

RESEARCH ARTICLE

Calcium Ferrite Reinforced Starch Nanovesicles—Application in Anticancer Drug Delivery

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ABSTRACT

Recently, the formulation of multifunctional nanoparticles has significantly reduced various limitations associated with drugs and drug carriers. In this study, a novel multilamellar hybrid nanovesicle system was developed using a combination of a polysaccharide and a lipid. Starch forms the core of hybrid nanoparticles, while stearic acid with polyethylene glycol containing calcium ferrite nanoparticles form the shell. The drug, zidovudine (AZT), was encapsulated within the hybrid nanovesicle system for anticancer drug delivery. The calcium ferrite nanoparticles were characterized by transmission electron microscopy (TEM) and x-ray diffraction (XRD) analyses. The morphology of the hybrid nanoparticles was analyzed using TEM, which showed a multilamellar morphology with an average size of 179 ± 18.23 nm. The drug loading and release behavior of these nanoparticles were assessed, and they showed high encapsulation efficiency (93%) and a sustainable release of AZT over 48 h. The cytocompatibility of the hybrid nanosystem was analyzed in human fibroblasts and keratinocytes, and the nanoparticles demonstrated no significant cytotoxicity toward these cell lines. The anticancer activity was tested against human breast cancer cell lines, which showed excellent cytotoxicity toward cancer cell lines. The investigation is promising for designing a multilamellar hybrid nanosystem-based anticancer delivery system.

1 | Introduction

On main grounds of fatality worldwide, cancer is dangerous and even incomprehensible disease since it extends through multistep carcinogenesis, which engrosses many cellular physiological systems. Despite advances in cancer therapy, chemotherapy remains the most effective treatment option. However, one of the biggest challenges in chemotherapy is multidrug resistance (MDR), which limits its effectiveness. Owing to their low molecular

weight, anticancer drugs often face issues of very low bioavailability in tissues, fast blood clearance, nonspecific selectivity, and low accretion in tumors, which enable cancer to persist against these anticancer drugs. Nanoparticles have been introduced as magic probes for in-depth study of pathogenesis at the molecular level. A futile therapeutic response has also been reported due to low stability of nanoparticles, poor drug solubility, chemical resistance, poor bioavailability, etc. To conquer these constraints, diverse sorts of drug delivery systems (DDSs) have been developed [1–4].

Azidothymidine or zidovudine (AZT) is the first antiretroviral drug that has been successfully used as a competent therapy for AIDS, either on its own or with other antiretroviral drugs. However, the clinical use of this drug is hampered by its serious side effects. Reverse transcriptase inhibitors (RTIs), such as AZT, may also have potential benefits in cancer treatment by inhibiting the growth of cancer cells through suppression of telomerase activity. AZT, originally designed as an antitumor agent, inhibits cancer cell propagation by targeting the reverse transcriptase enzyme, making it a promising chemotherapeutic drug [2–4].

Magnetite nanoparticles (MNPs) have attained key interest in the biomedical field due to their scientific impact in applications including cellular signaling, magnetic resonance imaging (MRI), magnetic hyperthermia (MHT), and drug delivery [1–4]. Magnetic nanoparticles also play a vital role in diagnosis and therapeutic applications. When exposed to an external magnetic field (MF), MNPs can be directed to specific targets within the body, opening possibilities for targeted drug delivery. The use of magnetic nanoparticles improves drug efficiency by allowing real-time monitoring of treatment progress. The prime requirement for the above is that nanoparticles should be non-toxic, biocompatible, uniform, and well dispersed in the liquid phase. The use of MNPs in in vivo applications will be hampered by drawbacks like poor half-life in blood and human tissues and non-specific targeting. Hence, it is necessary to formulate MNPs that are non-toxic, blood compatible, and stable under insensitive physiological conditions. Another limitation associated with the use of magnetic nanoparticles is that they tend to agglomerate in the nanosize range. This can be avoided by combining the magnetic nanoparticles with biological or synthetic polymers. The polymers avert aggregation and enable interactions with drugs, and can shield the carrier against detection by the immune system. Hence, coatings with suitable polymeric molecules formulate magnetic nanoparticles acquiescent to drug loading via covalent or noncovalent interactions and also result in stimuli-responsive drug release by the use of an external MF [5].

The fabrication of metal ferrite particles in the nanometer range and their dispersion in conjugated polymers have been employed in many drug delivery applications. A number of strategies have been employed for the synthesis of metal ferrite-reinforced hybrid nanoparticles. In this study, a simple, cost-effective, and environmentally friendly emulsion solvent evaporation method was used to synthesize MNPs. The incorporation of magnetic nanoparticles imparted magnetic properties to the drug carriers, enabling them to be directed by an external MF. The main advantage of these cargo molecules is that they contain a sufficient volume that can be loaded with guest molecules. Biocompatibility is one of the vital criteria to be considered for magnetic nanoparticles in biomedical applications. Here, the biocompatibility of hybrid system depended mainly on the compatibility of magnetic materials used for the synthesis, acting as the driving force for targeted drug delivery because the movement of the drug-loaded system in blood can be controlled by an external MF [6–9].

Starch is another excellent biocompatible drug delivery platform for many pharmaceuticals known for its in vivo degradability, non-toxicity, and non-immunogenicity that can enhance bio-

compatibility. Along with stearic acid (SA), polyethylene glycol (PEG) imparts stealth properties to formulated nanoparticles. PEG also prevented the adsorption of blood proteins when the nanoparticles came into contact with the bloodstream due to the strong hydrogen bond formation with lipids. This PEG-SA coating also extended the circulation time of the nanoparticles in the bloodstream. Numerous studies have been conducted to investigate the potential of PEG-lipid conjugation for drug delivery. PEG conjugation is a rapidly growing approach to elucidate barriers to therapeutic delivery and is being used as an additional tool in conventional drug delivery practices. The conjugation of lipids with PEG is of great interest because of its superior therapeutic performance compared to that of conventional liposomes. These conjugated lipids remain in blood circulation for an extended period of time and circulate through an organism moderately evenly, with a maximum dose enduring in the blood and a very small dose ending up in the liver [10–13].

In the present work, starch-based hybrid nanosystem was synthesized with the help of PEG-SA shell, protecting the nanoparticles from blood protein adsorption and extending in vivo blood residence times.

The aim of this study was to fabricate a suitable nanocarrier for the delivery of AZT to targeted tissues to enhance its anticancer activity. These magnetic nanoparticle-reinforced DDSs release AZT into cancer cells in response to an external MF. Lipid-polymer hybrid nanoparticles were successfully formulated and used as drug delivery carriers in our previous studies [14–16]. Here, calcium ferrite nanoparticles coated with PEG and SA became the shell of hybrid nanovesicles and AZT-encapsulated starch as the core moiety. This novel nanovesicle drug delivery platform provided better encapsulation efficiency and excellent stability because of the multilamellar shell comprising lipid-PEG. To date, no studies have reported the use of such a magnetic hybrid nanoparticle system containing PEG, lipids, and starch for successful encapsulation of AZT, making this study a significant contribution to the field.

