A DISSERTATION ON

Casein mediated Gold Nanoparticles and their antibacterial activity

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TO WHOM IT MAY CONCERN

This is to certify that **Mr. Atif Khan**, a student of M.Sc. Biotechnology (IV semester), Integral University has completed his four months dissertation work entitled "***Casein mediated Gold Nanoparticles and their antibacterial activity"*** successfully. He has completed this work from 2 Feb to 2 June 2022 at the Department of Biosciences, Integral University, under the guidance of **Dr. Salman Khan**.

The dissertation was a compulsory part of his M.Sc. degree. I wish him good luck and a bright future.

(**Dr. Snober S. Mir**)
Head,
Department of Biosciences,
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CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Mr. Atif Khan**, during the months 2 Feb to 2 June 2022 reported in the present thesis was under my guidance and supervision. The results reported by him are genuine and the script of the thesis has been written by the candidate himself. The thesis entitled ***"Casein mediated Gold Nanoparticles and their antibacterial activity"*** is, therefore, being forwarded for acceptance in partial fulfillment of the requirements for the degree award of the student of M.Sc. Biotechnology (IV semester), Department of Biosciences, Integral University, Lucknow, (U.P).

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Atif Khan **Date**

Introduction

Nanotechnology

Human perception and creative mind even more now and again would be the reason for greeting new science and innovation. Nanotechnology, a 21st-century boondocks, was the essential justification for such dreams. Nanotechnology is characterized as the comprehension and control of issues at aspects somewhere in the range of 1 and 100 nm where exceptional peculiarities empower novel applications. Albeit human openness to nanoparticles has not been new and it happened all through mankind's set of experiences. Nonetheless, it unexpectedly expanded while the modern and clinical transformations. The Nobel Prize Laureate Richard sigmondy originally proposed the idea of the "nanometer". He made the term nanometer transparently for describing molecule size, besides, he was quick to gauge the size of particles, for example, gold colloids utilizing a magnifying instrument [1]. Moreover, modern nanotechnology was the brainchild of Richard Feynman, who was a Nobel Prize Laureate in 1965 for material science. American Physical Society meeting at Caltech was held in 1959 where he introduced a talk named.

"There's Plenty of Room at the Bottom". On that occasion, he made sense of and presented the idea of controlling matter at the nuclear level. This original thought showed better approaches to thinking and Feynman's speculations have since been demonstrated right. Thus, consequently, he is viewed as the dad of current nanotechnology [2].

Roughly after long periods of Feynman's talk, a Japanese researcher, Norio.

Taniguchi was quick to utilize "nanotechnology" to depict semiconductor processes that happened arranged by a nanometer. He made sense that nanotechnology contains the handling, division, solidification, and twisting of materials by one particle or one atom. During the 1980s the most significant and brilliant time of nanotechnology started when Kroto, Smalley, and Curl found fullerenes and Eric Drexler at the Massachusetts Institute of Technology (MIT) [3]. The Coming Era of Nanotechnology." Drexler proposed the possibility of a nanoscale

"constructing agent" which would have the option to fabricate a duplicate of itself and different things of inconsistent intricacy. Later on Drexler's vision of nanotechnology is frequently called "atomic nanotechnology." The study of nanotechnology progressed further when Iijima, another Japanese researcher, created carbon nanotubes [4].

In a restricted period of around 50 years, nanotechnology has turned into the base for outstanding modern applications and dramatic development. For example, in the drug business, nanotechnology mindfully affects clinical gadgets, demonstrative biosensors, drug conveyance frameworks, and imaging tests. In the food and beauty care products enterprises, the utilization of nanomaterials has improved decisively for advancements underway, bundling, the period of usability, and bioavailability. Zinc oxide quantum dab nanoparticles show antimicrobial movement against food-borne microscopic organisms, also, nanoparticles are these days utilized as food sensors for recognizing food quality and wellbeing [5].

The well-being of customer items containing nanomaterials was an early broad concern. It was normal that the gamble appraisal methods utilized for drugs and harmful synthetic substances would be utilized for the gamble assessment of nanomaterials. Be that as it may, reports of enormous information holes demonstrated the need to increase regular poisonousness testing techniques.

Nanoparticles

Classification of Nanoparticles

There are various approaches to the classification of nanomaterials. Nanoparticles are classified based on one, two, and three dimensions. Because of their tiny size, nanoparticles have an extremely huge surface region to volume proportion when contrasted with mass material, like powders, plates, and sheets. This component permits nanoparticles to have unforeseen optical, physical, and compound properties, as they are adequately little to confine their electrons and produce quantum results. For example, copper is viewed as a delicate material, with mass copper bowing when its particles group at the 50nm scale. In this way, copper nanoparticles more modest than 50nm are viewed as an exceptionally hard material, with very unique flexibility and pliability execution when contrasted with mass copper [8]. Moreover, changes in size can likewise influence the liquefying qualities; gold nanoparticles dissolve at much lower temperatures (300 °C for 2.5 nm size) than mass gold (1064 °C). Also, retention of sun-powered radiation is a lot higher in materials made out of nanoparticles than in slight movies of ceaseless sheets of material [9].

One-dimension nanoparticles: One-layered frameworks, like flimsy film or fabricated surfaces, have been utilized for quite a long time in gadgets, science, and design. The creation of slim movies (sizes 1-100 nm) or monolayer is currently a normal spot in the field of sun-powered cells or catalysis. These slight movies are utilized in various mechanical applications, including data stockpiling frameworks, compound, and natural sensors, fiber-optic frameworks, the magneto-optic and optical gadgets [10].

Two-dimension nanoparticles: Carbon nanotubes are a hexagonal organization of carbon particles, 1 nm in breadth and 100 nm long, as a layer of graphite moved up into a chamber. CNTs are of two kinds, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). The little components of carbon nanotubes joined with their striking physical, mechanical and electrical properties,

make their Three-dimension nanoparticles: Fullerenes are round confines containing from 28 to more than 100 carbon particles, and contain C 60. This is an empty ball made out of interconnected carbon pentagons and hexagons, looking like a soccer ball. Fullerenes are a class of materials showing one-of-a-kind actual properties. They can be exposed to outrageous tension and recapture their unique shape when the strain is delivered. These atoms don't join, in this way giving them the significant potential for application as greases. They have fascinating electrical properties and it has been proposed to involve them in the electronic field, going from information capacity to the creation of sunlight-based cells. Fullerenes are offering likely applications in the rich area of nanoelectronics. Since fullerenes are unfilled designs with aspects like a few naturally dynamic particles, they can be loaded up with various substances and find potential clinical applications [12] with remarkable materials. They show metallic or semi-conductive properties, contingent upon how the carbon leaf is twisted on itself. The ongoing thickness that nanotubes can convey is incredibly high and can arrive at one billion amperes for every square meter making them a superconductor. The mechanical strength of carbon nanotubes is multiple times more prominent than the best steel. Carbon nanotubes have an extraordinary limit concerning sub-atomic ingestion and deal with a three-layered setup. In addition, they are synthetically and artificially truly steady [11].

