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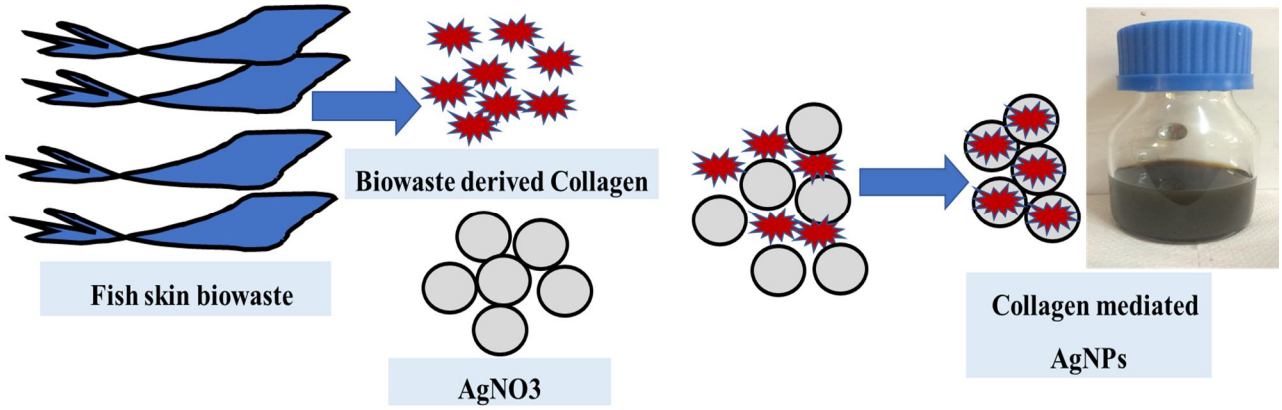
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Facile Synthesis and Characterization of Eco-friendly Collagen based silver Nanoparticles and its Biological Activities

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Abstract: ***For the past few decades, the natural polymer Type-I Collagen becomes more attention due to its biomedical applications like scaffold preparation, tissue engineering and nanomaterial production. Fish skin bio waste has been recognized as economical, widely accepted alternative to Collagen derived sources. This present study deals with the effective use of underutilized bio waste resource. Collagen was extracted and confirmed as Type-I Collagen from the fish skin bio waste. After that the AgNPs were synthesized from the Collagen. The Protein profile of extracted Collagen was analysed in the SDS-PAGE confirms the type of Collagen. The morphology of type-I collagen was revealed under SEM. The silver nanoparticle was synthesized by using silver nitrate as a reducing and Capping agent for the Collagen along with sodium borohydride ($NaBH_4$). The results of UV-Vis Spectroscopy confirmed the formation of AgNPs by showing a sharp absorption peak at 418nm. FTIR spectral peak shows the presence of alkynes, carboxylic acids and anhydrides compound which binds with Ag and also reduces and capped on Ag ions for the conversion of Ag+ into AgNPs. The DLS and zeta potential results positively shows the stability of synthesized nanoparticles. The morphology of the resulted AgNPs exhibited spherical shape with less than 50nm size under SEM. The antimicrobial activity possess the suitable material for the biomedical applications, the Cytotoxicity of the Collagen derived AgNPs were evaluated under the MG-63 cell line and the toxicity results having an Dose dependent manner. Key words: Collagen , AgNPs, Characterizations , Antimicrobial activity, MTT.***

Graphical Abstract



I. INTRODUCTION

Collagen is the most abundant protein which contains about 30% of total protein and 6% of animal body weight [1] .Collagen scaffold has been widely used in biological experiments for introducing chemical and pharmaceutical substances in Nanotechnology. Collagen is the main structural protein in connective, skin, and osteoarticular tissues of animal organisms The characteristic rope structure of collagen fibrils are organized by the union of basic units of collagen, formed by three subunit protein chains which are arranged as a triple helix and form large fibril stretches, which confer this protein its essential role in support tissues .Collagen synthesis is essential for maintaining different body structures, such as skin, bones, and cartilage. Fibroblast cells are the main responsible for the synthesis of extracellular matrix (ECM) which contains, among other proteins, collagen.

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Skin appearance depends on the collagen skeleton, for instance, wrinkle formation has been associated to decreased collagen synthesis and increased collagenase activity. Dermal fibroblasts usually present a higher collagen synthesis rate than other tissues, since collagen plays an important role in the maintenance of skin integrity Some approaches to prevent or retard the apparition of wrinkles in humans are to use cosmetics or to intake nutritional supplements which help to maintain collagen molecules in the skin at optimum.[2-3] Besides, sustainable socioeconomic and environmental principles promote the integral use of natural resources; this also applies to the fishery industry, which may represent an important source of valuable raw materials which are not always fully used. These raw materials, such as unused skins, are suitable for obtaining different products, such as collagen, gelatin, and collagen hydrolysates [4-5]. In this regard, the processes and technologies for the recovery of fish waste and its sustainable conversion to highly valued bio (nano) materials are more important .