2 | Materials and Methods

2.1 | Materials

Calcium nitrate ($\text{Ca}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) (98%) and ferric nitrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$) (99.99%) were purchased from Fisher Scientific Chemicals. Citric acid anhydrous (97%) and PEG (6000) were purchased from Merck, India. AZT was obtained from Mylan Pharmaceuticals Inc., India. SA and starch (amylose, M_w 1.01×10^6) were purchased from Sigma-Aldrich (Bangalore, India). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and poly(ethylenimine) (PEI) (average molecular weight 25 000 kDa) were obtained from Sigma-Aldrich. Trypsin-ethylenediamine tetraacetic acid and Dulbecco's Modified Eagles Media (DMEM) were supplied by Himedia (India). All other chemicals and reagents were of analytical grade and were used as received. Purified water was collected from MilliQ Plus (Millipore). The biological experiments were done with the consent of Institute's ethics committee.

2.2 | Synthesis of CaFe₂O₄ Nanoparticles

The conventional sol–gel method was adopted for the synthesis of calcium ferrite (CaFe₂O₄, CFO) nanoparticles. The pre-cursors were calcium nitrate (Ca (NO₃)₃·6H₂O) and solution of ferric nitrate (Fe (NO₃)₃·9H₂O), and anhydrous citric acid was used as the raw material. 0.1 M aqueous solution of calcium nitrate and 0.2 M aqueous solution of ferric nitrate were prepared and sonicated. To this mixture, citric acid solution and ethylene glycol were added. The solution was magnetically stirred (750 rpm) at 80–90°C. During heating, the solution first transforms into a viscous brown gel, which eventually transforms into a dried gel. The obtained gel was dried properly, and the resulting sample was ground well and washed thoroughly with ethanol and distilled water. The powder was then calcined at 500°C to yield calcium ferrite nanoparticles as the final product.

2.3 | Fabrication of Nanoparticles

Calcium ferrite-reinforced AZT-encapsulated hybrid nanoparticles were fabricated using an emulsion-solvent evaporation technique. Initially, calcium ferrite nanoparticles (50 mg) were coated with PEG (200 mg) in 100 mL distilled water, which acted as the aqueous phase of the secondary emulsion. An oil phase containing 100 mg of SA in 20 mL chloroform was added. The emulsion was obtained by mixing the two phases using a mechanical stirrer with a stirring speed of 850 rpm for 10 min. AZT of three different (100, 150, and 200 mg) concentrations were dissolved in the primary aqueous phase consisting of starch (200 mg in 40 mL of ultrapure water). The secondary aqueous phase was added to the primary emulsion and heated at 55°C for half an hour to form the second emulsion. After continuous stirring for 1 h at a constant speed of 700 rpm, the drug-loaded colloidal particles were formed. When the organic solvent was completely evaporated, the formulated nanoparticles were dispersed in distilled water and used in further studies. The unencapsulated free AZT in the synthesized nanoparticle solution was eliminated by centrifugation at 10 000 rpm for 20 min, and the residue was dispersed in water. This solution was lyophilized and used for physicochemical characterization. The formulations containing different weight percentages of AZT (100, 150, and 200 mg) were labeled as CLPNs1, CLPNs2, and CLPNs3, respectively.

2.4 | Physicochemical Studies of Synthesized Nanoparticles

2.4.1 | Particle Size and Zeta Potential

The dynamic light scattering (DLS) technique has been employed for the determination of size and zeta potential of the synthesized hybrid nanoparticles. The particle size and zeta potential of hybrid CLPNs were analyzed using DLS (90Plus, Brookhaven Instruments Co., USA) method by using the suspended sonicated CLPNs sample. Surface charge analysis of the CLPNs is also carried out using the same DLS method. To get an appropriate scattering intensity, the formulations were diluted with Millipore water. The zeta potential was also measured using the same instrument. An average of 10 measurements of each formulations was carried out at an angle of 90°.

2.4.2 | FTIR Spectroscopy

The structural analysis of all the samples was carried out in a Perkin Elmer, Spectrum 400 FTIR Spectrometer. Spectra were recorded in a range from 400 to 4000 cm⁻¹. The lyophilized samples of AZT, SA, PEG, starch, and CLPNs were analyzed in triplicate. The structure of CLPNs was confirmed from the peaks obtained compared with the spectra of individual components.

2.4.3 | Morphological Analysis on CLPNs

The morphology of synthesized CLPNs was observed using a JOEL JEM 2100 light resolution transmission electron microscope, which is operated at 100 kV. To perform transmission electron microscopy (TEM) analysis, the nanoparticle suspension was placed in a 200 mesh Formvar-coated copper grid and air dried. The average size of CLPNs was obtained with Image J software.

2.4.4 | XRD Analysis

X-ray diffraction (XRD) analysis was conducted using high-power XRD Philips X'Pert PRO XRD diffractometer with a Cu-Kα line (wavelength 1.54 Å) source to determine the chemical and atomic nature of a crystal. In the current study, XRD was used to measure the purity of calcium ferrite by studying the intensity of diffracted x-ray when it encounters the calcium ferrite crystals. The intensity peak varies according to the composition of the crystal. The average particle size of CFO nanoparticles calculated from the Scherrer equation, $D = k\lambda/\beta\cos\theta$ was found to be 11 nm.

2.4.5 | Drug Loading and Encapsulation Efficiency

For the three different formulations of CLPNs, the drug loading and encapsulation efficiency were measured using a UV spectrophotometer absorbance at 265 nm (Shimadzu double-beam spectrophotometer model 1700).

The drug loading and encapsulation efficiency were calculated using the following equations:

$$\text{Loading efficiency (\%)} = \frac{W_a - W_s}{(W_a - W_s) + W_l} \times 100 \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = \frac{W_a - W_s}{W_a} \times 100$$

where W_a is the weight of drug added to the formulation, W_s is the weight of unencapsulated drug in ultra-filtrate, and W_l is the weight of lipid.

2.4.6 | In Vitro Release of Zidovudine

Drug release profiles of AZT from CLPNs were investigated using a dialysis method. Lyophilized sample of 5 mg of CLPNs was dispersed in phosphate buffer saline (PBS) of pH 7.4 and pH 5.

The dispersed sample was sealed in a dialysis bag and engrossed into 50 mL of PBS. The analysis was performed at 37°C using a magnetic stirrer. At each time interval, 3 mL of dissolution medium was taken out to measure the AZT content and replaced with fresh PBS to avoid drug saturation. The released AZT from CLPNs was measured using a UV-Visible spectrophotometer at a wavelength of 265 nm ($n = 3$).

