSA NPs**

Particle size, DL and EE were optimised through the response surface method. In the CCD-RSM, 13 experiments were conducted for two factors at three levels. From the experimental results of the CCD, a quadratic polynomial equation was obtained (Z the CCD, a quadratic polynomial equation was obtained (Table 1). A quadratic polynomial equation was obtained (Z

| Table 1. Number of runs and the actual values from central composite design. |
|---|---|---|---|---|---|
| Factors | Actual factors | Responses |
| S.No | A | B | Amount of polymer (mg) | Glutaraldehyde conc | Particle size (nm) | Drug loading (%) | Entrapment efficiency (%) |
| 1 | 0 | 0 | 25 | 5 | 143 | 7.2 | 74.12 |
| 2 | –1 | 1 | 12.5 | 7.5 | 503.8 | 4.6 | 41.6 |
| 3 | 0 | 0 | 25 | 5 | 143 | 7.2 | 74.12 |
| 4 | 0 | 1.414 | 25 | 10 | 271 | 6.08 | 66.8 |
| 5 | –1 | –1 | 12.5 | 2.5 | 161 | 0.8 | 6.78 |
| 6 | –1.414 | 0 | 1 | 5 | 293 | 8.51 | 51.5 |
| 7 | 0 | –1.414 | 25 | 1 | 134 | 1.3 | 14.7 |
| 8 | 1 | –1 | 37.5 | 2.5 | 208.6 | 1.03 | 14 |
| 9 | 1 | 1 | 37.5 | 7.5 | 333 | 3.9 | 67.4 |
| 10 | 0 | 0 | 25 | 5 | 143 | 7.2 | 74.12 |
| 11 | 0 | 0 | 25 | 5 | 143 | 7.2 | 74.12 |
| 12 | 0 | 0 | 25 | 5 | 143 | 7.2 | 74.12 |
| 13 | 1.414 | 0 | 50 | 5 | 261 | 5.21 | 83.5 |
Drug loading $= 7.20 - 0.67A + 1.70B - 0.28AB - 0.83 A^2 - 2.42B^2$

Entrapment efficiency $= 74.12 + 9.78A + 20.24B + 4.65AB - 8.73A^2 - 22.10B^2$

A represents the amount of polymer and B represents the glutaraldehyde concentration. The main effects of variables on the responses are presented in Supporting Information Figure S1. The predicted $Z_{avg}$, % DL and % EE using the polymer amount (25 mg) and glutaraldehyde volume (5 µL) were 143 nm, 7.2% and 74.12%, respectively. The observed experimental values of $Z_{avg}$, DL% and EE% were 142.3 nm, 7.3% and 74.35%, respectively (Table 2).

The particle size of the HSA NPs and OLA@HSA NPs were 127.8 ± 0.32 and 143.5 ± 0.43 nm, respectively, as measured by the dynamic light scattering technique (Figure 1A). The zeta potential was $-8.27 ± 1.23$ for blank and $-11.27 ± 3.25$ mV for OLA@HSA NPs (Figure 1B). The TEM analysis of the sample proved the nanosize of both blank and drug-loaded NPs (Figure 1C and Supporting Information Figure S5A). The morphology of the prepared NPs was determined using scanning electron microscopy and found to be spherical (Figure 1D and Supporting Information Figure S5B). The % EE and DL in OLA@HSA NPs were 76.01 ± 2.53 and 6.76 ± 0.22, respectively (Supporting Information Figure S6).

The stability of OLA@HSA NPs on storage at 4 °C and the room temperature was determined by analysing the particle size and % DL (Supporting Information Figure S2B). The particle size and DL were 212.32 ± 2.61, 7.12 ± 1.21%, respectively, for OLA@HSA NPs after 30 days on storage at 4 °C, compared to 655.40 ± 2.1, 0.56 ± 0.05% at room temperature. An eventual slight decrease in the olaparib signal over 1–30 days was observed for OLA@HSA NPs stored at 4 °C (Supporting Information Figure S2A). The XRD analysis of the OLA@HSA NPs revealed the amorphous nature of olaparib in the NPs with no characteristic peaks of OLA (Figure 1F).