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Characterization of Nanoparticles

Nanoparticles are by and large portrayed by their size, morphology, and surface charge, involving such high-level minuscule procedures as examining electron microscopy (SEM), transmission electron microscopy (TEM), and nuclear power microscopy (AFM). The typical molecule width, size conveyance, and charge influence the actual strength and the in vivo dissemination of the nanoparticles. Electron microscopy strategies are exceptionally valuable in learning the general state of polymeric nanoparticles, which might decide their harmfulness. The surface charge of the nanoparticles influences the actual soundness and dispersibility of the polymer scattering as well as their in vivo execution [16].

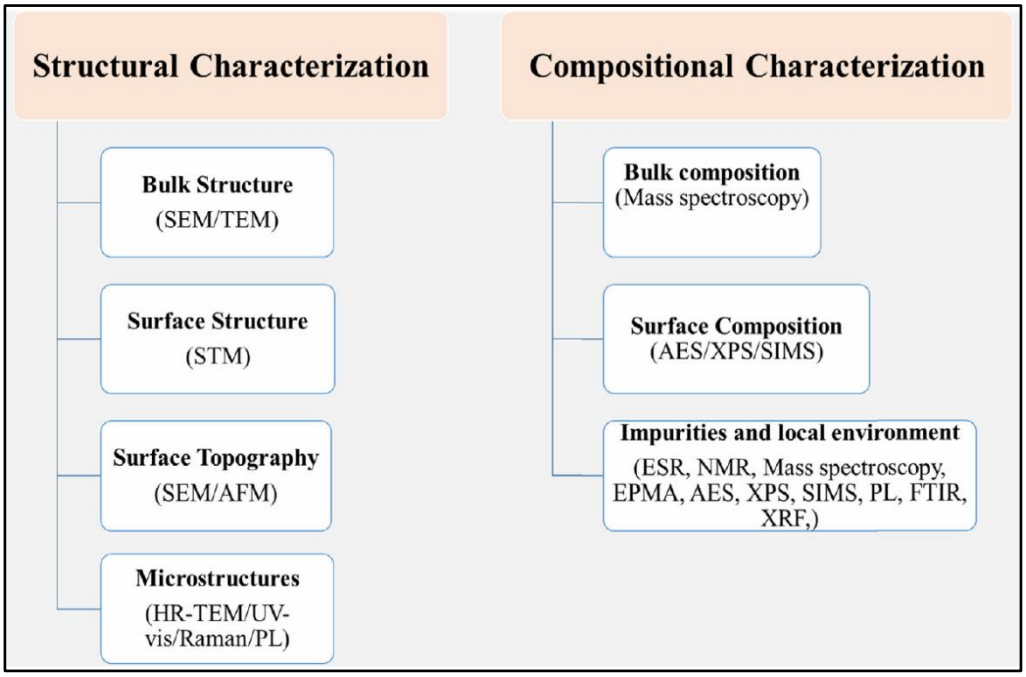

Fig. 1. General classification of characterization techniques for nanomaterials/nanostructures.

Particle size: Particle size distribution and morphology are the most important parameters for the characterization of nanoparticles.

Morphology and size are measured by electron microscopy. The major application of nanoparticles is in drug release and drug targeting. It has been found that particle size affects drug release. Smaller particles offer a larger surface area [17].

Dynamic light scattering (DLS): Currently, the fastest and most popular method of determining particle size is photon-correlation spectroscopy (PCS) or dynamic light scattering (DLS). DLS is widely used to determine the size of Brownian nanoparticles in colloidal suspensions in the nano and submicron ranges. Shining monochromatic light (laser) onto a solution of spherical particles in Brownian motion causes a Doppler shift when the light hits the moving particle, changing the wavelength of the incoming light [18].

Scanning Electron microscopy: Scanning electron microscopy (SEM) is giving morphological examination with direct visualization. The techniques based on electron microscopy offer several advantages in morphological and sizing analysis; however, they provide limited information about the size distribution and true population average [19]. Transmission electron microscope: TEM operates on a different principle than SEM, yet it often brings the same type of data. The sample preparation for TEM is complex and time-consuming because of its requirement to be ultra-thin for the electron transmittance [20].

Atomic force microscopy: atomic force microscopy (AFM) offers ultra-high resolution in particle size measurement and is based on a physical scanning of samples at the sub-micron level using a probe tip of atomic scale. The instrument provides a topographical map the of sample based on forces between the tip and the sample surface.

Surface Charge: The nature and intensity of the surface charge of nanoparticles are very important as it determines their interaction with the biological environment as well as their electrostatic interaction with bioactive compounds. The colloidal stability is analyzed through the zeta potential of nanoparticles. This potential is an indirect measure of the surface charge. It corresponds to the potential difference between the outer Helmholtz plane and the surface of shear. The measurement of

the zeta potential allows for predictions about the storage stability of colloidal dispersion [21]. High zeta potential values, either positive or negative, should be achieved to ensure stability and avoid aggregation of the particles. The extent of surface hydrophobicity can then be predicted from the values of zeta potential. The zeta potential can also provide information regarding the nature of material encapsulated within the nanocapsules or coated onto the surface.

Review of literature

Nanotechnology

The term nanotechnology was first defined by Tokyo Science University Professor Norio Taniguchi in 1974 as ‘’Nanotechnology mainly consists of the processing of separation, consolidation, and deformation of materials by one atom or one molecule” [22]. The development of experimental techniques for the synthesis of NPs with regulated sizes, shapes, and properties is known as nanotechnology.

Materials differences at the nanoscale’s scales and their implications and potential benefits to human health. Nanotechnology is the science that deals with matter at the scale of 1 billionth of a meter (i.e., 10^{-9} m = 1 *nm*), and is also the study of manipulating matter at the atomic and molecular scale. It is an emerging field of science that includes the synthesis and development of various nanomaterials. Nanoparticles can be defined as objects ranging in size from 1-100 nm that due to their size may differ from the bulk material. Presently, different metallic nanomaterials are being produced using copper, zinc, titanium, magnesium, gold, alginate, and silver. Nanoparticles are being used for diverse purposes, from medical treatments, used in various branches of industrial production such as solar and oxide fuel batteries for energy storage, to wide incorporation into diverse materials of everyday use such as cosmetics or clothes. Nanotechnology (sometimes shortened to "nanotech") is the science that deals with the synthesis, characterization, exploration, and application of Nano-sized materials for the development of science. Nanotechnology, alongside nanostructured materials, plays an ever-increasing role in science, research, and economics everyday life, as more and more products based on nanostructured materials are introduced to the market [55,56]. It is a field that has arisen as a crossroads of biotechnology and nanotechnology for developing novel biosynthetic devices and environmentally friendly nanomaterial synthesis technology [23].