2.5 | Biological Evaluation of CLPNs

2.5.1 | Blood Compatibility Studies: Red Blood Cell Aggregation and Hemolysis Tests

The antithrombogenicity of the prepared nanoparticles was analyzed using RBC aggregation studies. In brief, anticoagulated blood (100 μ L) diluted with saline in the ratio 1:4 was equilibrated with the CLPNs (100 μ L) along with positive control (PEI) and negative control (saline). The samples were incubated at 37°C for 30 min. After incubation, the images of RBC cells were captured using a phase contrast microscope (Leica DMIRB, Germany)

Hemolysis experiment was performed on CLPNs as follows. Diluted blood of 100 μ L (100 μ L blood with 800 100 μ L saline) was incubated with 100 μ L of CLPNs along with respective controls. Normal saline was used as the negative control (0% lysis), and distilled water was used as the positive control (100% lysis). All the samples were incubated for half an hour at 37°C. After incubation, the samples were centrifuged, and absorbance was measured at 541 nm by using UV-Vis spectrophotometer (Varian). The percentage of hemolysis was calculated as follows

$$\text{Hemolysis (\%)} = \frac{(\text{OD of sample}) - (\text{OD of -ve control})}{(\text{OD of +ve control}) - (\text{OD of -ve control})} \times 100 \quad (2)$$

2.5.2 | Cell Culture Studies

2.5.2.1 | In Vitro Biocompatibility Testing. 3T3 Fibroblast and HaCat keratinocyte cell lines were used to evaluate the anticancer activity of the hybrid nanoparticles. Cells were seeded on 24-well plates at 20 000 cells/cm² and cultured with DMEM (Gibco, USA) containing fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin solution (Gibco, USA) for 24 h. After 24 h, the media was replaced, and various amounts of the optimized hybrid nanoparticle (0.05 mg/mL) of 50, 100, and 200 μ L. Blank controls without any treatment were also maintained. To understand the effect of hybrid nanoparticles on cell morphology and proliferation, culture plates were observed under a phase contrast microscope, and images were taken after every 24 h interval. After 24 and 48 h of treatment, Live/Dead and MTT cell viability assays were performed as detailed below.

2.5.2.1.1 | Live/Dead Assay. Live/Dead cell staining was performed to understand the effect of hybrid nanoparticles on 3T3 Fibroblast and HaCat keratinocyte cell death according to the manufacturer's protocol (Invitrogen). In brief, 50 μ L of calcein

and ethidium mixture was added to cell culture plates after media removal. The plate was then incubated for 30 min in a carbon dioxide incubator. The images of the cells were taken with a fluorescent microscope (Olympus, FV300). The live and dead cells appeared as green and red in color, respectively. The percentage of dead cells was calculated using Equation (3).

$$\text{Dead cells (\%)} = \frac{\text{No. of dead cells}}{\text{Total No. of cells}} \times 100 \quad (3)$$

2.5.2.1.2 | MTT Cell Viability Assay. In addition, to determine the viability of 3T3 Fibroblast and HaCat keratinocyte cell lines, which were cells grown over the nanoparticles, MTT cell viability assay was carried out as per the procedure obtained from the manufacturer (Thermo Fisher Scientific, USA). After incubation, the cell culture media was replaced with fresh one. The cells were treated with 100 μ L of MTT dye solution and incubated at 37°C for a period of 4 h. Later, 500 μ L of DMSO solution was added to dissolve the formed formazan crystals. Finally, 30 μ L of above solution was transferred in to 96-well plates and absorbance was recorded at a wavelength of 570 nm with a TECAN, infinite F200 PRO (Tecan Austria GmbH) microplate reader. The absorbance was converted into percent cell viability by using Equation (4).

$$\text{Cell viability} = \frac{\text{OD of sample}}{\text{OD of control}} \times 100 \quad (4)$$

3 | Results and Discussion

3.1 | Synthesis of Multilamellar Structured Hybrid Nanovesicle System

The present study demonstrated the formation of multilamellar structure of a hybrid nanovesicle of starch-lipid reinforced calcium ferrite nanoparticles for efficient antiviral drug delivery. Our study was based on the formation of core-shell hybrid nanoparticles of different polymers and lipids for antiviral drug delivery in our previous investigations [18–20]. Here, the multilamellar structure was obtained by carefully adjusting the amounts of lipid and polymer in the pre-formulation studies. In our previous studies, we dissolved the polymer in water along with the drug; the lipid-containing organic solvent was added to the water phase, which was stirred to form the oil-in water emulsion. The lipid, having an amphiphilic nature, acted in the role of surfactant to facilitate the formation of core-shell structures. But in the present study, calcium ferrite added on the PEG layer minimized the repulsion between lipid bilayers, and this may lead to the formation of multilamellar structures on the surface of starch-containing drug [17–18]. A schematic representation of the possible formation of multilamellar structures of lipid-polymer hybrid nanoparticles is shown in Figure 1.

3.1.1 | Particle Size and Zeta Potential

In order to characterize different formulations of CLPNs, the particle size and zeta potential were measured using DLS analysis. The size obtained in DLS measurement is in the range of 3239–325 nm, and it is much higher than the observed size in TEM analysis. This is due to the fact that in DLS analysis, we have measured the hydrodynamic diameter of the particle. The size,

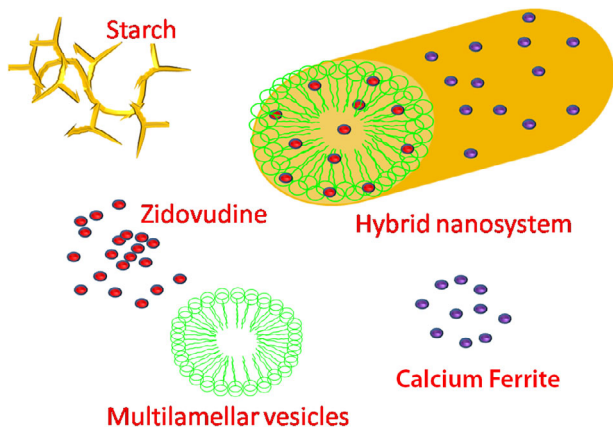


FIGURE 1 | Schematic representation of the formation of multilamellar hybrid nanovesicle.

TABLE 1 | Size and zeta potential of CLPNs.

Name of formulation	Particle size (nm)	Zeta potential (mV)	PDI
CLPNs1	339 ± 2.8	-50.9 ± 0.06	0.181
CLPNs2	329.4 ± 2.2	-52.3 ± 1.8	0.172
CLPNs3	325.5 ± 4.34	-54.6 ± 1.9	0.196

polydispersity index, and zeta potentials of all the formulations are within a similar range (-50 – 54 mV), indicating that there is no significant aggregation observed in the course of synthesis of the hybrid nanosystem.

