A DISSERTATION ON

One-step synthesis of Gold Nanoparticles by using bromelain as a reducing and capping agent and their bioconjugation with Levofloxacin

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BY

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TO WHOM IT MAY CONCERN

This is to certify that **Mr. Shawaz**, a student of M.Sc. Biotechnology (IV semester), Integral University has completed his four months dissertation work entitled ***"One-step synthesis of Gold Nanoparticles by using bromelain as a reducing and capping agent and their bioconjugation with Levofloxacin"*** 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 her M.Sc. degree. I wish him good luck and a bright future.

(**Dr. Snober S. Mir**)
Head,
Department of Biosciences,
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IU/DBS/S00192/2022/CIN/221112 **June 2022**

CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Mr. Shawaz**, during the months 2 Feb to 2 June 2022 reported in the present thesis was under my guidance and supervision. The results reported by his are genuine and the script of the thesis has been written by the candidate himself. The thesis entitled ***"One-step synthesis of Gold Nanoparticles by using bromelain as a reducing and capping agent and their bioconjugation with Levofloxacin"*** 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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My acknowledgment will be incomplete if I do not mention my parents with whose blessing, I was able to achieve my goal successfully. There are no words to express my feelings toward them. I silently acknowledge my debt to them.

Shawaz **Date**

LIST OF ABBREVIATIONS

AuNPs	Gold nanoparticles
LVN	Levofloxacin
BRN	Bromelain
PBS	Phosphate buffer
M	Molarity
mM	Milli Molar
DLS	Dynamic Light Scattering
OD	Optical density
M deg	Milli degree
SPR	Surface Plasma Resonance
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
UV-Vis	Ultraviolet-Visible Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
ZP	Zeta potential
nm	Nanometer

Introduction

Introduction of nanotechnology

The prefix nano in the word nanotechnology means a billionth ($1x10^{9}$). Nanotechnology deals with various structures of matter having dimensions of the order of a billionth of a meter. While the word nanotechnology is relatively new, the existence of functional devices and structures of nanometer dimensions is not new, and such structures have existed on Earth as long as life itself (Poole Jr, C. P., & Owens, F. J. 2003).

Nanotechnology is generating a lot of attention these days and therefore building great expectations not only in the academic community but also among investors, the governments, and industry. Its unique capability to fabricate new structures at the atomic scale has already produced novel materials and devices with great potential applications in a wide number of fields (Serrano, E.,2009). Nanotechnology, or systems/device manufacture at the molecular level, is a multidisciplinary scientific field undergoing explosive development. The genesis of nanotechnology can be traced to the promise of revolutionary advances across medicine, communications, genomics, and robotics. On the surface, miniaturization provides cost-effective and more rapidly functioning mechanical, chemical, and biological components. Less obvious though is the fact that nanometre-sized objects also possess remarkable self-ordering and assembly behaviors under the control of forces quite different from macro objects. These unique behaviors are what make nanotechnology possible, and by increasing our understanding of these processes, new approaches to enhancing the quality of human life will surely be developed. A complete list of the potential applications of nanotechnology is too vast and diverse, but without doubt one of the greatest values of nanotechnology will be in the development of new and effective medical treatments (i.e., nanomedicine) (Emerich, D. F., &Thanos, C. G. 2003) The worldwide emergence of nanoscale science and engineering was marked by the announcement of the National Nanotechnology Initiative (NNI) in January 2000. Recent research on biosystems at the

nanoscale has created one of the most dynamic science and technology domains at the confluence of physical sciences, molecular engineering, biology, biotechnology, and medicine. This domain includes a better understanding of living and thinking systems, revolutionary biotechnology processes, the synthesis of new drugs and their targeted delivery, regenerative medicine, neuromorphic engineering, and developing a sustainable environment. Nano biosystem research is a priority in many countries and its relevance within nanotechnology is expected to increase in the future (Roco, M. C. 2003).

Nanotechnology as defined by size is naturally broad, including fields of science as diverse as surface science, organic chemistry, molecular biology, semiconductor physics, energy storage [3] [4], engineering, [5] microfabrication [6], and molecular engineering [7]. The associated research and applications are equally diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, [8] from developing new materials with dimensions on the nanoscale to direct control of matter on the atomic scale. Scientists currently debate the future implications of nanotechnology. Nanotechnology may be able to create many new materials and devices with a vast range of applications, such as in nanomedicine, nanoelectronics, biomaterials energy production, and consumer products. On the other hand, nanotechnology raises many of the same issues as any new technology, including concerns about the toxicity and environmental impact of nanomaterials [9], and their potential effects on global economics, as well as speculation about various doomsday scenarios. These concerns have led to a debate among advocacy groups and governments on whether special regulation of nanotechnology is warranted.

Review of literature

Nanoparticles

A nanoparticle is a small particle that ranges between 1 to 100 nanometres in size. Undetectable by the human eye, nanoparticles can exhibit significantly different physical and chemical properties than their larger material counterparts.

Nanoparticles are usually distinguished from microparticles (1-1000 μm), “fine particles” (sized between 100 and 2500 nm), and “coarse particles” (ranging from 2500 to 10,000 nm), because their smaller size drives very different physical or chemical properties, like colloidal properties and ultrafast optical effects [4] or electric properties.

Being more subject to the Brownian motion, they usually do not sediment, like colloidal particles that conversely are usually understood to range from 1 to 1000 nm.

Being much smaller than the wavelengths of visible light (400-700 nm), nanoparticles cannot be seen with ordinary optical microscopes, requiring the use of electron microscopes or microscopes with lasers. For the same reason, dispersions of nanoparticles in transparent media can be transparent [5], whereas suspensions of larger particles usually scatter some or all visible light incident on them. Nanoparticles also easily pass-through common filters, such as common ceramic candles [6], so separation from liquids requires special nanofiltration techniques.

The properties of nanoparticles often differ markedly from those of larger particles of the same substance. Since the typical diameter of an atom is between 0.15 and 0.6 nm, a large fraction of the nanoparticle’s material lies within a few atomic diameters of its surface. Therefore, the properties of that surface layer may dominate over those of the bulk material. This effect is particularly strong for nanoparticles dispersed in a medium of different composition since the interactions between the two materials at their interface also becomes significant [7],

Nanoparticles occur widely in nature and are objects of study in many sciences such as chemistry, physics, geology, and biology. Being at the transition between bulk materials and atomic or molecular structures, they often exhibit phenomena that are not observed at either scale. They are an important component of atmospheric pollution, and key ingredients in many industrialized products such as paints, plastics, metals, ceramics, and magnetic products. The production of nanoparticles with specific properties is a branch of nanotechnology.

In general, the small size of nanoparticles leads to a lower concentration of point defects compared to their bulk counterparts [8], but they do support a variety of dislocations that can be visualized using high-resolution electron microscopes [9]. However, nanoparticles exhibit different dislocation mechanics, which, together with their unique surface structures, results in mechanical properties that are different from the bulk material [10][11][12].

Non-spherical nanoparticles (e.g., prisms, cubes, rods, etc.) exhibit shape-dependent and size-dependent (both chemical and physical) properties (anisotropy) [13][14]. Non-spherical nanoparticles of gold (Au), silver (Ag), and platinum (Pt) due to their fascinating optical properties are finding diverse applications. Non-spherical geometries of nano-prisms give rise to high effective cross-sections and deeper colors of the colloidal solutions [15]. The possibility of shifting the resonance wavelengths by tuning the particle geometry allows using them in the fields of molecular labeling, biomolecular assays, trace metal detection, or nanotechnical applications. Anisotropic nanoparticles display a specific absorption behavior and stochastic particle orientation under unpolarized light, showing a distinct resonance mode for each excitable axis. This property can be explained by the fact that daily new developments are being made in the field of synthesis of these nanoparticles for preparing them in high yield [15].

Liposomes

The first NP platform was the liposomes. Liposomes were first described in 1965 as a model of cellular membranes. Since then, liposomes have moved from a model in biophysical research to one of the first NP platforms to be applied for gene and drug delivery. Liposomes are spherical vesicles that contain single or multiple bilayered structures of lipids that self-assemble in aqueous systems (Torchilin, V. P. 2005). Unique advantages imparted by liposomes are their diverse range of compositions, ability to carry and protect many types of biomolecules, as well as their biocompatibility and biodegradability (Torchilin, V. P. 2005). These advantages have led to the well-characterized and wide use of liposomes as transfection agents of genetic material into cells (lipofection) in biology research. Lipofection generally uses a cationic lipid to form an aggregate with the anionic genetic material. Another major application of liposomes is their use as therapeutic carriers since their design can allow for the entrapment of hydrophilic compounds within the core and hydrophobic drugs in the lipid bilayer itself. To enhance their circulation half-life and stability in vivo, liposomes have been conjugated with biocompatible polymers such as polyethylene glycol (PEG). Liposomes can also be functionalized by targeting ligands to increase the accumulation of diagnostic and therapeutic agents within desired cells. Today, there are twelve clinically approved liposome-based therapeutic drugs.

Albumin-bound

Albumin-bound NPs (nab) use the endogenous albumin pathways to carry hydrophobic molecules in the bloodstream. Albumin naturally binds to the hydrophobic molecules with non-covalent reversible binding, avoiding solvent-based toxicities for therapeutics. As a result, this platform has been successfully adapted as a drug delivery vehicle. Abraxane, 130-nm nab-paclitaxel was approved by the FDA in 2005 for the treatment of metastatic breast cancer (Harries, M., et al., 2005). Abraxane concentrates in cells through albumin receptor (gp60)-mediated transport in endothelial cells. It may also target the albumin

binding protein SPARC (secreted protein acidic and rich in cysteine), which is over-expressed in certain tumors. Further understanding of the mechanism of action may lead to better targeting and development of novel therapeutics using the nab platform.

Polymeric

Polymeric NPs formed from biocompatible and biodegradable polymers have been extensively investigated as therapeutic carriers. Polymeric NPs are formulated through block-copolymers of different hydrophobicity. These copolymers spontaneously assemble into a core-shell micelle formation in an aqueous environment. Polymeric NPs have been formulated to encapsulate hydrophilic and/or hydrophobic small drug molecules, as well as proteins and nucleic acid macromolecules (Wang, A. Z., et al., 2008). The NP design can allow for the slow and controlled release of drugs at target sites. Polymeric NPs are usually able to improve the safety and efficacy of the drugs they carry. Functionalizing polymeric NPs with targeting ligands for improved drug delivery has been an important area of investigation since polymeric NPs are unique in their ability to be tailored before particle assembly. The incorporation of targeting ligands on the NPs can lead to their increased uptake along with their cargo, leading to enhanced therapeutic outcomes.