The study of phenomena and manipulation is known as nanoscience. The material at the atomic and molecular level characteristics differs dramatically from those at a bigger scales scale. It is a multidisciplinary

science that spans several fields. Nanotechnology, like semiconductor technology, information technology, and cellular and molecular, is expected to have a significant impact on our economy and society in the early twenty-first century. Nanotechnology is the creation and utilization of physical, chemical, and biological nanomaterials. tiny atom or molecules to large-scale chemical and biological systems nanostructures into bigger structures, as well as their integration into submicron dimensions' systems. Nanotechnology science and technology research promises advancements in areas such as materials and manufacturing, Nanoelectronics medicine and healthcare, energy, and biotechnology are just a few examples.

National security and information technology nanotechnology is commonly believed to be the future.

The following Industrial Revolution A nanoparticle is defined as a collection of atoms linked together with a radius ranging from 1 to 100nm it usually has 10-105 atoms. The area of nanotechnology. Nanotechnology offers opportunities for the development of materials, particularly those for medical applications, where traditional methods, may have reached their limits. Drug delivery devices having a unique polymeric membrane are known as Nanocapsules. That is important in almost every field of science and technology. Nanotechnology has numerous scientific uses, and information technology as a result, we give a comprehensive review in this article a broad summary of NPs, their characteristics, and medicinal applications in several disciplines of science and technology, with a focus on their therapeutic possibilities [60, 61]. Nanotechnology has a wide range of scientific and technological applications. As a result, in this study, we provide a comprehensive overview of NPs in general, their properties, and therapeutic applications in various domains of science and technology, with a focus on therapeutic applications. It has already been applied in various fields, such as computer electronics, communication, energy production, medicines, and the food industry.

Nanotechnology can be applied by two different approaches, either “bottom-up” or top-down’. The top-down approach is achieved by employing physical processing of the food materials, such as grinding and milling.’’ Bottom-up’’ is the term used to describe starting with a single atom and molecules to build nanostructures. The concept describes technologies enabling the construction of methods and tools to budding small components starting from larger regular matter.

Nanotechnology and Nanoscience got an improvement in the early 1980s with two main developments including both the birth of cluster science and the invention of the Scanning Tunneling Microscope (STM) in 1981. These developments caused the discovery of Fullerenes in 1985 and when Carbon Nano-tubes were advanced by Japanese scientists in 1991. In the medial of 1980s and beginning 1990s, several significant discoveries were made, which had an essential impact on the additional development of nanotechnology. For example, in 1991, the first Nano technological program of the National Scientific Fund was started to operate in the USA, and then in 2001, the National Nano technological Initiative (NNI) of the USA was approved. Since then, lots of technical research developments and scientific departments have taken place all over the world, particularly in some countries such as England, Japan, China, Germany, France, South Korea, and recently in the CIS countries [24].

History and Development of Nanotechnology

The term nanotechnology has been explained by a wide spectrum of numerous technologies that nanotechnology covers, which are based on many kinds of chemical, physical, and biological processes realized at the Nano-level [10]. The accurately established period for the beginning of nanotechnology development is demonstrated by the fact that nanotechnology has its background in the remote past when was used by people without knowledge of it.

The name “nanotechnology” was introduced by Norio Taniguchi for the first time in Tokyo in 1974 at the International Conference on Industrial Production to explain the super-thin processing of materials with

nanometer accuracy and the establishment of Nano-sized mechanisms. Concepts of the Nanotechnological approach were put forward by Richard Feynman (identified as the Father of Nanotechnology) in 1959 in his lecture delivered at the session of the American Physical Society and were developed in 1986 by Eric Drexler.

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Consequently, the nanotechnology pattern was formed at the turn of the 1960s, whereas the 1980s and 1990s are the starts of the growth of nanotechnology in its own right. According to the light of the toxicity testing in the 21st century suggested by the US National Research Council (NRC), high-throughput screening of nanomaterials looks promising and may be potential in the not too distant future. Despite the complex nature of the nanomaterials which makes the development of their safety assessment challenging, the future of nanotechnology sounds to be bright [13-15].

Even though nanotechnology seems like a budding aspect of science, its utilization by humanity isn’t novel at all. The history of nanomaterials usage in construction dates back to 4500 years ago when natural asbestos nanofibers were utilized for ceramic matrices. One of the

oldest, richest, and most progressive cultures globally, Egyptians, realized the capabilities of nanomaterials 4000 years ago. The journey of nanomaterials and nanotechnology made throughout history before millennial technological advances used was given briefly in tabular form.

Nanoparticles

The prefix Nano is derived from the Greek word Nanos meaning “dwarf” or extremely small18. Nano-sized materials, known as NPs, possess unique and improved properties because of their larger surface area to volume ratio. NPs can be broadly grouped into two, namely, organic NPs and inorganic NPs which include noble metal NPs (like silver and gold), and semi-conductor NPs (like titanium oxide and zinc oxide).

In general, nanoparticles used in the field of biotechnology range in particle size between 10 and 500 nm, seldom exceeding 700 nm. The nanosize of these particles allows various communications with biomolecules on the cell surfaces and within the cells in a way that can be decoded and designated to various biochemical and physicochemical properties of these cells. Similarly, its potential application in drug delivery systems and noninvasive imaging offered various advantages over conventional pharmaceutical agents. To utilize nanoparticles at their full throttle, the Nanoparticulate systems must be stable, biocompatible, and selectively directed to specific sites in the body after systemic administration. More specific targeting systems are designed to recognize the targeted cells such as cancer cells.

This can be achieved by conjugation of the nanoparticle with an appropriate ligand, which has a specific binding activity concerning the target cells. In addition, nanoparticles provide a platform to attach multiple copies of therapeutic substances to it and hence increase the concentration of therapeutic and diagnostic substances at the pathological site. Also, the concentration and dynamics of the active molecule can be varied by controlling the particle size of nanoparticles (>3–5 *nm*). This control in particle size in conjugation with surface coating with stealth ligand allows them to veil against the body’s immune system, enabling them to circulate in the blood for a longer period.

