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Synthesis, characterization, and molecular docking of ES/CA/Cs nanocomposite as a potential nanocarrier for gene delivery: a preliminary study

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Abstract

Nanotechnology has emerged as a promising approach in biomedical fields, including diagnosis and treatment. Gene therapy is a technique that delivers therapeutic genes into the targeted cells as an alternative to drugs or surgery, potentially minimizing harm to patients. The combination of gene therapy techniques and nanotechnology offers new opportunities to improve clinical outcomes. The present study aimed to develop a promising gene delivery carrier based on Eggshell/Citric Acid/Chitosan (ES/CA/Cs) nanocomposites synthesized via the co-precipitation method. The FTIR spectrum confirmed the chemical structure of the prepared nanocomposite. TEM analysis revealed plate-like structures with an average size of 300-400 nm, whereas DLS indicated hydrodynamic diameters ranging from 700 to 4000 nm. In silico molecular docking was performed using the MVD method within Schrodinger software. The results predicted favorable interactions between the synthesized nanocomposite and F218V mutant of the AtRCCR enzyme, with a binding affinity of -137.39 KJmol^{-1} at the active site. These findings suggest that the ES/CA/Cs nanocomposite may serve as a potential carrier for gene delivery and warrants further in vivo investigation for renal cell carcinoma treatment. However, this study is limited to physicochemical characterization and in silico predictions, and no in vitro or in vivo biological validation was conducted. Therefore, future studies should include cytotoxicity assays on renal cell carcinoma cell lines, evaluation of gene loading and release profiles, cellular uptake studies, and in vivo investigations in appropriate animal models to validate the gene delivery potential of this nanocarrier.

Keywords: Nanocomposites, molecular docking, gene therapy, biocompatible materials, chitosan, Renal cell carcinoma.

1. Introduction

Nanotechnology has provided new opportunities for researchers across various fields of science and technology. Nanomaterials offer diverse applications due to their small size (1-1000 nm), high stability, favorable conductivity, high carrier capacity, and optical properties [1, 2]. They enable significant improvements in the detection, diagnosis, and treatment of human cancers [3]. Additionally, nanomaterials are actively developed for in vivo applications, biomolecular profiling of cancer biomarkers, and targeted gene and drug delivery [3, 4].

Drug delivery systems can be generally classified into five established generations [5], as presented in Table 1. The first generation includes conventional medications such as tablets, capsules, etc. The second generation comprises repeat-action and prolonged-actions. The third generation consists of the osmotically and swelling-controlled systems. The fourth generation includes targeted drug delivery system and the emerging fifth generation refers to gene therapy, nanorobots, etc [5, 6].

Table 1 The generations of drug delivery systems

Generations of Drug Delivery Systems				
First Generation	**Second Generation**	**Third Generation**	**Fourth Generation**	**Fifth Generation**
Tablets and Capsules	Repeat action tablet	Osmotically controlled system	Targeted drug delivery	Gene therapy
Suspension	Prolong action tablet	Swelling-controlled system	Modulated drug delivery	Nanorobots
Emulsion	Enteric-coated tablet	Diffusion controlled system	Self-regulated drug delivery	Long-term delivery system

Among all the methods, gene therapy is technique that utilizes therapeutic genes to prevent or treat disease [7, 8]. It addresses genetic deficiencies by delivering functional genes to target cells, offering a less harmful alternative to drugs of [7-9]. The interaction of small molecules with DNA has gained significant attention across various fields, including biochemistry, medicinal chemistry, life science, and cancer therapy [10, 11]. These interactions underlie many intracellular processes and can induce predictable changes in DNA transcriptions and replications [12, 13], which are relevant to studying cell death, proliferation, gene mutations, cancer causes, and treatment [4, 12-14]. DNA-binding compounds exhibit a wide range of potential anticancer, antiviral, or carcinogenic effects [12, 13, 15, 16], making it a key target for many antitumor drugs [17-19]. Covalent binding between drugs and DNA is irreversible, leading to cell death through complete inhibition of DNA processes. Non-covalent DNA binders, such as groove binders and intercalators, are less cytotoxic and reversible compared to covalent agents [20, 21]. Intercalation involves π-π interactions between the planar aromatic moieties and DNA nitrogenous bases without disrupting hydrogen bonds [22]. Intercalators insert deeply between DNA base pairs, altering nucleotide structure and disrupting replication and transcription [23]. Studying DNA-compound interactions aids in designing more effective drugs [12, 15], understanding DNA structure changes [15], and investigating protein-nucleic acid complexes. Most available drugs cause substantial damage to both healthy and target tissues [3, 24]. These side effects limit their clinical use and reduce therapeutic efficacy [15, 24]. Due to emerging drug resistance and severe side effects, many chemotherapeutic agents pose life-threatening risks and remain a leading cause of death worldwide [3, 14-16, 24, 25].