Dendrimers

Another type of polymeric NP is dendrimers. Dendrimers are regularly branched macromolecules made from synthetic or natural elements including amino acids, sugars, and nucleotides. They have a central core, interior layers of branches, and an exterior surface. The varied combination of these components can yield dendrimers of well-defined size, shape, and branching length/density. As a result of their unique design, dendrimers can be developed as sensors as well as drug and gene delivery carriers. Dendrimers can be loaded with small molecules in the cavities of the cores through chemical linkage, hydrogen bond, and or hydrophobic interaction. The exterior surface can also be readily

modified to produce chemical functional groups for molecular targeting groups, detecting and imaging agents, and therapeutic attachment sites (Mintzer, M. A., et al., 2005).

Iron oxide

Iron oxide NPs are widely studied as passive and active targeting imaging agents as they are mainly superparamagnetic. The superparamagnetic iron oxide NP (SPION) generally has an iron oxide core with a hydrophilic coat of dextran or another biocompatible compound to increase its stability. The most widely used SPIONs consist of a magnetite (Fe_3O_4) and/or maghemite (γFe_2O_3) core. These NPs exhibit size-dependent super para-magnetism, which allows them to become magnetized with the application of an external magnetic field and exhibit zero net magnetization upon removal of the magnetic field. SPIONs have been successfully used as T2-weighted magnetic resonance (MR) contrast agents to track and monitor cells. SPIONs have several advantages over conventional gadolinium-chelate contrast agents including decreased toxicity and increased imaging sensitivity and specificity. SPIONS can also be degraded to iron and iron oxide molecules that are metabolized, stored in cells as ferritin, and incorporated into hemoglobin. Currently, two SPIO agents, ferumoxides (120–180 nm) and feru-carbotran (60 nm) are clinically approved for MRI. SPIONs have also been used in molecular imaging applications such as the detection of apoptosis and gene expression. SPIONs can be functionalized with magnetic, optical, radionuclide, and specific targeting ligands for multimodal imaging. They can also potentially be used as non-invasive diagnostic tools and as drug delivery vehicles (Mahmoudi, M., et al., 2011).

Quantum dot

First discovered in 1980, quantum dots (QDs) are semiconductor particles that are less than 10 nm in diameter. QDs display unique size-dependent electronic and optical properties. Most QDs studied consist of a cadmium selenide (CdSe) core and a zinc selenide (ZnS) cap. The

absorption spectra of these particles are very broad and emission is confined to a narrow band. QDs can also emit bright colors, have long lifetimes, high efficiencies, and are stable against photobleaching. They can be generated to have different biochemical specificities and can be simultaneously excited and detected. As a result, QDs have several significant advantages over many organic fluorophore dyes for optical applications. They are widely used in biological research as fluorescence imaging tools for applications such as cell labeling and biomolecule tracking. The small size of quantum dots also enables them to be suitable for biomedical applications such as medical imaging and diagnostics (Michalet, X., et al., 2005).

Gold

Gold NPs offer many size-and-shape-dependent optical and chemical properties, biocompatibility, and facile surface modification. Gold NPs can strongly enhance optical processes such as light absorption, scattering, fluorescence, and surface-enhanced Raman scattering (SERS) due to the unique interaction of the free electrons in the NP with light. These properties have enabled the realization of gold NPs in many applications such as biochemical sensing and detection, biological imaging, diagnostics, and therapeutic applications. Sensing techniques include the use of gold NPs in colorimetric arrays and the use of gold NPs as substrates in SERS to significantly enhance Raman scattering, allowing for spectroscopic detection and identification of proteins and single molecules at the NP surface. Gold NP probes have also been used to detect heart disease and cancer biomarkers. They can also transform absorbed light into heat and therefore, have a high potential for infrared phototherapy (Huang, X., et al., 2006).

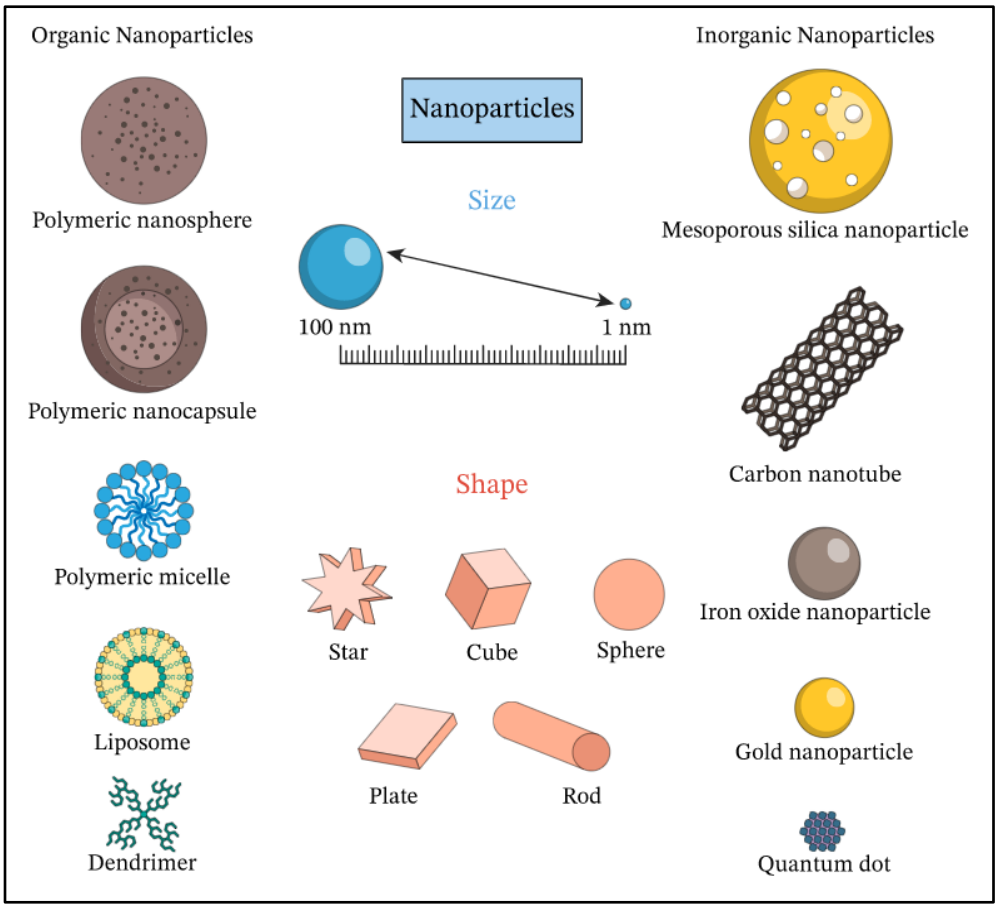

Fig. 1. Types of nanoparticles.

Synthesis of nanoparticles

Nanofabrication methods can be divided roughly into two groups: top-down and bottom-up methods (Love, J. C., et al., 2001). Top-down methods start with patterns made on a large scale and reduce their lateral dimensions before forming nanostructures. On the other hand, bottom-up methods begin with atoms or molecules to build up nanostructures, in some cases through smart use of self-organization (Mijatovic, D., et al., 2005.).

Top-Down Methods

In this method, a destructive approach is employed. Starting from the larger molecule, which decomposed into smaller units, and then these units are converted into suitable NPs. Examples of this method are grinding/milling, CVD, physical vapor deposition (PVD), and other decomposition techniques (Iravani, 2011). This approach is used to

synthesize coconut shell (CS) NPs. The milling method was employed for this purpose and the raw CS powders were finely milled at different intervals of times, with the help of ceramic balls and a well-known planetary mill. They showed the effect of milling time on the overall size of the NPs through different characterization techniques. It was determined that with the time increases the NP's crystallite size decreases, as calculated by the Scherer equation. They also realized that with each hour increment the brownish color faded away due to the size decrease of the NPs. The SEM results were also in an agreement with the X-ray pattern, which also indicated the particle size decreases with time (Bello et al., 2015). One study revealed the spherical magnetite NPs synthesis from natural iron oxide (Fe_2O_3) ore by a top-down destructive approach with a particle size that varies from 20 to 50 nm in the presence of organic oleic acid (Priyadarshana et al., 2015). A simple top-down route was employed to synthesize colloidal carbon spherical particles with control size. The synthesis technique was based on the continuous chemical adsorption of polyoxometalates (POM) on the carbon interfacial surface. Adsorption made the carbon black aggregates into relatively smaller spherical particles, with high dispersion capacity and narrow size distribution as shown in Fig. 5 (Garrigue et al., 2004). It also revealed from the micrographs, that the size of the carbon particles becomes smaller with sonication time. A series of transition-metal dichalcogenide nanodots (TMD-NDs) were synthesized by a combination of grinding and sonication top-down techniques from their bulk crystals. It was revealed that almost all the TMD-NDs with sizes <10 nm show an excellent dispersion due to narrow size distribution (Zhang et al., 2015). Lately, highly photoactive active Co_3O_4 NPs were prepared via top-down laser fragmentation, which is a top-down process. The powerful laser irradiations generate well-uniform NPs having good oxygen vacancies (Zhou et al., 2016). The average size of the Co3O4 was determined to be in the range of 5.8 nm} 1.1 nm. (Khan, I., Saeed, K., & Khan, I. (2017). Nanoparticles: Properties, applications, and toxicities. Arabian Journal of Chemistry.)

In analogy with micromachining, top-down methods for nanomachining can be subdivided into 3 categories:

- ❖ Bulk film machining.
- ❖ Surface- machining
- ❖ Mold-machining

A) **Bulk-/film-machining**

In bulk-/film-machining the channel is created by etching trenches in the substrate wafer or the film deposited on the substrate. This is done typically by standard photolithography followed by wet or dry etching of the substrate in the case of substrate (bulk) etching and usually chemical etching of the film in the alternative approach, i.e., film etching (Jansen, H. V., et al., 2004).