The current scenario of renewable origin and 'made to be made again' concept are most welcome in this pandemic situation. This not only has a positive impact on the environment, but builds long-term consistency. However, collagen stabilized with metal nanoparticles is attractive for biocompatibility and antibacterial properties. Metal nanoparticle, especially those made of noble metals, show excellent properties for biotechnology applications. In particular, AgNPs have established a broad range of applications in the majority of biomedical studies , due to their antibacterial ability and selective toxicity to microorganisms . In addition, AgNPs are widely used in various medical and industrial fields for venous catheters coating; vascular prostheses manufacturing; wound dressing manufacturing treatment for chronic wounds and ulcers or as a constituent incorporated into cement for the realignment of bone fractures in to water purification filter and into wall paint for providing an aseptic environment to hospital patients. [6]

The unique properties of metal nanoparticles involve enhanced mechanical stability and resistance against enzymatic degradation when incorporated into tissue scaffolds, easy incorporation of antibodies, growth factors and peptides at the surface of metal nanoparticles, enhanced biocompatibility, anti-inflammatory and antimicrobial properties. So these metallic nanoparticles such as AgNPs might be used in gel form by incorporated with the collagen scaffolds for many biomedical applications. Thus, nanocomposite hydrogel containing metal or metal-oxide nanoparticles are extensively used as imaging agents, drug delivery systems, conductive scaffolds, switchable electronics, actuators, and sensors [7]. The silver nanoparticles have a large area of interest as they have a large number of application such as nonlinear optics, spectrally selective coating for solar energy absorption, bio-labelling, intercalation materials for electrical batteries as optical receptors, catalyst in chemical reactions, antibacterial materials, chemically stable materials and good electrical conductors. They are ahead of time the interest of researchers for their novel method for synthesis of silver nanoparticles. Silver is well known for possessing an inhibitory result toward many bacterial strains and microorganisms commonly present in medical and industrial processes . The present study deals with the rapid synthesize of Silver nanoparticles from collagen extracted from Bio-wastes. The synthesized silver nanoparticles confirmed through UV-visible spectrophotometer, size and stability confirmed through DLS & zeta potential. The FT-IR spectral values shows the components present in the silver nanoparticles , The morphology of the sample confirmed through SEM , And the antimicrobial activity carried out by agar well diffusion method the cytotoxicity were performed on the MG-63 Cells .

II. MATERIALS AND METHODS

A. *Materials*

All used chemicals were of analytical grade and purchased from Sisco Research Laboratories (SRL) ,SIGMA unless otherwise specified. Collagen type I was enzymatically extracted in our lab from fish skin, purified by NaCl precipitation and dialyzed against distilled water and lyophilized, as previously described (Nagai *et al.*, 1999) [8]. The obtained gel consisted of nondenatured molecules of collagen with triple helix conformation.

B. *Methods*

1) *Preparation of Biowastes Collagen*

Collagen was extracted by acid solubilized and pepsin solubilized methods, purified and lyophilized (Nagai & Suzuki *et al.,* 2002) [9] .The entire experiment was done at 4ºC. The wastes of Fish (Skin) was extracted with 0.1 N NaOH to remove non-collagenous proteins, then washed with distilled water and lyophilized. The skin was defatted by soaking in 10% butyl alcohol for 1 day, washed with distilled water and lyophilized. The insoluble matter was extracted with 0.5 M acetic acid for 3 days, and the extract centrifuged at 20,000 x g for 1 h. The skin contents were re-extracted with the same solution for 2 days, and the extract was centrifuged in the same conditions. Each viscous solution was mixed and salted out by adding NaCl to a final concentration of 0.9

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M, followed by precipitation of the collagen by addition of NaCl (final concentration of 2.6 M) at neutral pH (in 0.05 M Tris ± HCl, pH 7.5). The resultant precipitate was obtained by centrifugation at 20,000 x g for 1 h and dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water, and then lyophilized .The extracted collagen was further taken for characterisation. Protein patterns of collagen samples were analysed with SDS-PAGE following the method as described by Laemmli (1970).Viscosity of the collagen sample (1g in 10 mL) was determined by rotary viscometer test method, using Viscometer (Model Brookfield)). In this test method, the solution was placed in a glass tube, housed in an insulated block at room temperature. A metal spindle was then rotated in the solution at 100 rpm, and the torque required to rotate the spindle is measured. Based on the internal resistance to rotation provided by the shear stress of the solution, the solution's absolute viscosity was determined. Absolute viscosity is represented in centipoise (cP).Viscosity of the collagen sample (1g in 10 mL) was determined by rotary viscometer test method, using Viscometer (Brookefield Digital).

2) Synthesis of AgNPs from Biowastes Collagen

Silver nanoparticles synthesized from Collagen ,To carry out the synthesis of silver nanoparticles, a solution of silver nitrate at 0.4M was prepared, and Bio-wastes collagen isolated from fish skin sample was prepared at a concentration of mg/ml, and a solution of Sodium borohydride (NaBH4) at 3.78 mg/ml was prepared using ultrapure water at 4°C. The $AgNO_3$ solution was added to the collagen in the same volume and agitated for 10 minutes to homogenise it. The $NaBH_4$ solution was jetted into the homogenised Col-AgNO3 solution. This solution was stirred for 10 minutes to homogenise. Following that, the reaction mixture was centrifuged at 3600 rpm for 15 minutes, and the final solution supernatants were removed.