These advances in the field of biotechnology have opened an endless opportunity for molecular diagnostics and therapy. Once targeted (active or passive), these nanocarriers can be designed in a way to facilitate them to act as imaging probes using a variety of techniques such as ultrasound (US), X-ray, computed tom(CT), positron emission tomography (PET), magnetic resonance imaging (MRI),), optical imaging, and surface-enhanced Raman imaging (SERS) [68].

Hence, these so-called “molecular imaging probes” can noninvasively provide valuable information about differentiate abnormalities in various body structures and organs to determine the extent of disease, and evaluate the effectiveness of treatment. Thus short molecular imaging enables the visualization of the cellular function and the follow-up of the molecular process in living organisms without perturbing them.

Over the year nanoparticles such as magnetic nanoparticles (iron oxide), gold and silver nanoparticles, nanoshells, and nanocages have been continuously used and modified to enable their use as diagnostic and therapeutic agents. Thus, in this particular review article, we have introduced iron oxide, gold, and silver nanoparticles along with newer nanoshells and nanocages. These are then briefly discussed for their method of development and some cite recent examples which utilize their intrinsic properties as diagnostic and/or therapeutic agents for diseases, mainly cancer [25].

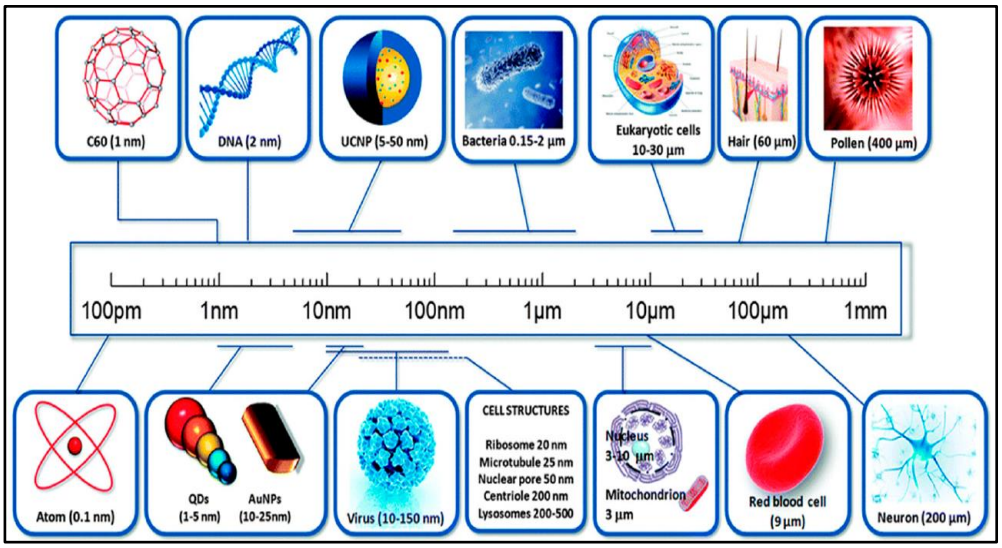

Fig. 2. A comparison of sizes of nanomaterial

Gold Nanoparticles Synthesis:

With regards to the union of gold nanoparticles, likely the most helpful and generally utilized blend strategy is the alleged citrate course grew over a long time back. The response is exceptionally straightforward and includes simply gold chloride, sodium citrate as a decreasing and settling specialist, and water as a dissolvable. They got gold nanoparticles to show a circular morphology with a somewhat thin size conveyance (20 ± 1.5 nm). Albeit the citrate course is still extremely well known for the readiness of watery gold nanoparticle sols, numerous different methodologies have been created, acting in unambiguous natural solvents or the presence of various sorts of surfactants and decreasing specialists to adjust the every one of these nanoparticles have different optical properties. On account of gold nanorods with fluctuating viewpoint proportions, this impact is noticeable to the unaided eyes. The optical ingestion spectra show a shift of the band at longer frequencies, relating to the retention and dissipating of light along with the long pivot of the nanorods, from the noticeable to approach infrared with expanding lengths, and the shade of the comparing scatterings change from pink to blue, green, and brownish.

Properties and application of gold Nanoparticles

Spherical AuNPs have helpful characteristics, for example, size-and shape-related optoelectronic properties, enormous surface-to-volume proportion, great biocompatibility, and low poisonousness. These properties make AuNPs a significant device in bionanotechnology. Significant actual properties of AuNPs incorporate surface plasmon reverberation (SPR) and the capacity to extinguish fluorescence. Round AuNPs display a scope of varieties (e.g., brown, orange, red, and purple) in the watery arrangement as the center size increments from 1 to 100 nm, and by and large show a size-relative retention top from 500 to 550 nm. This ingestion band emerges from the aggregate wavering of the conduction electrons because of the thunderous excitation by the occurrence of photons which is known as a "surface plasmon the band" [27].

Sensing: AuNPs are readily conjugated with recognition moieties such as antibodies or oligonucleotides for the detection of target biomolecules, allowing *in vitro* detection and diagnostics applications for diseases such as cancer. As an example, AuNPs play a critical role in the “bio- barcode assay”, an ultrasensitive method for detecting target proteins and nucleic acids. The principle of the “bio-barcode assay” utilizes AuNPs conjugated with both barcode oligonucleotides and target-specific antibodies, and magnetic microparticles (MMPs) functionalized with monoclonal antibodies for the target moiety. These complexes produce a sandwich complex upon detection of the target molecule that releases a large number of barcode oligonucleotides, providing both identification and quantification of the target.

Therapeutics: The transport of therapeutic agents to the cells by AuNPs is a critical process in biomedical treatment. Several research groups have used functionalized AuNPs to investigate the interactions with the cell membrane to improve delivery efficiency.

Imaging: The versatile optical and electronic properties of AuNPs have been employed for cell imaging using various techniques, including computed tomography (CT), dark-field light scattering, optical coherence tomography (OCT), photothermal heterodyne imaging technique, and Raman spectroscopy.

Casein

Milk, a fluid produced by female mammals for the nutrition of their young, is intended to be consumed as a liquid, but man has exploited its rather unique gelling properties for at least 8000 years in the production of a wide variety of fermented foods that fall into two general categories: cheeses and fluid fermented products. Taken together, these products probably represent the longest-established and most widely produced food protein-based gels: c. 10^7 t of cheese are produced worldwide annually from c. 10^8 to (10^{11} liters) of gelled milk. Milk proteins are used in many other food gel systems but, although these are quite significant in their own right, they are, quantitatively, trivial in comparison with cheese and fermented milk. However, gelation of milk proteins is not

always desirable, e.g. age gelation of sterilized kinds of milk and coagulation/gelation of milk, especially concentrated milk, during sterilization [1]. Caseins may be present in other food gel systems, e.g. meat products, but they are not the primary gelling agent; hence, the definition of ' casein gels' is to some extent subjective.