The CD spectra of HSA and OLA@HSA NPs showed two negative bands, at 208 and 222 nm, characteristic of α-helix. The mean residue ellipticity values at 208 and 222 nm decreased in OLA@HSA NPs compared to native HSA. The α-helix and β-sheet values were calculated to be 63.65 ± 0.88 and 7.52%, respectively, for HSA and 50.22 ± 0.32 and 14.22% for OLA@HSA NPs (Figure 2A). The SDS PAGE bands for HSA and OLA@HSA NPs matched the protein marker at 66 KDa. The bands were of equal thicknesses (Figure 2B).

Next, the haemolysis study was performed to determine the maximum cytocompatible NPs concentration. The assay result indicated that the percent haemolysis increased with increasing NPs concentration in the plasma. The highest tested concentration of OLA@HSA NPs (250 µg/mL) was observed to be as high as 5.1%.

Table 2. Mean and predicted values of the optimised OLA@HSA NPs formulation.

| Response                  | Predicted mean | Observed mean | Standard deviation | n value |
|---------------------------|----------------|---------------|--------------------|--------|
| Particle size (nm)        | 143            | 142.3         | 50.2               | 3      |
| Drug loading (%)          | 7.2            | 7.3           | 1.51               | 3      |
| Entrapment efficiency (%) | 74.12          | 74.35         | 11.85              | 3      |

![Figure 1](image-url). Physiochemical characterisation of OLA@HSA NPs. Particles size distribution of HSA NPs, OLA@HSA NPs (A); zeta potential of HSA NPs, OLA@HSA NPs (B); transmission electron microscopic image of OLA@HSA NPs (C); Scanning electron micrograph of OLA@HSA NPs (D); UV absorbance spectra of olaparib using OLA@HSA NPs in methanol, water (E); and X-ray diffraction pattern of OLA, OLA@HSA NPs (F).
haemolytic, considering the positive control to be 100% haemolytic (Figure 2C). The cumulative olaparib release from OLA@HSA NPs under three different pH conditions, pH 7.4, 6.5 and 5.5 at different time points up to 48 h, is represented in Figure 2D. The drug release was highest at pH 5.5 (83.3%) and the least at pH 7.4 (39.2%) after 48 h. The UV absorption spectrum indicated increased absorbance of OLA@HSA NPs at pH 5.5 than pH 6.5 and 7.4.

In vitro cell-based assays in monolayers

Cell viability
A time and concentration-dependent decrease in cell viability was observed following the treatment of both the cancer cells with olaparib and OLA@HSA NPs in MTT assay (Figure 3 and Supporting Information Figure S7). In general, the cytotoxicity produced by OLA@HSA NPs was higher compared to the free olaparib treatment. OLA@HSA NPs demonstrated cell viability of 32.21 ± 2.60% (24 h), 21.21 ± 3.85% (48 h), OLA 43.66 ± 2.43% (24 h), 30.25 ± 3.42 (48 h) in 4T1 cells. Similarly, in MDA MB 231 cells, OLA@HSA NPs demonstrated cell viability of 25.36 ± 1.25 (24 h), 12.36 ± 2.65 (48 h) compared to 39.36 ± 2.34 (24 h), 25.32 ± 1.35 (48 h) after free olaparib treatment at the highest drug concentration (100 µg/mL). The IC50 values of OLA@HSA-Rh NPs towards 4T1 were calculated as 10.52 and 6.54 µM following 24 h and 48 h of treatment, respectively. The IC50 values of OLA@HSA NPs towards MDA-MB-231 cells improved NPs cellular association observed in the incremental geometric mean values of 982.36 ± 24.59, 2381.74 ± 48.53 and 3456. 65 ± 51.32, at 1, 4 and 8 h, respectively.

Annexin V/PI assay
Annexin V assay measured the extent of cellular apoptosis following treatment with olaparib and OLA@HSA NPs (Figure 5A). The concentration-dependent increase in apoptosis and necrosis was observed for OLA@HSA NPs. The total apoptotic/necrotic 4T1 cells populations in Q2 and Q4 quadrants were 12.32 ± 1.24%, 29.41 ± 0.85% and 43.02 ± 0.25, for olaparib (conc 25 µg), OLA@HSA NPs (12.5 µg) and OLA@HSA NPs (25 µg), respectively. Likewise, the percentage apoptotic/necrotic MDA-MB-231 cells populations for olaparib, OLA@HSA NPs (12.5 µg), OLA@HSA NPs (25 µg) were 15.02 ± 1.15%, 36.02 ± 0.32% and 45.02 ± 0.25, respectively.