B) **Surface-machining**

In surface-machining (Gajar, S. A., et al., 1992), first, a bottom layer is deposited on the wafer followed by the deposition of the sacrificial layer and its patterning. Then, the top layer is deposited on top of the sacrificial layer and patterned (often with irrigation holes, which provide the access to the sacrificial layer). The nanochannel is finally formed by removing, i.e., etching the sacrificial layer leaving the bottom and the top layer to form the walls of the nanochannel. The bottom layer is not always required. It is mainly introduced to form the channel of one material (the same material as the top layer).

C) **Mold-machining**

In principle, in mold-machining first, the mold in the inverse shape of the desired structure is formed. This is filled with a structural material and then the mold can be etched or removed leaving the desired structure behind. The mold machining is mainly performed by soft lithography. In soft lithography, the mold is usually made by producing a pattern (master) in a layer of photoresist on the surface of the silicon wafer by photolithography or electron beam lithography (EBL) (Love, J. C., et al., 2001). Then a liquid precursor to polydimethylsiloxane (PDMS) is poured over it and cured into the rubbery solid. The PDMS stamp is then peeled off the master. The fabrication of the master is

rather expensive due to use of the electron-beam lithography or other advanced techniques. Copying the pattern on the PDMS stamp as well as the use of the stamp is, however, cheap and easy.

Bottom-up methods

In such methods, the atoms and molecules are assembled into the smallest nanostructures (dimensions of typically 2 to 10 nm) by carefully controlled chemical reactions, which make this technique cheaper as compared to the lithographical methods. This approach is employed in reverse as NPs are formed from relatively simpler substances, therefore this approach is also called the building up approach. Examples of this case are sedimentation and reduction techniques. It includes sol-gel, green synthesis, spinning, and biochemical synthesis. (Iravani, 2011). Mogilevsky et al. synthesized TiO_2 anatase NPs with graphene domains through this technique (Mogilevsky et al., 2014). They used alizarin and titanium isopropoxide precursors to synthesize the photoactive composite for photocatalytic degradation of methylene blue. Alizarin was selected as it offers strong binding capacity with TiO_2 through their axial hydroxyl terminal groups. The anatase form was confirmed by the XRD pattern. SEM indicates that with temperature elevation, the size of NPs also increases (Mogilevsky et al., 2014). Well-uniform spherical-shaped Au nanospheres with monocrystalline have been synthesized via laser irradiation top-down technique (Liu et al., 2015a, 2015b). Liu et al. selectively transform the octahedra morphology to a spherical shape by controlling the laser treatment time and other reaction parameters. Fig. provides the SEM and TEM of the prepared Au nanospheres, which showed an average diameter of 75} 2.6 nm of Au nanospheres (red column Fig. 6e) and 72} 3.1 in edge length of Au octahedra per particle. More recently, the solvent-exchange method is used to achieve limit-sized low-density lipoprotein (LDL) NPs for medical cancer drug delivery purposes by Needham et al. In this method nucleation is the bottom approach followed by growth which is the up approach. The LDL NPs were obtained without using phospholipid and possessed high hydrophobicity, which is essential for drug delivery

applications (Needham et al., 2016). The monodispersed spherical bismuth (Bi) NPs were synthesized by both top-down and bottom-up approaches (Wang and Xia, 2004). These NPs have excellent colloidal properties. In the bottom-up approach, bismuth acetate was boiled within ethylene glycol, while in the top-down approach the bismuth was converted into molten form, and then the molten drop was emulsified within the boiled diethylene glycol to produce the NPs. The size of the NPs obtained by both methods varied from 100 nm to 500 nm (Wang and Xia, 2004). The details of this study are provided in Scheme 3. Green and biogenic bottom-up synthesis attracts many researchers due to the feasibility and less toxic nature of the processes. These processes are cost-effective and environmental friendly, where synthesis of NPs is accomplished via biological systems such as using plant extracts. Bacteria, yeast, fungi, Aloe vera, tamarind, and even human cells are used for the synthesis of NPs. AuNPs have been synthesized from the biomass of wheat and oat (Parveen et al., 2016) and using the microorganism and plant extracts as a reducing agent (Ahmed et al., 2016).

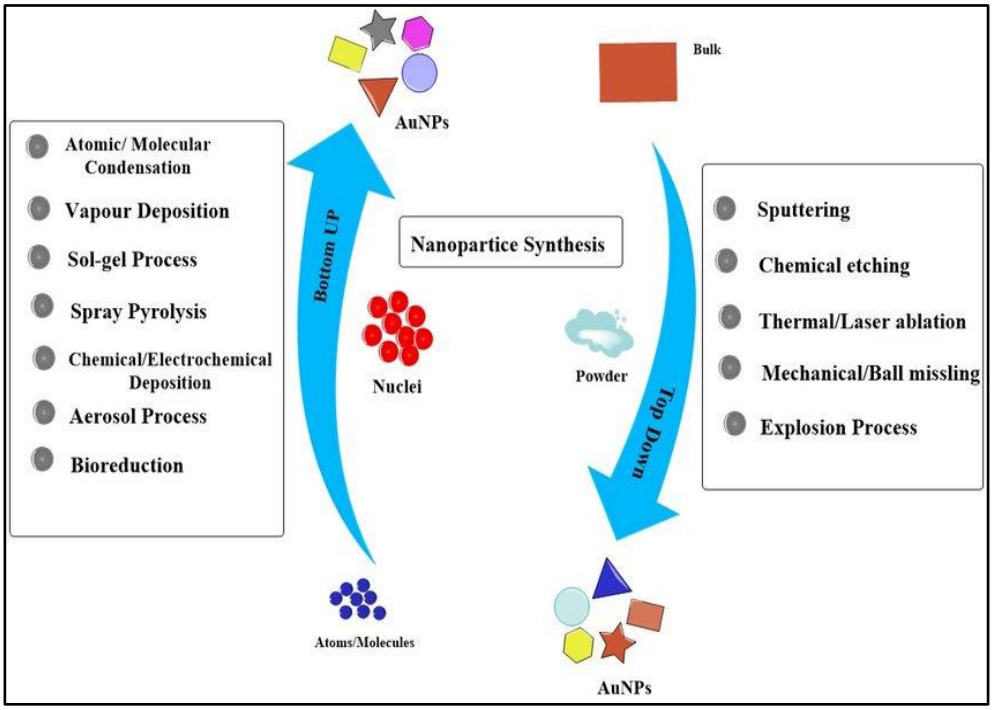

Fig. 2. Top-down and bottom-up approaches exploiting different physical, chemical, and biological methods for the synthesis of AuNPs

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Application of nanotechnology

Medicine

The scientific and scientific analysis areas have utilized the exclusive qualities of nanomaterials for various programs (e.g., comparison providers for mobile pictures and therapeutics for the treatment of cancer). Conditions such as biomedical nanotechnology, bio

nanotechnology, and nanomedicine are used to explain these multiple areas. Features can be included in nanomaterials by interfacing them with scientific elements or components. The size of nanomaterials is just like that of most scientific elements and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical analysis and programs. Thus far, the incorporation of nanomaterials with chemistry has led to the growth of analytic gadgets, comparison providers, systematic resources, actual physical rehabilitation programs, and medication distribution automobiles (Singh, K., et al., 2014).

Diagnostics

Nanotechnology-on-a-chip is one more sizing of lab-on-a-chip technological innovation. Attractive nanoparticles, limited to an appropriate antibody, are used for brand-specific elements, components, or harmful bacteria. Silver nanoparticles marked with short sections of DNA can be used for the recognition of inherited series for example (Connolly, J. M., et al., 2014). Multicolour visual programming for scientific assays has been obtained by embedding different-sized huge spots into polymeric microbeads. Nanopore technological innovation for research of nucleic chemicals transforms post nucleotides straight into digital signatures.

Drug-delivery

The overall medication intake and side effects can be reduced considerably by depositing the effective broker in the melancholy area only and in no higher amount than needed. This highly particular strategy decreases costs and human struggle. An example can be found in dendrimers and nano porous materials. They could hold little medication elements moving them to the preferred location. Another perspective is based on little electromechanical systems; NEMS are being examined for the effective launch of the medication. Some possibly important programs include cancer therapy with metal nanoparticles or silver seashells (Hsiao, I. L., et al., 2014). A focused or customized medication decreases the medication intake and therapy costs leading to an overall social benefit by decreasing the costs to the

public health system. Nanotechnology is also starting up new possibilities in implantable distribution techniques, which are often much better than the use of injectable medication because the latter frequently displays first-order kinetics (the blood focus goes up quickly but drops considerably over time). This fast increase may cause complications with poisoning, and medication effectiveness can reduce as the medication focus drops below the focused range.

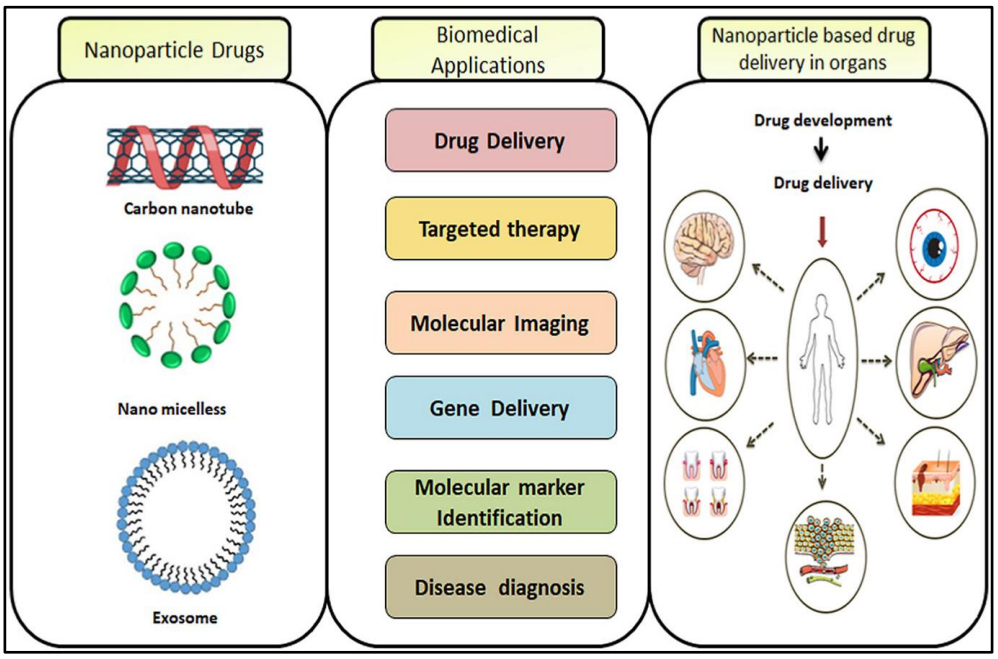

Fig. 3. Nanoparticles are used in drug development, drug delivery, and treatment of several diseases.

