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CHITOSAN AND CARBOXYMETHYL CHITOSAN (CMC)-COATED MAGNETITE NANOPARTICLES FOR DRUG DELIVERY TO CANCER CELLS: EFFECT OF MOLECULAR WEIGHT ON DRUG RELEASE

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ABSTRACT: Novel effective delivery systems for chemotherapeutic drugs are of utmost demand in nanomedicine. Herein, we prepared magnetite (Fe_3O_4) nanoparticles (NPs) coated with either low molecular (LM) or high molecular (HM) weight Chitosan or Carboxymethyl Chitosan (CMC) and assessed their drug loading and release efficiencies. Chitosan/CMC-coated MNPs were fully characterized by various physicochemical techniques including TEM, XRD, DLS, FTIR, and VSM. These techniques clearly elucidated the nanostructure, morphology, surface charge, and magnetization of all functional MNPs. We then loaded them with the anticancer drug Dox to achieve biocompatible, biodegradable, and pH-responsive drug nanocarriers. Drug release was evaluated at both neutral and acidic pH to mimic endosomal/lysosomal conditions. HM Chitosan-NPs or CMC-NPs showed faster and more sustained controlled release ($\sim 90 - 99\%$) at acidic pH, while LM NPs exhibited slightly lower drug release ($\sim 60 - 65\%$). Notably, almost negligible release was found at neutral physiological pH for all MNP formulations, which is crucial for successful pH-triggered delivery system. The delivery of Dox using CMC-NPs was then evaluated in human monocytic leukemia THP-1 cancer cells. Remarkably, while the unloaded CMC-NPs were non-toxic to the tested cell lines, Dox@CMC-MNPs were found to be highly potent causing apoptotic cell death to cancer cells ($\text{IC}_{50} = 16 \mu\text{g/mL}$), as evident from live confocal images. These results show great promise for the utilization of Chitosan/CMC-MNPs for potential *in-vivo* cancer theranostics.

INTRODUCTION: Magnetic iron oxide nanoparticles (MNPs) have been widely used for biomedical applications including imaging, biosensing, catalysis, magneto-guidance, and drug delivery¹⁻³.

Among the various MNPs, magnetite (Fe_3O_4) NPs are the most widely investigated, due to their low distinctive features including low toxicity, easy magnetic separation, stability, cost-effectiveness, simple synthesis, and versatile surface functionalization⁴.

In particular, the preparation of colloidal polymer-coated MNPs for biomedical applications remains challenging, primarily due to issues related to stability, biocompatibility, toxicity, and achieving consistent reproducible material properties⁵. For such applications, it is desirable to modify the

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surface of iron oxides with a protective coating layer, as the NP performance is closely linked to their optimized structures, sizes, magnetic properties, and enhanced stabilities. Many polymers are well known to coat MNP surfaces including but not limited to polyethylene glycol (PEG), polyacrylic acid (PAA), polyvinylpyrrolidone (PVP), Hyaluronic Acid (HA), Starch, Chitosan *etc.* The addition of a biocompatible complexing polymer provides protection, enhances colloidal stability, and reduces agglomeration in the NP formulation, thereby preventing degradation or oxidation of the core material over time. Specifically, polysaccharides such as HA, Starch, Chitosan, and their derivatives are amphiphilic, water-soluble, biocompatible, and non-toxic polymers that have been utilized for drug delivery to cancer cells ⁶⁻⁹.

Chitin, poly (β -(1 $\rightarrow$ 4)-N-acetyl-D-glucosamine), is a naturally occurring polysaccharide of significant importance, first identified in 1884 ¹⁰. This biopolymer is synthesized by a wide range of living organisms and is considered the second most abundant natural polymer after cellulose. In nature, chitin exists as highly ordered crystalline microfibrils that serve as structural components in arthropod exoskeletons and in the cell walls of fungi and yeast ¹¹. Despite its widespread natural occurrence, crab and shrimp shells remain the primary commercial sources of chitin **Fig. 1A**. In industrial processing, chitin is extracted from crustaceans through acid treatment. When the degree of deacetylation reaches $\sim$ 50%, the material becomes soluble in aqueous acidic media and is referred to as chitosan. Chitosan is, thus, the N-deacetylated derivative of chitin, although this N-deacetylation is almost never complete ¹². Chitosan becomes soluble through protonation of the $-\text{NH}_2$ group at the C-2 position of the D-glucosamine repeat unit, converting the polysaccharide into a polyelectrolyte in acidic media. As one of the pseudo-natural cationic polymers, chitosan exhibits unique properties that highlight its wide range of applications. Recently, chitosan has attracted considerable attention due to its promising properties for pharmaceutical applications, particularly its biodegradability, biocompatibility, and ability to enable controlled drug release ¹³⁻¹⁵. Furthermore, chitosan exhibits mucoadhesive and penetration-enhancing properties, making it

especially attractive for drug delivery ^{14, 16}. The cationic nature of the polymer allows interaction with negatively charged biological membranes, thereby enhancing drug absorption and retention. Although numerous efforts have been reported to prepare functional chitosan derivatives through chemical modifications, solubilities in aqueous solvents and some binary solvent systems are still a major concern which underscores a limitation in their reactivity and processability. Chemically-modified chitosan structures resulting in improved solubility have been reported by many workers ¹⁷. For instance, carboxymethyl chitosan (CMC) has been widely studied due to its ease of synthesis, amphiphilic nature, and increased solubility ¹⁸⁻²⁰. Chitosan exhibits low solubility at physiological pH ($\sim$ 7.0), which limits its use in systems requiring higher solubility or controlled drug release. Chemical modification of the hydroxyl and amino groups in the chitosan chains with carboxymethyl moieties can significantly alter its properties **Fig. 1B**. The water solubility of CMC across different pH conditions is determined by the degree of carboxymethylation. Another limitation of chitosan is its rapid water absorption and high swelling in aqueous media, which can lead to accelerated drug release ²¹. Thus, chitosan and its derivatives are considered suitable functional materials due to their excellent characteristics, including biocompatibility, biodegradability, non-toxicity, high nitrogen content, and chelation/adsorption capabilities. They are well-suited for the fabrication of films, tablets, gels, and nanotechnology delivery systems, offering prospects for commercialization and industrial-scale production.

Because Chitosan- and CMC-based formulations exhibit enhanced solubility in aqueous media, they represent an attractive alternative platform and good potential for the development of controlled-release drug delivery systems ²². In fact, Chitosan and CMC-NPs serve as useful drug delivery carriers in cancer chemotherapy ^{23, 24}. For instance, Doxorubicin (Dox) is frequently used as a frontline chemotherapeutic drug against a variety of cancers including breast, ovary, bladder, thyroid, acute lymphoblastic leukemia (ALL), and acute monocytic leukemia (AML). However, there are major pitfalls of Dox, including serious side-effects such as cardiotoxicity, myelosuppression, extravasation during administration, and

unselective delivery^{25, 26}. Consequently, liposomal formulation of Dox (Doxil) has been FDA approved for the treatment of ovarian cancer in patients who have failed conventional chemotherapy²⁷. This liposomal nanoformulation encapsulates the drug in tiny, fat-like particles (liposomes) to improve tumor delivery, reduce heart toxicity (cardiotoxicity), and prolong circulation *via* PEGylation, exploiting the EPR (enhanced permeability & retention) effect for better cancer treatment with fewer side effects than free drug²⁸. These formulations use pH gradients for stable loading and are a key nanotechnology advance, with newer versions (*i.e.* TLD) aiming for even smaller sizes and better tumor penetration²⁹. Chitosan NPs have been also used for successful and promising Dox delivery to MCF-7 and Dox-resistant MCF-7 breast cancer cells, because of their biocompatibility, high drug encapsulation efficiency, and target specificity³⁰. Chitosan and CMC-based NPs form spherical structures where the positively charged Chitosan binds to negatively charged iron-based NPs capable of carrying therapeutic molecules. In these systems, drugs like Dox can be either physically entrapped, attached, or encapsulated within the chitosan through complexation, allowing the drug to be held securely within the structure. The hydrophilic chitosan stabilizes the NPs in aqueous environments and protects the drug from premature degradation. This strategy of complexing macromolecules with chitosan is well-suited for the design of controlled-release drug delivery systems. While the majority of research utilizing chitosan and iron oxide NPs have focused on solid tumors, a sparse of literature has examined their utilization in hematological malignancies, specifically acute leukemias.

Leukemia constitutes a large group of hematological malignancies that are ominous for their rapid proliferation and rapid clinical deterioration. Drug delivery for this specific subset of disease remains unmet and demands urgent clinical need. To the best of our knowledge, no prior study has comprehensively investigated how the molecular weight of Chitosan/CMC-coated MNPs influences drug delivery performance, with a particular focus on leukemia cell models.