Free DNA is rapidly degraded by nucleases in the blood. Moreover, repulsive electrostatic interactions between negatively charged naked DNA and cell membrane limit cellular entry [26-28]. Gene transfer vectors are generally classified into viral and nonviral methods [28]. Viral vectors have major disadvantages, including complex production, quality control challenges, and safety concerns due to toxicity and immune responses [28-31]. Non-viral vectors have several advantages over viral vectors, including improved safety, high gene loading capacity, simple preparation, and lack of immunogenicity [28, 32-36]. nonviral gene delivery systems generally consist of three main components: (1) a plasmid-based gene expression system to control the function of a gene in the target cell, (2) a gene encoding a therapeutic protein, and (3) a delivery system that targets specific body locations [37-41].

Renal cell carcinoma (RCC) ranks among the ten most prevalent cancers worldwide and encompasses diverse tumors originating from renal tubular epithelial cells [42, 43]. Over the past 20 years, significant developments in the histopathological and molecular characterization of RCC have resulted in substantial changes to the disease's classification [43, 44]. Clear cell RCC (ccRCC) [45], papillary RCC (pRCC) [46], and Chromophobe RCC (chRCC) [47] are the major subtypes each with an incidence of ≥5%. The remaining subtypes have a combined incidence of less than 1% of cases, making them extremely unusual [48]. Tumors that do not match any diagnostic subtype are classified as unclassified RCC (uRCC; ~4% overall incidence) [49]. The majority of kidney cancer deaths are caused by ccRCC, the most prevalent subtype. [44]. Tumors with non-clear cell histology are categorized as "nccRCC" for clinical trial feasibility, given the prevalence of clear cell histology in metastatic disease (83-88%) [50, 51]. Recent genomic studies have revealed significant intra- and inter-tumor heterogeneity in ccRCC [52, 53], which may contribute to the varied clinical outcomes observed. Treatment options for localized RCC include ablation (using heat or cold to destroy the cancerous tissue), partial or radical nephrectomy (kidney removal) [54], or active surveillance (routine radiographic monitoring of tumor progression) [55]. Approximately 30% of patients with localized ccRCC later develop metastases, requiring systemic therapy and associated with significant mortality even after curative-intent nephrectomy. Although targeted treatments against the mTOR and VEGF pathways are available, most patients eventually experience disease progression despite variable treatment responses [44].

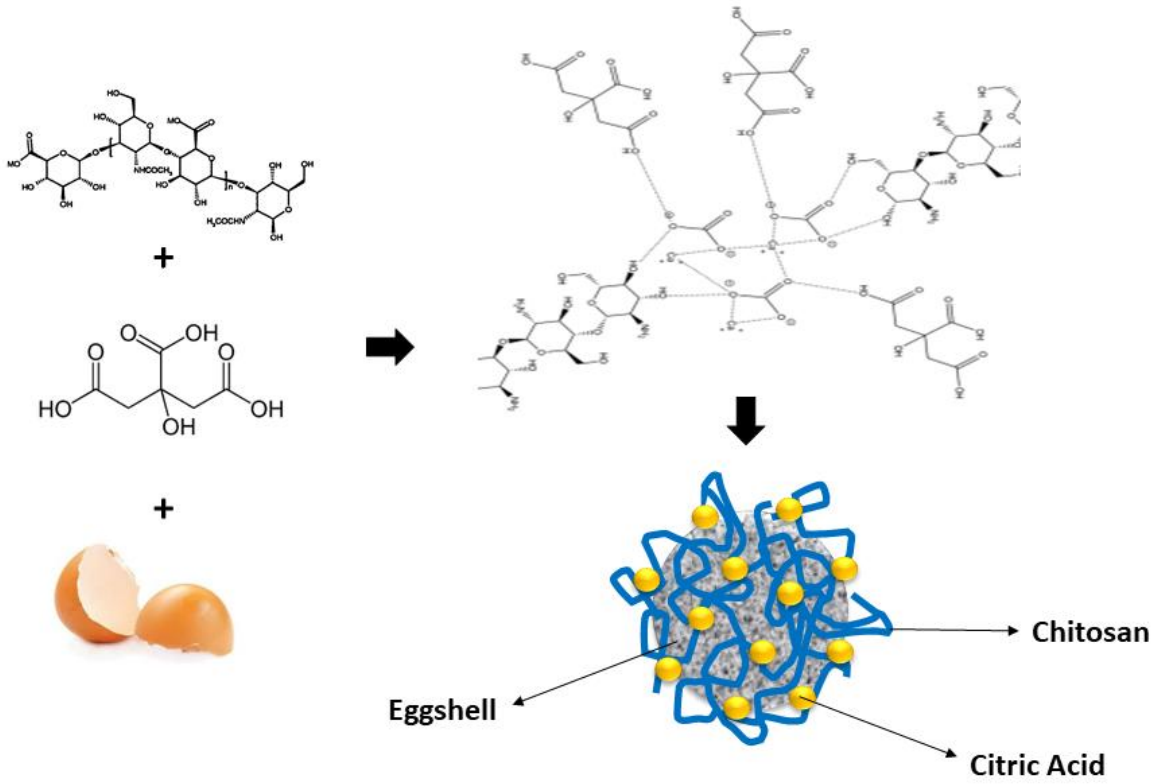
Recently, various nanomaterials - including ceramic, iron oxide, titanium oxide, and polymeric nanoparticles - have found widespread applications in clinical and biomedical fields [56-58]. Eggshell (ES) is a biocompatible material with diverse applications [59, 60], including drug delivery [61], biomedicine [62], bone replacement [59], catalysis [60, 63, 64], polymer industries, and nanocomposite production [65]. This material possesses a mesoporous nanostructure [60, 64, 66, 67] and its properties can be enhanced through combination with other materials. Eggshell waste consists of 90% calcium carbonate and exhibits superior properties in composites compared to mineral-derived carbonate [65]. Chitosan (Cs) is one of the most commonly used biopolymers for producing valuable compounds [68]. Chitosan is a polymer derived from chitin under alkaline conditions [66], and is the most abundant aminopolysaccharide [66, 68, 69]. The chemical and electrochemical binding of chitosan to metal ions leads to the stabilization of the compounds [70, 71]. Due to its high biocompatibility, low toxicity, biodegradability, and antibacterial properties, chitosan is used in tissue engineering, drug delivery, and gene delivery [70, 72, 73]. Chitosan can bind strongly to plasmids, adequately protecting nucleic acids from nuclease degradation in the blood [4, 74]. Citric acid (CA) acts as a crosslinking agent between eggshell and chitosan,