III. CHARACTERIZATION OF SYNTHESIZED SILVER NANOPARTICLES

The bio-reductive synthesis of AgNPs was characterized using a UV-Vis spectrophotometer . The UV-Vis spectra were recorded between 200 and 800 nm as a function of temperature for the bio-reductive and capping property of Bio-wastes collagen sample. The presence of functional groups in Col-AgNPs was identified by Shimadzu -8400 FTIR Spectrometer using KBr pellet technique. Scanning electron microscope-energy-dispersive spectra (SEM-EDX) analysis was carried out using Hitachi SN-344, Japan. Thin films of the sample were prepared on a carbon-coated copper grid by just dropping a very small amount of the sample on the grid. The samples were performed to determine the morphology of the Nanoparticles. The particle size and stability measured by DLS and ZETA potential .collagen conformed through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

IV. RESULTS AND DISCUSSION

A. Viscosity of the collagen and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)-Results

The acid-solubilized Collagen and Pepsin-solubilized Collagen were recovered from biowaste with yields of 8.2 percent (w/v) (wet weight basis) and 17.5 percent (w/v) (wet weight basis), respectively. SDS-PAGE was used to examine the protein profile of the isolated collagen, with commercially available BSA as a control [11]. The SDS-PAGE pattern showed that fish skin Collagen had a doublet pattern for α1 and α2 chains at corresponding to 97 kDa, and 116 kDa, respectively, and a β chain (150 kDa). The density for α1 was twice as much as α2, (Figure 4.1) Based on the patterns of α1 and α2, it suggested that the fish collagen has a composition of (α1), α2 heterotrimer, a type I collagen. This explained that the molecular weight of α2 chain was much smaller than that of α1 chain. The viscosity of collagen solution was 36 cP .

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins can also be separated based on their molecular size and charge, because these factors determine how fast they pass through the gel. SDSPAGE involves the use of sodium dodecyl sulfate (SDS) detergent to denature proteins. And also use electric current to pull them through the polyacrylamide gel, this process is called polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gel is made by polymerizing acrylamide monomer and appropriate crosslinking agent monomer. The most commonly used crosslinking agent is N, N-methylene bis-acrylamide. By linking together randomly growing polyacrylamide chains, a three-dimensional network is formed. The acrylamide concentration and crosslinking agent used determine the length and degree of crosslinking of the polymer, thereby affecting the physical properties of the polymer. Gel like density, elasticity, brittleness and most importantly pore size. Macro-porous gels can allow giant proteins to work faster, while high-density gels can slow down large proteins by better separating smaller molecules. The best way to analyse a single protein without knowing its size range in advance is to use a trial-and-error method, which involves testing different concentrations of gel [12-13]. The SDS-PAGE pattern showed that fish skin collagen had a doublet pattern for α1 and α2 chains at corresponding to 97 kDa, and 116 kDa, respectively, and a β chain (150 kDa).

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The density for α1 was twice as much as α2, based on the patterns of α1 and α2, it suggested that the fish collagen has a composition of (α1) 2α2 heterotrimer, a type I collagen. This explained that the molecular weight of α2 chain was much smaller than that of α1 chain.

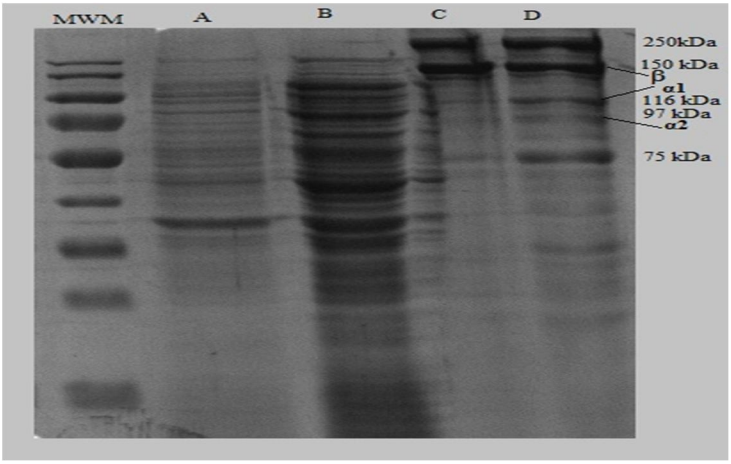


Figure 4.1 SDS-PAGE gel showing the different types of collagens, (A) BSA bovine serum albumin (B) Collagen peptide (C) ASC and (D) PSC, MWM-Protein marker10-250 KDa.

B. Scanning Electron Microscopy analysis of fish collagen

SEM pictures of isolated Collagen from stingray skin are shown in Figure 4.2. Under various magnifications, the structure and organisation of both extracted collagens were clearly visible. The illustration depicts the creation of separate fibres of uniform thickness. The diameter of the fibre was discovered to be around 878 nm. Furthermore, Collagens uniform and regular network structures make them suitable for drug delivery and wound dressing applications. As a result, the microscopic structure of fish skin collagens may provide a suitable biomaterial for a variety of biomedical applications [14].