In this study, different molecular weights (Mws) of Chitosan-or CMC-coated MNPs were prepared by KHB methodology and then loaded with the anticancer drug Dox to achieve a biocompatible, biodegradable, and pH-responsive drug nanocarrier. The as-prepared functionalized MNPs were examined by various electronic and spectroscopic techniques, including TEM, XRD, FTIR, and VSM, which provided detailed insights into the structure, morphology, and magnetization of the chitosan/CMC-coated MNPs. Drug release profiles were then evaluated at both neutral and acidic pH for Chitosan-MNPs and CMC-MNPs of different Mws. Finally, cellular internalization and cytotoxic effects of CMC-MNPs and Dox@CMC-MNPs were investigated on THP-1 leukemic cancer cells, indicating that they are promising nanocarriers as pH-triggered delivery system. It is to be noted that this work represents a systematic investigation of addressing the effect of different Mws of Chitosan and chemically-modified Chitosan MNP formulations on the drug release profiles, making this exploration a novel contribution in the field. The potential promise of using such unique Chitosan-ylated MNPs will be a great addition to the drug delivery toolbox.

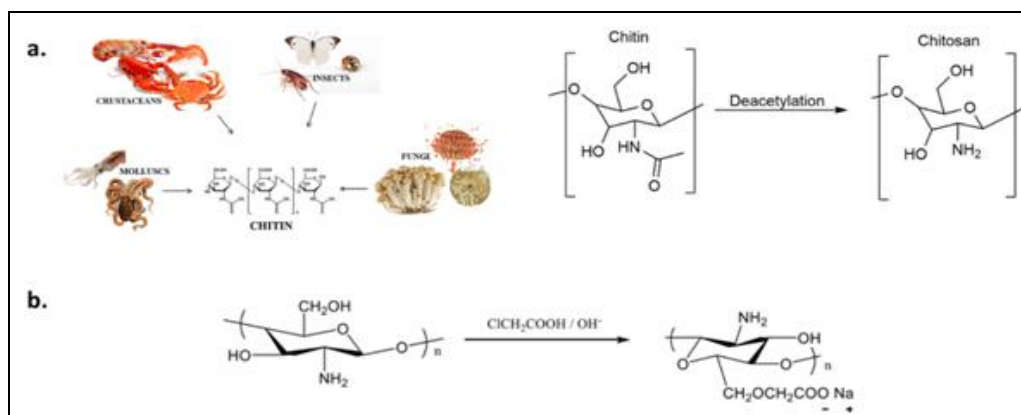


FIG. 1: (A) THIS FIGURE SHOWS THE PREPARATION OF CHITOSAN FROM CHITIN, (B) PREPARATION OF CARBOXYMETHYL CHITOSAN (CMC) FROM CHITOSAN

MATERIALS AND METHODS: All reagents and solvents were obtained from commercial suppliers and used as received, without further purification. Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) (98-102%), iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) ($\geq 99\%$), Chitosan (Low Mw (LM) and High Mw (HM)), Doxorubicin Ready-Made (Dox, 2 mg/mL), monochloroacetic acid, ethanol ($\text{C}_2\text{H}_5\text{OH}$) (Assay 96%), 2-Propanol ($\text{C}_3\text{H}_8\text{O}$) (Assay 99.8%), and 28% ammonium hydroxide (NH_4OH) were all purchased from Sigma-Aldrich. All reactions were carried out under an inert nitrogen atmosphere. For biological assays, phosphate buffered saline (PBS), Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS), Hoechst 33342 stain, L-glutamine, and Penicillin/Streptomycin (PS) were all purchased from Invitrogen. MTT (thiazolyl bluetetrazolium bromide) was purchased from Bioworld, USA. Cancer cell lines were purchased from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium supplemented with 10% FBS and 1% PS. Human cancer cells used in this study are THP-1 human monocytic cell lines derived from an acute monocytic leukemia patient.

Characterization: X-ray diffraction (XRD) was performed using Rigaku Ultima IV equipped with $\text{Cu-K}\alpha$ radiation source (0.15418 nm) with angle ranging from 20° to 80° and the crystal structure parameters were obtained through Rietveld analysis. The morphology and high-resolution structural features of the samples were investigated using a Talos L120C G2 transmission electron microscope (TEM) (Thermo Fisher Scientific, USA) at an accelerating voltage of 120 kV. Prior to imaging, the nanoparticle samples were dispersed in water, sonicated for 5 minutes, and drop-casted onto carbon-coated copper grids (TEM grid from EMS-Electron Microscopy Science, USA). High-resolution TEM (HR-TEM) was subsequently performed to examine the crystalline structure of the nanoparticle cores at the atomic scale. Clear lattice fringes were resolved, enabling measurement of interplanar (d -spacing) values, which were used to assess crystallinity and phase characteristics. Dynamic light scattering (DLS) measurements were assessed on Malvern Zetasizer Nano ZS instrument. Fourier transform infrared (FTIR) spectra ($400\text{--}4000\text{ cm}^{-1}$) were recorded as

KBr pellet forms using Shimadzu IRAffinity-1. Magnetic characterizations were performed using vibrating sample magnetometer (VSM) with 1.8 T magnets at ambient temperature. UV-vis absorption spectra were measured using ORION AQUAMATE 8000 UV-vis spectrophotometer in quartz cuvette cell over the wavelength range 200–800 nm.

Preparation of Carboxymethyl Chitosan (CMC): CMC was prepared following a previous procedure, with slight modification³¹. First, either LM or HM Chitosan (0.5 g) was dissolved in 20% NaOH (2 g NaOH + 10 ml distilled water) in a 100 ml beaker and stirred for 15 minutes. Monochloroacetic acid (5.29 mmol, 0.5 g) was then added in batches to the solution with continuous stirring for two hours in a water bath at 40°C . After cooling the solution to room temperature, it was neutralized with 2% acetic acid to obtain a neutral medium. An excess of 70% ethanol (20 ml) was poured into the neutral solution and then filtered, followed by extensive washing with distilled water and methanol to get rid of the non-reactive substances. Finally, the desired product “CMC” was obtained as a solution to be utilized in the preparation of coated NPs.

Chitosan/CMC-coated Fe_3O_4 NPs: All polymer-coated MNPs were prepared using our previously reported Ko-precipitation Hydrolytic Basic “KHB” method. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.3 g) was mixed with 2 mL of Chitosan solution (0.2 g Chitosan for every 10 ml water), followed by addition of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g) and stirred for few minutes at 80°C under nitrogen gas. NH_4OH ($\sim 3\text{ mL}$) was slowly added until $\text{pH} = 12\text{--}14$, where the solution turned from brown to black-brick colored depicting the formation of magnetite NPs and stirring was continued for an additional 3 hrs. The MNP suspensions were subsequently purified by centrifugation (4500 rpm, 5 min), washed multiple times with isopropanol, ethanol, and water, and finally dried at 80°C to produce $\sim 150\text{ mg}$ ($\sim 90\%$ yield) of Chitosan-coated MNPs. For the preparation of CMC-coated NPs, similar procedure was followed using 3 ml of the as-prepared CMC precursor yielding $\sim 140\text{ mg}$ of CMC-MNPs.

Drug Loading and Release: A total of 1 mg of Chitosan/CMC-coated MNPs was loaded with Dox

by incubating them with 1 mL of Dox solution (20 µg/mL), followed by continuous shaking for 24 hrs to ensure maximum drug loading. The drug-loading and release behavior of Dox-loaded Chitosan/CMC-coated NPs was then evaluated using UV-vis spectroscopy. First, the characteristic peaks of free Dox were recorded as a reference and calibration curve of Dox was generated. Drug loading was confirmed by the disappearance of the red color from the supernatant and reduction of Dox absorbance in the UV-vis spectrum at 480 nm. The drug loading efficiency (content) was calculated using the following equation:

$$\text{Loading efficiency} = W_L/W_0 \times 100$$

Where W_L is the amount of Dox loaded onto MNPs and W_0 is the amount of Dox in the initial solution. The amount of Dox loaded onto the MNPs was determined by calculating the difference between the initial Dox concentration and the concentration remaining in the supernatant.

For the release study, the loaded MNPs were dispersed separately in two acetate buffer media: an acidic (pH ~ 4.5) buffer to simulate endosomal/lysosomal conditions, and neutral (pH ~ 7.4) to assess release under physiological conditions at room temperature.

Samples were shaken on a rotary shaker for selected periods of time and the Dox-loaded MNPs were isolated from the solution using hand-held magnet where aliquots were analyzed by UV-vis spectroscopy. Three replicates for each release experiment were performed and the results are plotted as mean $\pm$ S.D.

Cell Viability Assays: Non-adherent THP-1 monocytic cancer cells were used as a representative cell type for leukemia. The cells were plated in flat-bottom 96-well plates at a density of $\sim 10^4$ cells/well in 100 µL of their respective growth medium. Serial dilutions of the various NP formulations (0.5 – 50 µg/mL) were added to cell plates (200 µL per well) in triplicates in a cell culture medium (RPMI supplemented with 10% FBS and 1% PS). Wells at the edges were left empty to minimize edge effects. After 48 hrs of incubation at 37 °C with 5% CO₂, the medium was removed, and cells were washed with PBS. Cell viability was assessed using the MTT assay.

