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Synthesis of a 17-Beta Estradiol Nano-Formulation as a Promising Wound Healing Agent

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Abstract. This is the first report of an estradiol reduced silver nanoparticle synthesis and characterization. Estrogen has a significant role on wound regeneration. 17-beta Estradiol, being a highly mitotic wound regenerative compound with reported wound healing activity is widely used as cutaneous applicant and as gels in degenerate wounds. Estrogen (E2) is involved in regulating inflammation, re-epithelialization, vascular organization, collagen deposition, fibroblast and keratinocyte migration in wounds. Even though the topical application of Estradiol is of high regeneration value the complications of systemic absorption limits their utility as an external applicant. The study concentrate to develop a noval combination of Estradiol and nano silver , a promising wound regenerative nano drug formulation-17β-Estradiol-reduced silver nanoparticle (ESNP) which may release the hormone slowly in very low concentrations on wounds, simultaneously, protecting the wound from microbial attack and biofilm formation due to the action of nano-silver. ESNP synthesis has been optimized and characterized by UV-VIS spectroscopy, TEM, SEM, DLS, XRD and FT-IR. Previous reports on other Estradiol Nano formulation has been exhibiting an induced neuronal regeneration in Spinal Cord Injury models (SCI), further experimentation of the drug may reveal its potential as a topical wound regenerative drug.

Key words: 17- beta Estradiol, Diabetic foot ulcer, Silver nanoparticles, Regenerative wounds, Nanodrug delivery, Wound healing

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1 Introduction

Cutaneous wound repair is highlighted by overlapping and continuous phases like — hemostasis, inflammation, proliferation, and remodelling—coordinated by a set of molecular players, events and pathways. Disruption or change in any of the mentioned key factors may derail healing and drive chronicity [1].
Among the reported regenerative wound healers, 17β-estradiol (E2)—consistently emerge as a modulator that correct estrogen-deficiency phenotypes and enhance repair across phases. Its role in the reduced deposition of type I/III collagen and elasticity in postmenopausal skin, and effect of topical application of E2 resulting in the increase in hydroxyproline, collagen/elastic-fiber morphology is well known [2].

Looking at the action of estrogen at molecular level, the initial step is the suppression of macrophage migration inhibitory factor (MIF) by E2 —elevated early just after injury and is exaggerated by ovariectomy. Estrogen is also reported to promote an alternative (repair-associated) macrophage (M2) activation with reduced TNF-α, promoting wound regeneration [3]. Interestingly, these effects are strictly ER-dependent and without estrogen receptor-α (ERα), wounds retain iNOS+ macrophages and heightened Cxcl1/Ccl2 chemokine axes [4].

E2 was found to accelerate re-epithelialization through two complementary programs. The first one is ERβ-dependent motility/attachment of fibroblasts and keratinocytes. Epithelial ER beta has a significant role in healing as shown by experiments using selective ERβ agonists (DPN), which increased keratinocyte migration even when inflammation is already damped [5]. Moreover, in estrogen-deficient states, ERK→AKT cascade mediated keratinocyte proliferation and PCNA was affected, lagging re-epithelialization [6].

Extra cellular matrix turnover. And its balance by estrogen is another important are of research as Classic fibroblast/matrix data show E2 shift remodeling toward collagen preservation by proline generation for collagen synthesis; which also decreases pro-collagenase and pro-stromelysin while increasing TIMPs [2, 7]. The decline in ERβ receptor also reduced collagen deposition accompanying delayed healing [5].

The third pillar of estradiol's action is Vascular restoration. An E2 dependent elevation in SDF-1/CXCR4 E2 is critical to home and form vessels by mobilizing bone-marrow–derived endothelial progenitor cells (EPCs) to accelerate re-endothelialisation [8]. To summarise estradiol maps onto each repair pillar beginning with ERα-dependent early immune restraint (↓MIF; M2 macrophages), secondly, EPC-centered vascular repair, followed by ERβ-driven epithelial migration with ERK→AKT-supported proliferation, matrix preservation via TIMP↑/MMP↓ and collagen synthesis [4, 5, 6, 7].