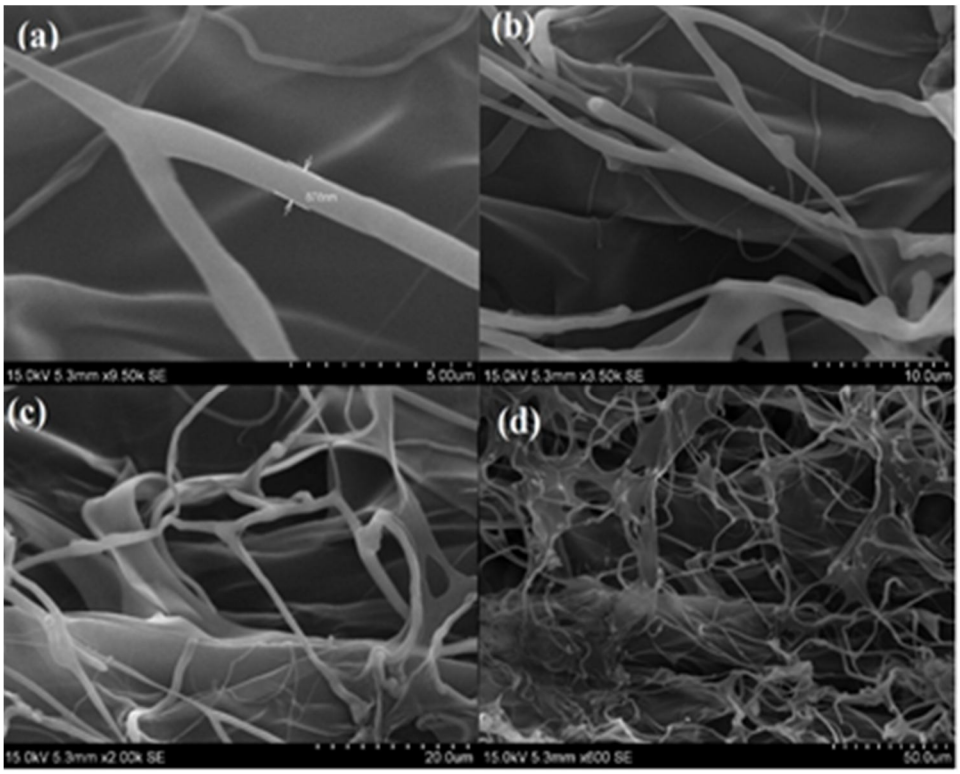


Figure 4.2 SEM micrographs of Collagen extricated from bio waste at various magnifications

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C. UV-Visible Spectrophotometer Analysis

The synthesized AgNPs were preliminary characterized by UV-visible-spectroscopy. A strong and broad surface plasmon peak located at 418 nm was observed and it showed the indication Conformation of AgNPs. The absorption band observed at 418 nm is the characteristic peak of Ag nanoparticles . The formation of AgNPs during the reduction process is indicated by the colourless solution turning into a visually identifiable dark brown. The colour change and the appearance of absorption peaks correspond to surface plasmon resonance (SPR) in the UV-Vis spectrum. The confirmation of the synthesis of silver nanoparticles provides a qualitative assessment of the size, shape, and yield, as well as the agglomeration state of suspended AgNPs due to its unique optical properties. The characteristic peak of the SPR band of silver nanoparticles was observed in the range of 418 nm. Figure 4.3 shows the UV-Vis spectrum of silver nanoparticles synthesized from collagen samples and the similar silver nanoparticles UV-Visible absorption were found in the [14-15].

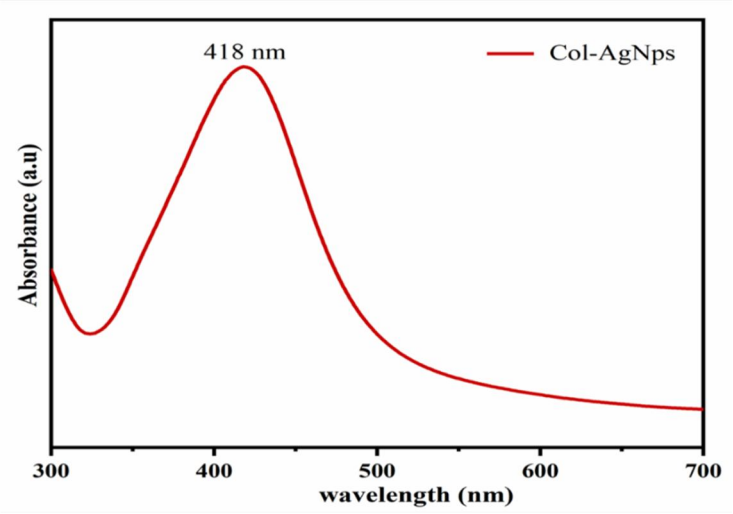


Figure.4.3 Shows the UV-Visible Absorbance of AgNPs synthesized from Collagen.