Cell cycle analysis
The cell cycle analysis plot is represented in Figure 5B. The maximum arrest of cells in the G2-M phase by OLA@HSA NPs treatment was observed compared to olaparib treatment, with 8.42% for control cells, 16.23, 21.23 and 34.36% for 4T1 cells and 15.32, 22.32 and 35.32% for MDA-MB-231 cells for free olaparib, OLA@
Figure 3. In vitro evaluation of the anticancer activity of OLA, OLA@HSA NPs. The dose–response curve for the determination of IC_{50} values of free OLA, OLA@HSA NPs (24 and 48 h) in cultured 4T1 (A, B) and MDA-MB-231 cancer cells (C, D).

Figure 4. Cellular uptake of rhodamine-labelled, OLA@HSA-Rh NPs in 4T1 cell, MDA-MB-231 cell lines (olaparib concentration, 25 μg/mL) (A and B). Red and Blue signals present cells stained by rhodamine and DAPI, respectively; assessment of the geometric mean of fluorescence of the 4T1 (C), MDA-MB-231 cells (D) at 1, 4, 8h incubation by histogram plots and bar graphs. The data in bar graphs represent mean±standard deviation, calculated from three sets of experiments.
HSA NPs at concentrations, 12.5 and 25 µg/mL, respectively. Migration of cell population from G1 phase to G2/M phase predominantly was observed with the OLA@HSA NPs treatment at both the tested concentrations.

DNA fragmentation/nuclear staining assay
The appearance of fragmented DNA bands with a ladder-like pattern was more prominent in OLA@HSA NPs-treated cells total DNA than in free olaparib treated DNA extract (Figure 6A,C). OLA@HSA NPs treated cells exhibited more nuclear fragmentation (NF) and cytoplasmic shrinkage (CS) than olaparib treatment. Blebbing in the cell membranes indicative of late apoptosis was observed in OLA@HSA NPs-treated cells (Figure 6B,D).

Mitochondrial membrane potential assay
The fluorescence micrograph of control cells treated with JC-1 exhibited strong red fluorescence, which merged with the green fluorescence of the cells with olaparib treatment giving rise to orange and yellow signals in olaparib-treated 4T1 and MDA-MB-231 cells, respectively (Figure 7A,B). The OLA@HSA NPs-treated cells showed a bright green signal under the fluorescence microscope, indicating the presence of depolarised mitochondria. The flow cytometry data supported the fluorescence spectroscopic observation (Figure 7C). The fluorescence emission shift from red to green was observed with the highest shift of cell population from Q2-1 to Q4-1 in OLA@HSA NPs-treated cells than free olaparib treated cells. The JC-1 monomer/aggregate ratio (green/red cell populations) was 35% in olaparib-treated cells, and 75% in the
Figure 6. Analysis of DNA fragmentation. DNA extracted from 4T1 cells, MDA-MB-231 cells viewed on ethidium bromide-stained gel. DNA from untreated cells (Control), free olaparib, OLA@HSA NPs-treated cells (A and C). Nuclear staining of 4T1 cells, MDA MB231 cells with acridine orange (AO) (stained in green color) and DAPI (stained in blue color). Arrows indicate cytoplasmic shrinkage (CS) and nuclear fragmentation (NF) (B and D).

Figure 7. Detection of mitochondrial membrane potential by JC-1 staining in OLA, OLA@HSA NPs treated cells, 4T1 and MDA-MB-231 by fluorescence microscopy (A and B) and flow cytometry (C). Nuclei stained with DAPI was visualized under laser, ex/em. 358/461. The JC-1 fluorescence was visualised under a laser, ex/em. 488/530 nm in both fluorescence microscopy and flow cytometry. Gated cell population. 10,000 for the flow cytometry analysis.
OLA@HSA NPs showed intense red fluorescence compared to the spheroid-induced cytotoxicity. The spheroids treated with OLA@HSA NPs showed a geometric mean of fluorescence, 2800±18.65, compared to 1425±17.63 after olaparib treatment. Similarly, a significant increase in the ROS generation was observed in MDA-MB-231 cells treated with OLA@HSA NPs (than free olaparib, with the geometric mean of fluorescence of 3452±30.41 and 1635.±18.71 for OLA@HSA NPs and free olaparib, respectively.