Structure

The majority of milk-based gels depend on either of two characteristics of the casein system: insolubility in the region of their isoelectric points (c. pH 4·6), which is exploited in the production of fermented milk and some fresh cheeses, and instability in the presence of Ca^{2+} following specific, very limited, proteolysis by selected proteinases (rennets), which is exploited in the manufacture of most cheeses. Compared to most other proteins, the caseins are very heat stable at pH> 6·5 and, therefore, do not normally form thermally-induced gels, although they may do so under certain conditions, which may cause problems in the manufacture of sterilized, concentrated products. It appears appropriate to commence this chapter with a brief review of the principal molecular and physicochemical properties of the caseins.

Heterogeneity of the Milk Protein System

Bovine milk contains c. 3-5% protein, which, like the milk of apparently all other mammals, falls into two principal groups: the caseins, i.e. phosphoproteins insoluble at pH 4·6 (the isoelectric pH) at 20°C, and the whey (or non-casein) proteins, which are soluble under these conditions. The ease with which isoelectric casein, which represents c. 80% of the total nitrogen of bovine milk, can be prepared, made it the subject of study by the pioneer protein chemists, e.g. Hammerstein (1880-1890). Isoelectric (acid) casein was considered to be homogeneous until the pioneering work of Linderstrom-Lang (1925-1930), Pedersen (1936) and Mellander (1939) showed that it contained at least three proteins, designated a, f3 and y in order of decreasing electrophoretic mobility. These proteins were isolated to apparent homogeneity by Hipp et al. (1952) and a-casein was fractionated by

Waugh and von Hippel (1956) into Ca-sensitive (a.) and Cainsensitive (K) fractions.

Properties of casein

Casein is the major protein (80%) in milk and is obtained as a coproduct during the production of skim milk. Attempts have been made to utilize casein for various applications. Casein has been made into films and the effects of various crosslinking agents on the properties of the casein products have been studied. Casein precipitated using CO_2 was made into films and the tensile properties, water vapor permeability, and solubility in water were found to be better than similar films made from commercially available calcium caseinate. Two-dimensional films and 3D matrices were developed from casein and crosslinked with glyceraldehyde, glutaraldehyde, and silane. Crosslinking was reported to improve mechanical properties and water stability. Casein-based materials have also been crosslinked with enzymes, especially transglutaminase. Transglutaminase was found to substantially increase the molecular weights and mechanical properties of casein films [18,19]. Ultrasound processes were found to improve the mechanical strength of edible films developed from casein. A wet spinning approach was also used to prepare films from casein. It was reported that the pH of the solution was critical for film properties and the films with the highest tensile strength and breaking elongation and low solubility in water were obtained using pH 9 solutions.

Objectives

Objectives

- Biosynthesis of AuNPs by using casein as a reducing and capping agent.
- Characterization of synthesized AuNPs by UV- vis spectroscopy. FTIR, TEM, DLS, and zeta potential.
- Antibacterial activity of synthesized C-GNPs Against both gram-positive and gram-negative pathogens.

Materials and method

Materials

Tetra chloroauric acid ($HAuCl_4$) was purchased from Sigma Aldrich. Phosphate buffer salts (Na_2HPO_4) and (NaH_2PO_4) were purchased from HIMEDIA. Double distilled waterhas been used as an aqueous medium for all experiments. All buffers were filtered with 0.2µm filter paper immediately after they were prepared.

Synthesis of casein capped gold nanoparticles

In vitro synthesis of GNPs of different sizes at varying concentrations were performed as follows: A total of four different reaction mixtures, containing 3µl of 1.0mM $H[AuCl_4]$ (prepared in 50mM Phosphate buffer) in 3ml of different concentrations of freshly prepared casein (0.33mg/ml, 0.66mg/ml, 1.66mg/ml, and 3.33mg/ml) were incubated at 40^oC temperature for 48 hours. In vitro synthesis of GNPs of different sizes at varying temperature were performed as follows: A total of four reaction mixtures, containing 3µl of 1.0mM $H[AuCl_4]$ (prepared in 50mM Phosphate buffer) and 3ml of freshly prepared casein solution of 0.33mg/ml were incubated at different temperature (40°C, 50°C, 60°C and 70°C) individually. A reaction performed in the absence of casein was used as a control. Samples were removed at regular intervals and analyzed by UV-Vis spectroscopy to ensure the formation of nanoparticles. On completion of the reaction, GNPs were collected by centrifugation (30,000g, 30min), washed twice with Milli Q water and the excess Bromelain was removed by treating with 50% v/v of ethanol and used for further characterization.

Characterization of synthesized gold nanoparticles and bioconjugated gold nanoparticles

UV-Vis spectrophotometry measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC).

Transmission Electron Microscopy (TEM) analysis was performed

on TecnaiTM G 2 Spirit BioTWIN, FEI Company, operated at an accelerating voltage of 80kV, Scanning Electron Microscopy (SEM) analysis was performed on JEOL JSM 5200. The mean size of GNPs was measured in a dynamic light scattering (DLS) particle size analyzer (Zetasizer Nano-ZS, Model ZEN3600, Malvern Instrument Ltd, Malvern, UK). Zeta potential was measured using a Zetasizer Nano-ZS, Model ZEN3600 (Malvern Instrument Ltd, Malvern, UK). *Fourier transform infrared spectroscopy of 'BRN capped Au-NPs' and* Au-BRN-LVN- NPs.

The Fourier transform infrared (FTIR) spectra of 'BRN capped Au-NPs' and Au-BRN-LVN-NPs were recorded using Perkin- Elmer Spectrum Two FTIR (Perkin Elmer Inc., Tres Cantos, Madrid) equipped with a Universal attenuated total reflectance sampling device and scanned at room temperature in transmission mode over the wave number range of 4000-650 cm^{-1} at a resolution of 4 cm^{-1}

Evaluation of antibacterial efficacy of casein

Determination of Antibacterial Activity by Disc Diffusion Method

The disc diffusion method was used to determine the antibacterial properties of casein GNPs. For antibacterial assay analysis, pure cultures of Staphylococcus aureus, Escherichia coli and were obtained from American Type Culture Collection. However, *Bacillus subtilis* and *Proteus Vulgaris* were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC). All the microorganisms were incubated at 37^{o}C for 24 h in nutrient broth. The culture suspensions were prepared. Muller Hilton agar (20 mL) was poured into each sterilized petri dish (10 mm×100 mm diameter) and allowed to solidify. After solidification, the bacterial culture was swabbed in nutrient agar plates. During the experiment, 50 µL of various concentrations of casein GNPs (10, 20, 40, 60, and 80 mg/ml) and various concentrations of casein protein (10, 25, 50, 75, and 100 mg/ml), negative control (PBS) and positive control levofloxacin (25 mg/ml) were added to the wells of MH agar plates. The agar plates

were incubated overnight at 37 ◦C. Following that, the diameter of the inhibitory zone was determined.