D. Fourier Transform Infrared Spectroscopy [FT-IR]

The FT-IR measurements used to identify potential molecules present and possible for the formation of Collagen-based silver nanoparticles. FTIR results show that the main absorption bonds in the Collagen spectrum are located at 710, 1642, 2138, and 3356 cm^{-1} . After reacting with Silver ion solution , the peak shifts to the side with the longest wavelength, such as , 663, 883, 1013, 1265, 1395, 1725, 2618, 3201 cm-^{1}. Due to the strong bending of the aromatic OH compound, the peak at 613 cm^{-1} in the synthesized AgNPs rises to 710 cm^{-1}. Since the peak at 1725 cm^{-1} is replaced by an ester from the weak olefin compound at 1642 cm^{-1}, the synthesized AgNPs has changed. The change of 2618 cm^{-1} is due to the large amount of carboxylic acid forming Ag^{+} ions the similar Functional groups response for the bio reduction capping were demonstrated in the [16].

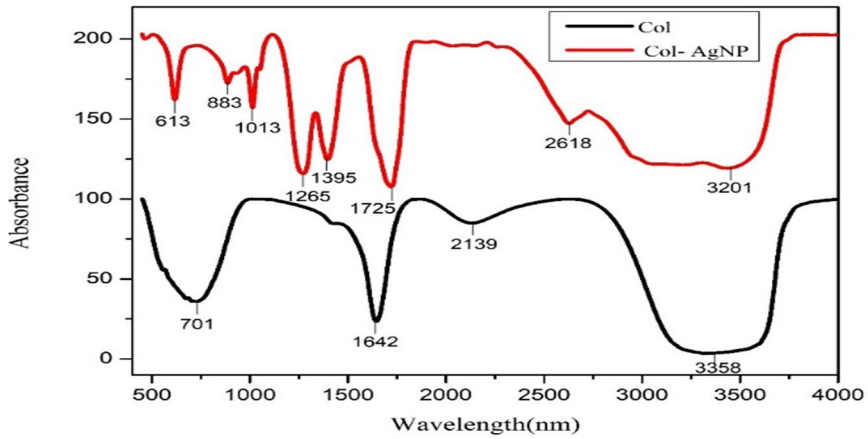


Figure 4.4 FT-IR results of Acid Soluble collagen and AgNPs Synthesized from Collagen

E. Dynamic Light Scattering & ZETA potential analysis

The hydrodynamic particle size and surface charge potential value of AgNPs were further measured using the DLS and ZETA potential measurement. The observed plot depicts the formed AgNPs were highly monodispersed, and the average size of the AgNPs was less than 80 nm, while the size of the highest percentage of AgNPs is 100nm. The zeta potential value -10.8mV with the polydispersity index 0.07 which indicating that they in monodisperse in nature and AgNPs were stable and suitable material for the biomedical applications the similar DLS and ZETA potential spectrum were found in the [17].

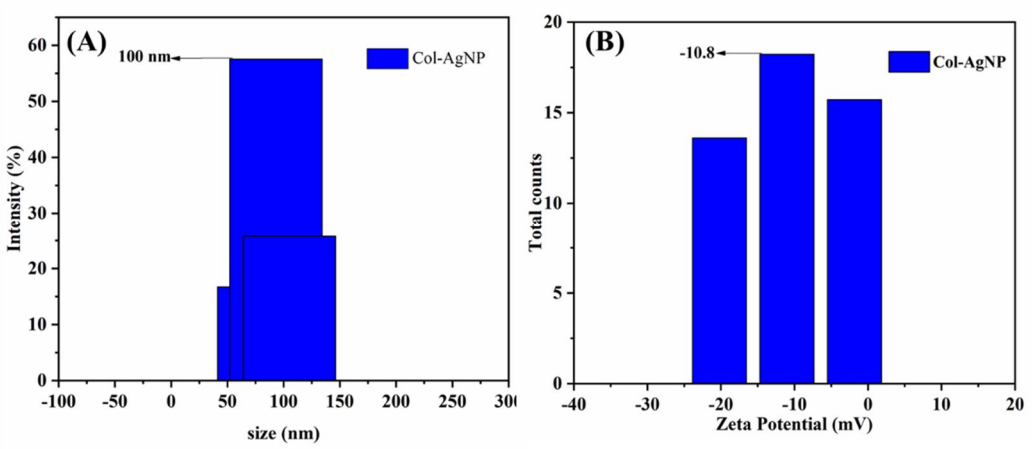


Figure 4.5 DLS and ZETA potential images of Collagen-AgNps

F. Scanning Electron Microscopy analysis of synthesized silver nanoparticles

Scanning electron microscopy was employed to visualise the size and shape of AgNPs. The SEM image reveals the size distribution of AgNPs ranges from 35-50 nm. SEM image showed well dispersed small and large sized, non- agglomerated and irregularly shaped silver nanoparticles. The EDAX involves Qualitative and Quantitative analysis of elements present in the nanoparticles and it shows no other impurities were presented. The elemental analysis shows count at 3keV confirm presence of silver nanoparticles, similar work demonstrated on [18-19] shows the similar spherical shaped Silver nanoparticles.