Briefly, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hrs at 37 °C. The supernatant was then discarded, and the resulting formazan crystals were dissolved in DMSO. Absorbance was measured at 590 nm using an iMark microplate reader. The percentage of viable cells was calculated by comparing the absorbance of treated wells to that of untreated control wells, which were set at 100% viability. IC₅₀ values were determined from dose-response curves generated using polynomial dose-response fitting.

Live Confocal Microscopy Imaging: Non-adherent THP-1 monocytic leukemia cells were suspended in the respective media in 8-well dish and were exposed to Dox@Chitosan-MNPs (10 µg/mL NPs) and further incubated for different periods of times (i.e. 6 and 48 hrs). Hoechst 33342 stain was then added. The cells were allowed to settle down for 20 min before microscopic visualization. To mimic physiological conditions, no fixation of cells was conducted.

RESULTS AND DISCUSSION:

Preparation and Characterization of Chitosan/CMC-MNPs: Chitosan/CMC-coated MNPs with relatively uniform and homogeneous sizes were synthesized using our previously reported Ko-precipitation Hydrolytic Basic (KHB) approach,^{32, 33} as depicted in **Fig. 2**. The approach relies on sequential basic hydrolytic in situ co-precipitation (Ko-precipitation) of inexpensive iron salts (Fe³⁺: Fe²⁺; 2:1) compartmentalized by the stabilizing polymers (i.e. Chitosan/CMC) in the presence of a base (i.e. NH₄OH). This allowed the formation of stabilized aqueous colloidal dispersions of Chitosan/CMC-coated MNPs. Delightfully, the synthetic methodology for the MNPs demonstrated good reproducibility, as repeated syntheses consistently yielded comparable percentage yields ($\sim 90 - 95\%$) and good aqueous dispersity.

It is to be noted that two different chitosan samples were used: (i) LM Chitosan with an average Mw range of 50–190 kDa and a degree of deacetylation (DDA) of $\sim 75-85\%$, and (ii) HM Chitosan presenting an average Mw of 310–375 kDa and a DDA of $\geq 75\%$. TEM, XRD, DLS, FTIR, and VSM analyses confirmed the structure, morphology,

surface charge, and magnetic properties of the as-synthesized MNPs. TEM images revealed that Chitosan/CMC-coated MNPs exhibited a uniform, quasi-spherical morphology **Fig. 3**, with average particle sizes of approximately 8–10 nm. HR-TEM image **Fig. 3F** revealed a well-defined lattice fringe

with an interplanar spacing (*d*-spacing) of ~ 0.445 nm, corresponding to the most prominent (111) crystallographic plane. To further elucidate the crystalline structure and phase composition of the as-synthesized MNPs, X-ray diffraction (XRD) analysis was performed.

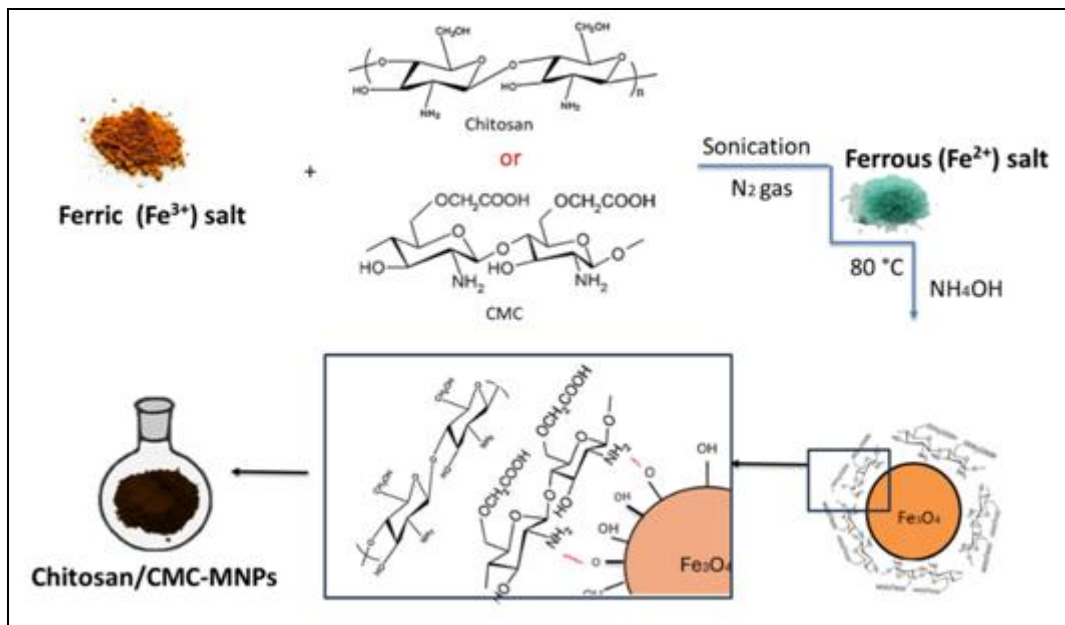


FIG. 2: SCHEMATIC ILLUSTRATION FOR THE PREPARATION OF CHITOSAN OR CMC-COATED MNPS USING KO-PRECIPIATION HYDROLYTIC BASIC (KHB) METHOD

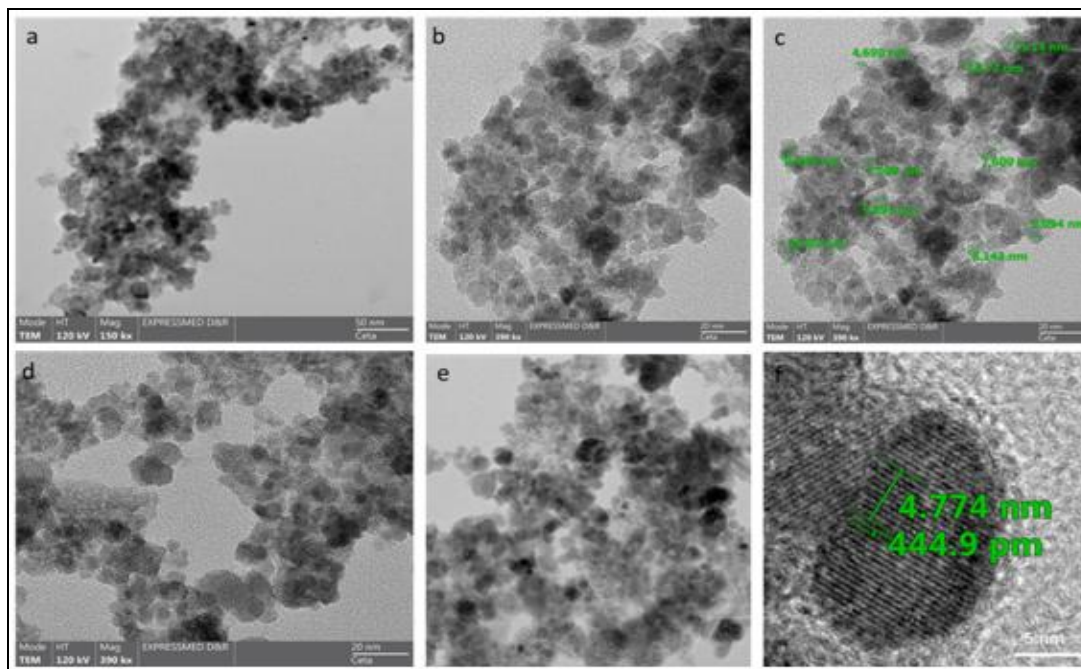


FIG. 3: TEM IMAGES AT DIFFERENT MAGNIFICATIONS OF (A-C) CHITOSAN-COATED MNPS AND (D-E) CMC-COATED MNPS SHOWING UNIFORM AVERAGE SIZES ~ 10 NM. (F) HR-TEM IMAGE WITH AN AVERAGE INTERPLANAR DISTANCE (*D*-SPACING) ~ 0.4449 NM, DEPICTING THE EXCELLENT CRYSTALLINITY OBTAINED FOR THE AS-PREPARED MAGNETITE NPS

The XRD patterns indicated that all samples possessed well-crystallized, single-phase inverse spinel structures **Fig. 4**. Each spectrum exhibited

well-defined peaks, indicative of the crystalline nature of the samples. Main peaks were identified at (31), (37), (44), (54), (57), (63), and (75)

2θ corresponding to (220), (311), (400), (422), (511), (440), and (533) which are related to the spinel structure of pure Fe_3O_4 cubic spinel phases (JCPDS # 01-088-0315). No additional peaks were observed, suggesting that no other iron oxide phases are formed. Inverse spinel magnetite has a face-centered cubic (FCC) crystalline lattice where oxygen and metal ions are positioned on the tetrahedral (A) and octahedral sites (B), where the tetrahedral A sites are occupied exclusively by ferric ions (Fe^{3+}), while the octahedral B sites contain an equal mixture of ferric (Fe^{3+}) and ferrous (Fe^{2+}) ions in a 1:1 ratio³⁴. Rietveld refinement of the XRD patterns confirmed the formation of pure Fe_3O_4 cubic inverse spinel phases with space group $\text{Fd}\bar{3}\text{m}$, in good agreement with JCPDS #01-088-0315. In the Rietveld refinement, the quality of agreement between the experimentally observed X-ray diffraction patterns and the calculated patterns is evaluated using several statistical parameters, including the profile

R-factor (R_p), weighted profile R-factor (R_{wp}), expected R-factor (R_e), goodness-of-fit (S), and chi-square (χ^2). The goodness-of-fit (S) is calculated as the ratio between R_{wp} and R_e ($S = R_{wp} / R_e$) and indicates how closely the refinement approaches the statistically expected agreement. A value of S close to 1 indicates that the calculated model adequately describes the experimental data. χ^2 is the square of the goodness-of-fit ($\chi^2 = S^2$) and is commonly used as an overall indicator of refinement quality; values approaching unity indicate a statistically acceptable refinement. Herein, the Rietveld refinements exhibited S values close to unity (1.08–1.11) and χ^2 values near 1 (1.17–1.24), indicating excellent agreement between the observed and calculated diffraction patterns. Importantly, Rietveld analysis perfectly corroborated XRD and TEM results with lattice parameters ($a = b = c = 8.36/7$) for all samples and crystallite sizes between 7 and 10 nm **Table 1**.

