Lysozyme microspheres incorporated with anisotropic gold nanorods for ultrasonic activated drug delivery

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ABSTRACT

We report on the fabrication of lysozyme microspheres (LyMs) incorporated with gold nanorods (NRs) as a distinctive approach for the encapsulation and release of an anticancer drug, 5-Fluorouracil (5-FU). LyMs with an average size of 4.0 ± 1.0 μm were prepared by a sonochemical method and characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and Fourier-transform infrared spectroscopy (FTIR). The LyMs were examined using hydrophobic (nile red) as well as hydrophilic (trypan blue) dyes under confocal laser scanning microscopy (CLSM) to obtain information about the preferential distribution of fluorescent molecules. Notably, the fluorescent molecules were accumulated in the inner lining of LyMs as the core was occupied with air. The encapsulation efficiency of 5-FU for LyMs-NR was found to be ~64%. The drug release from control LyMs as well as LyMs incorporated with NRs was investigated under the influence of ultrasound (US) at 200 kHz. The total release for control LyMs and LyMs incorporated with gold NRs was found to be ~70 and 95% after 1 h, respectively. The density difference caused by NR incorporation on the shell played a key role in rupturing the LyMs-NR under US irradiation. Furthermore, 5-FU loaded LyMs-NR exhibited excellent anti-cancer activity against the THP-1 cell line (~90% cell death) when irradiated with US of 200 kHz. The enhanced anti-cancer activity of LyMs-NR was caused by the transfer of released 5-FU molecules from bulk to the interior of the cell via temporary pores formed on the surface of cancer cells, i.e., sonoporation. Thus, LyMs-NR demonstrated here has a high potential for use as carriers in the field of drug delivery, bio-imaging and therapy.

1. Introduction

In recent years, ultrasonically synthesized protein microspheres (Ms) have shown great promise in the field of nanomedicine because of their potential for high encapsulation efficiency, controlled as well as targeted release, encapsulation of multiple drugs, enhanced biocompatibility and prolonged circulation in the blood (i.e., stealth behaviour against the immune system) [1]. Further, they are safer, non-invasive and better candidates for targeting specific body parts [2]. Although there are many methods to generate Ms such as the emulsification method, mechanical agitation, spray drying, emulsion solvent evaporation, and cross-linking polymerization [3], the sonochemical approach is widely used for Ms fabrication [4,5]. When a liquid is exposed to US, dissolved gas nuclei oscillate, grow and violently collapse, a phenomenon referred to as acoustic cavitation [6,7]. Extreme physical and chemical effects are generated by the acoustic cavitation process. As a result, the sonochemical approach has not only received a great interest in the synthesis of drug delivery carriers but is also used for releasing the loaded molecules by rupturing the shell [7,8]. The possibility of using proteins and other biocompatible biological molecules for drug delivery carrier formation is also reducing the issues related to cytotoxicity in in-vivo experiments. Hence, continuous efforts are being explored to generate stable and biocompatible Ms via a sonochemical approach for various biological applications.

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In our previous study, we have reported the synthesis of stable, narrow sized liquid-encapsulated lysozyme microspheres via a sonochemical approach [1]. Lysozyme, a hydrolyzing enzyme protein present in many animal tissues as an innate immunity component, has four intramolecular disulfide linkages that can be modified with the help of reducing agents such as dithiothreitol (DTT). The partial denaturation of lysozyme by DTT facilitates the flexibility of protein to form complex cross-linking of disulfide bonds under the effect of an applied acoustic field and aids to build new disulfide bonds enhancing inter protein cross-linking [1]. Based on these mechanisms, gas-filled LyMs, i.e. microbubbles, have also been successfully synthesized using high-intensity probe sonication [8–10]. The core gas typically defines the structural stability of microbubbles [1,11]. Even though various gases such as CO2, N2, and PF6 have been reported for microbubbles formation, the stability of microbubbles was found to be poor along with non-uniform size owing to their highly reactive nature [10,12]. To circumvent these limitations, perfluorocarbon gases are being used to fabricate stable and narrow sized Ms with the size range of 1–7 µm. These gases are well known to improve the stability and echogenicity of microbubbles because of their inertness and insubility in nature in water, hence, making them less likely to be leaked out [13,14]. Notably, the use of perfluorodecalin (PFD) resulted in more stable Ms with a size range of 2.5–3 µm in diameter. As it was smaller than the average size of RBCs (erythrocytes), prolonged circulation in in vivo conditions has been reported [15].

Gas-filled protein Ms are a sensitive echogenic system, which is efficient enough to be detected by a US tracking source [16]. At a particularly high acoustic pressure, the walls of Ms expand during an acoustic cycle, leading to the bursting of the shell that not only releases the encapsulated molecules but also ensures the uptake of released molecules into cells by sonoporation [17,18]. Few of the commercially available Ms are Definity (Lantheus Medical Imaging North Billerica, MA, USA), Sonovue (Bracco, Milan, Italy), Optison (GE Healthcare, Chalfont St Giles, UK), Sonozoid (GE Healthcare, Chalfont St Giles, UK) & Levovist (SHU 508, Schering, Berlin, Germany), which are currently being used as either echo contrast agents or delivery carriers [19–21]. Ms have been widely used for the encapsulation and release of several drugs/molecules such as paclitaxel and doxorubicin [8,22]. Indeed, high-frequency ultra-sonication has been shown to have an important role in enhancing protein degradation, thus triggering the release of encapsulated drugs [8]. Frequencies as high as 1–5 MHz have been reported to trigger 90% drug release through breakage of Ms [15,23,24]. However, such high frequencies can cause temperature rises that could damage healthy cells or can even cause genetic mutations [24].