Even though Estradiol regulates multiple facets of wound healing and proliferation, its systemic absorption from cutaneous application is a matter of concern led to the locally focused as well as controlled delivery via nanoparticles minimizing systemic Estrogen exposure [2, 7, 9]. Nanoparticle formulations operationalize that strategy by controlled slow and consistent release, limiting E2 at the wound site while keeping serum levels of Estradiol within physiologic range [10, 11]. It was indeed a landmark finding that in SCI models (Spinal cord injury models) at the molecular level nano-E2 swiftly reduced pro inflammatory cytokines like IL-6, GRO-KC, and MCP-1 and increased regenerative factors like VEGF (Vascular Endothelial Growth Factor) and GDNF (Glial cell line-derived neurotrophic factor), translating to better neurologic recovery [10,11].

Cutaneously, polymeric nanocapsules like PCL E2 nanocapsules in osteoporotic rats accelerated callus formation/maturation and, importantly, exhibited lower systemic exposure as observed as thinner endometrium than conventional E2 cream [12]. Together, these reports high light the E2 applications in Nano formulation with minimal systemic effects, at the same, enriching pro-angiogenic and neurotrophic signals [10, 11, 12]. These findings led to the hypothesis that the translation of nanosilver mediated local delivery systems in estrogen applications may benefit regenerative strategies to regulate inflammation, re-epithelialization, vascular organization, collagen deposition, fibroblast and keratinocyte migration and EPC homing by selective bioactives such as Macrophage migration Inhibitory factor (MIF) basic fibroblast growth factors (bFGF), along with minimal systemic exposure [13,14]. These findings of the estradiol wound healing effects promises a key component for drug formulation. Estradiol –silver nanoparticles not yet used for any advanced research areas for wound healing.

2 Materials and Methods

2.1 Materials Used

Silver nitrate ($AgNO_3$), 17-beta Estradiol cell culture grade (Sigma), Dimethyl sulphoxide (DMSO), 0.1N NaOH.
Equipment: magnetic/vortex stirrers, autoclave, pH meter, centrifuge, UV–Vis spectrophotometer, SEM, TEM, DLS, XRD and FT-IR.

2.2 Methodology

2.2.1 Synthesis of 17-beta Estradiol reduced Silver nanoparticles (ESNP)

Green synthesis of the 17-beta Estradiol Nano silver has been prepared by modifying standard protocols of silver nanoparticle green synthesis [15]. AgNPs were synthesized by the reduction of AgNO3 using the hormone 17-beta estradiol as a main reducing agent and stabilizing agent. The hormone was purchased from Sigma in cell culture grade and stock solutions were prepared in Dimethyl Sulfoxide (DMSO). The conditions for the synthesis were optimized by trial-and-error method.

2.2.2 Optimisation of ESNP Synthesis

Optimisation of the conditions required for the synthesis of 17-beta Estradiol reduced silver nanoparticles were done by optimising (i) molar ratios (1:1, 1:3, 1:10), (ii) pH (9.0, 9.5, 10.7, 12.8), (iii) temperature (40, 60, 100 °C), and (iv) heating time (10, 20, 40 min). UV–Vis spectra were recorded after each synthesis to assess plasmon peaks and stability.

2.2.3 Isolation of synthesized ESNP

Optimized ESNPs were isolated by an initial centrifugation at 5000 RPM for 5 minutes, in order to remove large sixed particles as well as macrostructures. ESNPs were ten isolated from the solution using high-speed cooling centrifugation (10,000 rpm, 15 min) with two water washes. The pellets were collected by discarding the supernatant. The synthesized AgNPs were used for the characterization and study the functional properties of ESNPs.

2.2.4 Characterization of synthesized ESNP

Ultra Violet (UV) - visible spectroscopy: The reduction of silver nitrate to silver nanoparticles by utilizing the hormone 17-beta Estradiol was monitored by recording UV-Visible spectrum, which is the most commonly, used primary characterization technique to confirm the surface plasmon resonance property of AgNPs.