stabilizing the nanocomposite structure. It also provides pH-sensitive properties that may facilitate endosomal escape and controlled release of genetic material in target cells [75, 76]. The combination of these three components creates a promising hybrid nanocarrier for gene delivery applications that leverages the high surface area of eggshell, the DNA-binding capacity of chitosan, and the crosslinking/stabilizing function of citric acid.

Computational methods play a fundamental role in various fields [77, 78]. Molecular docking, a bioinformatics-based method, has become an important technique in drug discovery [79]. Several studies have successfully combined in vitro experiments with molecular docking to evaluate bioactive compounds, including Vanitha et al. [62] who successfully combined in vitro and in silico protocols to assess microbicidal compounds from Plumbago zeylanica L and Arulmozhi et al. [80] who used Schrödinger software for docking analysis of plant extracts. During docking, two key steps are crucial: 1) recognizing the ligand's structure and characterizing its location and orientation within binding sites, and 2) assessing binding affinity. [79, 81]. Molecular docking simulations focus on predicting non-covalent binding modes of small molecules (ligands) within the active site cavities of the target macromolecules (receptors), such as proteins or DNA [79, 82, 83]. Molecular docking typically employs two main strategies. The first uses computer simulations to assess ligand-receptor fitting and interactions by calculating energy levels at various positions. The second analyzes shape complementarity between ligands and the binding pocket [79, 84]. The aim of this procedure is to generate stable structures with enhanced specificity and potential efficacy, facilitating the development of more effective drugs [85].

In recent years, extensive research has been conducted to develop more efficient structures for drug and gene delivery. Render et al. synthesized eggshell-derived calcium carbonate ($CaCO_3$) nanoparticles for enteric drug delivery system. [86]. Iravani Kashkouli et al. synthesized Fe_3O_4/chitosan biopolymer as a novel nanocarrier for targeted gene delivery, demonstrating high efficiency and potential applications in cancer therapy. [4]. Jayasree et al. synthesized carbonated calcium-deficient hydroxyapatite (ECCDHA) nanoparticles using a simple method as a potential drug delivery carrier [87]. Iravani Kashkouli et al. synthesized a novel functionalized chitosan nanocarrier as an efficient gene delivery system [72]. Muthu et al. synthesized mesoporous carbonated hydroxyapatite from eggshell waste for biomedical applications such as drug/protein delivery, bone fillers, and tissue engineering [88]. Atabaki et al. synthesized Fe_3O_4/chitosan nanocomposite with potential applications in drug delivery systems [89]. Ranjbar et al. synthesized anti-cancer curcumin derivatives and investigate their properties against HepG2 cell line [90].

In the present study, ES/CA/Cs nanocomposites were synthesized using the mild co-precipitation method. This composite exhibits potential for biomedical applications due to the presence of calcium carbonate in eggshell and chitosan biopolymer [64, 72, 86]. Based on the literature, the combination of eggshell and chitosan may serve as a promising carrier system for drug and gene delivery. The structure, morphology, and size of the nanoparticles were characterized by FTIR, TEM, and DLS. The binding of the ES/CA/Cs nanocomposites with the F218V AtRCCR enzyme was modeled using molecular docking (MVD method in Schrödinger software). However, while in silico results are promising, further in vitro and in vivo studies are required to validate this nanocomposite as a suitable nanocarrier for renal cell carcinoma treatment. Scheme 1 presents a schematic representation of synthesized nanocomposite.