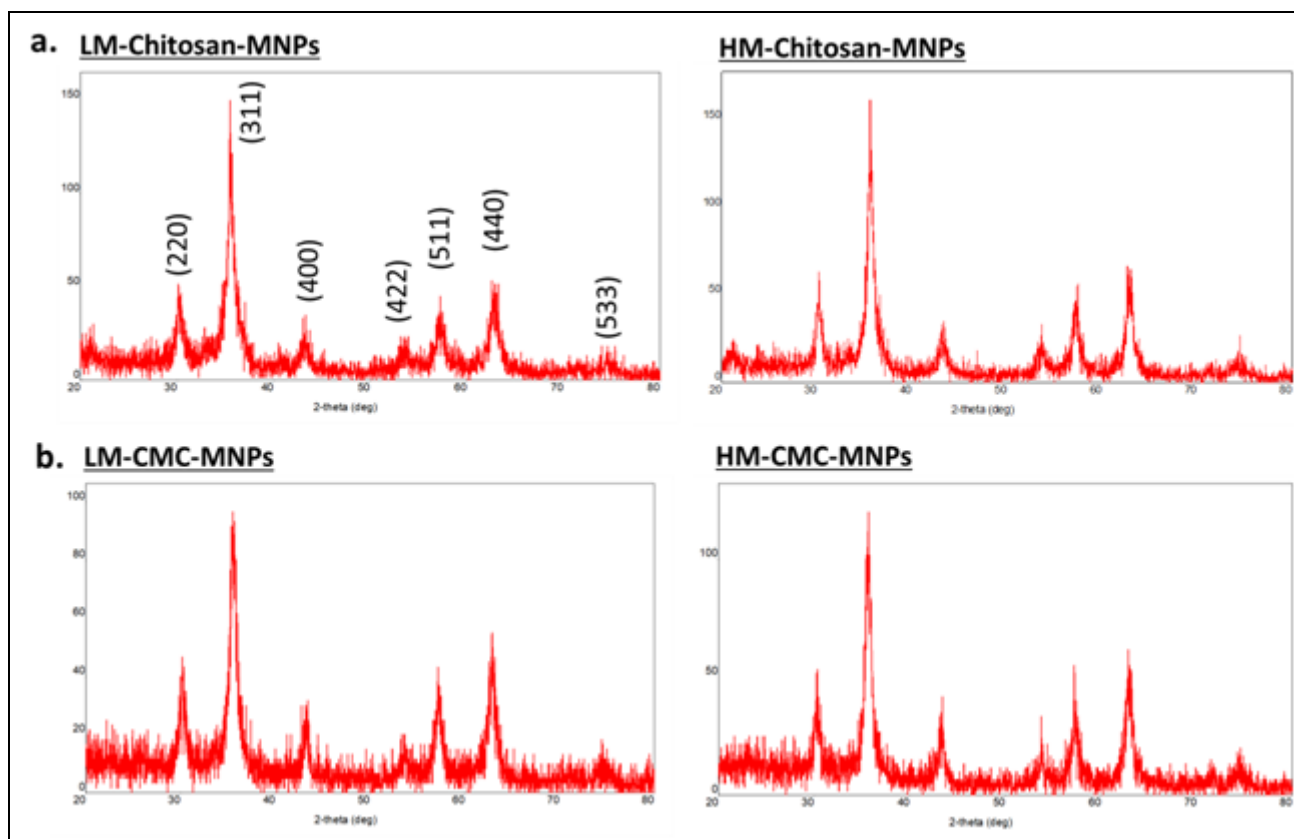


FIG. 4: XRD DIFFRACTION PATTERNS OF THE VARIOUS MW (LM AND HM) CHITOSAN/CMC-COATED MNPS, A) CHITOSAN-MNPS AND B) CMC-MNPS, CLEARLY SHOWING THE FORMATION OF PURE CRYSTALLINE PHASES OF Fe_3O_4

Next, DLS measurements of aqueous dispersions of Chitosan/CMC-MNPs were recorded. While TEM measures the core sizes of the MNPs, DLS records

the hydrodynamic radii in water. Chitosan-MNPs revealed hydrodynamic sizes (D_H) between 275 and 434 nm with uniform and narrow size distributions

as evident from the sharp peaks and low polydispersity index (PDI) values for all samples **Fig. 5A**. It is to be noted that the hydrodynamic sizes of CMC-MNPs (~275 - 301 nm, PDI = 0.423 - 0.497) were found to be slightly smaller than Chitosan-MNPs (365 - 434 nm, PDI = 0.633 - 1). HW Chitosan-MNPs (PDI = 1) shows higher PDI than LW Chitosan-MNPs (PDI = 0.633), while HW CMC-MNPs (PDI = 0.497) and LW CMC-MNPs (PDI = 0.423) illustrated slightly better PDI. This is likely attributable to the successful carboxymethyl substitution of chitosan, which resulted in MNPs with a narrow size distribution.

All the functionalized MNPs exhibited good stability in aqueous dispersion, even after prolonged storage. No significant changes in particle size were observed over time, further confirming their excellent colloidal stability. Zeta potential measurements indicated positive charge surfaces for all samples ($\xi \geq +5$ mV), further confirming coating with the positively charged Chitosan/CMC **Fig. 5B**. Notably, the average surface charges of Chitosan-MNPs were found to be slightly higher ($\xi = +12$ and 18 mV) than CMC-MNPs ($\xi = +6$ and 8 mV), due to carboxymethylation of Chitosan. Moreover, zeta potential of bare Fe_3O_4 MNPs was found to be equal to $\xi = -12.5$ mV, further confirming

Chitosan/CMC coating the magnetite surfaces. FTIR analysis was then conducted to validate the successful coating of Chitosan/CMC onto MNPs **Fig. 6**. The presence of iron oxide (Fe_3O_4) core is clearly evident by the Fe-O stretching bands appearing at $\sim 560 - 620 \text{ cm}^{-1}$. The broad peaks at $\sim 3400 \text{ cm}^{-1}$ are ascribed to the O-H and N-H stretching vibrations of hydroxyl and amino groups on MNPs. Symmetric and asymmetric C-H stretching vibrations clearly appear at $\sim 2850 \text{ cm}^{-1}$ and 2920 cm^{-1} . The presence of a strong peak at $\sim 1150 \text{ cm}^{-1}$ is a characteristic signature of the C-O-C stretching vibrations. Another major peak is the stretching vibration of C=O carbonyl evident at $\sim 1635 \text{ cm}^{-1}$, which coincides with the N-H and O-H bending vibrations occurring $\sim 1600-1650 \text{ cm}^{-1}$. [35, 36] Peaks at 1601 cm^{-1} (due to symmetric axial deformation of $-\text{COOH}$) and 1392 cm^{-1} (due to asymmetric axial deformation of $-\text{COOH}$) confirm the existence of carboxymethyl groups on chitosan³⁷. All these functional vibrations provide clear evidence of the successful functionalization of Fe_3O_4 MNPs with Chitosan or CMC, suggesting strong interactions and stability of the chitosan/CMC-MNPs probably via electrostatic/hydrogen bonding interactions between amino, hydroxyl, and carboxyl groups of chitosan or CMC and the hydroxyl groups on MNP surfaces.