Using DLS, the particle size and zeta potential of the three formulations of CLPNs are determined, and it is depicted in Table 1. At the optimal formulation, the average size exhibited by the CLPN is 325 ± 4.34 nm.

The stability and surface charge of the drug-loaded nanovesicles were investigated using zeta potential. The zeta potential value of ± 30 generally accepted as the stable formation of nanoparticles. In the present study, the three formulations have an average zeta potential values of -50.9 ± 0.06 , -52.3 ± 1.8 , and -54.6 ± 1.9 , indicating the stability of synthesized nanovesicles.

The average particle size of nanovesicles was studied using DLS analysis. The particle size was found to be 339 ± 2.8 , 329.4 ± 2.2 , and 325.5 ± 4.34 respectively for Formulations 1, 2, and 3. The hydrodynamic diameter and particle size distribution indicate the hydrophilic nature of the nanovesicles. Furthermore, the particle size obtained from TEM analysis was different from that of DLS analysis because of this hydrophilic nature [19–24].

Zeta potential is the measure of surface charge that determines the stability of colloidal dispersions. The AZT-loaded CLPNs have a negative zeta potential in all three formulations, as indicated in Table 1. The negative zeta potential indicates the good dispersion stability, which minimizes the undesired rapid elimination of AZT-loaded CLPNs from the circulation.

3.1.2 | Morphology Analysis

The morphology of bare calcium ferrite nanoparticles and calcium ferrite-reinforced AZT-loaded hybrid nanosystem, CLPNs, its lamellar structure, and hollow sphere inside the lamellar morphology are shown in Figure 2A–D, respectively. Imaging showed that the bare nanoparticles of calcium ferrite showed an aggregated morphology with an average size of 15 ± 3.2 nm. However, after the formation of the hybrid nanosystem, a clear distinct multilamellar morphology in cylindrical form with a large hollow core of nanoparticles with an average size of 179 ± 18.23 nm was observed. Here, the polymer forms a cylindrical core and multilamellar lipid layers shell on the surface of the polymer. The starch-containing AZT was successfully encapsulated in multilamellar lipid layers. Here, the reinforcement of calcium ferrite facilitated the successful formation of multilamellar structures in the hybrid nanosystem. These results displayed the formation of multilamellar layers of lipids on polymers without the need for an external crosslinking agent. Normally, multilamellar structures are formed by crosslinking of lipids with suitable crosslinking agents. However, here in this study, calcium ferrite acts as a reinforcing agent that stabilizes the formation of multilamellar structures with lipids. This multilamellar structured hybrid nanosystem resulted in a high loading efficiency of the antiviral drug AZT compared to the normal core-shell structure of the hybrid nanosystem [25].

3.1.3 | FTIR Spectroscopy

The FTIR spectrum of hybrid system is shown in Figure 3A. From this, the IR spectrum of AZT shows a strong peak at 3460 cm^{-1} due to the stretching vibration of NH. The C–N (amine) stretching peak of AZT observed at 1110 cm^{-1} . The C–O stretching at 1682 cm^{-1} and azide group stretching at 2080 cm^{-1} were exhibited. A strong peak at 2934 and 2812 cm^{-1} due to C–H stretching and C–H deformation (CH_3) was shown at 1463 cm^{-1} . In the spectrum of SA, symmetrical and asymmetrical C–H stretching bands were exhibited at 2955 and 2948 cm^{-1} . The carbonyl stretching of CO groups appeared at 1695 cm^{-1} and C–H deformation showed at 1463 cm^{-1} .

A broad peak of starch, at 3385 cm^{-1} corresponding to –OH stretching, C–H stretching bands were observed at 2922 and 2854 cm^{-1} . The sharp peaks at 1743 and 1643 cm^{-1} were due to C–O stretching. O–H bending peaks were observed at 1460 and 1348 cm^{-1} .

The FTIR spectrum of CLPNs showed O–H stretching band at 3464 cm^{-1} . The C–H stretching band appeared at 2883 cm^{-1} . The characteristic azide group of drug AZT was exhibited at 2104 cm^{-1} . This confirms the presence of encapsulated drug in the hybrid nano system. The C–O stretching bands appeared at 1691 and 1664 cm^{-1} . C–H deformation of the hybrid nanosystem appeared at 1469 cm^{-1} . C–H bending peaks were at 1344 and 1274 cm^{-1} . The C–N stretching of AZT exhibited peaks at 1097 cm^{-1} . The characteristic peaks at 842 and 765 cm^{-1} were due to Fe–OH stretching and bending of calcium ferrite molecules. The observed peaks confirmed the formation of drug-loaded calcium ferrite reinforced hybrid nanosystem.

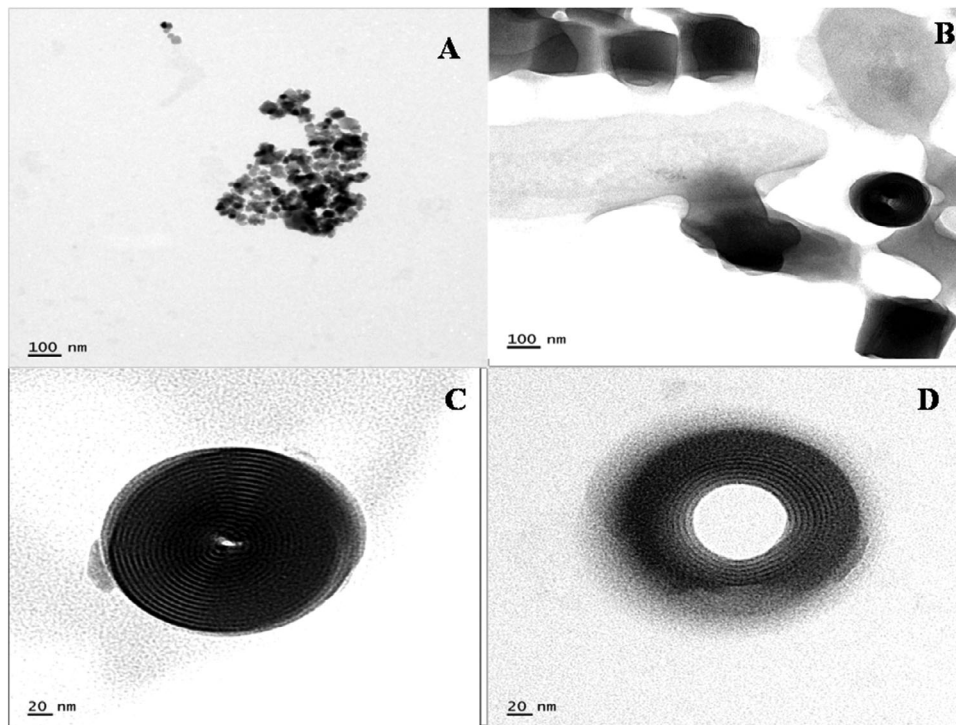


FIGURE 2 | Transmission electron microscopy (TEM) images of CLNPs (A) calcium ferrite nanoparticles, (B) CLNPs, (C) multilamellar vesicles in CLNPs, and (D) appearance of hollow spheres in CLNPs.