Minimal Inhibitory Concentration (MIC) Determination

The antibacterial efficacy of casein was determined by evaluating the minimum inhibitory concentration (MIC) of casein and pure LVN against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. Casein GNPs were serially diluted in 50 µL of LB medium in 96-well microtitre plates to achieve the desired concentrations with bacterial inoculums (5×104 CFU/well) and were incubated at 37 °C overnight. The MIC was taken as the lowest GNPs concentration at which growth was inhibited. The lowest concentration at which there is no visible bacterial growth indicates 99.5 % killing of the original inoculums. The absorbance of each well was measured at a wavelength of 600 nm by a microtitre plate reader (Bio-Rad laboratories Inc., India) and compared with a control. PBS was used as a negative control for each experiment. The procedure was repeated for the pure levofloxacin alone too.

Results

Results

Fig. 3. Showing the ruby red color of synthesized casein GNPs.

Characterization of Gold Nanoparticles:

Characterization of C-GNPs

The plasmon band was observed for the wine red colloidal gold nanoparticles at 532nm in the UV-visible spectrum. The colloidal solution containing casein capped GNPs had shown a very intense and characteristic pink-red color.

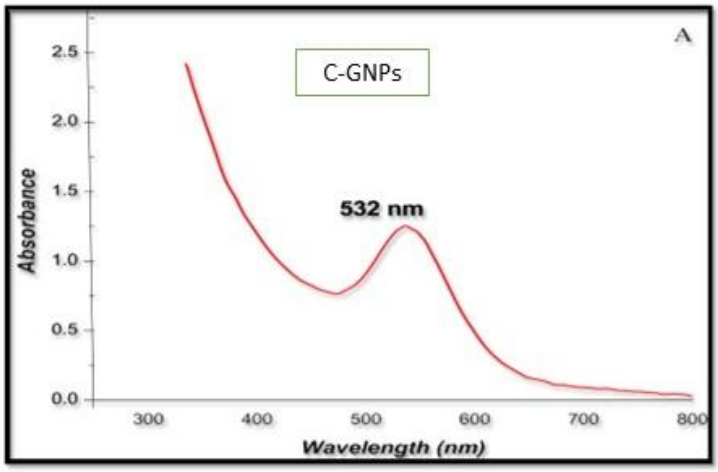

Fig. 4. UV-Vis absorbance of synthesized C-GNPs shows the absorbance at 532 nm.

Dynamic Light Scattering (DLS)

The hydrodynamic diameter of the C-GNPs was studied using DLS and it was found that the hydrodynamic diameter appeared to be larger. The hydrodynamic diameter for C-GNPs was 56.0.

DLS is having a limit to differentiating particles whose diameters are having differences of less than 30 nm. It provides hydrodynamic radii of particles present in the solution whereas TEM provides an inorganic core without hydration. Therefore, particle size obtained in DLS is bigger than in TEM but DLS can measure a large number of particles (in Millions) than TEM (a few Hundred). Therefore, DLS is appropriate for calculating the polydispersity index.

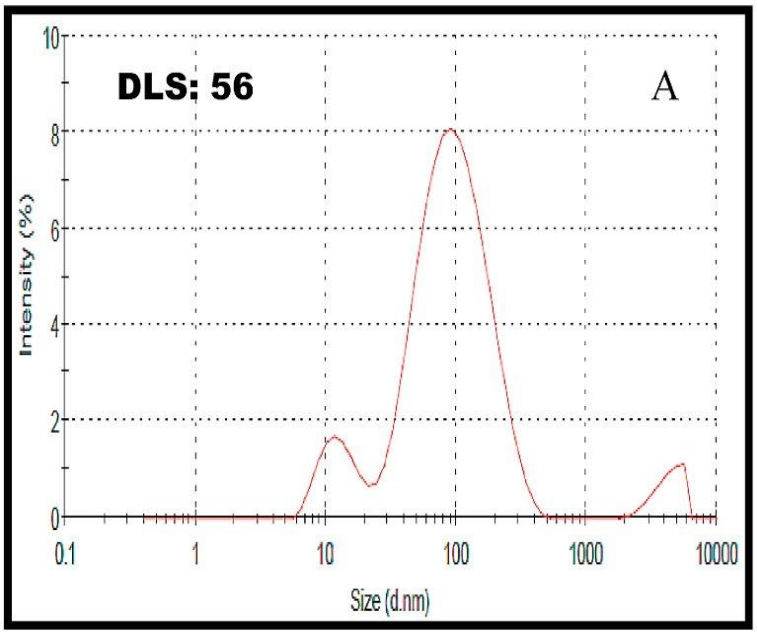

Fig. 5. Size determination of casein encapsulated gold nanoparticles by Dynamic Light Scattering (DLS) 56 d. nm.

Zeta potential

The zeta potential studies of C-GNPs were conducted to predict the stability of the respective nano-emulsions. The zeta potential of C-GNPs was found to be -19.5mV, with an anionic charge. These values are within the range required to form a stable emulsion. The stability of the emulsions and colloids isn't always predicted by the zeta potential alone. Since zeta potential doesn't consider Vander Waal forces and it only relies on ionic repulsive forces. Therefore, a stable colloid can also have

low zeta potential and vice versa. However, the Hamaker constant can explain the involvement of Vander Waal attractive forces which indirectly corresponds to the difference between the refractive index of the particles and the dispersant. Therefore, weak van der Waals forces (reflected by Hamaker constant).