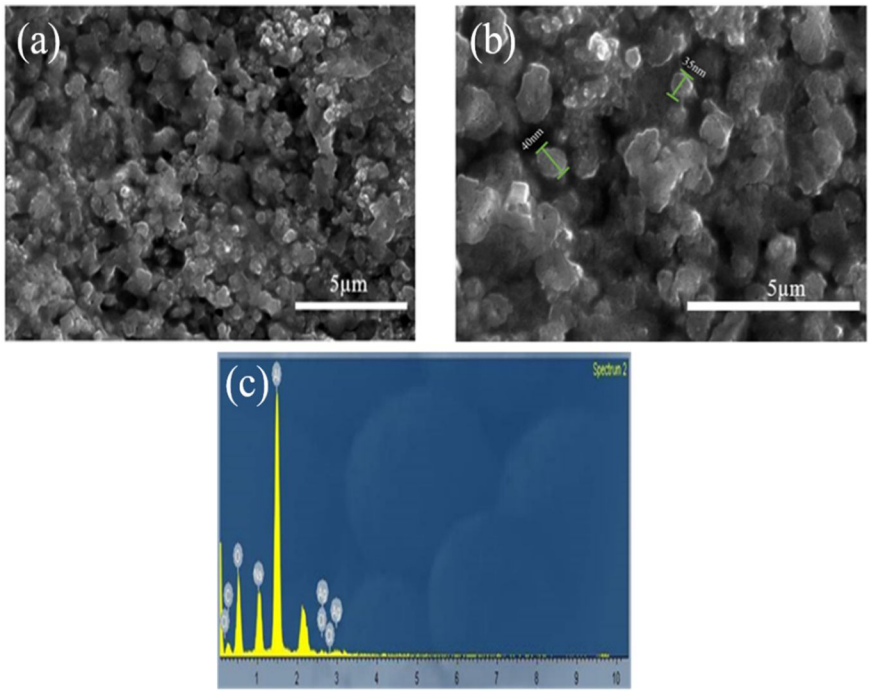


Figure 4.6 (a, b) SEM images and (c) EDS images of AgNPs synthesized from Collagen

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G. Cytotoxicity results of Collagen mediated AgNPs on MG-63 cancer cell line

The effect of collagen mediated AgNPs on cell viability of osteoblastic MG63 cells have been determined using the colorimetric MTT assay. The nanoparticles displayed growth inhibition properties and reduced the MG-63 cell viability with IC_{50} values of 42.2 ±3.9 µg/ml. The results obtained from MTT assay revealed that the treatment of AgNPs showed dose- and time- dependent decrease in cell viability in both cell lines compared to control group. Here, the control (untreated) group is referred as 100% of viable cells. Data are presented as the mean ±standard deviation. The figure 4.8 shows the morphological changes in the cell line MG63 cells.

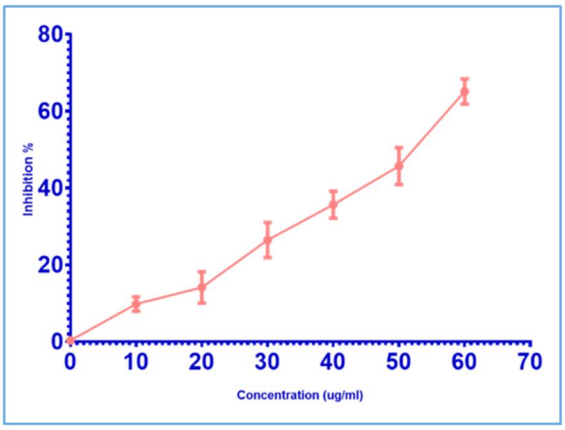


Figure 4.7: MTT assay showing inhibitory effect of different concentration of Collagen -AgNPs with various concentrations (10-60µg/ml) for 24 h on proliferation of bone cancer cells

Photomicrograph (20x) represents morphological changes in MG-63 cells such as shrinkage, detachment, membrane blabbing and distorted shape induced by sample Collagen -AgNPs treatment (40 & 50 µg/ml for 24 h) as compared with control. Control (untreated) showed normal intact cell morphology and their images were captured by light microscope. Collagen -AgNPs showed dose-dependent cytotoxicity against MG63 osteoblast cells through activation of the lactate dehydrogenase (LDH), reactive oxygen species (ROS) generation, eventually leading to cytotoxicity the similar cytotoxicity of the Collagen mediated silver nanoparticles are developed and discussed in the previous work [20-23] and the results suggests more over similar to out cytotoxicity of the collagen mediated silver nanoparticles .