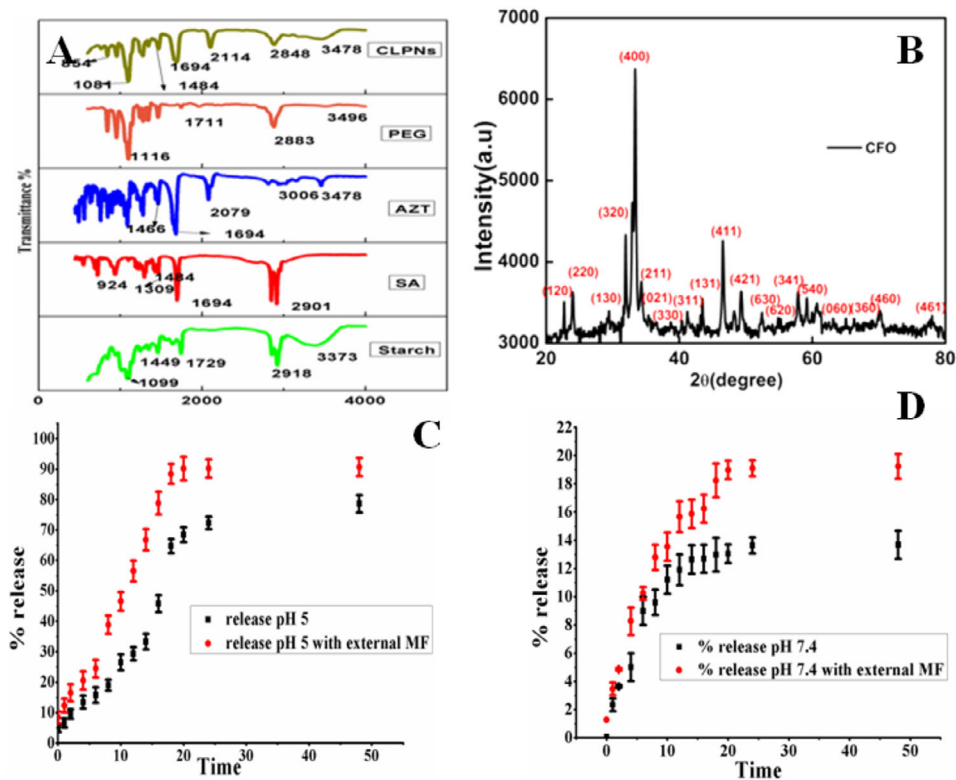


FIGURE 3 | (A) FTIR spectrum of CLNPs, (B) XRD analysis of calcium ferrite nanoparticles, (C) AZT release at pH 5, and (D) AZT release at pH 7.4. AZT, zidovudine; FTIR, Fourier transform infrared; XRD, x-ray diffraction.

TABLE 2 | Drug loading and encapsulation efficiency of CLNPs.

Name of the formulation	SA (mg)	Starch (mg)	AZT (mg)	Drug loading (%)	Encapsulation efficiency (%)
CLPN1	100	100	100	48.21 ± 2.01	93.10 ± 2
CLPN2	100	100	150	57.11 ± 1.08	88.79 ± 0.91
CLPN3	100	100	200	65.28 ± 3.2	94.33 ± 2.001

3.1.4 | XRD Analysis of CFO Nanoparticles

XRD pattern of CaFe_2O_4 nanoparticles is shown in Figure 3B. The given XRD pattern was well matched with the JCPDS pattern 74-2136. The above XRD pattern of CaFe_2O_4 comprised of sharp peaks, which reveal that the material is highly crystalline, and the absence of impurity peaks confirms that the obtained CaFe_2O_4 was highly pure. The reflection planes corresponding to each XRD peak are indexed in Figure 3B. Comparing the XRD pattern of CaFe_2O_4 nanoparticles with the standard JCPDS data sheet orthorhombic structure of CFO was identified with lattice parameters $a = 10.66 \text{ \AA}$, $b = 9.2 \text{ \AA}$, $c = 3.01 \text{ \AA}$.

3.1.5 | Drug Loading and Encapsulation Efficiency

Table 2 depicts the different drug loading and encapsulation efficiencies of the three different formulations of hybrid nanoparticles. The fabricated nanoparticles were stable in water and PBS. The particles showed a high drug loading efficacy. Different formulations were made in order to obtain a high loading and encapsulation efficiency. For this, the weight of lipid and polymer was kept constant while varying the amount of AZT. The highest drug loading percentage of $65.28 \pm 11.5 \text{ mg}$ was obtained with an encapsulation efficiency of $94.33 \pm 12.01 \text{ mg}$. The highest observed loading efficiency is due to the presence of calcium ferrite nanoparticles present in the system. Calcium ferrite facilitated the formation of multilamellar structure on hybrid nanosystem, and the drug can be loaded both the lamellar vesicles along with the polymeric core.

The enhanced loading efficiency of AZT is due to the presence of increased space between the polymeric core and the surface lamellas. The presence of magnetic nanoparticles enhanced the space between the polymeric core and surface, and as a result, the AZT molecules bind with ease on the surface of the hybrid system [26, 27].

3.1.6 | In Vitro Release of Zidovudine

The advantage of calcium ferrite-reinforced hybrid nanosystem in AZT delivery is the release of drug that can be controlled by an external MF. Sustained release of AZT from the hybrid nanosystem is an effective strategy to avail the maximum potential of the drug. Obviously, the diffusion of drug molecules into the PBS medium was monitored to study the in vitro drug release profile of the CLPNs with respect to the function of time. The result indicated that the synthesized hybrid nanoparticles were found to be an efficient carrier for AZT which diminishes the

premature burst release. We expect an enhanced drug release by the application of an external MF. The improved release was due to the alignment of calcium ferrite nanoparticles by the external MF, which were in turn swelling the hybrid nanosystem that allows more drug molecules to be released into the medium.

Release of AZT from magnetic nanoparticles was found to be in a sustained manner (Figures 3C and 3D) due to the easy diffusion of drug into PBS medium. The presence of magnetic nanoparticles employed an external MF that facilitates drug release. The external application of an MF aligns magnetic particles in the hybrid system, which causes the polymer core and lamellar surface to expand and enhances the release of drug to the medium.