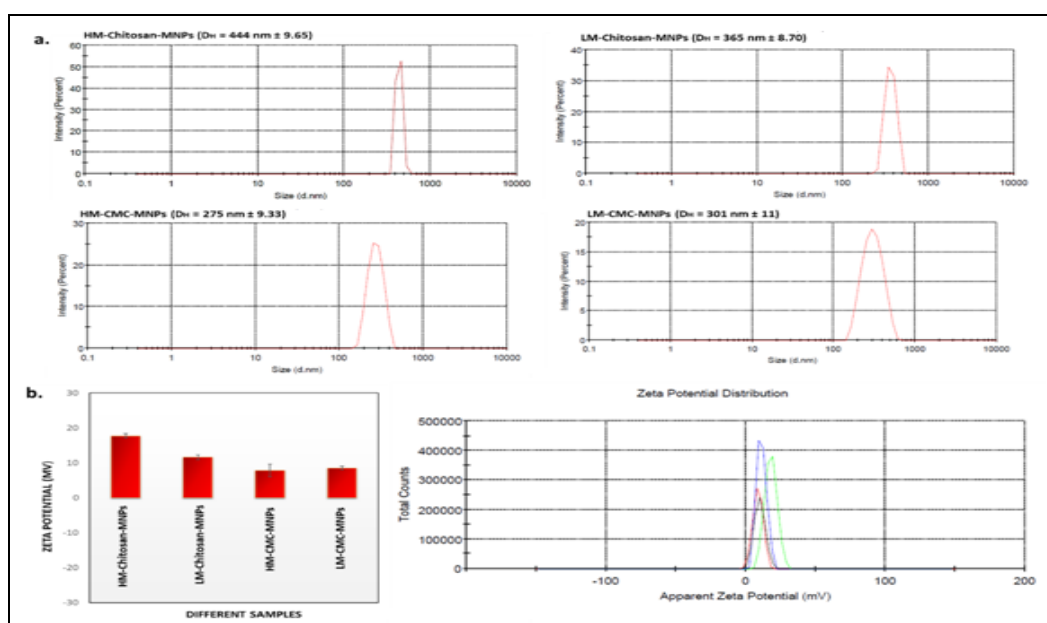


FIG. 5: DLS MEASUREMENTS FOR THE DIFFERENT MWS OF CHITOSAN/CMC-COATED MNPS DISPERSED IN WATER. A) REPRESENTATIVE SIZE DISTRIBUTION PLOTS; B) AVERAGE ZETA POTENTIAL (ξ) SURFACE CHARGES REPRESENTED AS BAR GRAPHS ALONG WITH A REPRESENTATIVE ZETA POTENTIAL DISTRIBUTION FOR THE DIFFERENT CHITOSAN/CMC-COATED MNPS. ERROR BARS REPRESENT THREE INDEPENDENT MEASUREMENTS REPORTED AS AVERAGE MEANS $\pm$ S.D.

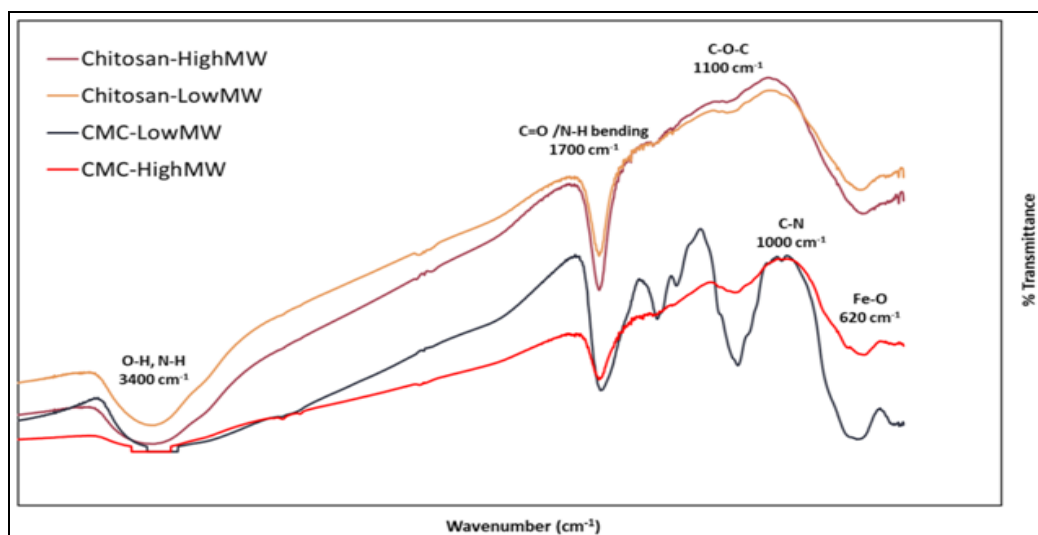


FIG. 6: FTIR FOR THE DIFFERENT AS-PREPARED CHITOSAN/CMC-COATED MNPS. THE FTIR SPECTRA CLEARLY SHOW THE SUCCESSFUL CHITOSAN AND CMC COATING THE IRON OXIDE CORE

Magnetic Properties: Magnetically-responsive MNPs are composed of elements possessing magnetic properties such as iron, nickel, cobalt, or metal oxides. Consequently, there is a strong demand for the preparation of stabilized polymer-coated MNPs with high saturation magnetization (M_s) using simple, scalable, cost-effective, and reproducible methods. The preparation of colloidal and well-dispersed MNPs with controlled particle

sizes and biocompatible polymer coatings have recently revealed an intriguing capability to mediate drug delivery applications. Therefore, designing MNPs that have optimal magnetization for magnetic-guided drug delivery applications is highly demanded. The significance stems from preparation of high-performance water-dispersible magneto-responsive NPs and tuning their physicochemical parameters.

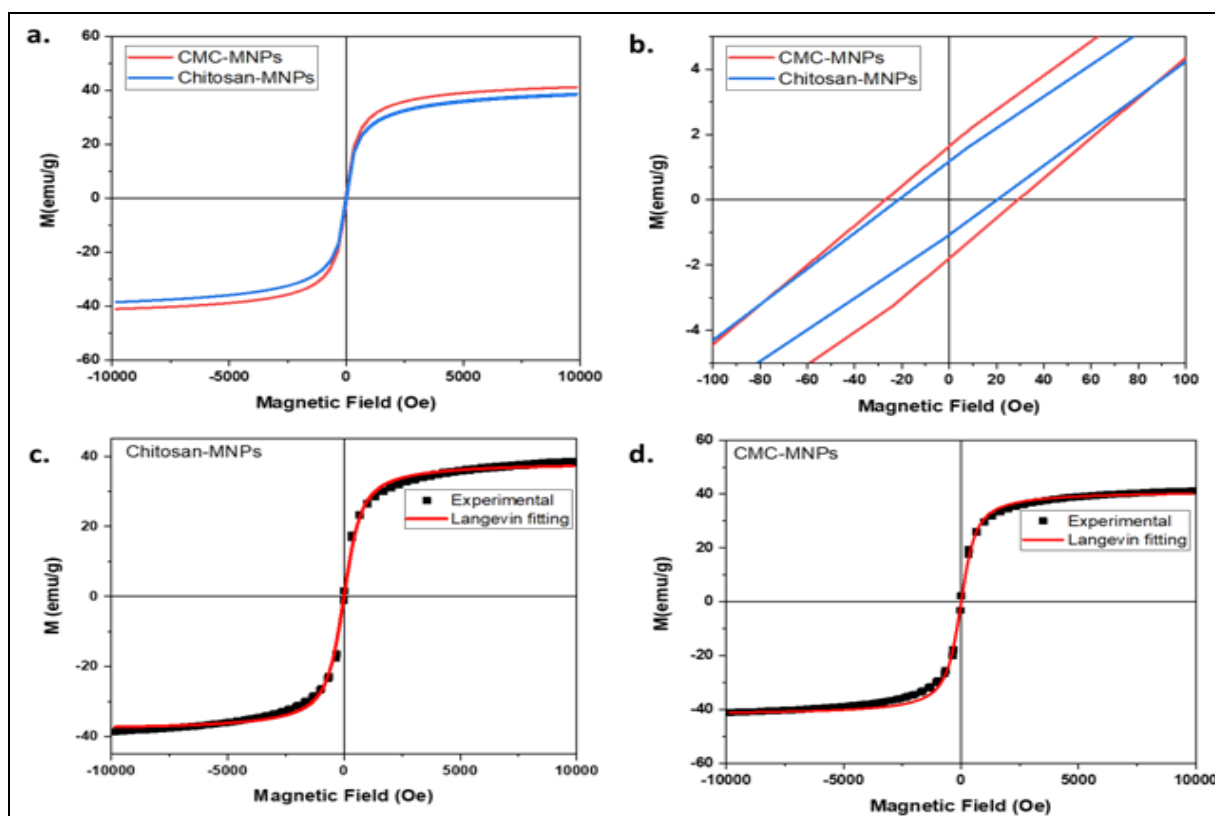


FIG. 7: FIELD-DEPENDENT MAGNETIZATION HYSTERESIS LOOPS AT ROOM TEMPERATURE FOR (A,B) CHITOSAN-MNPS AND CMC-MNPS AND (C,D) LAW OF APPROACH TO SATURATION (LAS) FITTINGS

To determine the magnetic behavior for the as-prepared Chitosan/CMC-coated MNP samples, field-dependent magnetizations were conducted. As depicted in **Fig. 7**, the hysteresis loops (M - H) as a function of applied magnetic field at room temperature are recorded. From the magnetization curves, the saturation magnetization (M_s), coercivity (H_c), and remanence (M_r) values were extracted and are summarized in **Table 2**. It can be observed that the MNPs show super paramagnetic characteristics with very small H_c (< 30 Oe) and almost negligible M_r . The M_s magnetization obtained for the Chitosan-MNPs and CMC-MNPs were found to be equal to 38.77 and 41.25 emu/g, respectively. The magnetic measurements revealed relatively high saturation magnetizations, reflecting a high degree of magnetic ordering and crystallinity in the samples. To further assess their distinctive magnetic behavior, the effective anisotropy

constant (K_{eff}) was calculated, as it is a key parameter directly influencing magnetization. The law of approach to saturation (LAS) was employed to determine K_{eff} , using the following equation applied to the experimental magnetization data.

$$M(H) = M_s (1 - b/H^2)$$

Where b is a parameter which is deduced from the fitting of experimental magnetization with the above equation. K_{eff} is then determined by:

$$K_{\text{eff}} = \mu_0 M_s \sqrt{(15b/4)}$$

Fitting the equation to the experimental magnetization data showed that all M - H curves were well described by the LAS model, yielding very high K_{eff} values $\sim 1.2 - 1.3 \times 10^5$ erg/cm³ **Fig. 6B**.