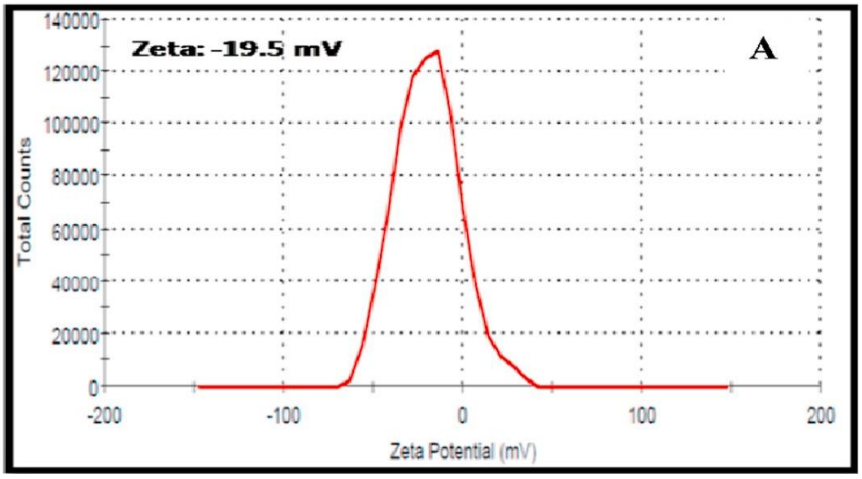

Fig. 6. Zeta Potential of C-GNPs -19.5mV.

Transmission Electron Microscopy (TEM)

The topographical studies of the C-GNPs were conducted using high-resolution TEM. It also determines the exact size and surface structure of particles without the hydration layer. The diameters of C-GNPs are 15nm, respectively. Gatan digital micrograph software was used to estimate the size of nanoparticles manually. The particles appeared to be spherical.

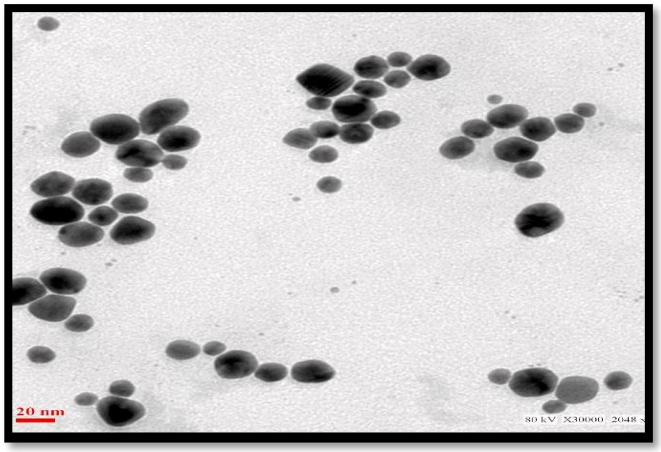

Fig. 7. Transmission Electron Microscopy (TEM) of C-GNPs (15nm).

Fourier-transform infrared spectroscopy (FTIR)

The confirmation of CS-GNPs was done by FTIR spectroscopy. Analysis of coupling of casein. The obtained spectra were found to show broadband contour at 3600–3000 cm^{-1} in each coupling reaction which corresponds to the –NH stretch of the peptide bond. Further confirmation of coupling can be drawn through CO anhydride stretching vibration for peptide bond at around 1640 and 1570 cm^{-1}.

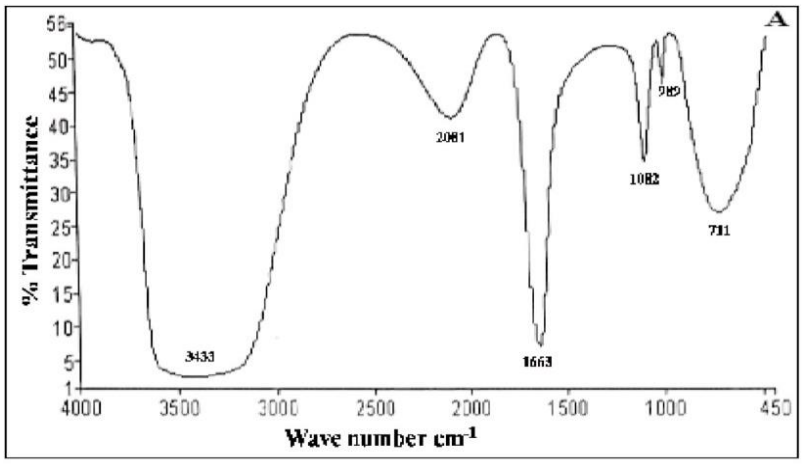

Fig. 8. FTIR spectra of synthesized C-GNPs.

Evaluation of antibacterial efficacy of casein

Determination of Antibacterial Activity by Disc Diffusion Method

The antibacterial activity of casein and C-GNPs was investigated using the disc diffusion method against gram-negative *(E. coli)* and gram-positive (*Staphylococcus aureus*) bacterial strains.

Maximum efficacy against *E. coli and Staphylococcus aureus was reported at a concentration of* 50µg/well casein protein with inhibitory zones of 16 and 11 mm. Furthermore, the measured zone of inhibition in the instance of levofloxacin (50µg/well) 19 and 13 mm against *E. coli and Staphylococcus aureus* all are resistant to this antibiotic. However, a similar concentration (50µg/well) of C-GNPs showed a considerably high zone of inhibition when compared to casein protein

and levofloxacin. The zone of inhibition was formed by C-GNPs 25 and 27 against *E. coli and Staphylococcus aureus* respectively.

Concentration	Zone of inhibition (mm)	
	E. coli	**S. aureus**
Control	**0**	**0**
Cs-GNPs	**25**	**27**
Casein	**16**	**11**
Antibiotic	**19**	**13**

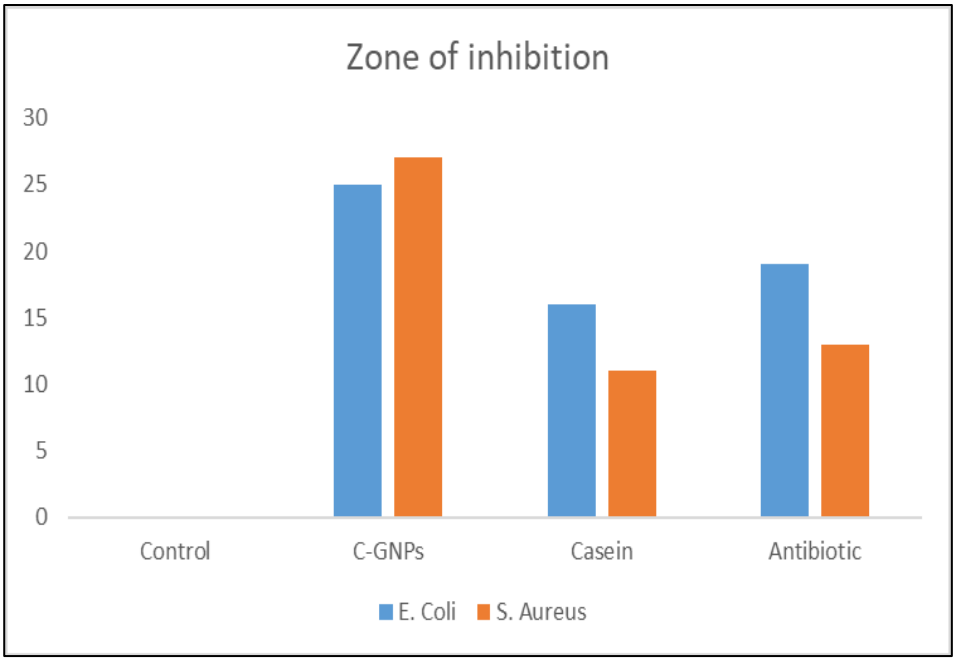

Fig. 9. Graph showing zone of inhibition of synthesized C-AuNPs against bacterial strains.