Scanning electron microscopy (SEM): The morphology and size of the synthesized ESNPs were examined by ZEISS Gemini SEM 300 operated at 5Kv, magnification 20K. The sample was prepared on an aluminium foil as thin film. The surface images of the sample were taken at different magnifications.

Transmisson electron microscopy (TEM): The size and shape of nanoparticles were characterized by FEI Tecnai G2 Spirit BIO-Twin TEM of accelerating potential 120KV.

Dynamic Light Scattering (DLS): Polydispersity and size distribution of ESNP were examined by Malvern Panalytical Zetasizer-ZS particle size analyzer.

FTIR Study of ESNP: FTIR spectroscopy was performed to study the functional groups and capping interactions of 17-beta estradiol- AgNPs using an FTIR Bruker ALPHA spectrophotometer in the wavelength range of 400-8000cm^{-1} with resolution of 4cm^{-1}.

X Ray Diffraction studies of ESNP: X-Ray Diffraction (XRD) of the precipitate was also carried out in order to see the crystalline nature of the material using a Rigaku- Miniflex 600 x-ray diffractometer.

2.2.5 Stability analysis of the synthesized ESNP molecules over time

In order to analyse the time dependent stability of the ESNP molecules, the UV-visible spectroscopy absorbance spectra of the synthesized Silver-17-beta estradiol nanoparticles have been taken up to 4 months period at room temperature at different time intervals after synthesis.

3 Results and Discussion

3.1 Green Synthesis of 17-beta estradiol reduced Silver nanoparticles

Reduction of silver ions have been carried out by the addition of silver nitrate and 17-beta estradiol as described in methodology (Fig 1). After synthesis the 17-beta estradiol reduced AgNPs were centrifuged at 5000 rpm for 5 minutes to discard the macromolecules and collected the supernatant for UV -Visible spectrophotometric analysis (Fig 2), quantified and used for physicochemical characterizations.

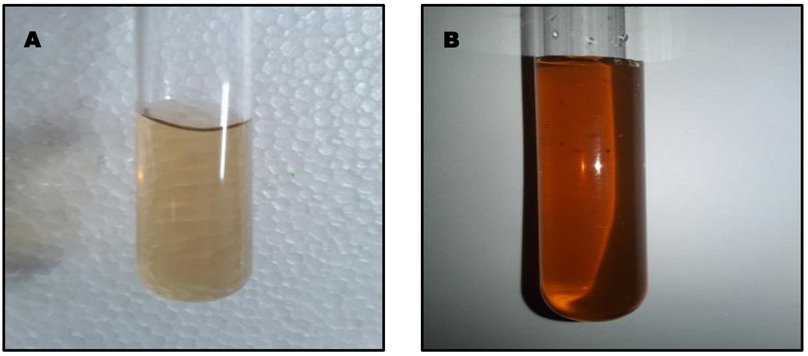


Fig. 1. Green synthesis of 17-beta Estradiol reduced Silver nanoparticles (ESNP) a visible change in colour on optimized. A) before optimization of ESNP ; B) after the optimization of ESNP.

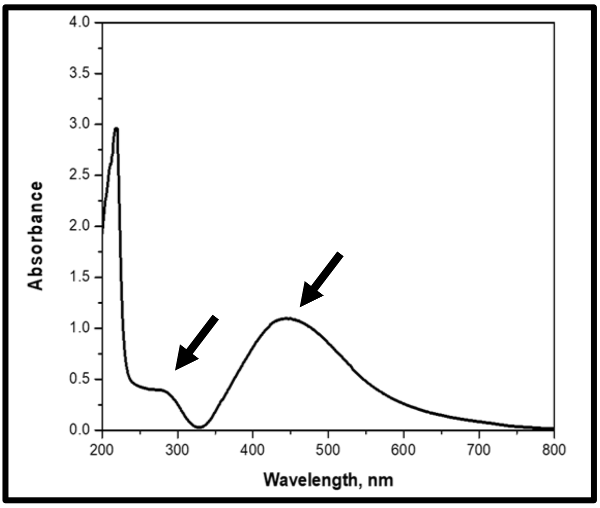


Fig. 2. UV-Visible spectra of 17-beta Estradiol reduced Silver nanoparticles (ESNP).A strong absorption peak characteristic of Silver nanoparticles at 430-450 nm and estradiol at 280nm.