The release of AZT was studied in PBS of two different pHs through the dialysis method to monitor the release under blood pH and under the microenvironment of tumor growth. As shown in Figure 3C, AZT was released more rapidly in buffer at pH 5. The result shows that after 48 h more than 90% of AZT was released in the presence of an MF. Even in the absence of an MF, the release was high as 78.66%. But in pH 7.4, Figure 3D, a very slow release was observed. Only 13.69% of drug was released after 48 h without the presence of an external MF. However, in the presence of MF improved drug release was observed (19.23% after 48 h). Slow release at pH 7.4 was due the AZT molecules stayed on the hybrid system over long period of time, which may not cause any severe side effects because of the presence of hybrid envelope. The fast release at acidic medium can cause the easy engulfment of drug-loaded particles of tumor cells via endocytosis process [28–30].

3.2 | Biocompatibility Studies of CLPNs

3.2.1 | Blood Compatibility Studies

Adsorption of plasma proteins is the first incident that occurs when a material approaches in contact with blood. The nature and quantity of proteins that get adsorbed will openly persuade the blood compatibility of the material. Hence, the blood compatibility of the material is of prime concern when the material is intended for clinical applications. Blood compatibility of the material depends on several factors that include surface charge, area, hydrophilicity, hydrophobicity, etc. The aggregation of blood cells and hemolysis activity on synthesized hybrid nanosystem were evaluated similar to our previous studies, Figure 4 shows the RBC aggregation studies of CLPNs. It showed no aggregation of RBCs on incubation of with CLPNs, similar to the negative control saline, whereas PEI showed significant aggregation on RBC [31–33].

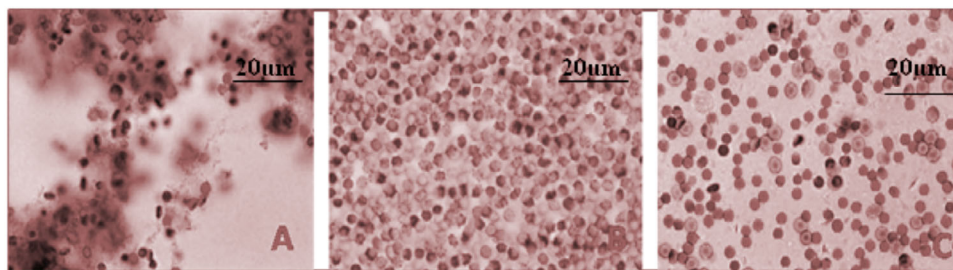


FIGURE 4 | Microscopic images of red blood cells in (A) poly(ethylenimine) (PEI), (B) normal saline, and (C) CLPNs.

Due to the chemical inhomogeneity of calcium ferrite nanoparticles, its surface may not have direct contact with blood, and hence the calcium ferrite-reinforced hybrid nanoparticles showed very good blood compatibility. This can be attributed to the fact that all the materials used for the fabrication of the hybrid nanosystem were biocompatible, and the calcium ferrite nanoparticles coated by this hybrid nanosystem prevented any thrombus formation tendency in the blood cells. A similar result was obtained in the case of hemolysis percentage too, where the results proved that the fabricated calcium ferrite nanoparticles reinforced hybrid nanosystem was blood compatible and could be used safely inside the human body. There was no lysis of RBC observed after incubation, and the percentage lysis of CLPNs was 0.4% which fit within the permitted limit of 1% [34].

3.2.2 | In Vitro Cell Culture Studies

Figure 5 shows the effect of hybrid nanoparticles on cell morphology and cell viability of 3T3 fibroblast and HaCat keratinocyte cells after 24 and 48 h of treatment. Treatment with 50 μL did not show a significant effect on cell morphology or cell number in both time periods (Figure 5A). However, 100 and 200 μL showed significant reduction in cell proliferation and led to the changes in cell morphology. In the case of 100 μL nanoparticles treated cells, several spherical dead cells were observed after 24 h treatment. The number of spherical and irregularly shaped cells was significantly increased at the 48 h treatment. Compared to 100 μL treatment groups, 200 μL hybrid nanosuspensions treated cells showed higher variation in cell morphology. In order to further verify the effect of nanoparticles on cells, a Live/Dead assay was performed, and the results are given in Figure 5B. Along with green-colored live cells, several red-colored dead cells were observed on culture plates treated with hybrid nanoparticles. In this study also, the number of dead and irregularly shaped cells was significantly increased at 48 h treatment with 100 and 200 μL nanoformulations. The percentage of dead cells significantly increased in 100L and 200 μL treatment groups compared to the controls (Figure 5C, D).

Cell viability of 3T3 fibroblast and HaCat keratinocyte cells upon treatment with hybrid nanoparticles was investigated using MTT assay. Cell viability after 24 and 48 h of treatment was determined and is given in Figure 6A, B. Compared to untreated control cells, viability of 50 μL nanoparticle treated cells was observed as $97\% \pm 5\%$ and $95\% \pm 7\%$ after 24 and 48 h of treatment, respectively, indicating that the nanoparticles had relatively low cytotoxicity at this concentration. However, at 100 μL nanoparticles content,

cells showed significant reduction in viability. As shown in Figure 6A, 100 μL nanoparticle treatment groups showed a $73\% \pm 7\%$ and $67\% \pm 8\%$ reduction in 3T3 cell viability after 24 and 48 h of treatment, respectively. However, 200 μL nanoparticles treatment group showed a very significant reduction in cell viability after 24 h ($62\% \pm 6\%$) and 48 h ($37\% \pm 5\%$) of treatment.

3.3 | In Vitro Anticancer Activity

In SK-BR-3 breast cancer cells, Figure 7 showed the influence of hybrid nanoparticles on cell morphology and cell viability after 24 and 48 h of treatment. Treatment with 50 μL did not show a significant effect on cell morphology or cell number in both time periods (Figure 7A). However, 100 and 200 μL showed a significant reduction in cell proliferation and led to the changes in cell morphology. In the case of 100 μL nanoparticles treated cells, several spherical dead cells were observed after 24 h treatment, and more cells were found dead after 48 h treatment. Compared to 100 μL treatment groups, 200 μL hybrid nanosuspensions treated cells showed higher variation in cell morphology. In order to further verify the effect of nanoparticles on cells, a Live/Dead assay was performed, and the results are given in Figure 7B. Along with green-colored live cells, several red-colored dead cells were observed on culture plates treated with hybrid nanoparticles. In this study also, the number of dead and irregularly shaped cells was significantly increased at 48 h treatment with 100 and 200 μL nanoformulation. The percentage of dead cells significantly increased in 100 and 200 μL treatment groups compared to the controls (Figure 7C).