Scheme 1 The chemical structure of ES/CA/Cs nanocomposite

2. Experimental Section

2.1. Materials and Methods

The morphology and particle size of the synthesized nanocomposite was examined by transmission electron microscopy (TEM) using a CM120 device (Philips, France) operating at an acceleration voltage of 80–120 kV and magnifications of 31X to 680KX for one hour at 20 °C. A diluted suspension of the sample was drop-cast onto a carbon-coated copper grid and allowed to dry at room temperature before imaging [91]. Fourier transform infrared (FTIR) spectroscopy was performed to identify the functional groups and chemical structure of the synthesized nanocomposite. The spectrum was recorded using the KBr pellet technique in the transmission mode over a wavenumber range of 400–4000 cm^{-1} using a Bruker FTIR spectrometer (Bruker, Germany). The KBr pellet was prepared by mixing a small amount of the sample with spectroscopic-grade KBr and pressing it into a transparent disk [92]. The hydrodynamic size of the structure was measured by dynamic light scattering (DLS) using a Nano DS Dual scattering device (CILAS, France). The measurements were performed at 25°C with a scattering angle of 90°. Prior to measurement, the sample was diluted with deionized water and sonicated for 10 minutes to ensure uniform dispersion [93]. Molecular docking studies were performed using the MVD method by Schrödinger software on a Core i-5 processor system (Schrödinger, United States). All chemical materials used in this research were of analytical grade and purchased from Merck (Germany).

2.2. Synthesis of Eggshell/Citric Acid /Chitosan

To synthesize the ES/CA/Cs nanocomposites, 0.5 g eggshell powder was dissolved in 20 ml water, sonicated for 30 min and stirred. Then 1 g Citric Acid was added to adjust the pH to 2.5 and the mixture was stirred for 24 hours

at room temperature. The following day, 0.1 g of chitosan was dissolved in 25 ml water and added dropwise to the solution. The mixture was then stirred for an additional 24 hours. Finally, the resulting precipitate was washed with distilled water and stored for further characterization.

2.3. Molecular Docking

The 3D structure of the enzyme and the compounds were downloaded from the PDB and PubChem databases, respectively (Fig. 1 (a, b)). All structures were optimized using Chem3D software before docking. Energy minimization and structural optimization were then performed using Chem3D. molecular docking was performed using the MVD method on a Core i-5 computer to predict the interactions between the enzyme's active site and the nanocomposite structure. The nanocomposite components and enzyme molecules were optimized and selected as inputs in MVD software. The molecular cavities of the enzyme were analyzed to identify the most suitable binding cavity (up to five cavities were detected). Docking was performed using the Moldock score with the Moldock search algorithm. The number of interactions was set to 10, and the grid center was positioned at the active site coordinates (X: 3.34, Y: 2.87, Z: -1.46). After docking in MVD, Schrödinger software was used to visualize the interactions between amino acid residues and the active site of F218V AtRCCR.

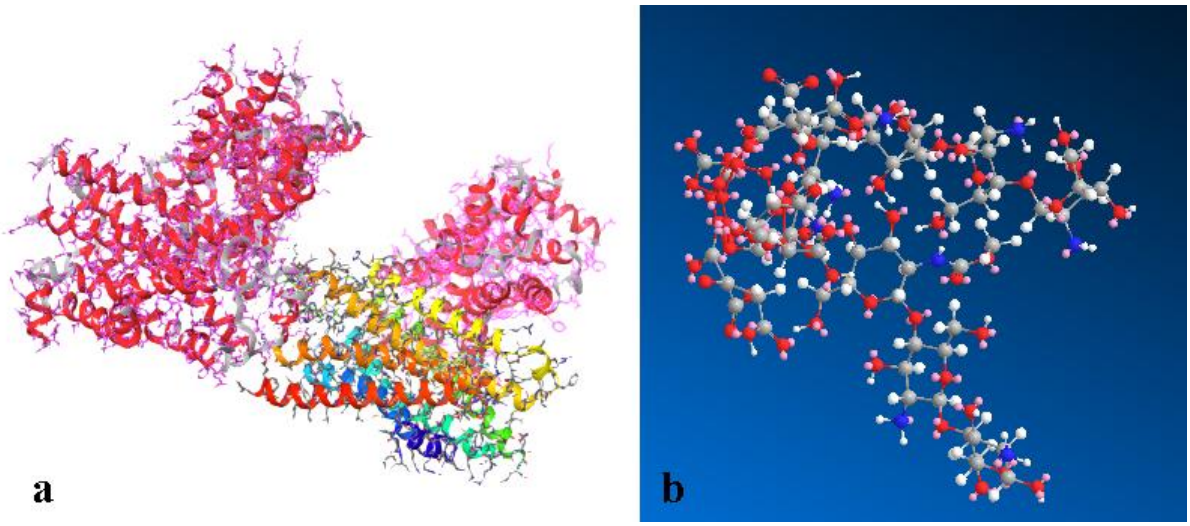


Fig. 1 (a) F218V AtRCCR Enzyme and the (b) ES/CA/Cs structure

3. Results and discussion

The ES/CA/Cs nanocarrier was successfully prepared via the co-precipitation method. Firstly, the CA was added to the eggshell solution at room temperature. After 24 hours of stirring, chitosan solution was added dropwise to the solution, and again after 2 hours of stirring, the final nanocomposites were made. The morphology, size, structure, and molecular docking of synthesized nanocomposites were studied.