**Spheroids study**

As shown in Figure 9A, the growth of the OLA@HSA NPs treated spheroids significantly reduced than the free olaparib group. Over time, an increment in the diameter of untreated spheroids (size reached up to about 925 µm) was observed, which was significantly bigger in volume than the spheroids treated with OLA@HSA NPs and free olaparib. The average diameter was found to be 925.23±22.61, 514.15±23.52 and 352.20±10.61 µm in control, free olaparib and OLA@HSA NPs treated spheroids, respectively, on day 4. Live/dead cell assay was performed to estimate the time-dependent spheroid uptake (Figure 9B). The Z-stacked images of spheroids treated with OLA@HSA-Rh NPs showed red fluorescence in the centre slices (50–70 µm) after 4h, indicating time-dependent spheroid uptake (Figure 9C).

**In vivo study**

In the tumour inhibition study, both the treatments, OLA@HSA NPs and free olaparib, suppressed the growth of the tumour to a significantly greater extent than control (no treatment). OLA@HSA NPs treatment showed higher tumour volume reduction than free olaparib treatment. The tumour volumes on day 21 were 1205.32±6.35, 639.22±5.2 and 205.32±2.5 mm³ for saline, free olaparib and OLA@HSA NPs treated animals groups (Figure 10A). The mouse body weight increased slightly during the treatment (Figure 10B). The average weight of the tumours for control, free olaparib and OLA@HSA NPs groups were 3.85±0.12, 1.66±0.32 and 0.56±0.22 g, respectively (Figure 10C,D). The luciferin D-mediated bioluminescence in 4T1-Luc tumours was captured in IVIS-Lumina live animal imaging facility, and the images have been displayed in a series (n=3) in Figure 10E. The spike in the signal in the region of interest in control groups indicated the highest tumour growth rate, followed by free olaparib groups and OLA@HSA NPs.

**Immunohistochemistry**

The TUNEL-positive cells, as indicated by the green fluorescence, were abundant in cryo-sectioned tumour tissues from the mice, which received the OLA@HSA NPs treatment (Figure 10F). The treatment with free olaparib caused a relatively greater extent of DNA fragmentation than saline treatment. The nuclear protein Ki-67 was reduced in OLA@HSA NPs-treated tumours (Figure 10G). The intense green fluorescence signal in control tumours indicated the presence of the proliferative marker, Ki-67, which was drastically reduced in the nanoparticles-treated tumour. The reduction in the rate of cell proliferation could be due to the induction of apoptosis to a greater extent in OLA@HSA NPs treatment groups, as evident further in the ROS production to a greater extent than free olaparib-treated groups (Figure 10H).

**Lung metastasis**

The breast cancer metastasis in the lungs was visualised by imaging the live animals using the in vivo imaging system (IVIS). The highest bioluminescence signal was evident in control, reduced in free olaparib-treated groups and practically invisible in the OLA@HSA NPs group (Figure 11A). The bar graph representation in Figure 11A compared the photon flux in the lung tissues of mice in three treatment groups. The bioluminescence was significantly lower following NPs-treatment than the free drug. The ex vivo images of lung samples dissected from the tumour-bearing mice supported the live animal imaging data (Figure 11B). The growth of cancer cells on lungs in colonies indicated by white nodules was visible in the control group, which decreased significantly in the treatment groups, including free olaparib and OLA@HSA NPs.
HSA NPs (Figure 11D). The histology of the lung of a normal mouse has been represented in Figure 11C as a control. The loss of alveoli and airway was distinct in the infected lung, whereas the shrinkage in the airways and denser cell growth as nodules were evident in the free olaparib-treated groups. The microscopic view of the lungs from the OLA@HSA NPs-treated groups showed no sign of hyperplasia or cancer metastasis. Colonising cancer cells to the lungs increased the size and weight proportionally. The weight of the lungs in the OLA@HSA NPs-treated group (0.213 g) was significantly lower than free olaparib (0.412 g) and saline-treated groups (0.652 g), respectively (Figure 11E).