Till date, many different systems have been explored by incorporating inorganic or inert NPs into the polymeric shell and using them for US mediated drug delivery. Hollow polyelectrolyte capsules made of poly(allylamine) (PAH)/poly(styrene sulfonate) (PSS)/Fe3O4 or PAH/PSS/Ag NPs were prepared by layer-by-layer (LbL) assembly and employed for ultrasonically activated release of loaded molecules [25–27]. To fabricate NP incorporated LbL capsules, the NPs are either used as layer components or synthesized on the surface of preformed hollow capsules [25,28]. Similarly, Jun et al. reported hydrogen-bonded tannic acid and poly(N-vinylpyrrolidone) capsules via LbL approach to improve both US imaging contrast and the ability to deliver the loaded cargo at low-power diagnostic (~100 W/cm2) or high-power therapeutic (~10 W/cm2) ultrasound irradiation [29]. Notably, LbL assembly has been shown to release the loaded molecules in the acoustic frequency range of 40–50 kHz with power as high as 105 W/cm2 [30]. The issues such as polycations induced cytotoxicity, low yield of capsules, capsule aggregation, biocompatibility, and longer preparation time limit their potential in cancer therapy and related drug delivery applications. Further, the attempts to encapsulate both hydrophilic and hydrophobic molecules in single carrier systems are being explored. Hence, we believe that the use of protein Ms developed via sonochemical approach eliminates the limitations of conventional methods on the one hand while providing the options to encapsulate both hydrophilic and hydrophobic molecules on the other hand.

In this work, we report the fabrication of LyMs using DTT and PFD as denaturation agents and core gas, respectively. These LyMs were incorporated with anisotropic gold nanorods (AuNRs) by mixing pre-formed NRs with protein solution during Ms formation. The incorporation of NRs within LyMs caused local density variation in the shell leading to its burst under US irradiation. As triggering of release is expected in a short exposure time at lower US frequencies, the effect of shear forces on normal cells is minimized and the cells can even recover from the shock after a few hours of irradiation. It can inflict major damage to cancer cells at concentration ~IC50 via sonoporation in combination with the chemotherapeutic action of the drug. To the best of our knowledge, this is the first report wherein anisotropic nanoparticles have been demonstrated to alter the US frequency and exposure time for a bubble burst. We also report successful encapsulation and in vitro release of anticancer drug, 5-fluorouracil (5-FU), under US exposure at 200 kHz. Further, the anti-cancer activity was also investigated on the THP-1 cell line at a drug concentration of ~IC50 value to demonstrate that the sonoporation mechanism could be effectively exploited to target cancer cells.

2. Materials and methods

2.1. Chemicals

Lysozyme from hen egg white lyophilized powder, nile red, 5-FU, DTT, gold(III) chloride trihydrate (HAuCl4·3H2O), cetyl trimethylammonium bromide (CTAB), L-ascorbic acid (AA) and PFD were purchased from Sigma–Aldrich. Sodium borohydride (NaBH4), sodium citrate (Na3C6H5O7), silver nitrate (AgNO3), phosphate-buffered saline (PBS), tris (hydroxyl methyl) aminomethane (tris buffer) and trypan blue were purchased from SRL Pvt. Ltd., India. Roswell Park Memorial Institute (RPMI-1640) medium was purchased from LONZA, propidium iodide (PI) from Thermofisher, acridine orange (AO) and ethidium bromide (EB) from HI MEDIA Laboratories, India. All experiments were performed with Milli-Q water with a resistivity greater than 18 MΩ cm.

2.2. Fabrication of LyMs

LyMs were fabricated in three steps via sonochemical assisted method [31], namely, emulsification, aggregation and chemical cross-linking. Briefly, the process involves the emulsification of the liquid into a suspension of microdroplets in an aqueous solution followed by the aggregation of lysozyme at the liquid (PFD in this case) water interface and chemical cross-linking of cysteine residues of the partially denatured lysozyme [32]. The protein solution of 5% (w/v) lysozyme was prepared in 2.5 mL of tris HCl buffer (0.1 M) at pH 8. It was partially denatured by adding 0.15 g of DTT under continuous stirring for 2 min [22]. After partial denaturation, 100 µL of PFD was then added to the upper surface of the protein solution as a phase separator. For the synthesis of protein Ms, a 10 mL beaker consisting of 2 mL of the reaction solution was irradiated with US at 20 kHz for 30 s at an applied acoustic power of 500 W/cm2 (SAISONICS SJIA-500 W, India). The probe was operated at 40% amplitude and the power delivered was estimated to be 200 W. By positioning the tip of a 6 mm high-intensity ultrasonic horn at the protein solution interface, the LyMs were formed by irradiating the suspension with US. Notably, the inter-protein cross-linking was caused by the superoxide generated during the acoustic cavitation process leading to the encapsulation of the gas. After formation, the LyMs were washed multiple times with excess Milli-Q water at 3000 rpm for 5 min in order to remove excess residual protein and reducing agents. The washed LyMs were stored at 4 °C for further use.
2.3. Synthesis of gold NRs and NPs