3.2 Optimization of conditions for ESNP

The following conditions provided optimal plasmon resonance and stability: molar ratio 1:3 (17β-estradiol:$AgNO_3$), pH 10.7, 60 °C, 20 min. Characteristic plasmon bands were observed at ~437 nm (molar-ratio optimization and pH = 10.7), ~433 nm (60 °C), and ~435 nm (20 min heating). Across room-temperature incubations, spectra remained stable within ~430–445 nm (Fig 3 A-D).

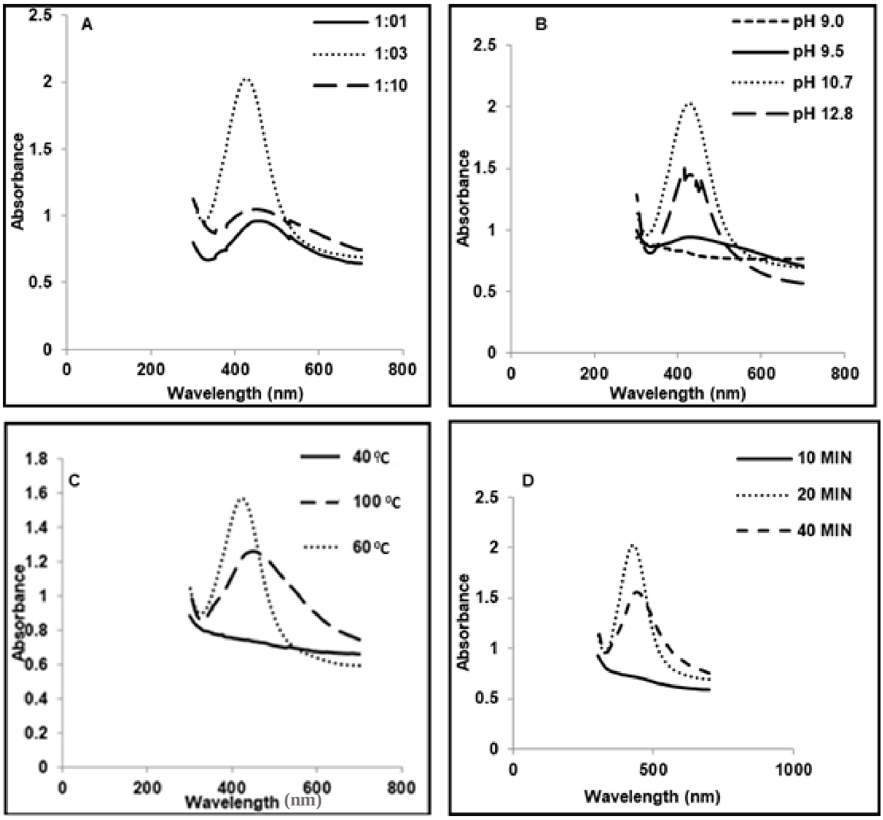


Fig. 3. Optimisation of ESNP synthesis (A) Molar concentrations of 17-beta estradiol : $AgNO_3$; (B) Varying pH (C) Varying temperature conditions and (D) Time dependent synthesis

3.3 Characterisation of ESNP by UV Visible spectroscopy, FT-IR, X-Ray diffraction and SEM analysis

3.3.1 UV-Visible spectroscopic analysis

It revealed a strong absorption peak characteristic of Silver nanoparticles at 430-450 nm, consistent with their surface plasmon resonance. Concurrently, 17-beta estradiol exhibited an absorption peak between 280 nm (Fig 2).