**Western blotting**

The expressions of PARP1, γH2AX and p53 proteins were analysed by the western blot technique. Treatment with OLA@HSA NPs significantly downregulated PARP1 expression level compared to free OLA. The γH2AX and p53 protein expressions were increased in the tumour tissues after treating with free olaparib, and OLA@HSA NPs, as evident from our immunoblotting experiment. The representative bar graph of proteins expressions, as quantified by Image J software were shown in Figure 12. A significant upregulation of γH2AX and p53 protein expressions (p < .001) in OLA@HSA NPs-treatment groups indicated the superiority of the nanoformulation over the free drug in developing apoptosis.

**Discussion**

The increased size of OLA@HSA NPs compared to HSA NPs was due to the olaparib entrapment in the NPs. The slight decrease in the zeta potential in OLA@HSA NPs-treated groups was due to the anionic nature of the drug. The spherical morphology was ascertained by SEM analysis. The slight decrease in the olaparib absorbance during 1 month of storage could be due to the degradation of the drug. The storage stability data indicated that the NPs could be considered stable for 5–10 days following reconstitution, after which the NPs increased the size with a decrease in DL. The stability of NPs at 4 °C was not compromised to the extent as NPs at 25 °C, indicating the need for refrigeration of the reconstituted product. The disappearance of sharp 2θ peaks of olaparib in the X-ray powder diffraction spectrum of OLA@HSA NPs was indicative of its entrapment of olaparib into the NPs. The two characteristic peaks at 11.8° and 25.6° is from the human serum albumin [34]. Similarly, the disappearance of the melting point of OLA in the DSC thermogram of OLA@HSA NPs indicated successful encapsulation. The endothermic peak at 94° is characteristic of HSA [35]. Therefore, the DSC thermograms supported the reliability of the NPs preparation procedure. The characteristic peaks of both OLA and HSA were observed in the FTIR spectra of OLA@HSA NPs. Hence, the analysis confirmed the chemical structure of the NPs.

The CD spectroscopic data determines the ligand binding with protein, influencing the protein’s secondary structure [36]. The
Figure 10. Assessment of therapeutic efficacy of OLA, OLA@HSA NPs in 4T1-Luc tumour-bearing BALB/C mice. Graphical representation of tumour volume vs. days during treatment (A); measurement of body weight during the treatment (B); the average weight of tumours isolated from various treatment groups (C); and representative tumours isolated from mice post-treatment. Data represent mean ± SEM, n=4. **p<.01 and ***p<.001 (D); Tumour growth was assessed at the indicated time points by whole animal bioluminescence imaging as shown in a representative mouse and bioluminescence expressed as photon flux/second (ph/s) (E); representative images of tumour sections after TUNEL staining (F); fluorescent images of tumour tissue sections for immune-histochemical analysis of Ki-67 (G); detection of ROS level in tumour tissues by fluorescence microscopy and flow cytometry analysis (H).

Figure 11. Suppression of metastasis of 4T1-Luc cells in lungs by OLA@HSA NPs treatment. Imaging of live mice to assess metastasis on day 7 following surgery (n=5) and the bar graph representation of bioluminescence (A); ex-vivo bioluminescence intensities in lungs tissues of free olaparib, OLA@HSA NPs-treated mice (B); tumour metastasis in lung sections detected using haematoxylin and eosin staining (marked with circles). Scale bar:100µm (C); visualisation of lungs of free olaparib and OLA@HSA NPs-mice groups to check the development of metastatic nodules of 4T1 cells, and bar graph representation of the number of nodules/lungs in the free olaparib and OLA@HSA NPs-treatment groups (D); bar graph representation of lung weight (E). Significance of difference was analysed by paired t-test, **p<.01 and ***p<.001.
decrease in the MRE values at 208 and 222 nm in OLA@HSA NPs indicated the decrease in α-helical content [37]. The decrease of α-helical contents by approximately 13.4% indicated the influence of glutaraldehyde conjugation or interaction with olaparib with HSA in OLA@HSA NPs. The slight conformational changes induced by the protein binding were observed. However, the secondary protein structure remained predominantly α-helical in OLA@HSA NPs. The SDS-PAGE analysis revealed the presence of HSA in OLA@HSA NPs. The molecular weight of HSA did not increase, which indicated that there was no aggregation, and the NPs formation did not change the overall protein charge.