Initially, the seeds were prepared by mixing the solutions of HAuCl₄ (5 mL, 0.0005 M), CTAB (5 mL, 0.2 M) and 0.6 mL of ice-cold 0.01 M NaBH₄. To grow the seeds into NRs, the growth solution was prepared by mixing the solutions of AgNO₃ (0.2 mL, 0.004 M), HAuCl₄ (5 mL, 0.001 M) and CTAB (5 mL, 0.2 M), followed by the addition of AA (0.07 mL, 0.0788 M). The NRs were prepared by adding 0.012 mL of seed solution into the growth solution under continuous stirring at 27–30 °C. The color change from yellowish-brown to violet after ~20 min of stirring indicated the formation of AuNRs. The formed NRs were washed many times with water and stored at 4 °C for further use.

Similarly, spherical gold NPs were also prepared by the seed-mediated method [34]. The seed suspension was prepared by mixing 0.6 mL of ice-cold 0.1 M NaBH₄ solution in 20 mL of aqueous equimolar solution (2.5 × 10⁻⁴ M) containing HAuCl₄ and trisodium citrate under continuous stirring. To grow spherical NPs, the seed suspension (1 mL) was added into growth solution consisting of 0.25 mL of 10 mM HAuCl₄, 0.05 mL of 100 mM NaOH, 0.05 mL of 100 mM ascorbic acid and 9 mL of 7.5 × 10⁻² M CTAB. After 5 min, 1 mL of formed NPs suspension was again added into a fresh growth solution as mentioned above. The suspension was turned into a deep purple in colour after 30 min of continuous stirring indicating the formation of larger sized spherical NPs. Finally, the formed NPs were washed multiple times with milli-Q water and stored at 4 °C for further use.

2.4. Fabrication of LyMs incorporated with NRs (LyMs-NR)

LyMs-NR preparation follows the same procedure as described above in Section 2.2, only with a little modification. The preformed AuNRs (~200 µL) were added to the initial solution of lysozyme along with DTT and PFD to form LyMs-NR. After high-intensity probe sonication for 30 s at 500 W/cm², the formed LyMs-NR were washed several times with Milli-Q water at 3000 rpm and stored at 4 °C for further use.

2.5. Morphological investigations of LyMs

The morphology of the fabricated LyMs, NRs and LyMs-NR such as shape and size was investigated by field emission scanning electron microscope (FE-SEM) and high-resolution transmission electron microscope (HR-TEM). For FE-SEM analysis, the samples were prepared by placing a drop of sample suspension on a fluorine-doped tin oxide glass plate and dried overnight in a desiccator to remove the moisture completely. After sputtering a thin gold layer, the samples were studied under FE-SEM at an accelerating voltage of 4 kV (Quanta, FEG 200 High-Resolution FE-SEM, FEL, Netherlands). For HR-TEM analysis, a drop of sample suspension was directly placed on a 300-mesh carbon-coated copper grid and air-dried overnight to remove the moisture completely. The analysis was performed at 200 kV on a field emission HR-TEM (TEM 2100 Plus, JEOL, Japan).

2.6. Neubauer chamber counting method

The LyMs concentration in one mL of suspension was determined by adding diluted LyMs suspension in a hemocytometer (Neubauer chamber) placed under an optical microscope at 20x magnification [24]. The number of intact LyMs present in a fixed volume of the sample suspension was estimated by manually counting the number of LyMs present in all the square boxes of the hemocytometer. The total LyMs population in the original solution was estimated by using the following equation:

\[
\text{Number of Ms per mL} = \text{Mean Value} \times \text{Dilution Factor} \times 10^3
\]

2.7. Confocal laser scanning microscopy (CLSM)

The degree of filling and preferential distribution of drug molecules in LyMs were investigated using a confocal microscope equipped with a 100×/1.4–1.7 oil immersion objective (LSM 510 META, Zeiss, Germany). Nile red and trypan blue were used as model fluorescent molecules to visualize the preferential distribution on the different parts of Ms to gain more detailed information about the encapsulation process. For CLSM imaging, the fluorescent probe solution (0.5 mg mL⁻¹) was directly mixed with the protein solution before irradiating with US. The dye loaded LyMs were washed twice with Milli-Q water and stored at 4 °C for further investigations in CLSM. 20 µL of dye loaded suspension was placed on a microscopic slide and visualized under CLSM at an excitation wavelength of 552 nm for nile red and 607 nm for trypan blue. It is important to mention that all experiments involved in fluorescent molecules were performed in a dark atmosphere to avoid the loss of fluorescence.

2.8. Fourier transform infra-red (FTIR) spectroscopy

The control and dye loaded LyMs were lyophilized and made into pellets by mixing with KBr powder. The spectra were acquired in transmission mode on a Cary 600 FTIR spectrophotometer.

2.9. Zeta potential measurements

The surface charge and stability of the Ms were investigated by measuring the zeta potential based on the dynamic light scattering technique. Dilute samples of LyMs, LyMs-NR and LyMs-NR-5FU were prepared in water and their potentials were directly estimated (Horiba Scientific SZ-100 Analyzer, Japan). The experiments were performed in triplicates and the number of scans for each measurement was maintained at 50.