3.3.2 FT-IR analysis

FTIR spectroscopy is one of the finest and economical functional characterization techniques to analyse the chemical compositions and interactions of AgNPs with different functional groups. FTIR spectrum shows the presence of different functional groups at various positions. FTIR analysis of ESNP reveals characteristic absorption bands related to the functional groups present on the nanoparticle surface, primarily those responsible for capping and stabilization. These peaks can vary depending on the synthesis method and capping agent used. Generally, for 17-beta estradiol, prominent bands are observed in the C=C (≈1653 cm^{-1}), =C–H (≈2990 cm^{-1}), N–H (≈3350 cm^{-1}), and O–H (≈3461 cm^{-1}). For ESNPs, notable bands included C=O (≈1739 cm^{-1}), =C–H (≈3177 cm^{-1}), and O–H (≈3455 cm^{-1}). The O–H shift supports strong association/capping of 17β-estradiol on the silver surface (Fig 4).

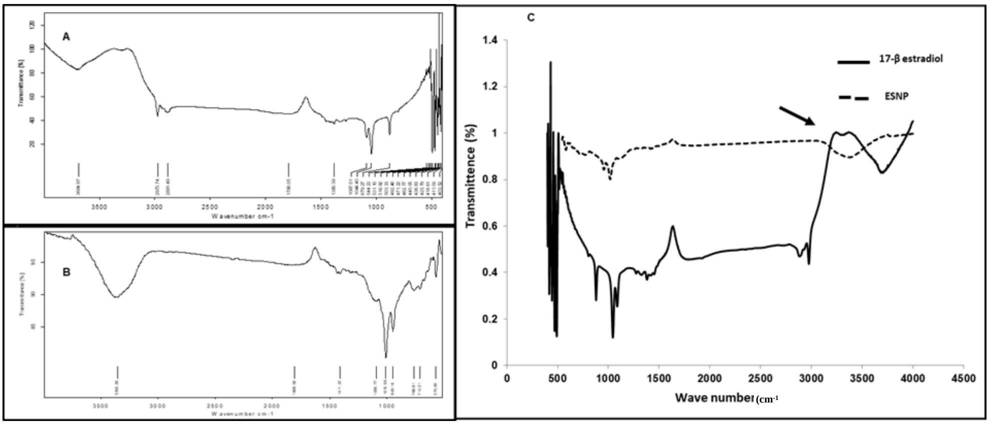


Fig. 4. FTIR analysis of 17-β estradiol (A) ESNP (B) and,combined graph (C) showing a shift in the OH bond indicating the association of 17-β estradiol and AgNO3 Ag+ is reduced to Ago by -OH group of 17-β estradiol.

3.3.3 X-ray diffraction studies (XRD)

XRD is a crystalline material characterization technique used to determine the properties like crystallographic structure, crystallite size, chemical composition and understanding their chemical properties. ESNP was ground in to fine, uniform particles and its average bulk composition is then measured. Each diffraction peak in the XRD shows a specific set of atomic planes in the lattice of the material. Likewise the Heights of a plane shows more x-ray get reflected from those planes, which indicate that more crystals have that plane of orientation in the material. The X-RAY diffraction spectra between the intensity of radiation and 2θ angle showed the characteristic peaks of Silver at 2θ angles 38.2, 44.1, 64.1 and 77 degrees. The resulting XRD pattern shows that the procedure successfully formed silver nanoparticles. The XRD peaks 38.2°, 44.1°, 64.1°, and 77.0° corresponding to the plane of metallic silver crystals (111), (200), (220), and (311), respectively, which indicate that the silver nanoparticles exhibit a face-centered cubic, and crystalline structure. There is no trace of contaminants or pollutants peaks were detected revealing the formation of pure crystalline silver (Fig 5).