Haemolysis is the damage to the red blood cells resulting in the release of haemoglobin in the plasma [38]. As the clinical use of NPs is increasing, a thorough understanding of the safety of the circulating NPs in the bloodstream is much needed. Haemolysis occurs if the red blood cell membranes are damaged by the NPs, which causes the release of haemoglobin into the bloodstream. The haemoglobin release could lead to severe side effects, including renal toxicity, hypertension and anaemia [39]. In our study, the haemolytic % of 5.1, as displayed by the maximum tested concentration of 250 µg/mL is considered to be slightly haemolytic.

OLA release from OLA@HSA NPs might involve two different mechanisms. The initial burst release within 10 h might be attributed to the diffusion/ph-dependent drug dissolution. Further, a sustained release might be related to polymer hydrolysis and erosion, as indicated previously [40].

According to the cell viability assay data, the OLA@HSA NPs showed superior cytotoxicity than OLA at all concentrations due to their ability to penetrate efficiently into the cells compared to free olaparib [41]. The formulations induced a higher level of apoptosis, which could be due to the enhanced cellular uptake of olaparib via the nanocarrier systems. Cellular uptake study revealed that the HSA NPs were taken up by the cancer cells effectively in a time-dependent manner [42]. More DNA damage was indicated by increased cell cycle arrest in G2/M by OLA@HSA NPs than free olaparib treatment. The increase in G2/M arrest by OLA@HSA NPs suggests the ability of the formulation to inhibit the cellular division at the mitotic phase and thus control the doubling time and proliferation rate [43]. The OLA-loaded HSA NPs caused significantly higher DNA fragmentation and cytoplasmic shrinkage in cells than free olaparib treatment. The formation of apoptotic bodies and chromatin condensation were more noticeable in OLA@HSA NPs. The DNA damage was more prominent in gel electrophoresis [33]. Cytotoxic intracellular ROS could cause damage to mitochondria in cells, resulting in cell death [44]. High levels of ROS could disrupt the mitochondrial permeability transition pore and destroy the integrity of the mitochondrial membrane, resulting in immediate dissipation of mitochondrial transmembrane potential and osmotic swelling of the mitochondrial matrix [45]. A remarkably higher level of mitochondrial damage was observed in OLA@HSA NPs groups than the free drug, as indicated by the strong green signal of monomeric JC-1.

Multicellular spheroids mimic the three-dimensional tumour growth pattern, unlike cells grown in monolayers [46]. Deeper tissue penetration of NPs was observed in a time-dependent manner. The NPs treatment showed improved tumour tissue damage/necrosis, as indicated by the strong red PI fluorescence. The cell death resulted in spheroid shrinkage significantly to both free drug/drug-loaded NPs-treatments compared to the untreated spheroids [47].

The in vivo experiment proved that the OLA@HSA NPs treatment was superior in producing a therapeutic effect than the free drug treatment. The solid tumour represents acidic condition (low pH), hypoxia and nutrient-deprived microenvironments, creating favourable conditions for drug release due to the PH-dependent activity of formulation. Moreover, the leaky tumour vasculature creates an environment for formulation to enter the tumour vascular space, allowing the facilitated delivery of macromolecular drugs into the extravascular space by the EPR effect [48]. There was a significant reduction in the tumour volume in the OLA@HSA NPs-treatment group than control and free olaparib treatments. The non-reducing body weights indicated the safe use of the NPs. The live animal imaging shed light on the tumour growth pattern over 21 days. The reduction in bioluminescence intensity in the OLA@HSA NPs treatment groups due to cell death indicated successful treatment outcomes. The immunohistochemistry of the tissue sections indicated a higher number of apoptotic nuclei in OLA@HSA NPs-tumour groups than untreated control and olaparib groups in TUNEL assay, where the free 3′-hydroxyl termini of DNAs are labelled. The higher labelling is indicative of the presence of more fragmented DNA by double strands break leading to apoptosis. The decreased
presence of proliferative marker, ki-67 and increased presence of ROS was also observed in the tumour tissue section of the OLA@HSA NPs treatment group, revealing the promising treatment outcome.