3.1. Transmission Electron Microscopy

Fig. 2 shows TEM revealing the morphology and average particle size of the ES/CA/Cs nanocomposites. Fig. 2 (a-d) display plate-like form of particles at scales of 70, 100, 200, and 500 nm, respectively. The presence of CA and chitosan biopolymer resulted in smooth and flat plate-like structures, as shown in Fig. 2 (a, b). Additionally, chitosan and CA contribute to the well-dispersion and colloidal stability of the nanocomposites (Fig. 2 (d)). The

average particle size of synthesized nanocomposite, as determined by TEM, was approximately 300-400 nm and the strong stability of the solution with this size is extraordinary.

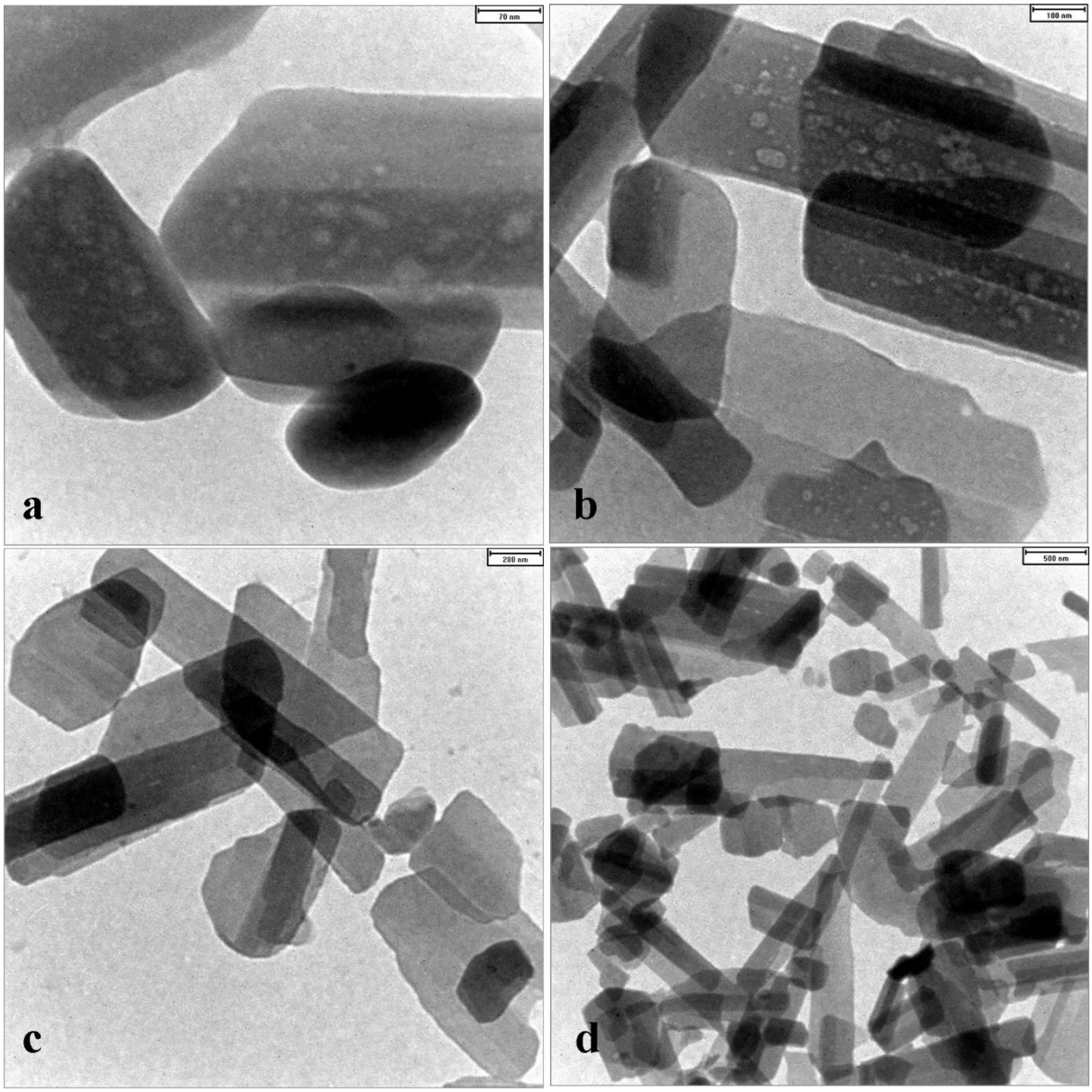


Fig. 2 TEM image of synthesized ES/CA/Cs nanocomposites

3.2. FTIR analysis

Fourier transform infrared spectroscopy (FTIR) spectrum, recorded using the KBr method in the range of 500 – 4000 cm^{-1}, is shown in Fig. 3. According to this figure, the spectrum reveals several broad bands between 3327 and 3456 cm^{-1} due to the overlapping stretching vibration of OH groups (from citric acid and chitosan) with N-H groups (from chitosan) [1, 2, 4, 94, 95]. The peak at 1557 cm^{-1} can be assigned to the bending vibration of N-H bands in chitosan [83, 84]. The stretching vibration of the C=O group in the CA structure appeared at above 1700 cm^{-1} [1, 94, 95]. The bands at 599 and 837 cm^{-1} correspond to Citric Acid [95, 96], while the bands at about 870, 1074, and 2938 cm^{-1} correspond to the $CaCO_3$ structure in the sample [60, 64, 97].