Tissue Engineering

Nanotechnology can help to reproduce or to fix broken tissues. “Tissue engineering” makes use of artificially activated mobile growth by using appropriate nanomaterial-based scaffolds and growth aspects. Tissue technology might alternative to today’s traditional treatments like whole body transplants or artificial improvements. Impressive types of tissue technology may cause lifestyle development. For patients with the end-state whole body unable, there may not be enough healthier tissues for growth and hair surgery in the ECM (extracellular matrix) (Tiwari, V., et al., 2014; Baccar, H., et al., 2014). In this situation, pluripotent control tissues are required. One potential source for these tissues is IPS (induced Pluripotent Control cells); these are common tissues from the

patient's own whole body that are reprogrammed into a pluripotent condition and have the benefits of avoiding being refused (and the possibly life-threatening problems associated with immunosuppressive treatments). Another potential source of pluripotent tissues is embryos, but this has two disadvantages:

- It needs that we fix the issue of cloning, which is officially very challenging (especially avoiding abnormalities).
- It needs the growing of embryos. Given that each one of us was once an embryo, this resource is legally challenging.

Chemistry and environment

Substance catalysis and purification methods are two popular illustrations where nanotechnology already performs a part. The functions provide novel components with designed functions and chemical properties: for example, nanoparticles with a unique chemical around (ligands), or particular visual qualities. In this feeling, the chemical makeup is indeed primary nanoscience. From a short-term viewpoint, chemical makeup will provide novel “nanomaterials” and in the long run, excellent procedures such as “self-assembly” will allow time and energy protecting methods (Ramesh Kumar, K., et al., 2014). In a feeling, all chemical functions can be recognized by nanotechnology, because of its capability to produce certain elements. Thus, chemical makeup types a platform for nanotechnology offering tailor-made elements, polymers, etc, as well as groups and nanoparticles.

Catalysis

Substance catalysis advantages especially from nanoparticles, due to the incredibly huge surface place to quantity rate. The program prospective of nanoparticles in catalysis varies from energy mobile to catalytic converters and photocatalytic gadgets (Swed, A., et al., 2014). Catalysis is also essential for the development of substances. Jewelry nanoparticles are now being regarded in the next creation of automobile catalytic converters because the very high place of nanoparticles could decrease the quantity of platinum needed. However, some issues have been brought up due to tests indicating

that they will automatically burn if methane is combined with the normal air. Continuous analysis at the Center Nationwide de la Recherché Scientifique (CNRS) in Italy may take care of their real effectiveness for catalytic programs. Nanofiltration may come to be an essential program, although upcoming analysis must be cautious to examine possible poisoning (Deiwick, A., et al., 2014).

Filtration

A strong influence of nanochemistry on waste-water therapy, air filtration, and energy storage gadgets is to be expected. Technical or chemical techniques can be used for efficient filtration techniques. One class of filtration techniques is based on the use of walls with appropriate hole sizes, whereby the liquid is pushed through the tissue layer (Raghavan, V., et al., 2014). Nanoporous walls are appropriate for an analog filtration with extremely small skin pores smaller than 10 nm ("nanofiltration") and may be consisting of nanotubes. Nanofiltration is mainly used for the removal of ions or the separating of different liquids. On a larger scale, the tissue layer filtration technique is named ultrafiltration, which works down to between 10 and 100 nm. One important field of application for ultrafiltration is medical reasons as can be found in kidney dialysis. Attractive nanoparticles offer an efficient and reliable method to remove metal pollutants from spending H_2O by making use of magnetic separating techniques. Using nanoscale contaminants increases the performance to process the pollutants and is relatively inexpensive compared to traditional rainfall and filtration techniques. Some water treatment gadgets integrating nanotechnology are already on the market, with more in development. Low-cost nanostructured separating walls techniques are efficient in producing safe and clean H_2O in a majority of folks (Bhandare, N., & Narayana, A. 2014).

Energy

The most advanced nanotechnology tasks related to power are: storage, transformation, manufacturing developments by reducing materials and process rates, power saving (by better heat-insulating

material for example), and improved alternative power (Pantidos, N., & Horsfall, L. E. 2014).

Reduction of energy consumption

A decrease in power intake can be achieved by better insulating material techniques, the use of more effective illumination or burning techniques, and the use of less heavy and more powerful materials in the transport industry. Currently used lights only turn roughly 5% of the power into mild (Cramer, S., et al., 2014). Nanotechnological techniques like light-emitting diodes (LEDs) or huge caged atoms (QCAs) could lead to a strong decrease in power intake for illumination.

Increasing the efficiency of energy production

Modern best solar panels have levels of several different semiconductors placed together to process mild at different efforts but they still only handle to use 40 percent of the Sun's energy. From the commercial perspective available solar panels have much lower effectiveness (15-20%). Nanotechnology could help increase the performance of mild transformation by using nanostructures with a procession of bandgaps (Davis, S. S. 1997). The level of efficiency of the internal combustion engine is about 30-40% currently. Nanotechnology could enhance loss by creating particular aspects with the enhanced place. In 2005, scientists at the University of Toronto designed a spray-on nanoparticle content that, when used in a place, instantly transforms it into a solar panel.

The use of more environmentally friendly energy systems

A case for an ecologically cordial manifestation of energy is the utilization of power modules controlled by hydrogen, which is preferably delivered by renewable energies. Most likely the most conspicuous nanostructured material in power devices is the impetus comprising of carbon upheld respectable metal particles with distances across of 1-5 nm. Suitable materials for hydrogen stockpiling contain countless nanosized pores. Consequently, numerous nanostructured materials like nanotubes, zeolites, or alanates are under scrutiny (West, J. L., et al., 2000). Nanotechnology can add to the further diminishment of

burning motor poisons by nanoporous channels, which can clean the fumes mechanically, by exhaust systems given nanoscale honorable metal particles, or by reactant coatings on barrel dividers and synergist nanoparticles as an added substance for energizes.

Recycling of batteries

Because of the moderately low energy thickness of batteries, the working time is constrained and substitution or reviving is required. The colossal number of spent batteries and collectors speaks to a transfer issue (Duncan, T. V. 2011). The utilization of batteries with higher energy substances or the utilization of rechargeable batteries or supercapacitors with a higher rate of reviving utilizing nanomaterials could be useful for the battery transfer issue.

Information and communication

Current high-innovation generation techniques are given customary top-down procedures, whereas nanotechnology has as of now been presented noiselessly (Wilkinson, J. M. 2003). The basic length size of incorporated circuits is as of now at the nanoscale (50 nm and underneath) concerning the entryway length of transistors in CPUs or DRAM gadgets.

Memory storage

Electronic memory plans in the past have to a great extent depending on the arrangement of transistors. On the other hand, research into crossbar switch-based gadgets has offered an option utilizing reconfigurable interconnections in the middle of vertical and flat wiring shows to make ultra-high thickness memories (Wong, Y. W. H., et al., 2006; Surendiran, A., et al., 2009). Two pioneers around there are Nantero which has added to a carbon nanotube-based crossbar memory called Nano-RAM and Hewlett- Packard which has proposed the utilization of memristor material as a future substitution of Flash memory.

Novel semiconductor devices

An illustration of such novel gadgets is in light of spintronics. The reliance on the safety of material (because of the twist of the electrons) on an outside field is called magnetoresistance. This impact can be

fundamentally increased (GMR-Giant Magneto-Resistance) for nanosized items, for instance when two ferromagnetic layers are divided by a nonmagnetic layer, which is a few nanometers thick (e.g. Co-Cu-Co). The GMR impact has prompted a solid increment in the information stockpiling thickness of hard plates and made the gigabyte range conceivable (Wickline, S. A., et al., 2006). The purported burrowing magnetoresistance (TMR) is fundamentally the same as GMR and in light of the twist ward burrowing of electrons through adjoining ferromagnetic layers. Both GMR and TMR impacts can be utilized to make a non-unpredictable fundamental memory for PCs, for example, the purported attractive arbitrary access memory or MRAM (Shen, Z., et al., 2004). In 1999, a definitive CMOS transistor created at the Laboratory for Electronics and Information Technology in Grenoble, France, tried the points of confinement of the standards of the MOSFET transistor with a distance across of 18 nm (roughly 70 molecules put side by side). This was very nearly one-tenth the extent of the littlest mechanical transistor in 2003 (130 nm in 2003, 90 nm in 2004, 65 nm in 2005, and 45 nm in 2007). It empowered the hypothetical incorporation of seven billion intersections on a €1 coin. Notwithstanding, the CMOS transistor, which was made in 1999, was not a basic examination investigation to study how CMOS innovation capacities, but instead a show of how these innovation capacities now that we are getting nearer and nearer to chipping away at an atomic scale. Today it would be difficult to ace the facilitated get-together of countless transistors on a circuit and it would likewise be difficult to make this on a mechanical level.

Novel optoelectronic devices

In the present-day correspondence innovation, conventional simple electrical gadgets are progressively supplanted by optical or optoelectronic gadgets because of their tremendous transfer speed and limit, individually. Two guaranteeing illustrations are photonic precious stones and quantum dabs. Photonic precious stones are materials with an occasional variation in the refractive file with a grid consistent that is a large portion of the wavelength of the light utilized.

They offer a selectable band hole for the spread of a certain wavelength, consequently, they take after a semiconductor, however for light or photons rather than electrons. Quantum spots are nanoscaled items, which can be utilized, among numerous different things, for the development of lasers. The preference of a quantum speck laser over the conventional semiconductor laser is that their radiated wavelength relies on the width of the dab. Quantum dab lasers are less expensive and offer a higher pillar quality than customary laser diodes.

Displays

The creation of showcases with low energy utilization could be fulfilled by utilizing carbon nanotubes (CNT). Carbon nanotubes are electrically conductive and because of their little distance across a few nanometers, they can be utilized as field emitters with a great degree of high proficiency for field discharge presentations (FED). The rule of operation takes after that of the cathode beam tube, yet on a much littler length scale.