Cell viability of SK-BR-3 human breast cancer cells upon treatment with hybrid nanoparticles was investigated using MTT assay. Cell viability after 24 and 48 h of treatment was determined and given in Figure 7D. Compared to untreated control cells, viability of 50 μL nanoparticle treated cells were observed as $97\% \pm 5\%$ and $95\% \pm 7\%$ after 24 and 48 h of treatment, respectively, indicating that the nanoparticles had relatively low cytotoxicity at this concentration. However, at 100 μL nanoparticles content, cells showed significant reduction in viability. As shown in Figure 7D, 100 μL nanoparticles treatment groups showed $73\% \pm 7\%$ and $67\% \pm 8\%$ reduction in viability after 24 and 48 h of treatment, respectively. However, 200 μL nanoparticles treatment group showed a very significant reduction in cell viability after 24 h ($62\% \pm 6\%$) and 48 h ($37\% \pm 5\%$) of treatment.

Several studies have proved that AZT has antitumor activity and interesting biological properties. Initial experiments to character-

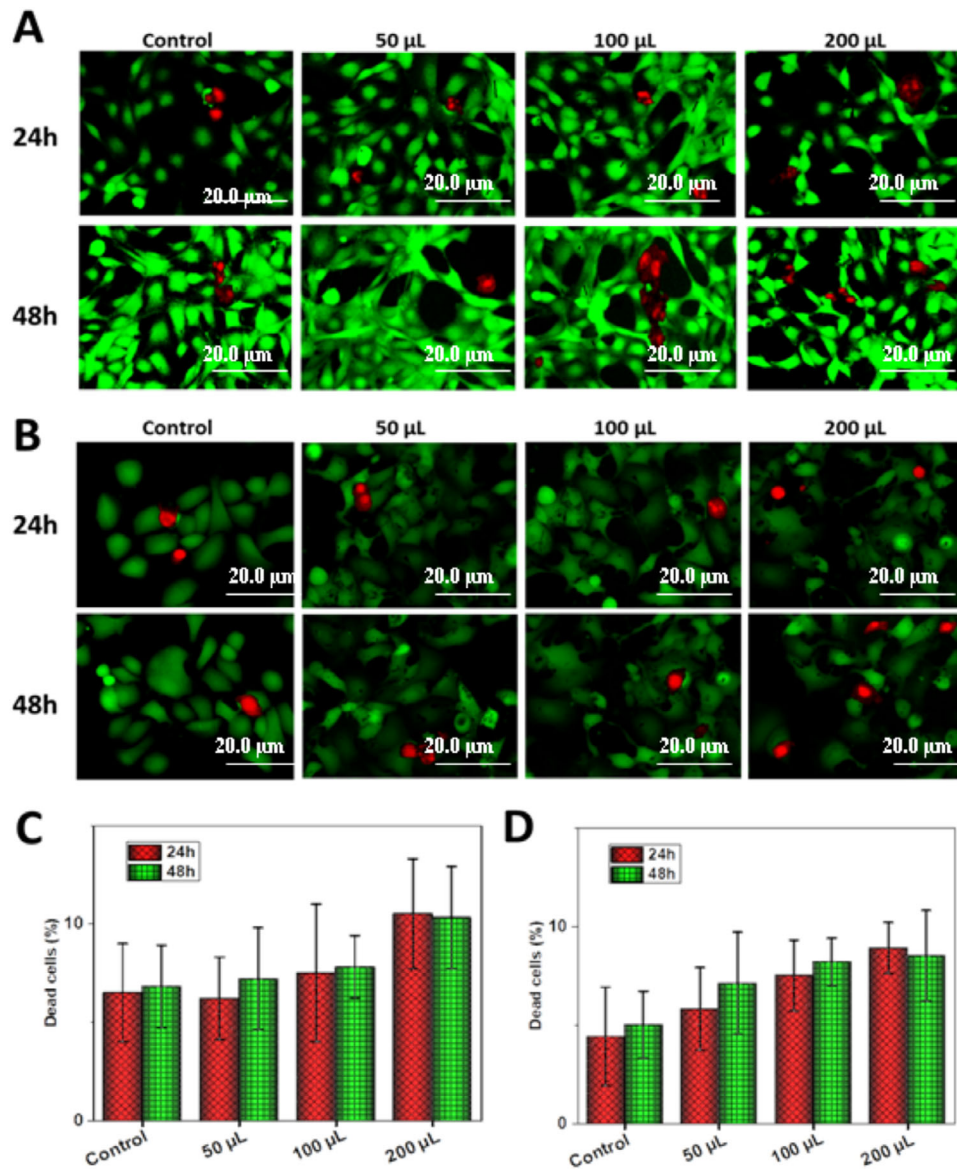


FIGURE 5 | Fluorescent microscopic images after Live/Dead staining showing the morphology of 3T3 fibroblast (A) and HaCat keratinocyte (B) in the presence of CLPNs after 24 and 48 h treatment. Percentage of dead cells calculated from the result of Live/Dead staining on 3T3 fibroblasts (C) and HaCat keratinocytes (D).

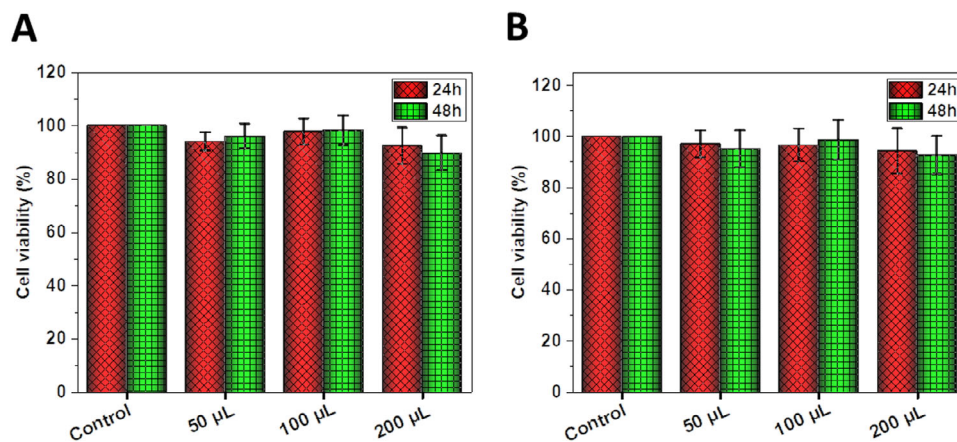


FIGURE 6 | Viability of 3T3 (A) and HaCat (B) cells based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in the presence of CLPNs.