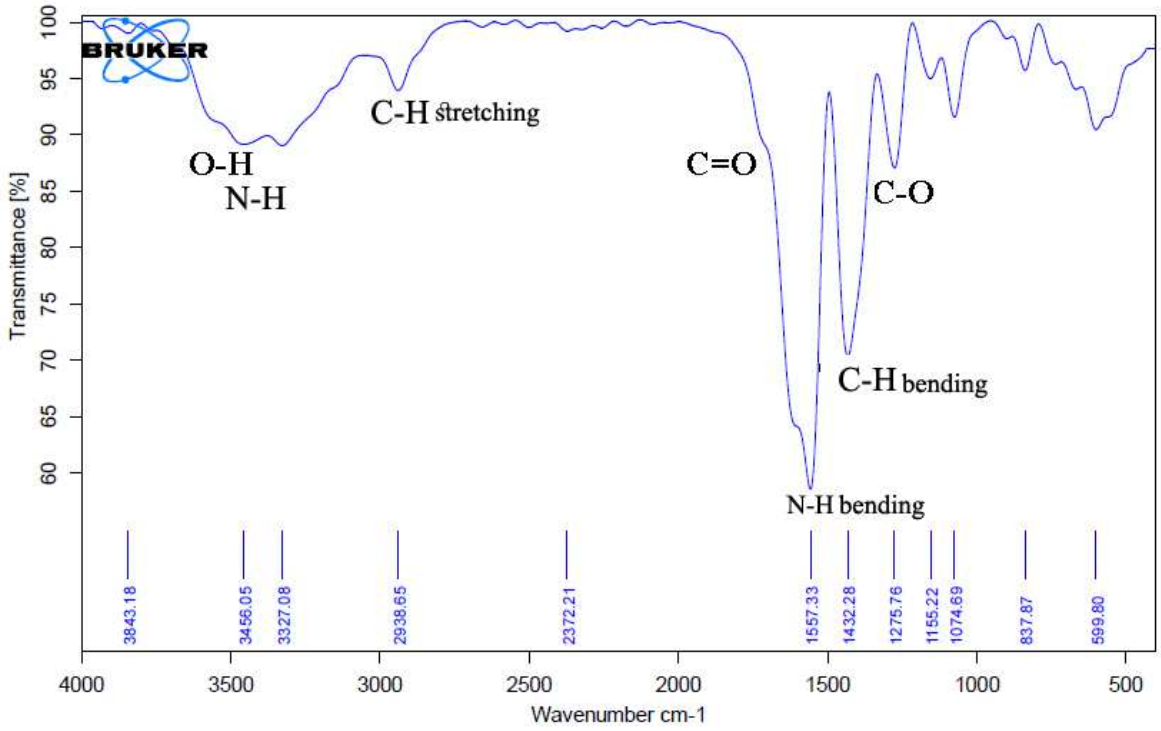


Fig. 3 FTIR analysis of synthesized ES/CA/Cs nanocomposites

3.3. DLS analysis

The ES/CA/Cs nanocomposite was synthesized via the co-precipitation method, washed, and prepared for DLS analysis. The washed particles exhibited polydispersity, with hydrodynamic diameters ranging from 449 to 4000 nm. The majority of particles fell within the range of 700-4000 nm (Fig. 4). The larger hydrodynamic diameters observed by DLS, compared to TEM, can be attributed to the formation of a hydrated layer around the nanoparticles. This is likely due to the mesoporous nature of eggshell-derived nanoparticles and strong hydrogen bonding with water molecules. Consequently, the hydrodynamic sizes measured by DLS are larger than the dry sized observed by TEM.

However, the broad size distribution (700-4000 nm) and the presence of large aggregates indicate limited colloidal stability. This polydispersity may negatively impact cellular uptake and systemic gene delivery, as nanoparticles larger than 200 nm are generally less efficient for cellular internalization. Future studies should focus on optimizing synthesis parameters (e.g., sonication time, surfactant addition, or centrifugation-based size fractionation) to achieve a more uniform size distribution suitable for in vivo applications.