Quantum computers

Altogether new methodologies for processing adventure the laws of quantum mechanics for novel quantum PCs, which empower the utilization of quick quantum algorithms (Shen, Z., et al., 2004). The Quantum PC has quantum bit memory space termed "Qubit" for a few calculations in the meantime. This office may enhance the execution of the more seasoned frameworks. Heavy Industry: An inescapable utilization of nanotechnology will be in the overwhelming industry. Aerospace: lighter and stronger materials will be of massive utilization to flying machine makers, prompting expanded execution. Space apparatus will likewise profit, where weight is the main consideration. Nanotechnology would help to decrease the measure of supplies and accordingly diminish fuel-utilization needed to get it airborne (Shen, Z., et al., 2004). Hang lightweight planes may have the capacity to split their weight while expanding their quality and durability through the utilization of nanotech materials. Nanotech is bringing down the mass of supercapacitors that will progressively be utilized to offer force to

assistive electrical engines for dispatching hang lightweight flyers off flatland to thermal-pursuing elevations (Qu, X., et al., 2013)

Construction

Nanotechnology can make development quicker, less expensive, more secure, and more changed. The computerization of nanotechnology development can take into account the formation of structures from cutting-edge homes to enormous high rises substantially more rapidly and at a much lower expense (Sawhney, A. P. S., et al., 2008).

Refineries

Utilizing nanotech applications, refineries delivering materials, for example, steel and aluminum will have the capacity to evacuate any contaminations in the materials they make.

Vehicle manufacturers

Much like aviation, lighter and stronger materials will be valuable for making vehicles that are both quicker and more secure. Burning motors will likewise profit from parts that are all the more hardwearing and more high-temperature safe.

Consumer goods

Nanotechnology is as of now affecting the field of shopper merchandise, giving items novel capacities running from simple to clean to scratch-safe. Cutting edge materials are wrinkle-safe and stain-repellent; in the mid-term garments will get to be "savvy", through installed "wearable hardware" (Ge, Z., & Gao, Z. 2008). Effectively being used are diverse nanoparticle-enhanced items. Particularly in the field of makeup, such novel items have a guaranteeing potential.

Foods

The complex set of designing and experimental difficulties in the nourishment and the bioprocessing industry for assembling top-notch and safe sustenance through productive and supportable means can be tackled through nanotechnology. Microorganisms' recognizable proof and nourishment quality observing utilizing biosensors; insightful, dynamic, and brilliant sustenance bundling frameworks; nanoencapsulation of bioactive nourishment mixes are a few cases of developing uses of nanotechnology for the nourishment business.

Nanotechnology can be connected to the creation, handling, security, and bundling of nourishment. A nanocomposite covering procedure could enhance sustenance bundling by putting against microbial specialists straightforwardly on the surface of the covered film. Nanocomposites could expand or decline gas penetrability of distinctive fillers as is required for diverse items (Seil JT, Webster TJ 2012). They can likewise enhance the mechanical and hotness safety properties and bring down the oxygen transmission rate. Exploration is being performed to apply nanotechnology to the location of synthetic and organic substances for sensing in nourishments.

Nano-foods

New consumer Products Emerging Nanotechnologies (PEN), taking into account a stock it has drawn up of 609 known or guaranteed nano-products. On PEN's rundown are three sustenances - a brand of canola cooking oil called Canola Active Oil, a tea called Nanotea, and a chocolate eating regimen shake called Nanoceuticals Slim Shake Chocolate. As per organization data posted on PEN's Web website, the canola oil, by Shemen Industries of Israel, contains an added substance called "nano drops" intended to convey vitamins, minerals, and phytochemicals through the digestive framework. The shake, as indicated by U.S. maker RBC Life Sciences Inc., utilizes cocoa mixed "NanoClusters" to upgrade the taste and medical advantages of cocoa without the requirement for additional sugar.

Household

The most unmistakable utilization of nanotechnology in the family unit is cleaning oneself or "simple to-clean" surfaces on pottery or glasses. Nanoceramic particles have enhanced the smoothness and hotness safety of normal family supplies, for example, the level of iron (Smith, D. M., et al., 2013).

Optics

The primary shades utilizing defensive and anti-reflective ultrathin polymer coatings are available. For optics, nanotechnology additionally offers scratch-safe surface coatings because of nanocomposites.

Nano-optics could consider an increment in the accuracy of student repair and different sorts of laser eye surgery.

Textiles

The utilization of engineered nanofibers as of now makes garments water- and stain repellent or wrinkle-free. Materials with a nanotechnological completion can be washed in less much of time and at lower temperatures. Nanotechnology has been utilized to incorporate small carbon particles film and ensure full-surface assurance from electrostatic charges for the wearer. Numerous different applications have been produced via research organizations, for example, the Textiles Nanotechnology Laboratory at Cornell University.

Cosmetics

One field of use is sunscreens. The customary substance UV security methodology experiences its poor long-haul steadiness. A sunscreen because of mineral nanoparticles, for example, titanium dioxide offers a few focal points (Smith, D. M., et al., 2013). Titanium oxide nanoparticles have a tantamount UV security property as the mass material, however, lose the cosmetically undesirable whitening as the molecule size is decline.

Agriculture

Uses of nanotechnology can change the whole agriculture part and nourishment industry anchor from generation to preservation, handling, bundling, transportation, and even waste treatment. NanoScience ideas and Nanotechnology applications can update the generation cycle, rebuild the preparation and protection forms and rethink the nourishment propensities for individuals. Real Challenges identified with agriculture like Low profit in cultivable regions, Large uncultivable ranges, Shrinkage of cultivable terrains, Wastage of inputs like water, composts, pesticides, Wastage of items, and Food security for developing numbers can be tended to through different utilizations of nanotechnology (Rashidi, L., &Khosravi-Darani, K. 2011).

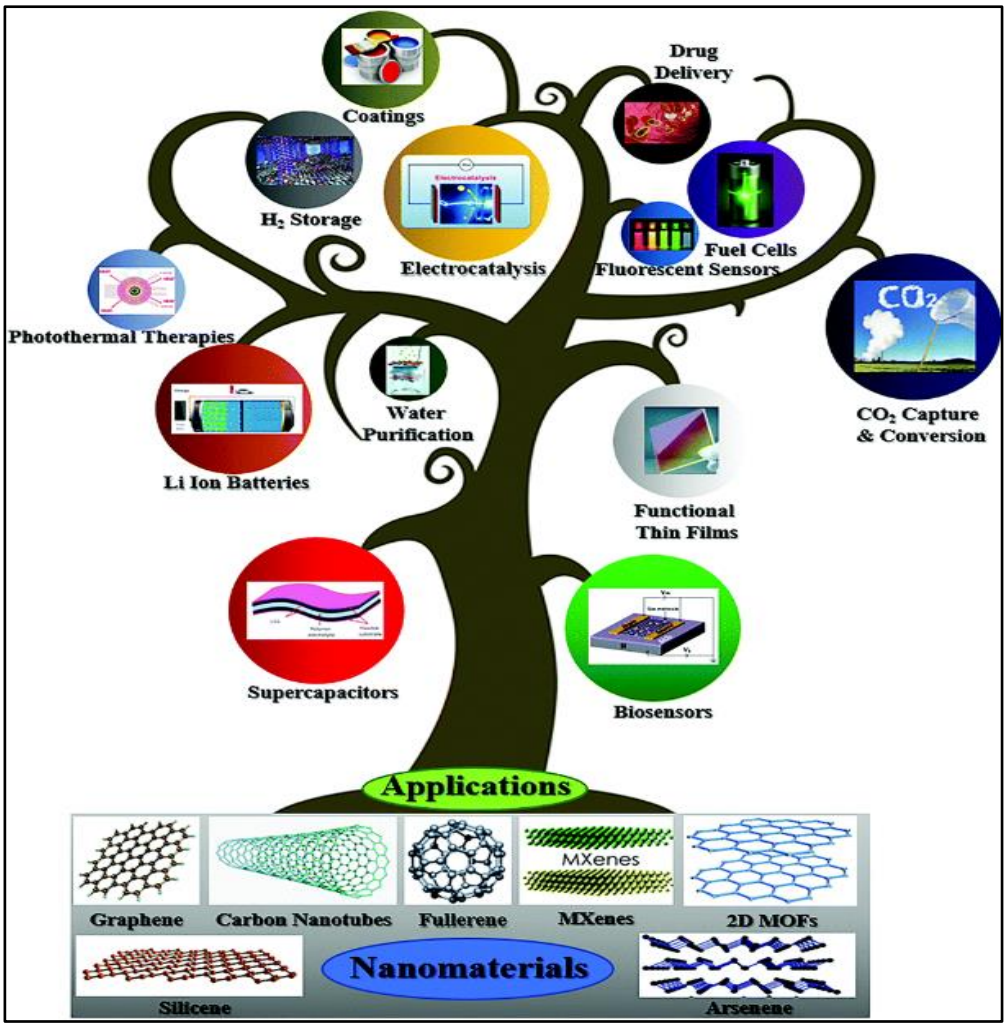

Fig. 4. Schematic representation of nanomaterials and their applications.

Characterization of nanoparticles

Optical microscopes are generally used for observing micron-level materials with reasonable resolution. Further magnification cannot be achieved through optical microscopes due to aberrations and limits in the wavelength of light. Hence, the imaging techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM/HRTEM), scanning tunneling microscopy (STM), atomic force microscopy (AFM), etc. have been developed to observe sub-micron size materials. Though the principles of all the techniques are different one common thing is that they produce a highly magnified image of the surface or the bulk of the sample. Nanomaterials can only be observed through these imaging techniques as the human eye as well as an optical microscope cannot be used to see dimensions at the

nano level. The basic principles and applications of all these imaging techniques used in nanotechnology research are described below.

Scanning electron microscopy (sem)

The scanning electron microscope is an electron microscope that images the sample surface by scanning it with a high-energy beam of electrons. Conventional light microscopes use a series of glass lenses to bend light waves and create a magnified image while the scanning electron microscope creates the magnified images by using electrons instead of light waves (Mills, K., et al., 2007).

Applications

The SEM shows very detailed three-dimensional images at many high magnifications (up to ×300000) as compared to a light microscope (up to × 10000). But as the images are created without light waves, they are black and white. The surface structure of polymer nanocomposites, fracture surfaces, nanofibres, nanoparticles, and nanocoating can be imaged through SEO with great clarity.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy is a microscopy technique whereby a beam of electrons is transmitted through an ultra-thin specimen and interacts as passes through the sample. An image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens, and appears on an imaging screen.