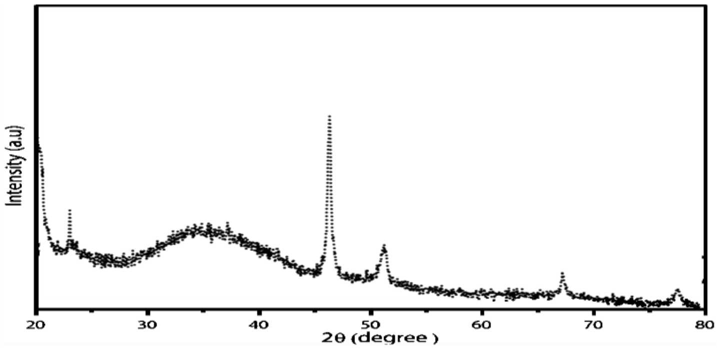
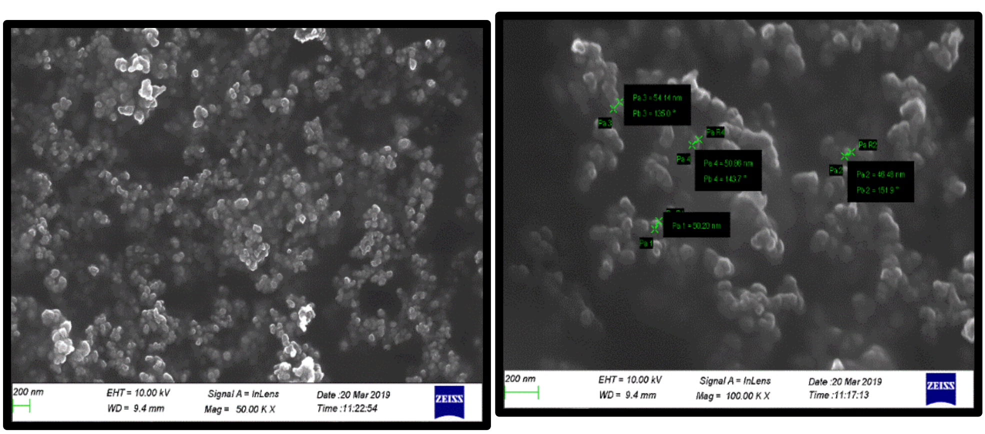


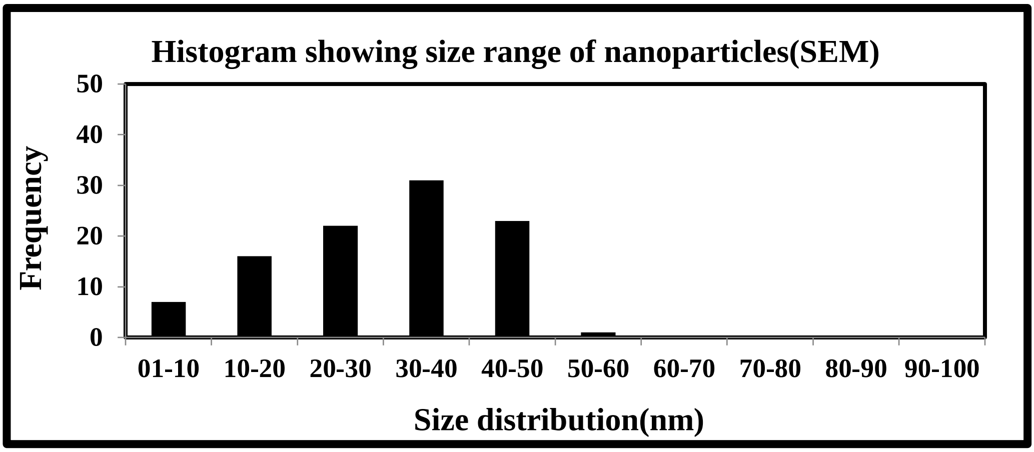
Fig. 5. X-Ray Diffraction profile of 17-beta Estradiol reduced Silver nanoparticles (ESNP)

3.3.4 Scanning Electron Micrographic analysis

SEM micrographs exhibited high-density AgNPs synthesized by Estradiol reduction (Figure 6). SEM images confirm the relatively spherical and uniform shape of AgNPs with diameter of 30-40 nm size. The SEM image of silver nanoparticles formed due to the interactions of hydrogen bond and electrostatic interactions between the bioorganic capping molecules bound to the AgNPs. Additionally, silver nanoparticles aren't responsible and not in direct contact for the aggregation, it indicates the stabilization of the nanoparticles by a capping agent, here the 17-beta estradiol itself acts as a capping and stabilizing agent. The presence of larger silver particles may be due to the accumulation or cluster formation of the smaller ones, due to the SEM measurements.