2.10. Drug encapsulation experiments

The encapsulation of 5-FU was carried out during the formation of Ms by adding 1.5 mg of the drug into the 2 mL of protein solution prior to probe sonication. After the sono-assisted encapsulation process, the drug-loaded LyMs and LyMs-NR were collected by centrifugation at 3000 rpm for 5 min. The amount of unencapsulated drug was estimated by measuring the absorbance of the supernatant at 265 nm using a UV-Vis spectrophotometer (UV-Vis) (NanoDrop Technologies, USA) [35]. The amount of drug encapsulated in the Ms was calculated by subtracting the amount present in the supernatant from the original amount of drug added for the encapsulation process. The encapsulation efficiency (EE%) was calculated by the following equation:

\[
\text{EE} (%) = \frac{\text{Amount of drug encapsulated in the LyMs suspension} \times 100}{\text{Total amount of the drug added initially during preparation}}
\]

2.11. In-vitro drug release experiments

The release of 5-FU loaded LyMs-NR was investigated under US irradiation and compared to that of LyMs. The release experiments were performed in 2 mL centrifuge tubes by irradiating the suspensions at 200 kHz and 0.45 W/cm² using an acoustic power bath sonicator (Kaijo Quava mini, Kaijo Shibuya America Inc., USA). The temperature of the suspensions was maintained at 35 to 37 °C. It is worth noting that the irradiation of US was stopped for every 10 min of exposure followed by a resting period of 5 min to prevent the overheating of Ms suspensions.

After specific time intervals (10, 20, ... 60 min), the sample was centrifuged at 3000 rpm for 5 min to separate the Ms. The supernatant was withdrawn and its absorbance was measured at 265 nm to estimate the amount of released drug. Then, the supernatant was exchanged with fresh pre-warmed Milli-Q water to maintain the concentration gradient
between supernatant (released) and drug encapsulated Ms. During the release process, the tubes were kept sealed to minimize the evaporative loss. The experiments were done in triplicates to ensure repeatability and consistency.

2.12. High-performance liquid chromatography (HPLC) experiments

To rule out the possibility of any interference of other components during UV-Vis measurements, a confirmatory analysis of 5-FU encapsulation and release from LyMs-NR was performed using HPLC (Shimadzu, LabSolutions). Similar experiments as mentioned in Sections 2.10 and 2.11 were conducted and supernatants were collected for measurements in HPLC. The amount of drug present in the supernatant after the encapsulation process as well as at different time intervals during the release process was estimated by introducing the supernatants into the HPLC column. The column temperature was maintained at 40 °C where the mobile phase was pumped at a flow rate of 0.8 mL/min. For LyMs-NR, the mobile phase used was a mixture of methanol and sodium acetate buffer (pH adjusted to 4.0) in the ratio of 30:70 (%vol.). The experiments were performed in triplicate to ensure repeatability and consistency.

2.13. Anti-proliferative activity investigations

Human monocytic cells (THP-1) obtained from National Centre for Cell Science, India were used for studying the anti-proliferative activity of 5-FU loaded LyMs. The cells were seeded in a 6 well plate containing RPMI media at 37 °C with 5% CO₂ and grew up to 80% confluency. The cultured cells (~1x10⁶ cells) were incubated with the samples for 24 h in RPMI media. To investigate the anti-proliferative activity, different samples of Ms (LyMs, LyMs-NR, LyMs-NR-SFU, pure 5-FU, and control) were added to the cell medium and exposed to 200 kHz US in the bath sonicator for 1 h. It is worth noting that a cooling period (incubation temperature 35–37 °C) of 5 min was given after every 10 min of US exposure to avoid cell death caused by the overheating of the medium. The temperature of medium containing cells was maintained between 35–37 °C using cold water and ice and monitored with the help of a thermometer. Additionally, normal THP1 cells without US exposure were also studied to investigate the influence of US on THP1 cells. The cells were then centrifuged, washed with 1x PBS and re-suspended in 500 µL of sterile PBS for 24 h. The cell suspension was then stained using 5 µL of PI solution (1 mg/mL in water) for 5 min and analyzed via fluorescence-activated cell sorting (FACS) on low mode (BD FACS Calibur, U.S.A.). The individual cells were detected by their forward angle scatter (FSC) and right angle scatters (SSC) at an incident wavelength of 488 nm. This light scattering data was used to establish a gated region that excluded cell clusters and debris from the fluorescence analysis. The light scattered gated PI fluorescence of individual cells was acquired by using a 630 nm band-pass filter (FL-2) and displayed in single parameter dot plots and histograms, by using preinstalled software (Cell Quest Pro). The fluorescence dot plots were investigated to determine the live and dead cells.

To gain more detailed information about the role of US irradiation on cancer cell cytotoxicity, THP-1 cells were exposed to US irradiation and their viability was investigated as a function of the time of irradiation. Separately, another batch of control cells was exposed to US for 1 h with a cool period of 5 min for every 10 min of exposure, and their viability is compared to cells exposed for continuous exposure for 1 h. To investigate the recovery of cells after US exposure, the cells were incubated in the medium over a time period of 0 to 24 h and their viability was estimated by FACS.