TABLE 1: RIETVELD XRD ANALYSIS OF THE DIFFERENT AS-PREPARED CHITOSAN/CMC-MNPS

Sample	Phase Composition (%)	Crystallite size (nm)	Microstrain (%)	Lattice parameters (Å)	Fit parameters
LM-CMC-MNPs	Fe ₃ O ₄ 100%(7)	8.51 (7)	0.36(3)	a= b=c= 8.357(6) $\alpha=\beta=\gamma=90$	$R_{\text{wp}}=44.27$ $R_p=33.55$ $R_e=40.23$ $S=1.0997$ $\chi^2=1.2094$
HM-CMC-MNPs	Fe ₃ O ₄ 100%(5)	8.82(6)	0.11(3)	a= b=c= 8.362(4) $\alpha=\beta=\gamma=90$	$R_{\text{wp}}=42.69$ $R_p=32.23$ $R_e=39.41$ $S=1.0827$ $\chi^2=1.1722$
LM-Chitosan-MNPs	Fe ₃ O ₄ 100%(6)	6.72(2)	0.425(18)	a= b=c= 8.361(7) $\alpha=\beta=\gamma=90$	$R_{\text{wp}}=41.81$ $R_p=31.11$ $R_e=37.54$ $S=1.1130$ $\chi^2=1.2388$
HM-Chitosan-MNPs	Fe ₃ O ₄ 100%(4)	8.99 (13)	0.303(6)	a= b=c= 8.369(17) $\alpha=\beta=\gamma=90$	$R_{\text{wp}}=41.32$ $R_p=30.83$ $R_e=37.91$ $S=1.0892$ $\chi^2=1.1864$

R_{wp} : Weighted profile R-factor; R_e : Expected R-factor; R_p : Profile R-factor; S : goodness of fit ($S=R_{\text{wp}}/R_e$); $\chi^2=S^2$

TABLE 2: MAGNETIC PARAMETERS OBTAINED FROM M-H CURVES AND LAW OF APPROACH TO SATURATION (LAS) FOR CHITOSAN/CMC-MNPS

Samples	H_c (Oe)	M_r (emu/g)	M_r/M_s	K_{eff}	Saturation M_s (emu/g)	
					Experimental	Lang in fitting
Chitosan -MNPs	29.7	1.64	0.04	1.20×10^5	38.77	38.37
CMC-MNPs	20.9	1.14	0.02	1.29×10^5	41.25	41.68

Drug Loading and Release: Significant pH variations exist at the systemic, tissue, and sub-cellular levels³⁸. This pH gradient has been utilized as an endogenous trigger for designing nanocarriers capable of selectively releasing their payloads in acidic tumor environments^{29, 39, 41}. For example, the extracellular milieu of tumor tissues is more acidic (pH 6.2–6.9) than that of normal tissues and systemic blood pH. Additionally, the acidic conditions within intracellular compartments such as endosomes and lysosomes (pH 4.0–6.5) have

been exploited to design pH-responsive nanocarriers that release their cargo intracellularly^{38, 40}. pH-responsive nanocarriers are typically constructed from polymers that undergo transitions between hydrophilic (swollen) and hydrophobic (collapsed) states in response to protonation and deprotonation of labile functional groups, such as amino and carboxylic acid groups, under varying pH conditions. This behavior provides a mechanism for pH-triggered payload release. Polymers containing amine groups have been

extensively investigated for such applications, as protonation enhances polymer hydrophilicity^{42, 43}. Furthermore, amine groups can effectively neutralize the acidic endosomal environment via protonation, raising the endosomal pH. This process facilitates ionic influx into the endosome, causing it to swell and rupture, ultimately releasing the internalized NPs - a phenomenon known as the proton-sponge effect⁴⁴. For instance, smart pH and thermoresponsive *N,O*-Carboxymethyl Chitosan-conjugated nanogels, possessing enhanced swelling behavior at lower pH, exhibited improved therapeutic efficacy of Dox in MCF-7 and MDA-MB-231 breast cancer cells⁴⁵.

To explore the possibility of Chitosan/CMC-coated MNPs as pH-responsive drug nanocarriers **Fig. 8A**, we first loaded them with the anticancer drug Doxorubicin (Dox). 1 mL of Dox solution (20 µg/mL) was incubated with the respective MNPs, followed by continuous shaking for 24 hrs to ensure maximum entrapment. Drug loading content of Dox@Chitosan/CMC-coated NPs was evaluated by UV-vis spectroscopy which was confirmed by the complete disappearance of the red color in the

supernatant along with a decrease in Dox absorbance. Almost quantitative amount of drug (i.e. 100% loading efficiencies) was successfully loaded onto all samples of Chitosan/CMC-coated MNPs (i.e. 20 µg Dox/mg MNPs), as evident from absorption spectroscopy **Fig. 8B**. These results were reproducible when using different batches of Chitosan/CMC-coated MNPs (1 mg/mL). Dox can be loaded into Chitosan/CMC-coated MNPs primarily through electrostatic interactions, hydrogen bonding, and Van der Waals interactions taking advantage of Chitosan's structure and Dox's properties. This physio-adsorption of Dox onto the coated MNPs offers the advantage of preserving both the structure of the NPs and the conjugated Dox. MNPs in which drug molecules are covalently conjugated to their surfaces typically show low drug entrapment efficiencies and limited drug release due to the covalent bonding. In addition, the proper orientation of the drug's active functional groups is critical for biological activity. Thus, we anticipated that simple physio-adsorption interactions would be a more suitable approach to preserve the activity of the drug on the MNPs.

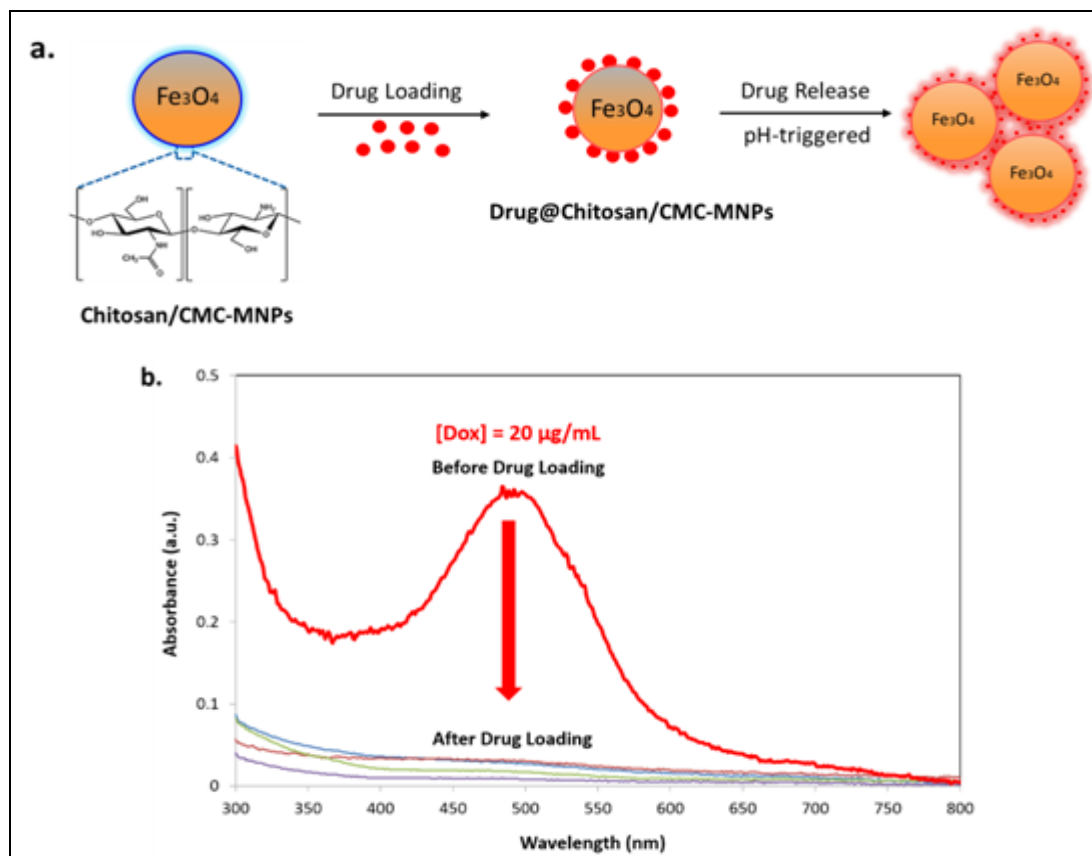


FIG. 8: (A) SCHEMATIC REPRESENTATION FOR THE DRUG LOADING AND PH-DEPENDENT DRUG RELEASE; B) UV-VIS SPECTRA BEFORE AND AFTER LOADING OF DOX ONTO THE DIFFERENT MNPs