Spontaneous metastasis is the hallmark of the delayed and advanced stage of cancer progression [49]. Lung tissue is the primary site of metastasis for 4T1 breast cancer cells miming human breast cancer. In the lung metastasis experiment, the PBS group mice revealed tumour lesions and protruding nodules compared to the free olaparib group. Metastatic nodules were hardly visible in OLA@HSA NPs-treated animals, as visualised by the naked eye and under the IVIS system via bioluminescence following luciferin treatment. The presence of nodules increased the lung weight, which was highest in the tumour-bearing untreated control group and lowest in the OLA@HSA NPs group. Similar observation was also reported elsewhere [50]. The histopathological observation revealed that the lung in the tumour-bearing untreated mice had a dense tissue population indicative of metastatic loci. However, lung histology of the OLA@HSA NPs-treated group was similar to the normal, non-tumour implanted mice.

Further, western blot analysis revealed the expression of several apoptosis-inducing cancer biomarkers. As indicated earlier, PARP1 protein mediates post-DNA damage repair and assists cells to pass through the G2/M phase. Downregulation of the PARP1 in the tumour tissues was significantly higher in OLA@HSA NP-treated mice compared to the control and free olaparib group. The suppression of the G2/M phase in the cell cycle could be due to PARP inhibition, which resulted in robust apoptosis in the NPs-treatment group [51]. The enhanced accumulation of olaparib in tumours of the OLA@HSA NPs-treatment group is due to the combination of active and passive targeting phenomena. The NPs accumulated to the tumour environment via the Enhanced Permeability and Retention (EPR) effect due to the nanosize [52]. Simultaneously, HSA has strong interaction with endothelial cells and tumour-microenvironment-associated SPARC protein and gp-60 receptors, resulting in an active nanocarrier targeting to the tumours [53]. The cleavage of the double standard DNA is initiated by phosphorylation of Ser-139 residue over histone H2AX, forming yH2AX. Increased expression of yH2AX is indicative of DNA damage. A significantly higher expression of yH2AX in tumour tissues was observed. Similarly, the tumour suppressor protein, p53 expression, was drastically increased in the OLA@HSA NPs-treatment group, indicating the triggering of apoptosis. Overall, the expressions of cancer-related biomarkers indicated the shifting of cancer cells' equilibrium towards apoptosis from replicative immortality.

Conclusion

Here, the PARP inhibitor, Olaparib-loaded, stable human serum albumin nanoparticles system was prepared by desolvation technique. The nanosized particles, OLA@HSA NPs were characterised to determine various physicochemical parameters. The particles exhibited low polydispersity, optimal DL, entrapment and sustained release at blood pH with accelerated release at tumour pH. The OLA@HSA NPs were stable at 4°C, non-haemolytic and demonstrated lower half-maximal inhibitory concentrations than free olaparib in mouse and human TNBC cell lines. The cellular uptake study revealed that the NPs were internalised time-dependently. A concentration-dependent increased expression level of the apoptotic marker phosphatidylserine was noted in OLA@HSA NPs-treated cells than free olaparib and untreated cells in both the cell lines. The OLA@HSA NPs caused the highest cell cycle arrest in the G2/M phase, DNA fragmentation, mitochondrial membrane depolarisation and ROS generation. The in vivo study demonstrated that the OLA@HSA NPs suppressed the tumour volume significantly compared to control and free olaparib-treated tumours. The isolated OLA@HSA NPs-treated tumours had increased expressions of fragmented DNAs, proliferative marker, Ki-67, p53, γH2AX and ROS, and reduced expressions of PARP-1. The lung metastasis study revealed that the OLA@HSA NPs-treatment slowed the cancer cells’ migration to distal organs. Overall, the OLA@HSA NPs induced a strong apoptotic response in vitro and in vivo, demanding further exploration, and revealing their potential for successful utilisation as chemotherapy in TNBC.

Disclosure statement

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