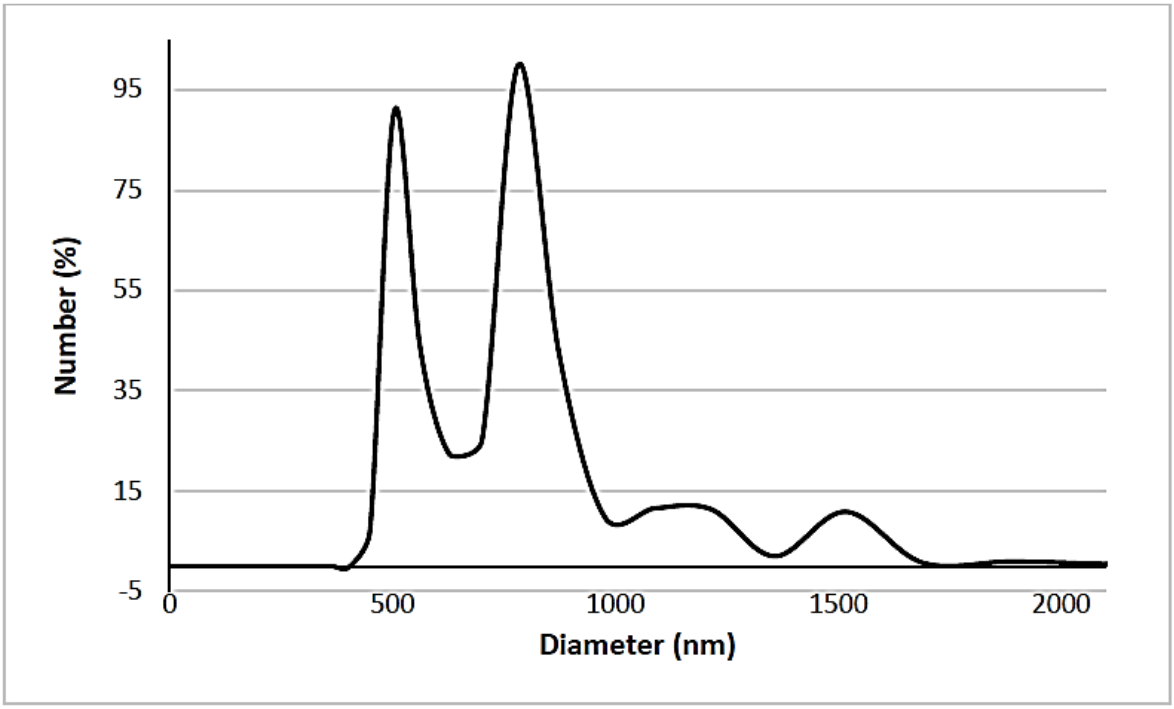


Fig. 4 DLS analysis of synthesized ES/CA/Cs nanocomposite

3.4. Molecular Docking Results

Molecular docking analysis of the nanocomposite components on the active site of F218V AtRCCR (Fig. 5) revealed that several amino acids residues, including Lys 147, Glu 262, Ile 266, Ser 145, and Val 259 interact with the active site of F218V AtRCCR (Fig. 6). The results suggest that the synthesized nanocomposite may inhibit the active site of F218V AtRCCR, based on in-silico predictions. The amount of binding affinity was -137.39 $KJmol^{-1}$. Binding affinity is a measure of the interaction strength between the compound and the enzyme's active site. A more negative binding affinity indicates stronger and more favorable interactions with the enzyme.

This enzyme was selected as a docking target because this mutation directly alters the structure of the active site [98]. It has a measurable effect on substrate binding and reaction outcome. Therefore, it serves as an appropriate model for molecular docking analysis and molecular design. Molecular docking alone does not confirm gene delivery functionality, it provides insights into binding energy and interaction types. Inhibition of this enzyme may improve gene therapy efficiency.