With Dox@Chitosan/CMC-MNPs in hand, we subsequently examined their *in-vitro* drug release profiles **Fig. 9**. One notable feature of chitosan is its pH-dependent solubility, which can be leveraged for targeted drug administration⁴⁶. Importantly, very few reports⁴⁷, if any explored the effects of different Mws of chitosan on drug release behavior. To conduct the release studies, all loaded MNPs were dispersed separately in two media: an acidic buffer (pH ~ 4.5) to simulate endosomal/lysosomal conditions, and neutral (pH ~ 7.4) conditions to assess release under physiological conditions. At selected time points, samples were analyzed by UV-vis spectroscopy to monitor the re-appearance of the characteristic Dox peaks, indicating the extent of drug release in each medium. We compared between LM and HM Chitosan- and CMC-coated MNPs in drug releasing experiments **Fig. 9A**. The release of drug was measured with time until 24 hrs under pH 4.5 and pH 7.4 for all the samples. For all formulations, at acidic medium (pH = 4.5), higher and faster drug release was observed compared to neutral pH = 7.4. Among the samples, HM-Chitosan MNPs exhibited the greatest release at acidic pH, giving almost quantitative release within 24 hrs, while the HM-CMC showed slightly lower release (~ 90 %) **Fig. 9B**. In contrast, LM-Chitosan and CMC MNPs showed lower drug release behaviour (~ 60 - 65%) at acidic pH compared to HM-Chitosan/CMC-MNPs **Fig. 9C**.

Remarkably, drug release profiles at neutral pH were markedly low ($\leq 20\%$) for all formulations, indicating the stability of Dox-loaded MNPs at neutral physiological pH. This enhanced release at acidic pH is attributed to decreased interactions between Dox and Chitosan bonds, which lead to faster diffusion of the anticancer drug. The initial burst phase of Dox release is ascribed to Dox located at the surface of the particles, while the remainder of the unreleased Dox is sustainably released as it is assumed to be entrapped within the Chitosan/CMC-MNPs. Chitosan and CMC are sensitive to pH, where the bonding between Dox and Chitosan/CMC will be protonated in acidic medium, leading to releasing the drug in a controlled sustained fashion. In endosomes and lysosomes (pH ~ 4 – 6.5), protonation of amine groups increases polymer hydrophilicity causing NP swelling, disassembly, and consequent drug

release. In general, CMC was found to be slightly more stable than chitosan, that means CMC show more controlled and a bit slower release profiles, where the carboxymethyl group is anticipated to interact with the drug stronger. This controlled yet sustained release of Dox is advantageous for achieving cytotoxic intracellular concentrations, in both *in-vitro* and *in-vivo* cancer therapy, consistent with previous observations⁴⁸. Together, these findings indicate that the physically bound Dox molecules in our system can be released rapidly, efficiently, and in a sustained manner, highlighting our Chitosan/CMC-MNPs as promising nanocarriers for imaging, drug delivery, and therapeutics.

One of the key benefits of using nano-based drug delivery systems is their ability to provide more targeted and less toxic solutions, effectively addressing the challenges of poor specificity and the dose-limiting toxicities often associated with conventional anticancer drugs. Thus, to better understand and determine the intracellular uptake of MNPs in cancer cells, live confocal laser scanning microscopy was employed. For these experiments, we chose the optimal HMC-MNP formulation, as it has excellent water solubility and colloidal dispersions, smallest sizes, positive surface charges, and suitable *in-vitro* drug release profiles.

A representative human monocytic leukemic cancer cell line (THP-1) was treated with Dox@CMC-MNPs and imaged at various time points **Fig. 10**. Notably, confocal images demonstrated that Dox is effectively delivered to the cytoplasm within relatively short time frames of 6 hrs **Fig. 10A**. Apoptotic features observed after 48 hrs of incubation with Dox-loaded MNPs clearly reveal morphological changes associated with effective nanoparticle-mediated drug delivery. As the NPs penetrate the cells and release Dox, the cells exhibit characteristic signs of apoptosis, including cell shrinkage, membrane blebbing, and the formation of irregular cellular fragments **Fig. 10B**. We then proceeded to evaluate, quantitatively, the cytotoxic potentials of CMC-MNPs and Dox@CMC-MNPs against this cell line using thiazolyl blue tetrazolium bromide (MTT) cell viability assay. While unloaded CMC-MNPs exhibited no cytotoxicity toward the cells ($IC_{50} >$

200 µg/mL), even at the highest NP concentrations tested, Dox@CMC-MNPs showed significant toxicity against THP-1 leukemic cells, killing the cancer cells with an $IC_{50} = 16 \pm 2.2$ µg/mL **Fig. 10C**. Although free Dox, at equivalent concentrations, exhibited more pronounced cytotoxicity against THP-1 cells than Dox@CMC-MNPs, the noted cytotoxicity of our formulation could prove superior for *in-vivo* situations. Dox is known to have initial rapid distribution to internal organs within 5 minutes. This rapid distribution can account for the systemic toxicity of the drug and to lesser exposure of leukemic cells to the drug in circulation. The current formulation is expected to have longer half-life in circulation, innate to its hydrodynamic size. This, in turn, can ensure longer exposure and selectivity to cancer cells that will

internalize the Dox-loaded CMC-NPs through phagocytosis/pinocytosis that is common to most hematological malignancy cells. We anticipate that the observed pronounced potency of Dox@CMC-MNPs primarily results from the selective and differential uptake of the NPs by cells, followed by the intracellular release of Dox over time in response to biochemical changes, particularly the pH-triggered conditions within endosomes and lysosomes. The drug payload subsequently undergoes sustained translocation to the nucleus, where it exerts its cytotoxic effect. Markedly, the Dox@CMC-MNP nanocarriers described here offer inherent advantages, as it minimizes the undesirable diffusion-associated side effects of the free drug and enables more selective and targeted drug delivery.