Applications

The TEM is used widely both in material science/metallurgy and biological sciences. In both cases, the specimens must be very thin and able to withstand the high vacuum present inside the instrument. For biological specimens, the maximum specimen thickness is roughly 1 micrometer. To withstand the instrument vacuum, biological specimens are typically held at liquid nitrogen temperatures after embedding in vitreous ice or fixated using a negative staining material such as uranyl acetate or by plastic embedding.

Ultraviolet-visible (uv-vis) spectroscopy

Ultraviolet spectrophotometers consist of a light source, reference and sample beams, a monochromator, and a detector. The ultraviolet

spectrum for a compound is obtained by exposing a sample of the compound to ultraviolet light from a light source, such as a Xenon lamp.

Applications

In certain metals, such as silver and gold, the plasmon resonance is responsible for their unique and remarkable optical phenomena. Metallic (silver or gold) nanoparticles, typically 40–100 nm in diameter, scatter optical light elastically with remarkable efficiency because of a collective resonance of the conduction electrons in the metal known as surface plasmon resonance. The surface plasmon resonance peak in UV absorption spectra is shown by these plasmon resonant nanoparticles. The magnitude, peak wavelength, and spectral bandwidth of the Plasmon resonance associated with a nanoparticle are dependent on the particle's size, shape, and material composition, as well as the local environment.

Bromelain

Bromelain belongs to a group of proteolytic enzymes which are used as drugs for the oral-systemic treatment of inflammatory, blood-coagulation-related and malignant diseases. Apart from the plant cysteine-proteinases bromelain and papain, the group comprises proteinases from animal organs such as trypsin and chymotrypsin. These enzymes offer a wide spectrum of therapeutic efficacies: they demonstrate, in vitro and in vivo, anti-edemateous, anti-inflammatory, antithrombotic, and fibrinolytic activities. They modulate the functions of adhesion molecules on blood and endothelial cells, and also regulate and activate various immune cells and their cytokine production. Indeed, these enzymes are used in the United States and Europe as an alternative or complementary medication to glucocorticoids, nonsteroidal antirheumatics, and immunomodulatory agents. Their very low toxicity makes them suitable tools for controlling chronic inflammatory diseases. For the therapy of inflammatory and malignant disorders, these proteinases are employed as additives for chemotherapy (to reduce side effects of drugs and to improve quality of life); additives for radiotherapy (to reduce inflammation and edema);

additives in surgery (to reduce edema and to improve wound healing); additives to prevent lymphedema by reducing lymph congestion, detritus, the viscosity of the extrudate and stimulation of phagocytosis of associated leukocytes. It should be added that clinical studies support these recommended indications only to a limited extent. Yet the large body of preclinical, pharmacological, and daily experience offers an important and worthwhile field for well-designed clinical studies to evaluate evidence-based medical indications.

Fig. 5. Bromelain

Pharmacology of bromelain: preclinical studies

Pharmacodynamics of bromelain from a variety of in vitro and animal experiments, mainly with rodents, as well as from clinical observations, based on uncontrolled and controlled studies, the general properties of bromelain may be summarized as follows [11–14].

- prevents edema formation and reduces existing edemas
- reduces the blood level of fibrinogen
- supports fibrinolysis
- activates plasmin

- prolongs the prothrombin and partial thromboplastin time (after relatively high doses)
- prevents aggregation of blood platelets
- prevents adhesion of platelets to endothelial cells of blood vessels – reduces the blood level of plasma kinins
- reduces the level of prostaglandin E2 and thromboxane A2 in exudates during acute inflammation
- acts as an antiinflammatory agent
- induces the secretion of interleukin (Il)-1, Il-6, Il-8, and tumor necrosis factor (TNF)-a from blood monocytes and granulocytes
- supports the oxidative burst and the cytotoxicity of granulocytes against tumor cells
- increases the tissue permeability of antibiotic drugs
- prevents metastases in a mouse model
- supports skin debridement of burns

Some effects of bromelain may result from its capacity to alter and modulate distinct cell surface structures by cleaving off peptides [15]. Thus, the bromelain-mediated modification of adhesion molecules on platelets and other normal and malignant tumor cells may inhibit their aggregation. The dissolution of cell membrane constituents and the effects on components of hemostatic processes may explain antiedemateous and fibrinolytic phenomena

Bacteria

Escherichia coli

Escherichia coli (E. coli) bacteria normally live in the intestines of healthy people and animals. Most types of *E. coli* are harmless or cause relatively brief diarrhea. But a few strains, such as *E. coli* O157:H7, can cause severe stomach cramps, bloody diarrhea, and vomiting. You may be exposed to *E. coli* from contaminated water or food especially raw vegetables and undercooked ground beef. Healthy adults usually recover from infection with *E. coli* O157:H7 within a week. Young

children and older adults have a greater risk of developing a life-threatening form of kidney failure.

Symptoms

Signs and symptoms of *E. coli* O157:H7 infection usually begin three or four days after exposure to the bacteria. But you may become ill as soon as one day after exposure to more than a week later. Signs and symptoms include:

- Diarrhea, which may range from mild and watery to severe and bloody Stomach cramping, pain, or tenderness
- Nausea and vomiting, in some people.

Causes

Only a few strains of *E. coli* trigger diarrhea. The *E. coli* O157:H7 strain belongs to a group of *E. coli* that produces a powerful toxin that damages the lining of the small intestine. This can cause bloody diarrhea. You develop an *E. coli* infection when you ingest this strain of bacteria.

Unlike many other disease-causing bacteria, *E. coli* can cause an infection even if you ingest only small amounts. Because of this, you can be sickened by *E. coli* from eating a slightly undercooked hamburger or from swallowing a mouthful of contaminated pool water. Potential sources of exposure include contaminated food or water and person-to-person contact.

Pathogenic activity

E. coli are pathogenic, meaning they can cause illness, either diarrhea or illness outside of the intestinal tract. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or contact with animals or persons.

Bacillus subtilis

Bacillus subtilis, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium, found in soil and the

gastrointestinal tract of ruminants, humans, and marine sponges. As a member of the genus *Bacillus, B*.

Bacillus species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, Gram-positive bacteria; in some species, cultures may turn Gram-negative with age. The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment.

Pathogenic activity

Bacillus subtilis is a Gram-positive, rod-shaped bacterium that forms heat-resistant, dormant spores. It is not pathogenic. It produces important commercial products.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a common encapsulated, Gram-negative, strict aerobic (although can grow anaerobically in the presence of nitrate), Rod-shaped bacterium that can cause disease in plants and animals, including humans.

Causes and Risk Factors of *Pseudomonas Aeruginosa*

P. aeruginosa is spread through improper hygiene, such as from the unclean hands of healthcare workers, or via contaminated medical equipment that wasn't fully sterilized. Common hospital-associated.

Disease caused by *Pseudomonas aeruginosa*

The most serious infections include malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia. The likelihood of recovery from *pseudomonas* infection is related to the severity of the patient's underlying disease process.

Pathogenic activity

The pathogenicity of *P. Aeruginosa* is largely caused by multiple bacterial virulence factors and genetic flexibility enabling it to survive in varied environments.

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive round-shaped bacterium, a member of the Bacillota, and is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin.

Staphylococcus aureus causes

It is the leading cause of skin and soft tissue infections such as abscesses (boils), furuncles, and cellulitis. Although most staph infections are not serious, *S. aureus* can cause serious infections such as bloodstream infections, pneumonia, or bone and joint infections.

Pathogenic activity

The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. The increase in the resistance of this virulent pathogen to antibacterial agents, coupled with its increasing prevalence as a nosocomial pathogen, is of major concern. *Staphylococcus aureus* is an extraordinarily versatile pathogen that can survive in hostile environmental conditions, colonize mucous membranes and skin, and can cause severe, nonpurulent, toxin-mediated disease or invasive pyogenic infections in humans.

Objectives

Objectives

- Biosynthesis of gold nanoparticles using bromelain as a caping and reducing agent.
- Characterization of synthesized BRN-GNPs and LVN-BRN-GNPs by UV-Vis Spectroscopy, DLS, Zeta potential, TEM, and FTIR.
- Bioconjugation of synthesized BRN-GNPs with Levofloxacin.
- Antibacterial activity of synthesized BRN-GNPs and LVN-BRN-GNPs.

Materials and methods

Materials and methods

Chemicals and reagents All the chemicals and solvents used in the present investigation were of analytical grade. Levofloxacin was procured from Yarrow Chem Products, Mumbai (India), and bromelain from Merck Chemicals Darmstadt, Germany.

Synthesis of 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 Bromelain (0.33mg/mL, 0.66mg/mL, 1.66mg/mL, and 3.33mg/mL) were incubated at 40^{o}C 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 bromelain 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 Bromelain 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.

Bioconjugation of gold nanoparticles to levofloxacin

Synthesized Au-NPs were bioconjugated to LVN by using 1-ethyl-3-(3-dimethyl- amino-propyl)-carbodiimide (EDC) as the activator (Timkovich, 1977). The 5 mM EDC was added to the 5 ml reaction mixture containing 250 µg of LVN, 250 µg of Au- NPs, and 50 mM HEPES buffer (HiMedia Laboratories, India) in aliquots within 3 h at 30 °C for the coupling process. The bioconjugates so formed were separated from unconjugated Au-NPs by passing the reaction mixture

through Biogel P-30 gel filtration column preequilibrated with 20 mM HEPES buffer (*pH* 6.0) containing 150 mM sodium chloride. The fractions were scanned between 200-900 nm and subsequently pooled. The pooled samples were then dia- lyzed against distilled water and used for fur- ther characterization (Khan et al., 2015b). The samples were removed at regular intervals and analyzed in UV-visible spectroscopy (Shimadzu dual-beam spectrophotometer, model UV-1601 PC, Japan) at a resolution of 1 nm to confirm the formation of 'BRN capped Au-NPs' and 'levofloxacin conjugated with BRN capped Au-NPs' (Au-BRN- LVN-NPs).)

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 FT-IR (Perkin Elmer Inc., Tres Cantos, Madrid) equipped with a Universal attenuated total reflectance sam- pling device and scanned at room tempera- ture in transmission mode over the wave number range of 4000-650 cm^{-1} at a resolu- tion of 4 cm^{-1}.