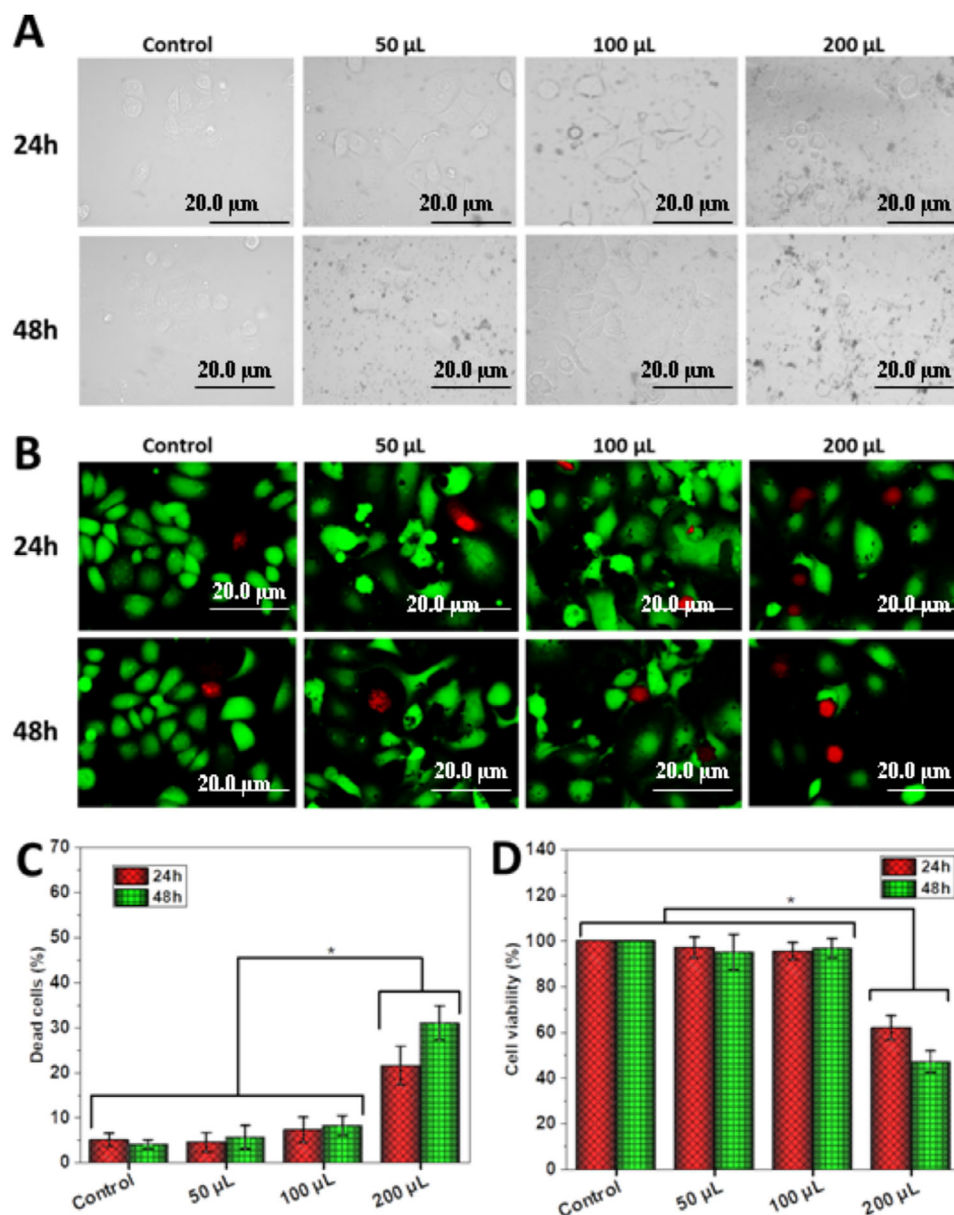


FIGURE 7 | SK-BR3 cell proliferation in the presence of hybrid nanoparticles. Phase contrast microscopic images showing the morphology of cells after 24 and 48 h treatment with hybrid nanoparticles (A). Fluorescent microscopic images after Live/Dead staining (B). Percentage of dead cells calculated from the results of Live/Dead staining (C). Cell viability based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in the presence of nanoparticles (D).

ize the anti-breast cancer activity of AZT focused on assessing the potency of the drug in vitro. As can be seen from the results shown in figure (Figure 7A), AZT showed considerable antitumor activity against the human breast cancer cell line, SK-BR3. Analysis of these data disclosed that AZT was able to inhibit the proliferation of SK-BR3 cells to a significant level and over a long time period. The MTT assay was also conducted to examine the cytotoxic effects of AZT on SK-BR3 cell line, and the results indicated that AZT was able to significantly increase the rate of growth inhibition in tumor cell lines. The results obtained from the Live/Dead assay confirmed the results obtained by MTT assay. SK-BR3 cells treated with AZT showed a low percentage of viability. AZT induced an apoptotic rather than a necrotic type of cellular death. AZT has been widely used as a forged alternative for viral reverse transcriptase and

has been employed almost entirely as an antiretroviral agent; it has multiple pharmacological actions, including prevention of human telomerase reverse transcriptase component. It has been established that AZT, alone or in combination with other anticancer agents, is able to reduce the in vitro cell growth of several cancer cell lines [35–39].

4 | Conclusion

Self-assembled multilamellar vesicles were investigated as potential carriers for AZT to treat breast cancer. The starch-based hybrid system overcomes solubility issues of the lipophilic drug through their encapsulation into a nanocarrier, achieving AZT loadings of more than 90%. Here, the antiviral drug, AZT, was

utilized as a strong persuader of the anticancer in-vitro drug model. The FTIR, TEM, and XRD analyses confirmed the successful synthesis of calcium ferrite-reinforced AZT encapsulated multilamellar hollow nanovesicles. The in vitro release study indicated that the synthesized nanoparticles were capable to release the drug within the cancer cells. Our suggested DDS considerably enhances the drug concentration in cancer cells, and afterwards decreases the dose-dependent side effects. Therefore, in normal cells, very small amount of loaded drug will be released. Based on in vitro results, 3T3 fibroblast and HaCat keratinocyte cells possessed more than 95% cell viability in the presence of these nanospheres. However, these nanoparticles due to the incorporation of AZT showed higher cytotoxic activity against SK-BR3 breast cancer cells. Hence, this study highlights that the improved anticancer activity of calcium ferrite-reinforced AZT loaded hybrid system of starch is a promising therapeutic approach for the treatment of advanced-stage breast cancer patients.

Author Contributions

Joshy K S: Conceptualization, methodology, writing original article, **Robin Augustine:** Cell culture studies, **Anshida Mayeen, Jiya Jose, Annu Thomas, Aiswarya Sathian:** formal editing, **Sana Kab-drakhmanova, Nandakumar Kalarikal:** Funding resources, **Sabu Thomas, Anwarul Hasan:** supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy or ethical restrictions.

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