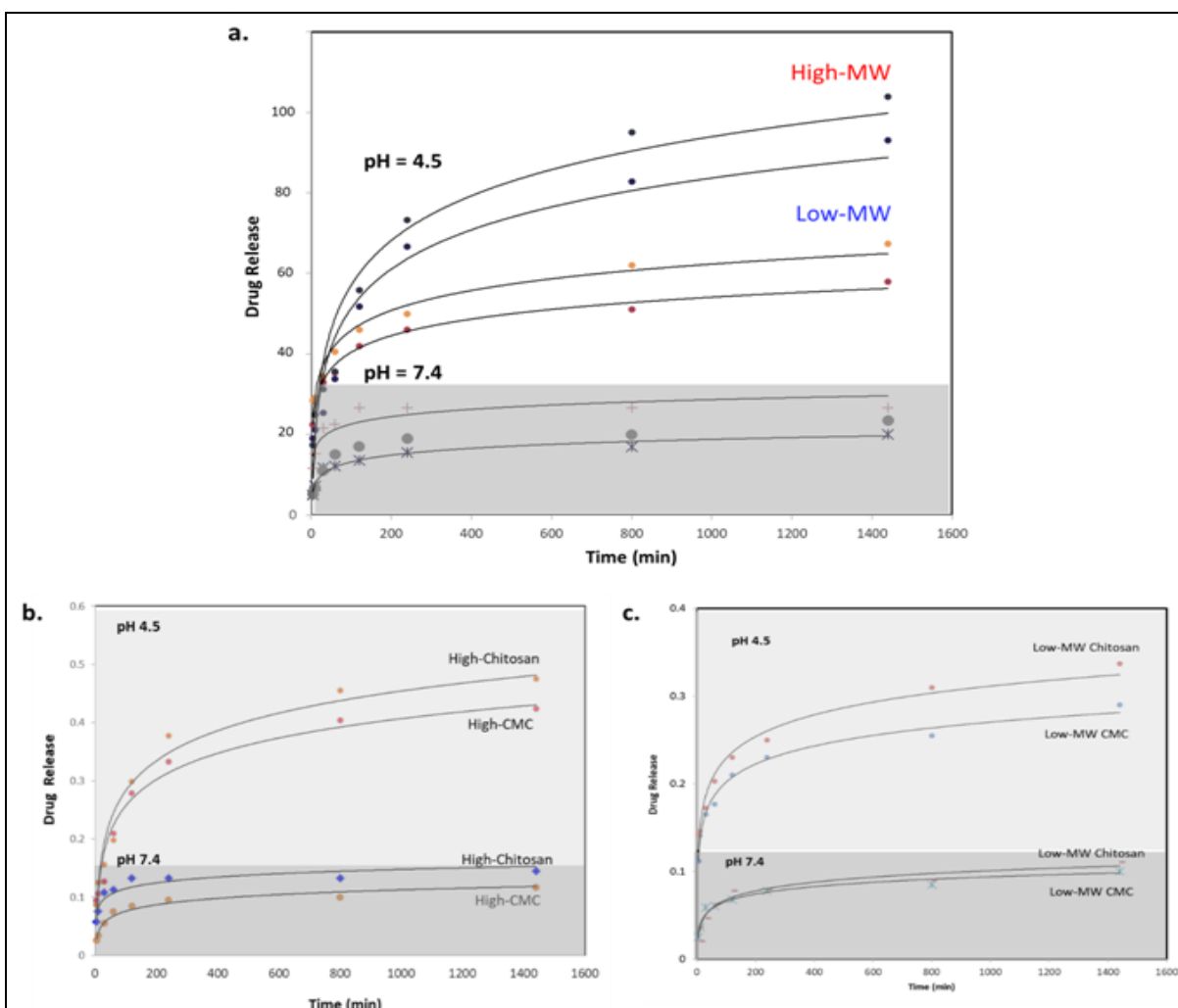


FIG. 9: THIS FIGURE SHOWS A) DOX RELEASE PROFILES FOR HM AND LM CHITOSAN/CMC-COATED MNPS UNDER ACIDIC AND NEUTRAL MEDIA. B) DOX RELEASE FROM HM CHITOSAN AND CMC MNPS UNDER ACIDIC AND NEUTRAL PH. C) DOX RELEASE FROM LM CHITOSAN AND CMC MNPS UNDER ACIDIC AND NEUTRAL pH. THE RESULTS SHOW HIGHER SUSTAINED DRUG RELEASE OF HM-CHITOSAN OR CMC COMPARED TO LM-CHITOSAN OR CMC AT ACID PH CONDITIONS, WHILE NONE OF THE FORMULATIONS EXHIBIT SIGNIFICANT RELEASE AT NEUTRAL pH

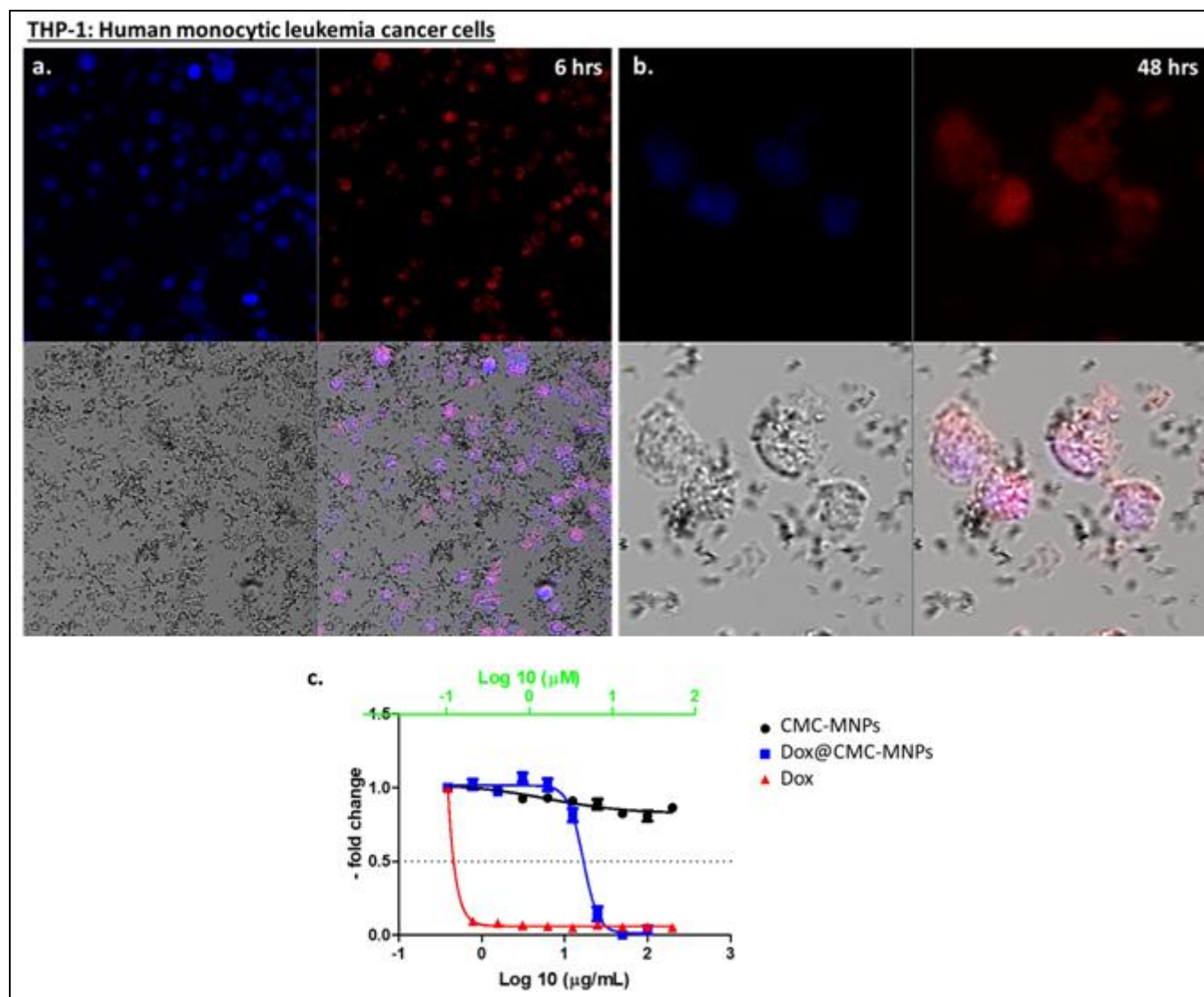


FIG. 10: CONFOCAL MICROSCOPY IMAGES OF THP-1 CELLS AFTER INCUBATION WITH DOX@CMC-MNPs (10 MG/ML MNPs; 0.37 MM DOX) FOR A) 6 HRS AND B) 48 HRS. (A,B) BLUE HOECHST CHANNEL SHOWING POSITIONS OF THE NUCLEI, RED DOX CHANNEL, AND OVERALL OVERLAY WITH TRANSMITTED LIGHT (TL). THE IMAGES CLEARLY SHOW SUCCESSFUL DELIVERY OF DOX TO THE CYTOPLASM, CAUSING APOPTOSIS WITH TYPICAL APOPTOTIC FEATURES INCLUDING CONDENSATION, SHRINKAGE, AND MEMBRANE BLEBBING. C) MTT CELL PROLIFERATION CYTOTOXICITY ASSAY FOR UNLOADED CMC-MNPs, DOX@CMC-MNPs, AND FREE DOX ON THP-1 LEUKAEMIA CELLS. X-AXIS: NP CONCENTRATIONS EXPRESSED IN $\mu\text{G/ML}$; Y-AXIS: ABSORBANCE AT 450 NM. EXPERIMENTS WERE CARRIED OUT IN TRIPLICATES AND AVERAGE RESULTS ARE PLOTTED

CONCLUSION: In conclusion, different Chitosan/CMC-coated MNPs with varying MWs were successfully prepared and utilized as nanocarriers for drug delivery to cancer cells. Carboxymethylation of chitosan proved to be effective in enhancing aqueous solubility while maintaining sustained and controlled drug-release profiles. By using HM or LM Chitosan or CMC-MNPs, pH-triggered Dox release was evaluated at both acidic and neutral pH conditions. In our system, a relatively fast initial burst release over the first 2 hrs was observed, followed by a more

controlled slower drug release, with almost quantitative discharge at acidic pH = 4.5, but almost no significant release at the physiological neutral pH. HM Chitosan/CMC-MNPs showed slightly higher drug release profiles compared to LM counterparts.

This behaviour is consistent with the increased protonation of functional groups at low pH. Confocal microscopy and viability cytotoxicity assays revealed pronounced killing of THP-1 cells using Dox@CMC-MNPs, causing apoptotic cell

death, but not when unloaded CMC-MNPs were utilized. This sustained release of Dox is distinctive and likely advantageous for achieving cytotoxic intracellular concentrations, benefiting both *in-vitro* and *in-vivo* cancer therapy. Therefore, drug-loaded chitosan-based nanocarriers may offer broad applicability and considerable potential for the development of controlled-release formulations and leukaemia management, warranting further *in-vivo* testing.

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Author Contributions: K.H.B. conceived, designed, and conceptualized the study. J.K.A. prepared all the Chitosan- and CMC-coated MNPs and performed the drug loading and release experiments. O.M.L. conducted magnetization and calculated magnetic parameters. A.M. carried out XRD and Rietveld analyses. K.G. helped with performing the MTT and confocal microscopy data as well as DLS measurements. K.H.B. and J.K.A. analyzed the experimental data and wrote the manuscript. All authors revised and approved the manuscript.

Data Availability: The authors confirm that the data supporting the findings of this study are available within the article. Further inquiries can be directed to the corresponding author, upon request.

Ethics Declaration: Not applicable

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