Evaluation of antibacterial efficacy of bio- conjugated levofloxacin, Au-BRN-LVN-NPs over pure levofloxacin alone

Determination of Antibacterial Activity by Disc Diffusion Method

The disc diffusion method was used to determine the antibacterial properties of BRN-NPs and Au-BRN-LVN-NPs. 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 R-AuNPs (10, 20, 40, 60, and 80 mg/mL) and various concentrations of crude RSALE (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^{0}C. Following that, the diameter of the inhibitory zone was determined.

Minimal Inhibitory Concentration (MIC) Determination

The antibacterial efficacy of bioconjugated LVN (Au-BRN-LVN-NPs) was determined by evaluating the minimum inhibitory concentration (MIC) of Au-BRNLvN-NPs and pure LVN against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. Au-BRN-LVN-NPs 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 Au-BRN-LVN-NPs 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 com- pared with a control. Autoclaved

water and ‘BRN capped Au-NPs’ was used as a negative control for each experiment. The procedure was repeated for the pure levofloxacin alone too.

Results

Results

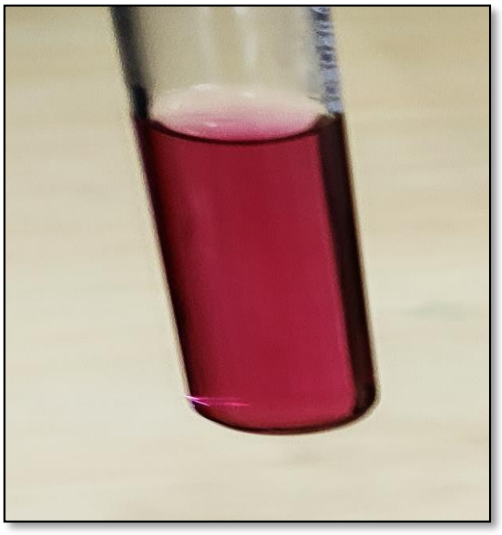

Fig. 6. Showing the ruby red color of synthesized BRN-GNPs.

Characterization of Gold Nanoparticles:

The plasmon band was observed for the wine red colloidal gold nanoparticles at 534 nm in the UV-visible spectrum. The colloidal solution containing 'BRN capped Au- NPs' had shown a very intense and characteristic pink-red color. Two peaks were observed in the Au-BRN-LVN-NPs spectrum at 538 nm during ultraviolet (UV) spectroscopy (Figure 7).

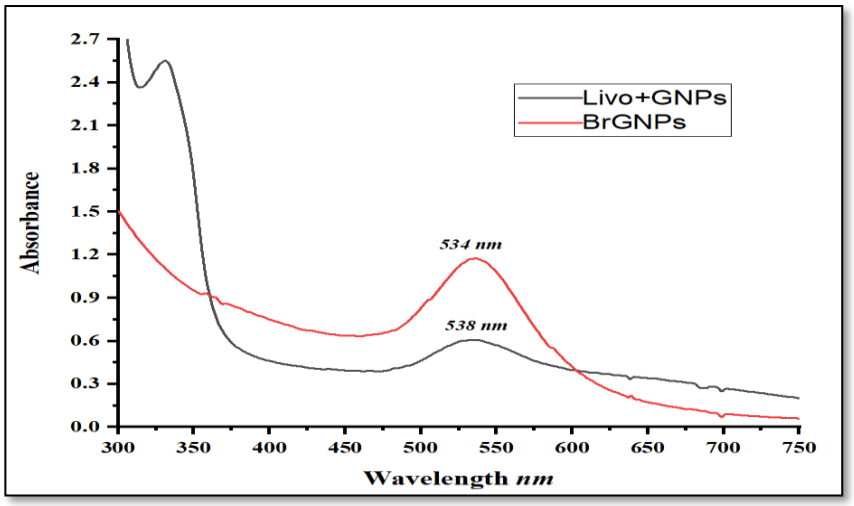

Fig. 7. Synthesized BRN-GNPs shows the absorbance at 534 nm, while bio conjugated LVN-BRN-GNPs shows the absorbance at 538nm.

Measurement of mean particle size and zeta potential of 'BRN capped Au-NPs' and Au- BRN-LVN-NPs

The diameter of Au-BRN-LVN-NPs was found to be 58.65 ± 2 nm while that of its unconjugated form 'BRN capped Au-NPs' was 38.11 ± 2 nm (Figure 3). The zeta poten- tial of Au-BRN-LVN-NPs was found to be -9.01 mV and that of its unconjugated form 'BRN capped Au-NPs' was -13.8 mV (Figure 4).

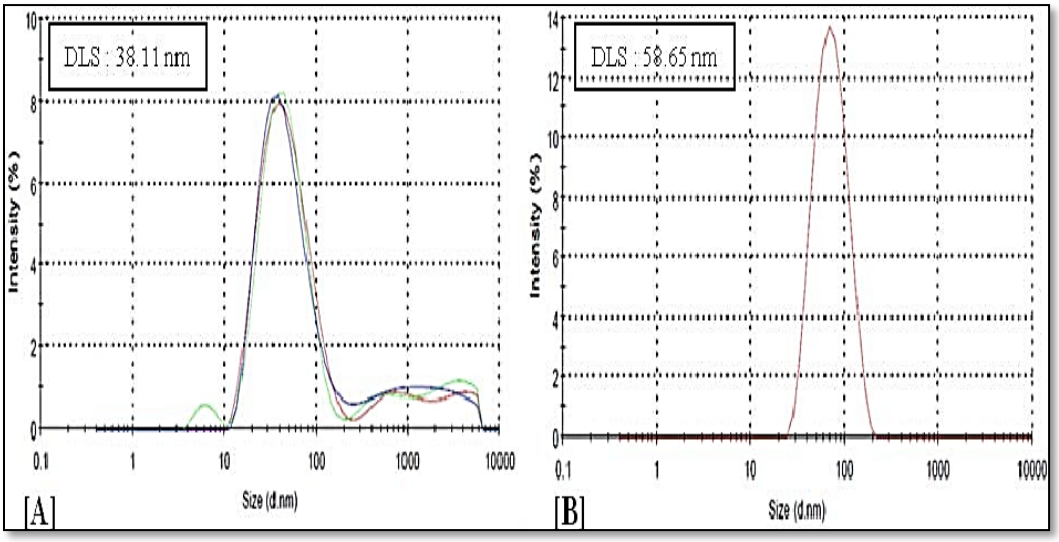

Fig. 8. Dynamic light scattering (DLS) analysis of [A] BRN capped gold nanoparticles (BRN capped GNPs), and [B] BRN capped gold nanoparticles conjugated with levofloxacin (Au-BRN-LVN-NPs).

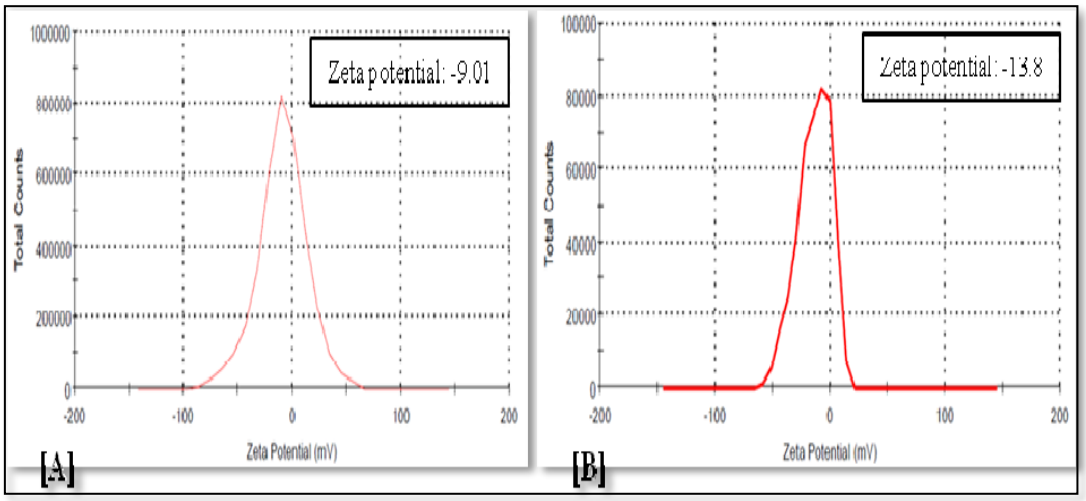

Fig. 9. Zetasizer analysis of [A] BRN capped gold nanoparticles conjugated with levofloxacin (Au-BRN-LVN-NPs), and [B] BRN capped gold nanoparticles (BRN capped Au-NPs).

Transmission electron microscopy of 'BRN capped Au-NPs' and Au-BRN-LVN-NPs

Surface morphological study of 'BRN capped Au-NPs' and Au-BRN-LVN-NPs showed the formation of spherical particles with the particle size of 11.4 nm of 'BRN capped Au-NPs'. The particle size of Au- BRN-LVN-NPs was

found to be ~13.2 nm and it retained the spherical shape (Figure 5).

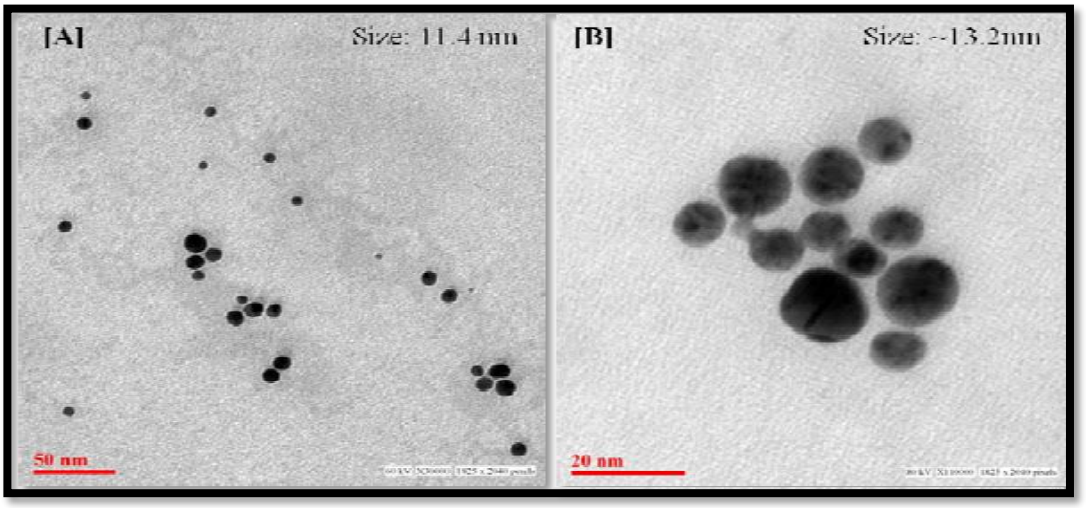

Fig. 10. Transmission electron microscopic (TEM) images of [A] BRN capped gold nanoparticles (BRN capped Au-NPs), and [B] BRN capped gold nanoparticles conjugated with levofloxacin (Au- BRN-LVN-NPs).