The different sample group treated cells were also visualized under the confocal microscope to understand the morphological changes. Another set of cells was treated with LyMs-NR-5-FU and pure 5-FU for 24 h at a concentration of < IC₅₀ value of 5-FU under US irradiation to understand more about the influence of sonoporation on the cancer cell cytotoxicity. The cells without US exposure were kept as control. These cells were then treated with PBS containing 50 µL of both acridine orange (AO) and ethidium bromide (EB) and visualized under CLSM to estimate the live (green) and dead cell (red) population at a wavelength of 526 and 650 nm, respectively.

3. Results and discussion

3.1. Synthesis of protein Ms

The LyMs were successfully fabricated by the sonochemical method using PFD and DTT as liquid core and cross-linking agents, respectively. The partial denaturation of lysozyme with DTT followed by phase separation in the presence of PFD under US irradiation at 200 kHz resulted in the formation of stable LyMs. During the phase separation, the flexible and partially denatured protein molecules undergo configurational reorganization by forming new disulfide bonds (inter-protein cross-linking) at gas/liquid interphase and stabilize Ms [36]. Notably, the LyMs were stable in both liquid and dried forms with a size range of about 3–5 µm in diameter, as shown in Figure 1. Optical microscopic investigations show that the suspension has good colloidal stability without any secondary aggregation of Ms (Figure 1a). The LyMs concentration estimated using hemocytometer was found to be 1.8 × 10⁸ LyMs mL⁻¹ (Experimental Section 2.6). Further, the percentages of intact and broken LyMs in one mL of suspension were also estimated by mounting a hemocytometer filled with a fixed volume of suspension under an optical microscope. It is worth noting that 67% LyMs were intact demonstrating the effectiveness of the sonochemical method. While SEM investigations show that the LyMs are maintaining their spherical shape in dried form, the TEM investigations revealed that NPs of 100 to 200 nm in size were observed in the shell of Ms, most likely caused by partial denaturation of lysozymes, as shown in Figures 1b and

![Figure 1. Morphological investigation of LyMs as studied by (a) optical microscope, (b) SEM, and (c) TEM.](image-url)
c. For the fabrication of LyMs-NR, the preformed AuNRs were added to the protein solution during the fabrication process prior to US exposure. To synthesize AuNRs, a two-step seed-mediated approach has been used as it provides better control over the shape and size of NRs [33,37]. It involves the initial formation of gold seeds of ~4 nm followed by the growth of these seeds into rod-shaped structures in the presence of fresh gold ions and shape directive agents. The formation of NRs was confirmed by UV–Vis which showed two characteristic plasmons (transverse and longitudinal) peaks at 514 and 729 nm, respectively, as shown in Figure 2b [37]. The diameter and length of the NRs were found to be ~10 and ~30 nm, respectively (Figure 2a). The US irradiation of protein solution in the presence of AuNRs, PFD and DTT resulted in the formation of LyMs-NR (Figure 2c). The shape, size and other morphological features of LyMs-NR were similar to control LyMs. Notably, uniformly distributed AuNRs with the size of 30 ± 5 nm could be seen clearly in the shell of LyMs along with other smaller NPs. The zeta potential value of LyMs-NR was found to be increased from 55 ± 2 (for LyMs) to 94 ± 4 mV after NR incorporation indicating the improved stability of the formed LyMs-NR (Supporting information, Figure S1). As Gold NRs have a positive surface charge (65 ± 4 mV) due to the presence of CTAB, Ag⁺ ions deposition and Ag⁺/CTAB complex, the incorporation of NRs into LyMs increases the surface potential of LyMs-NR, which in turn, increases the stability of LyMs-NR [38–39]. It is believed that NRs interact with thiol groups present on the surface of lysozyme during the fabrication of LyMs-NR [40]. Of note, similar experiments were performed with BSA instead of lysozyme to fabricate BSA-Ms, as shown in Supporting Information, Figure S2. Though optical microscopic investigations showed that the colloidal stability of aqueous suspension was good, the collapse of BSA-Ms was observed in the dried form. Notably, < 5% of intact spherical BSA-Ms were observed in the dried form as shown in TEM investigations. Previous investigations on BSA and lysozyme based Ms showed that the wall thickness values were 50 and 130 nm for BSA-Ms and LyMs, respectively [3,4]. This is corresponding to ~6 protein molecules for BSA and ~26 protein molecules for lysozyme when their hydrodynamic radii were considered for thickness calculation in unfolded forms. As a result, LyMs form a more compact and thick shell and thus reduced gas permeability. Hence, all our further experiments were performed with LyMs.

To gain more detailed information about the rupturing process of Ms under US irradiation, LyMs and LyMs-NR were exposed to US for 1 h and their morphological changes were investigated using TEM. During the experiments, the Ms were given 5 min of the cooling period for every 10 min of continuous US irradiation to prevent any temperature-induced morphological changes on Ms. After 1 h of US irradiation, the LyMs were intact and there was no sign of bursting and any other discontinuities on their surface (Figure 3a). However, the LyMs-NRs were completely collapsed and bursted at multiple points due to the incorporation of AuNRs, as shown in Figure 3b and Supporting Information, Figure S3. The presence of NRs on the surface of LyMs-NR creates a local density difference in the shell and reduces the elasticity of Ms, which plays a key role in making them more prone to bursting by acoustic cavitation. As a result, the rupture/bursting of Ms was observed at much lower frequencies than that of values reported in the literature, i.e., at 200 kHz. This eliminates the use of high frequency or high power ultrasonicators for rupturing of Ms. Many reports have shown frequencies in the range of 1–10 MHz for the complete bursting of Ms which could also affect adversely by causing genetic mutations [24]. Further, US with the intensity in the range between 1000 and 10,000 W/cm² has been commonly used to treat diseases like uterine fibroid treatment [41,42]. Such high-intensity US exposure can induce lesions or tissue necrosis at space cramped small locations deep inside the tissues when the local temperature exceeds 70 °C. As we have used US with a frequency of 200 kHz and 0.45 W/cm² to demonstrate the bursting of LyMs, this system will have a huge potential for use as a US-guided drug...
delivery system.