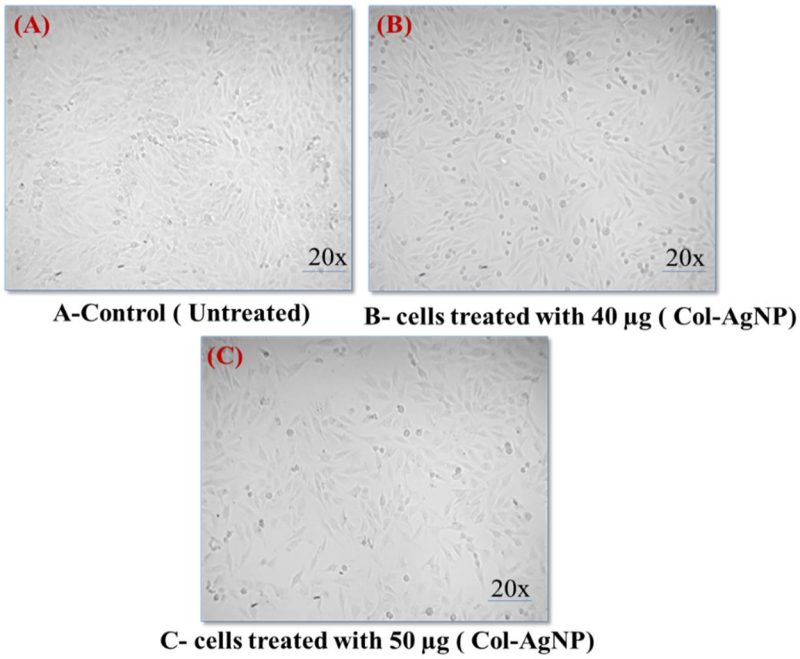


Figure 4.8: Morphological changes in Control (A untreated) and samples of B and C Collagen -AgNPs treated with bone cancer MG-63 cells for 24 h.

H. Anti -Microbial activity of AgNPs

The bacterial colonies [*Staphylococcus aureus* [ATCC7443] *Escherichia coli* [ATCC443] *Pseudomonas aeruginosa* [ATCC3163] had been grown in the medium of Nutrient broth. The antibacterial undertaking used to be examined the usage of agar well diffusion method. One hundred micro litters of the suspended culture were spread uniformly on Nutrient agar plates. The agar was then gently punctured with the help of cork borer to make wells, and 20,40,60,80,100 µL of the synthesized AgNPs used to be added in the respective well. The plates were incubated at room temperature for 24 hrs. The antibacterial exercise used to be assayed with the aid of measuring the diameter of the inhibition quarter shaped around the well ,the antimicrobial activity carried out by the [24,26]shows the similar results. The antimicrobial endeavour of synthesized AgNPs was carried out by agar Disk well diffusion technique and the maximum zone of inhibition was discovered at a 100µl, in *Staphylococcus aureus*, most inhibition observed at 80µl. The microbial synthesis of silver nanoparticles has comparatively low quarter of inhibition examine with collagen synthesis of silver nanoparticles.

Table 1.Antimicrobial Activity MIC of Collagen mediated AgNps

S. No	Name of the Organism	Zone of inhibition [mm in diameter]				
		20µl	40µl	60µl	80µl	100µl
1.	*Escherichia coli [MTCC443]*	4	7	15	17	21
2.	*Pseudomonas spp [MTCC3163]*	12	13	16	18	20
3.	*Staphylococcus aureus [MTCC7443]*	2	5	7	11	-

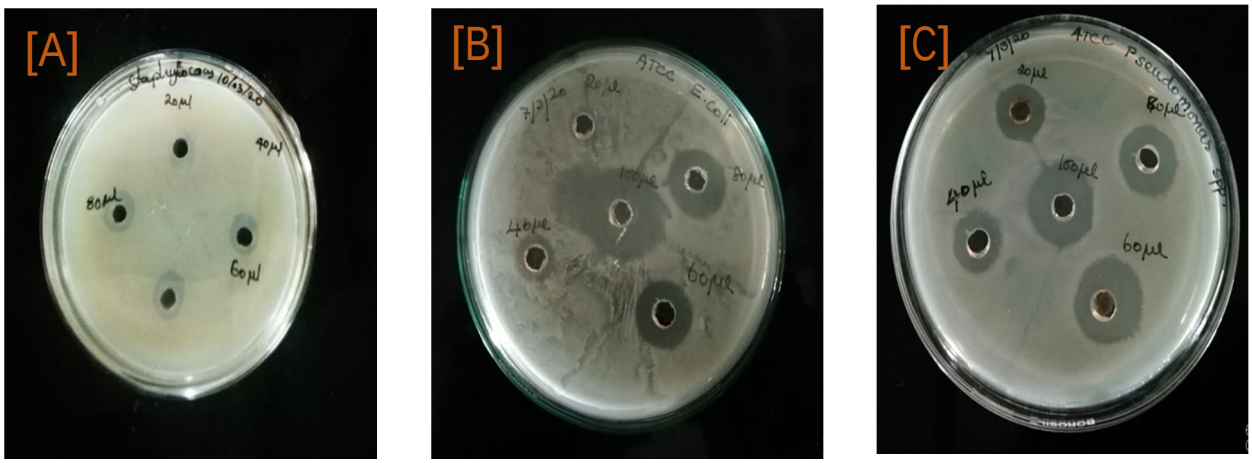


Figure 4.7 Antimicrobial effect of Collagen-AgNPs on different organisms, [A] *Staphylococcus aureus* [B] *Escherichia coli* [C] *Pseudomonas aeruginosa*