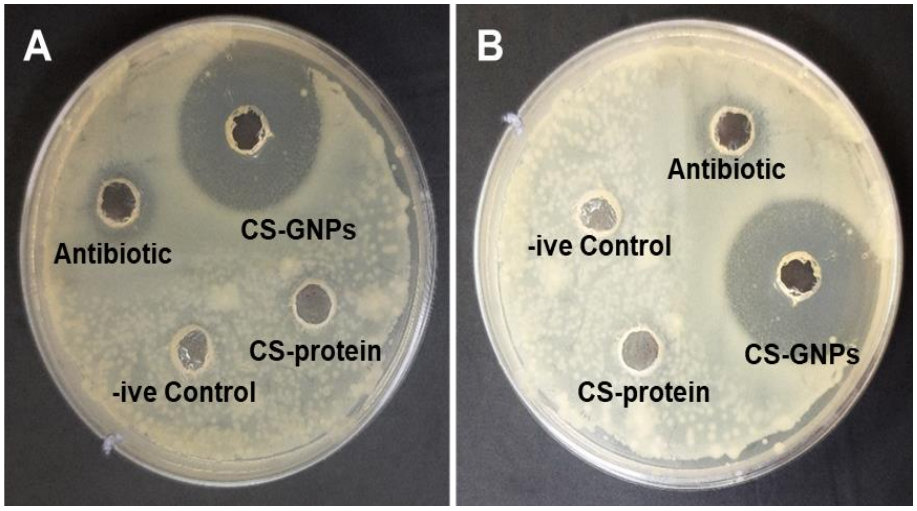

Fig. 10. Synthesized AuNPs shows antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Minimal Inhibitory Concentration (MIC) Determination

The MIC is the lowest concentration of casein protein and C-GNPs that completely inhibits bacterial growth, and MIC50 is the concentration of casein protein and C-GNPs that inhibits 50% of the bacterial population. The MIC_{50} of casein protein and C-GNPs against several Gram-negative and Gram-positive bacterial strains were recorded. However, levofloxacin was used as a standard antibiotic during the experiment. The quantified MIC_{50} values were *E. coli* 1.30 µg/mL and *Staphylococcus aureus*, 2.51 µg/ml for C-GNPs

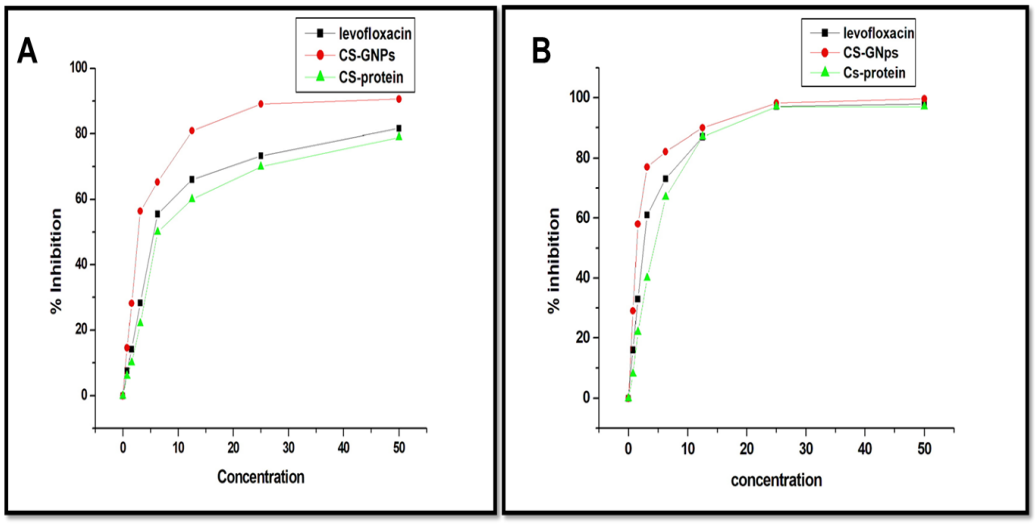

Fig. 11. The MICs (8µg/mL) of Casein protein, C-GNPs, and pure Levofloxacin and against (A) S. aureus, and (B) E. coli (96 well plate method).

Discussion and conclusion

Discussion

DLS was used to investigate the hydrodynamic diameter of C-GNPs, and it was discovered that the hydrodynamic diameter looked to be greater. C-GNPs have a hydrodynamic diameter of 56.0. C-GNP topography investigations were carried out utilizing high-resolution TEM. It also influences the precise size and surface texture of particles that are not covered by a hydration layer. C-GNPs have diameters of 15, respectively. C-GNP zeta potential experiments were carried out to forecast the stability of the various nano-emulsions. C-GNPs were discovered to have a zeta potential of -19.5mV. Furthermore, we have to check the C-GNP potential against pathogens. Optimum effectiveness against E. coli and Staphylococcus aureus was recorded at a casein protein concentration of 50g/well, with inhibitory zones of 16 and 11 mm, respectively. However, the measured zone of inhibition in the instance of levofloxacin (50µg/well) 19 and 13 mm against *E. coli and Staphylococcus aureus* all are resistant to this antibiotic. But as compared to casein protein and levofloxacin, C-GNPs at the same dose (50µg/well) displayed a much higher zone of inhibition. C-GNPs 25 and 27mm against E. coli and Staphylococcus aureus, respectively, created the zone of inhibition.

Conclusion

This study provides a novel approach for the synthesis of C-GNPs using a casein protein, casein as a reducing and capping agent. The C-GNPs were found to be more effective against *E. coli* and *S. aureus* than pure antibiotics due to their synergistic actions. However, realizing C-GNPs as practical drug delivery tools for clinical use can pose biological, technological, and study-design challenges, which require further research to be conducted.

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