From the perspective of using LyMs for drug delivery applications, the loading of different fluorescent probes into the LyMs shell was investigated by CLSM. Fluorescent probes such as nile red (hydrophobic) and trypan blue (hydrophilic) were used as model biological molecules of varying hydrophilicity to investigate the encapsulation behavior of LyMs as shown in Figures 4a and d, respectively. CLSM investigations revealed intense red and blue fluorescence coming from the interior of LyMs as shown in Figures 4a and d, respectively. CLSM investigations revealed intense red and blue fluorescence coming from the interior of LyMs. Notably, it was difficult to confirm that the encapsulation was observed in the shell or inner core of LyMs as LyMs were moving randomly in the suspension. To get more detailed information on the encapsulation process of LyMs, we performed Z-stacking separately on trypan blue and nile red loaded LyMs, as shown in Supporting Information, Figure S4. Interestingly, the fluorescence was seen in all planes of Ms in the fluorescent channel indicating successful encapsulation for both trypan blue and nile red. By comparing the overlapped image of fluorescent and bright field channels (Figure 4c and f), it can be concluded that the loading of the dyes is observed only in the shell as it appeared in either blue or red colour. The swollen darker core of LyMs also confirms that there is no encapsulation in the interior of Ms.

Given the successful encapsulation of trypan blue and nile red, as investigated by CLSM, and with a desire to further elucidate the structural information, the fluorescent molecule loaded LyMs were investigated by FTIR spectroscopy (Figure 5). Lysozyme spectrum shows three characteristic bands at 1635, 1548 and 1387 cm\(^{-1}\), which are corresponding to amide I (carbonyl (C-O) stretching), amide II and amide III of primary amide groups. The amide II band is resulting from the combination of N-H in-plane bending and C-N stretching while amide III

Figure 4. CLSM investigations showing LyMs incorporated with (a,b,c) nile red and (d,e,f) trypan blue.

Figure 5. FTIR investigations of LyMs before and after encapsulation with nile red and trypan blue.
consists of two complex vibrational modes at 1387 and 1316 cm\(^{-1}\). The additional peaks at 1191, 1105, 907 and 662 cm\(^{-1}\) correspond to C-N stretching, C–C–C bending, CH\(_2\) bending and C–H bending, respectively [43]. The characteristic peaks of LyMs incorporating nile red were observed at 1633 (C=O), 1538 (C-N), 1315 (C-N) and 1138 cm\(^{-1}\) (C–C–C), which were corresponding to characteristic peaks found in pure nile red spectra [43]. After encapsulation of nile red, the spectrum appeared same as major functional groups of nile red were also present in the lysozyme. Similarly, trypan blue shows three characteristic peaks at 3419, 2924 and 1633 cm\(^{-1}\), which are ascribed to O–H stretching, C–H symmetric and asymmetric stretchings, respectively [44]. Notably, the trypan blue loaded LyMs showed the characteristic peaks of both trypan blue and control LyMs. Hence, FTIR results confirmed the encapsulation of nile red and trypan blue into the LyMs.

3.2. Anti-cancer drug encapsulation and release experiments

The encapsulation of the anticancer drug (5-FU) was performed by adding a defined quantity of drug to the protein solution prior to US irradiation. The amount of encapsulation of the drug was estimated by measuring the difference in free (un-encapsulated) drug present in the supernatant prior to and after the encapsulation process. The encapsulation efficiencies were found to be ~46 and 57% for LyMs and LyMs-NR, respectively (Supporting Information, Figure S5). The amount loaded in LyMs and LyMs-NR was about 0.689 and 0.852 mg, respectively, in 1 mL of the aqueous suspension. The increase of encapsulated amount for LyMs-NR is possibly caused by either participation of incorporated NRs directly in the encapsulation process or their incorporation improves the porosity of the shell and enhances the encapsulation process.