V. CONCLUSION

In the present study, we demonstrated the synthesis of an AgNPs solution stabilized with collagen by using NaBH4 as a reducing agent. The synthesized AgNPs- col from collagen that resulted have a number of positive qualities, including the conformation of silver nanoparticles can be seen using a UV-Visible spectrophotometer, and the structural location of AgNPs-col confirmed.. The fish collagen is confirmed by SEM and SDS PAGE, and the size and surface morphology of the AgNPs-col are shown by scanning electron microscopy. Fourier Transform Infrared Spectroscopy shows the possible molecules present in the AgNPs-cols, DLS shows the diameter, positive to zeta potential, antimicrobial activity, it is found that the activity against bacterium is facilitated by the electrostatic interaction between the positively charged AgNPs-col and the negatively charged bacterial membranes. Collagen0-AgNPs showed dose-dependent cytotoxicity against MG63 osteoblast cells and showed its anti-cancer activity. Compare with other antimicrobial studies, the AgNPs-col antimicrobial activity have high zone of inhibition. Collagen mediated AgNPs showed dose-dependent cytotoxicity against MG-63 cells and showed its anti-cancer activity ,from the Cytotoxicity images its clearly suggest that the Collagen mediated AgNps having an Dose dependent manner with increasing concentration of toxicity against the MG-63 Cells.

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B. Conflicts of Work

- The authors declare that there is no conflict of work.
- The authors declare that they have no known competing financial interests.
- The authors declare there is no conflict of interest on the Ethical Clearances.

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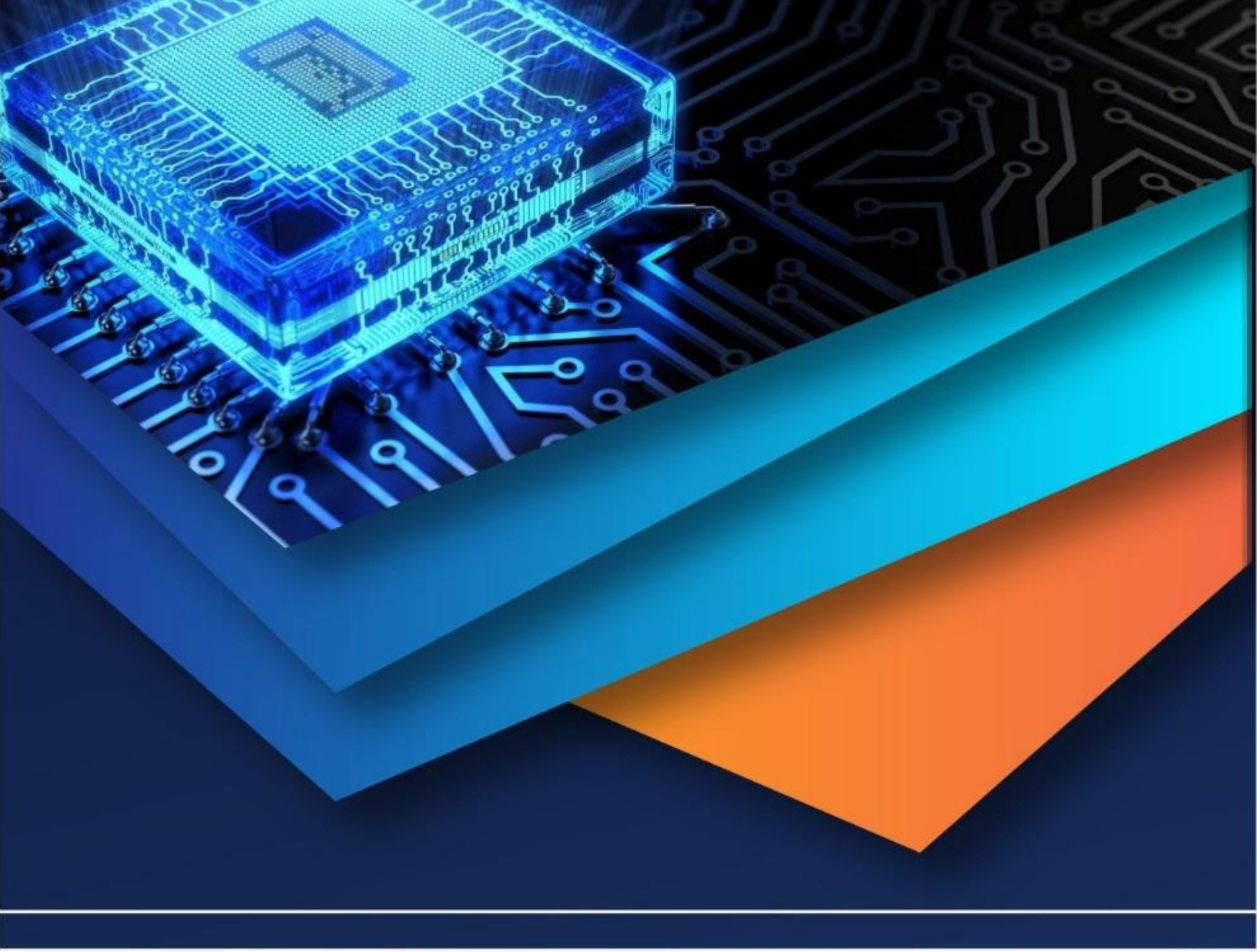
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