***Fourier transform infrared spectroscopy of* 'BRN capped Au-NPs' and Au-BRN-LVN- NPs**

FTIR spectrum of 'BRN capped Au-NPs' is shown in Figure 6(A) which has showed the characteristic C–N stretch vibration fre- quencies of monoalkyl guanidinium assigned to the observed IR bands at 1641, 1425-1256, and 1100 cm^{-1}. The band at 1760-1670 cm^{-1}(s) showed the presence of C = O groups (amides at ~1640cm^{-1}). FTIR spectrum of Au-BRN-LVN-NPs is shown in Figure 6(B) which has shown the presence of a peak at 1634.24 cm^{-1} due to the presence of C = O (str) of the amide I linkage and the presence of a peak at 3338cm^{-1} due OH stretching vibrations.

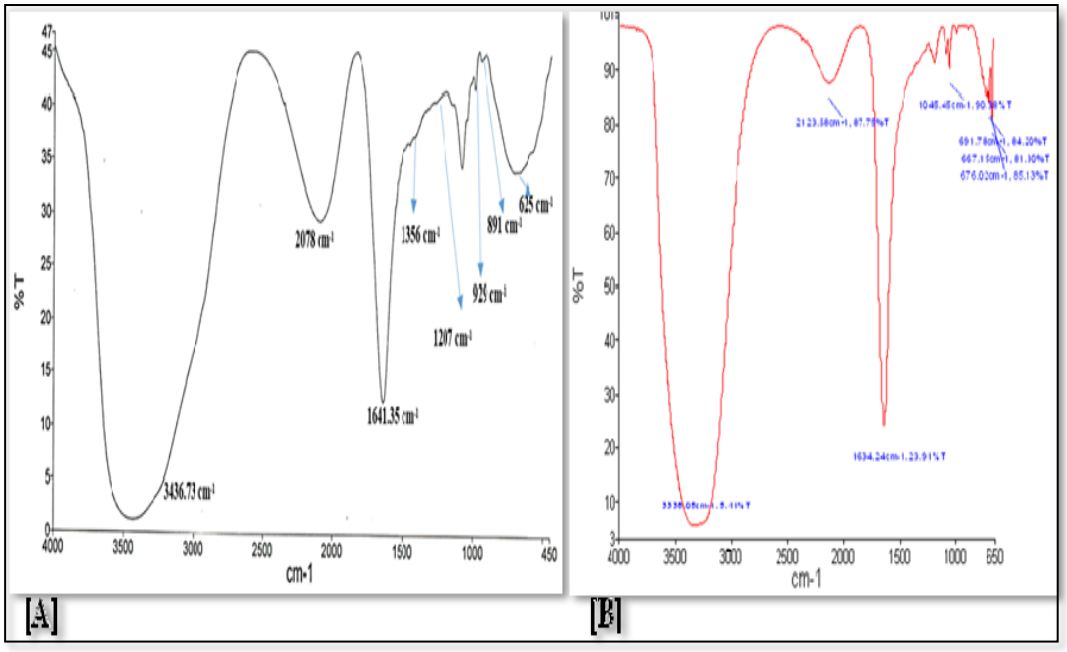

Fig. 11. Fourier transform infrared (FTIR) spectra of [A] Pure levofloxacin, and [B] BRN capped gold nanoparticles conjugated with levofloxacin (Au-BRN-LVN-NPs).

Minimal Inhibitory Concentration (MIC) Determination

The MIC is the lowest concentration of BRN capped gold nanoparticles and Au-BRN-LVN-NPs that completely inhibits bacterial growth, and MIC50 is the concentration of plant extract and R-AgNPs that inhibit 50% of the bacterial population. The MIC50 of BRN capped gold nanoparticles and Au-BRN-LVN-NPs against several Gram-negative and Gram-positive bacterial strains were recorded. However, levofloxacin was used as a standard antibiotic during the experiment.

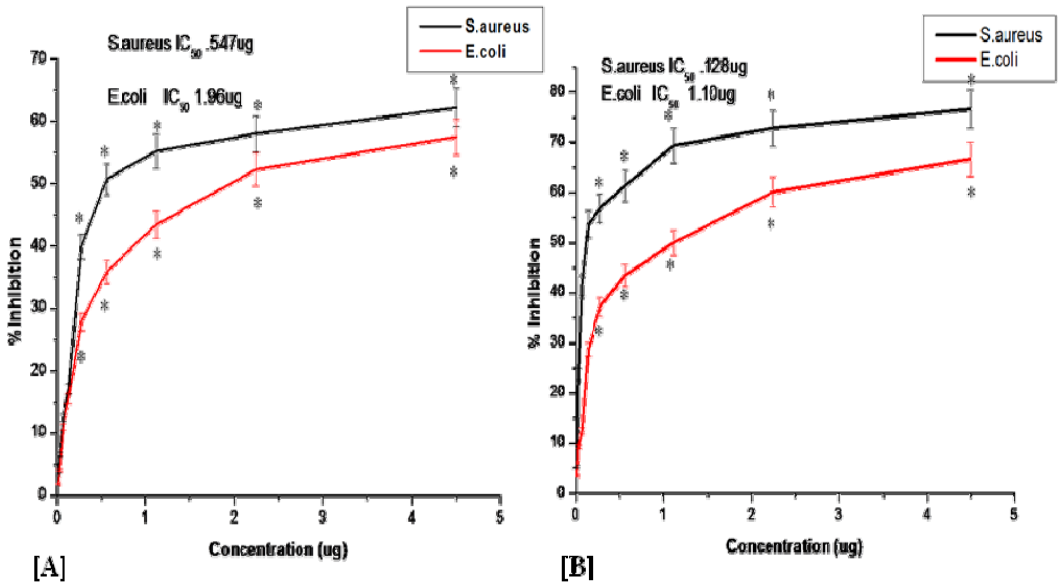

Fig. 12. The plot of percent inhibition of bacterial strains (S. Aureus, E. Coli) versus different concentrations of [A] pure levofloxacin, and [B] BRN capped gold nanoparticles conjugated with levofloxacin (Au-BRN-LVN-NPs).

Discussion and conclusion

Discussion

The plasmon band was observed for the wine red colloidal Au-NPs at 534 nm in the UV-visible spectrum which is characteristic of Au-NPs. The colloidal solution containing 'BRN capped Au-NPs' had shown a very intense and characteristic pink-red color confirming the formation of Au-NPs. However, two peaks were observed during UV-Visible spectral analysis of Au- BRN-LVN-NPs one at 307 nm due to aromatic acid transitions of LVN and another one at 538 nm due to redshift. This red shift in the plasmon band suggests a different dielectric environment which confers conjugation of LVN with 'BRN capped Au-NPs'. DLS provides hydrodynamic diameter which includes an inorganic core and the thin electric dipole layer of the solvent that adheres to the surface of the nanoparticles. The diameter of Au- BRN-LVN-NPs was found to be 58.65 ± 2 nm while that of its unconjugated form 'BRN capped Au-NPs' 38.11 ± 2 nm proving that bioconjugation of LVN has taken place to form Au-BRN-LVN-NPs. Zeta potential indicates the stability of a colloidal system against agglomeration. The zeta potential of Au-BRN-LVN-NPs was found to be -9.01 mV which is well in range to prevent agglomeration as compared to its unconjugated form 'BRN capped Au-NPs' which was found to be -13.8 mV supporting the absence of nanoparticle aggregation after conjugation. This change in zeta potential may be ascribed to the decrease in the number of carboxylic groups which are involved in the conjugation of LVN.

During a surface morphological study by TEM, the particle size of Au-BRN-LVN-NPs was found to be ~13.2 nm while that of 'BRN capped Au-NPs' was 11.4 nm. It is in coherence with the DLS and further corroborates bioconjugation of LVN with 'BRN capped Au-NPs' and supports the absence of aggregation of nanoparticles after bioconjugation.

From the results of the study, it was found that the percent inhibition of the bacteria *S. aureus* and *E. coli* were increasing with increasing doses of pure levofloxacin and Au-BRN-LVN-NPs suggesting their antibacterial activity against *S. aureus* and *E. coli* in a dose-dependent manner. The functionalized nanoparticles showed superior antibacterial activity

compared to pure LVN at a similar concentration. From the results of the study, it is also clear that there was a reduction in the IC_{50} values from 0.547 µg/ml and 1.96 µg/ml of pure LVN to 0.128 µg/ml and 1.10 µg/ml of Au-BRN-LVN-NPs against *S. aureus* and *E. coli* respectively may be attributed to disruptions in the cell wall and membrane structures of bacteria by it leading to loss of cellular integrity and finally cell death. The IC_{50} value for Au-BRN-LVN-NPs was less against *S. aureus* as compared to that of *E. coli* suggesting that Au-BRN-LVN-NPs are more effective against *S. aureus* bacterium. On the other hand, Au-BRN-LVN-NPs are also able to generate reactive oxygen species which may play a synergistic role in killing bacteria. The probable reasons for the superior antibacterial activity of Au-BRN-LVN-NPs may be its superior stability and transport of a huge number of LVN molecules into a highly localized area at the site of particle-bacterium contact.

Conclusions

This study provides a novel approach for the synthesis of GNPs using bromelain as a reducing as well as capping agent. We found that when the concentration of bromelain as well as incubation temperature increases, the size of GNPs also increases. While 0.33mg/mL concentration of bromelain at 40ºC was found to be most effective in producing GNPs of smaller size with higher monodispersed and higher zeta potential. In addition, GNPs synthesized by this method are highly stable and can be stored at room temperature for months. The results suggest that bromelain capped gold nanoparticles can be used as effective carriers for levofloxacin molecules. The bioconjugated bromelain capped gold nanoparticles exhibited superior antibacterial activity against both Gram-negative and Gram-positive bacteria compared to pure levofloxacin which may be due to its superior stability and transport of a huge number of LVN molecules into a highly localized area at the site of particle-bacterium contact.

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