The release of encapsulated 5-FU from LyMs and LyMs-NR was investigated under US exposure at 200 kHz using a UV–Vis. The release was continued over a period of 1 h and samples were withdrawn at specific time points to estimate the amount of release in the supernatant, as shown in Figure 6a and b. As explained in the experimental section, a cool-in period of 5 min was provided for every 10 min of continuous US irradiation to prevent the increase in local temperature. The release profile showed an initial burst release in the first 10–20 min followed by sustained release up to 1 h. The release in the burst phase was 32% for LyMs and it increased to 71% after 1 h of US irradiation. Notably, the release was 68% for LyMs-NR in the burst phase and increased to 96% after 1 h of US irradiation demonstrating an enhanced rupturing of Ms after NR incorporation. The acoustic cavitation bubbles formed during US irradiation collapse with greater energy in the suspension resulting in physical damage to Ms. This phenomenon plays an important role in the bursting of the Ms followed by the release of 5-FU. As discussed previously in TEM investigations of the rupture of Ms, the rupture caused by US irradiation increased the release of 5-FU from LyMs-NR. Thus, it can be concluded that the incorporation of NR into the shell most likely made the shell heavy and increased its rigidity, which made them vulnerable to acoustic cavitation. Of note, the encapsulation and release experiments were also performed using HPLC as it has better sensitivity than UV–Vis. During UV–Vis experiments, the peak of 5-FU absorbance lies at 265 nm and can interfere with the lysozyme absorbance peak at 280 nm. The HPLC investigations showed that the amount of encapsulation of 5-FU in LyMs-NR was found to be 64% (Supporting Information, Figure S6). Notably, the release estimated was ~76% after 1 h of US exposure at 200 kHz. As there is an overlap between the characteristic peaks of 5-FU (265 nm) and lysosome (280 nm), the interference contributes to a higher release in UV–Vis measurements. We have also
performed similar release studies with LyMs incorporated with spherical gold NPs of 50–100 nm in size to investigate the effect of the shape of NPs on US induced release. Notably, under similar experimental conditions, the estimated release was only 40% for Ms incorporated with spherical NPs, which is much lower than the 96% release observed for NRs incorporated LyMs (Figure S7 supporting information). We believe that the spherical NPs were unable to destabilize the LyMs surface at 200 kHz, thus lowering the release. Hence, all our further experiments were performed with LyMs-NR.

3.3. Anti-cancer activity investigations

The anti-cancer activity against THP-1 cells was performed in the presence of various samples such as pure 5-FU, LyMs-NR, LyMs-NR-5-FU and control cells under US exposure using FACS to evaluate their anti-cancer activity for in vivo applications. Prior to anti-cancer activity investigations with Ms, the IC50 value of 5-FU against THP-1 cells was estimated by measuring the viability of the cells as a function of concentration, as shown in Supporting Information, Figure S8. From the MTT assay, the IC50 value of 5-FU was found to be ~7 µM. As we have performed the rupture and drug release experiments of LyMs for 1 h of US exposure, a systematic study was designed to investigate the effect of US on THP-1 cancer cells. Although the total exposure of 1 h was maintained for cell line experiments, a recovery time of 5 min was given at different time intervals to investigate the role of US exposure on cells and their possible recovery during the incubation in medium, as shown in Figure 7a. After 60 min of continuous US irradiation (without break) at 200 kHz followed by incubation in the medium for 24 h, the cell viability was found to be 38%. It showed that the cells were not able to recover from the trauma caused by US even after 24 h of incubation in the medium. When the number of recovery breaks was higher during the irradiation time, the viability was high indicating a better possibility for recovering from the trauma caused by US. For instance, when a recovery period of 5 min was given for every 10 min of US exposure, the viability of cells was found to be 97% which is the same as that of control cells without US exposure. To get more detailed information about the recovery process, the US treated cells (recovery period of 5 min for every 10 min of exposure) were incubated in a medium and their viability was investigated as a function of incubation time (Figure 7b). Interestingly, the viability after exposure was about 43% and increased to 97% after 24 h of incubation in the medium. These investigations revealed that the cells might have been in shock after exposure and started behaving as dead cells (Figure 7b). After a sufficient recovery period of 24 h in medium, the cells were able to recover from the shock, thus improving the viability of the cells. Previous investigations demonstrate that the cells are getting repaired from the damage done by the US alone [45].

Until the DNA gets damaged, the cells can recover from any trauma and the ruptured cell membrane might get repaired when provided a sufficient incubation period. It is worth noting that the cells exposed continuously for more than 10 min were not able to recover from the trauma even after 24 h of incubation and showed lesser viability than control cells (Figure 7a). To get more insight into the recovery of cells after US exposure, the recovery process was investigated using a confocal microscope by studying the cell morphology as a function of recovery time. Notably, the cells appeared with porous and rough cell membrane just after US exposure transformed into healthy and active cells with smooth membrane after 24 h of incubation in the medium as shown in Figure 8. Notably, the cells were uniformly distributed in the suspension in all the time intervals revealing that the cells were alive and active. It should be noted that the dead cells start aggregating and appeared as clusters. Thus, it can be concluded that the cells were in some kind of transition state after US exposure for 1 h and transformed into healthy and active cells after sufficient time of incubation in the medium.

By taking the advantage of the transition state (trauma) of cancer cells after US exposure, the anti-cancer experiments were performed at a 5-FU concentration of 3.5 µM (i.e., half the IC50 concentration of 7 µM) as they might be more susceptible to chemotherapeutic drugs after US exposure. Various samples such as pure 5-FU, LyMs-NR, LyMs-NR-5-FU and control were treated with THP-1 cells under 1 h of US exposure (interval of 10 min) and their viability was investigated after 24 h of incubation in the medium (Figure 9 and Supporting Information Figure S9). Notably, LyMs-NR-5-FU treated cells showed cell death of 96% demonstrating the susceptibility of cancer cells under US exposure.

Figure 8. CLSM investigations show the morphology of the cells as a function of incubation time. After US exposure for 1 h (5 min recovery break after every 10 min), the cells were incubated in a medium for (a) 0 h, (b) 4 h and (c) 24 h.