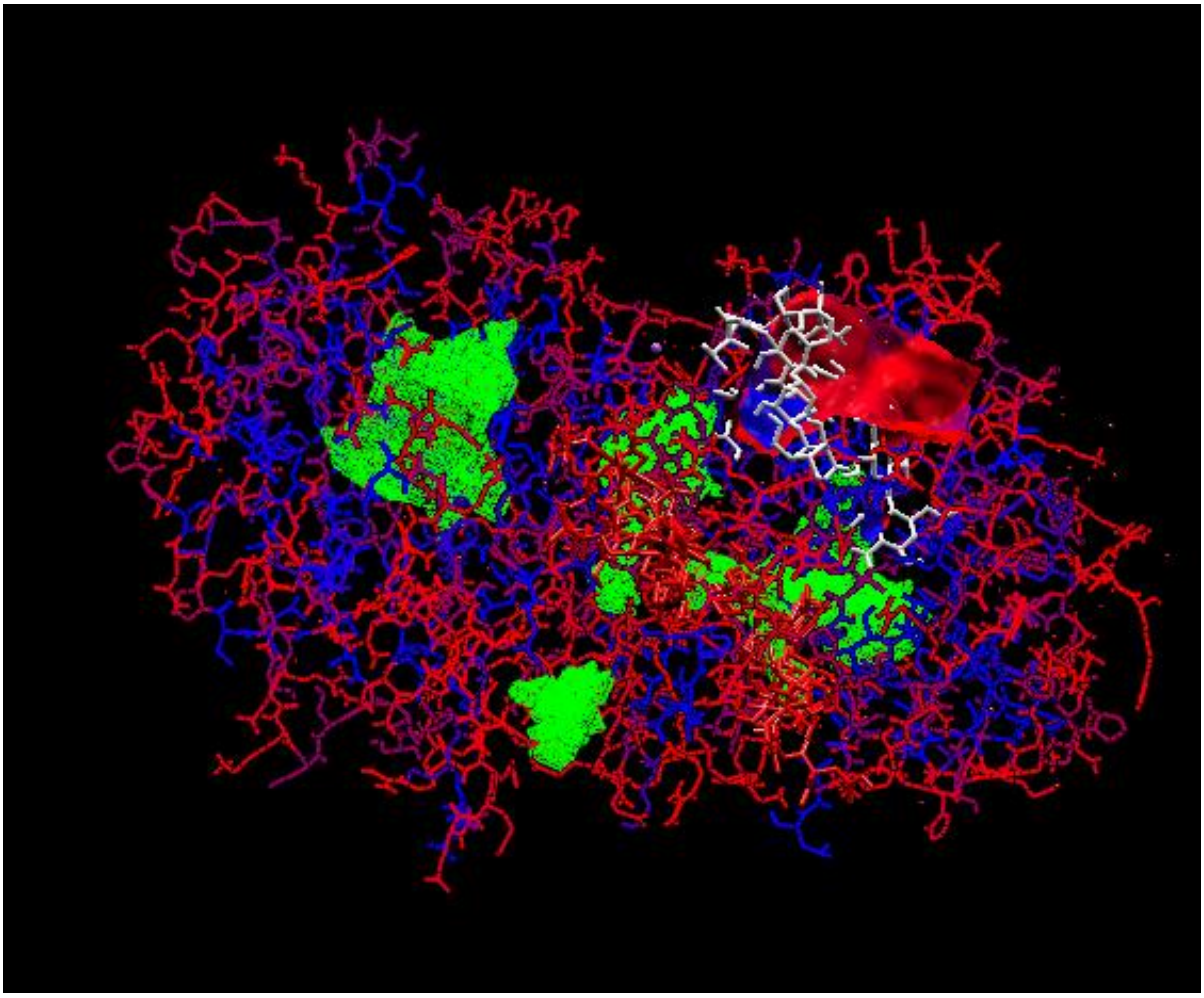


Fig. 5 The active site of Enzyme F218V AtRCCR

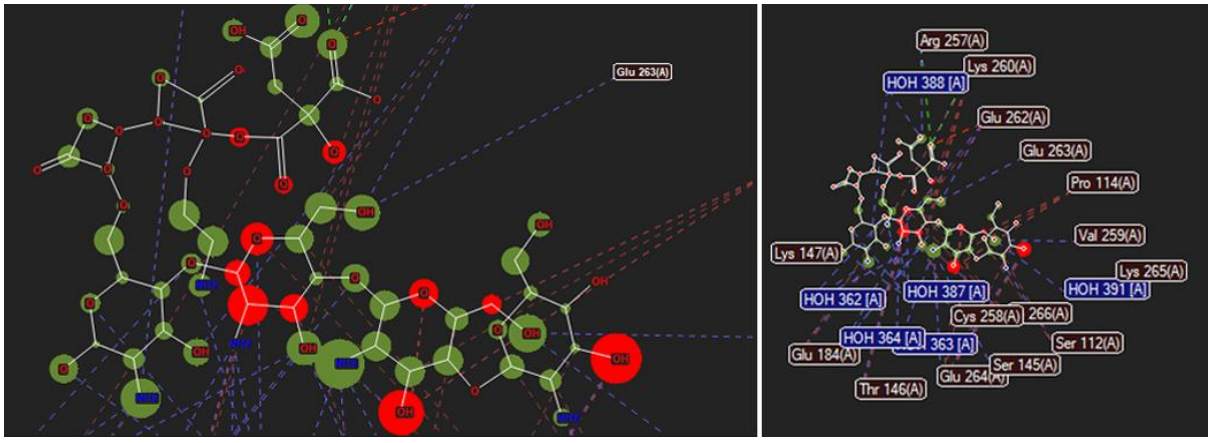


Fig. 6 The interaction of amino acids with the active site of enzyme F218V AtRCCR (Blue and red dotted lines indicate the hydrogen bonding and electrostatic interactions respectively)

Conclusion

This study aimed to synthesize a promising nanocomposite as a potential carrier for gene delivery applications. a key aspect of this study was to investigate the binding interaction of the synthesized nanocomposite with the F218V AtRCCR enzyme via molecular docking. The ES/CA/Cs nanocarrier was successfully synthesized using the co-precipitation method, and its chemical structure was confirmed by FTIR analysis. TEM analysis revealed

an average size of approximately 300-400 nm, while DLS showed a hydrodynamic size ranging from 700 to 4000 nm. Molecular docking was employed to evaluate the interaction between the nanocomposite and F218V AtRCCR. The in silico results predicted a favorable interaction between the nanocomposite and F218V AtRCCR, with a binding affinity of -137.39 $KJmol^{-1}$ at the active site. These findings suggest that the ES/CA/Cs nanocomposite may serve as a potential carrier for gene delivery and warrants further in vivo investigation. The broad size distribution observed by DLS (700-4000 nm) indicates polydispersity and potential aggregation, which may limit systemic gene delivery. Additionally, no comparison with a standard inhibitor was performed in the docking analysis, which is a limitation of this study. Moreover, only a single concentration of the components was evaluated in this study. Future studies should include optimization of synthesis conditions to reduce polydispersity, comparative docking with known inhibitors to further validate the binding affinity results, and design of experiments (DoE) approach to systemically optimize the concentration of drug and excipients and to evaluate their effects on nanocomposite properties..

Declarations

Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

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Consent to Publish Declaration: not applicable

Consent to Participate Declaration: not applicable

Ethics Declaration: not applicable

Reference

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