Figure 9. Cell viability of 5-FU, LyMs-NR, LyMs-NR-5-FU, control-US and control cells after 1 h of US irradiation.
irradiation. The possible reason could be the drug transfer mechanism from the bulk into the cell via temporary pores formed during the US exposure, as shown in Figure 10. As LyMs-NR-5-FU is a US responsive system, it could transfer the drug through a sonoporation mechanism with the help of the phenomenon known as acoustic microstreaming [46]. The temporary pores formed during the collapse of LyMs-NR-5-FU in response to high-frequency ultrasonication act as a passage for the drug to enter the cells without any interference [47]. Although, the cells exposed to pure 5-FU could also be affected by acoustic cavitation which induces temporary pores under US irradiation, but the uniformly distributed local drug concentration (3.5 µM) near the cells was much lower than the obtained IC$_{50}$ value of ~7 µmol (Supplementary information Figure S8). In other words, we believe that the high amount of drug released at localized regions from LyMs-NR via sonoporation increased the local concentrations beyond IC$_{50}$ value near the cell membrane and induced cell death. As a result, the viability was 81% for pure drug-treated cells. As the drug released locally from LyMs-NR-5-FU is higher than the IC$_{50}$ value, it resulted in 96% cell death. As expected, the control cells recovered quickly, and the viability reached 97%. Similar experiments were performed separately, and the morphology of cells was investigated by CLSM, as shown in Figure 11. Notably, the control cells were dispersed well in the suspension and exhibited intact cell membranes. The free drug-treated cells showed a smaller % of odd-shaped and aggregated cells indicating a lower % of cell death. Interestingly, LyMs-NR-5-FU treated cells showed complete cellular aggregation and loss of cellular integrity demonstrating higher cell death (Figure 11d).

To investigate the cellular apoptosis-associated morphological changes of the cell membrane, the cancer cells after treatment with different samples were stained with AO/EB to analyse the live and dead cell populations (Figure 12). AO is a cell-permeable cationic dye interacting with DNA and RNA by intercalation and thus, stains live cells. EB is also an intercalating agent commonly used as a fluorescent tag for nucleic acid staining. EB is not cell-permeable, thus its presence inside cells represents cellular membrane being compromised and identified as red fluorescence. As shown in Figure 12 (a, d and g), no significant cell death was observed in control cells as most of the cells were appeared in green color (i.e., stained with AO). On the other hand, 5-FU in US treated group showed moderate cell death (Figure 12 b, e, and h) indicating either the ineffectiveness of acoustic sonoporation or penetration of a lower amount of drug into the cell as mentioned in the earlier section. Notably, the LyMs-NR-5-FU with US treated cells showed the majority of the cells in red color (i.e., stained with EB as shown in Figures 12 c, f and i) indicating the enhanced anticancer activity. The orange color in few cells indicates damaged cells. Thus, these results confirm that LyMs-NR-5-FU could be used as US-guided drug carriers in cancer therapy.

4. Conclusions

We have demonstrated the ultrasonic synthesis of lysozyme microspheres (LyMs) incorporated with AuNRs for US-guided anti-cancer drug delivery. LyMs with an average size of 4.0 ± 1.0 µm were prepared by

Figure 10. Schematic representation showing the interaction of LyMs-NR with cancer cells via temporary pore formation.

Figure 11. CLSM investigations show the morphology of THP-1 cells after treatment with different samples. (a) Control cells with no US exposure, (b) control cells after 1 h of US exposure, c) free drug (5-FU) treated cells after 1 h of US exposure and (d) LyMs-NR-5-FU treated cells after 1 h of US exposure.
the sonochemical method and demonstrated for successful encapsulation and release of hydrophilic (trypan blue) and hydrophobic (nile red) model fluorescent molecules. The encapsulation efficiency of LyMs-NR was found to be ~64% for 5-FU. Under US irradiation at 200 kHz, the bursting of LyMs followed by the release of 5-FU was observed. The release was found to be 68% for LyMs which increased to 96% after incorporation with AuNRs. The viability of THP-1 cancer cells against US exposure is largely dependent on the exposure time and recovery time in between the exposures. When the cells were exposed to US for 1 h with a recovery period of 5 min for every 10 min of exposure, the cells recovered quickly and viability increased to 97% after 24 h of incubation in the medium. Under US irradiation, the temporary pores formed on the cell surface can be targeted via acoustic micro-streaming to improve the anti-cancer activity. Notably, LyMs-NR-5-FU showed 97% cell death against THP-1 cells even at half the IC_{50} concentration of 3.5 µmol. The LyMs showed unique and attractive characteristics needed for US guided-drug delivery, thus showing great promise in the area of cancer therapy.

**CRediT authorship contribution statement**

**Bharat Bhargawa**: Methodology, Writing – original draft. **Varsha Sharma**: Methodology. **Munuswamy-Ramanujam Ganesh**: Methodology. **Francesca Cavalieri**: Writing – review & editing. **Muthupandian Ashokkumar**: Writing – review & editing. **Bernardshaw Neppolian**: Conceptualization, Writing – review & editing. **Anandhakumar Sundaramurthy**: Conceptualization, Writing – review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Figure 12.** Anti-cancer activity experiments showing the live and dead cells after staining with AO/EB. a) Control, b) free drug and c) LyMs-NR-5-FU treated cells after US exposure of 1 h. The top, middle and bottom panels show green, red and overlapped channels, respectively. Note that the live cells appear in green colour while dead cells appear in red.