a)

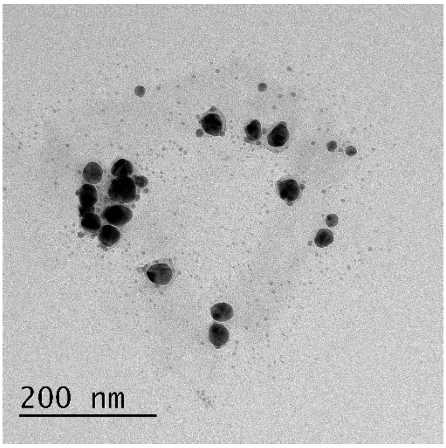


b)

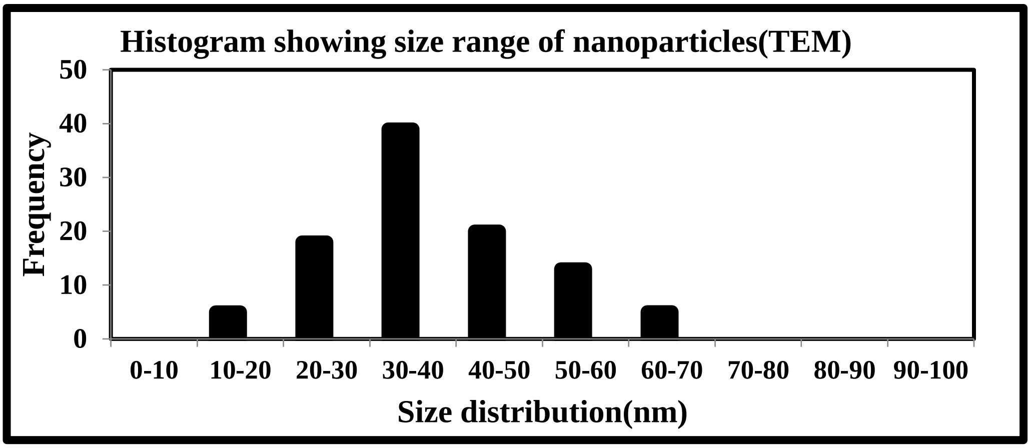
Fig. 6. SEM micrographs of ESNP showing spherical and uniform AgNPs a) SEM image of ESNP; b) Histogram showing the size range of ESNP

3.3.5 Transmission electron microscopic analysis

TEM analysis of ESNP were confirms the quantitative distribution of particles, size distribution and morphology. The TEM images can provide more resolution and capable for additional analytical measurements than SEM. TEM image show that the nanoparticle have spherical shape with minimal aggregation and uniform particle size distribution. The fig. 7 b) explains the histogram size distribution of ESNP with size ranging from 10-70nm. Majority of silver nanoparticles are in the size range of 30-40nm.



a)



b)

Fig.7. TEM images of ESNP showing spherical shaped uniformly distributed particles. a)TEM image of ESNP; b) Histogram showing the size range of ESNP.

3.3.6 Analysis of Dynamic light scattering of ESNP exhibited stability and Polydispersity of particles

The particle size distribution of silver nanoparticles was shown in the fig.8. The Polydispersity index (PDI) of the synthesized silver nanoparticles is 0.256 which means the silver nanoparticles are in good quality and uniformity in their particle size. The DLS analysis yielded a broad peak with z- average size of 94.29nm. DLS analysis is more sensitive to the presence of large particle size. The particle size determined by DLS was larger than the nominal size. So the result suggests that DLS is not an accurate method for measure/ determine the exact size for polydisperse silver nanoparticles. DLS analysis method only helps to measures hydrodynamic size of the particles not the exact physical size of the particles.

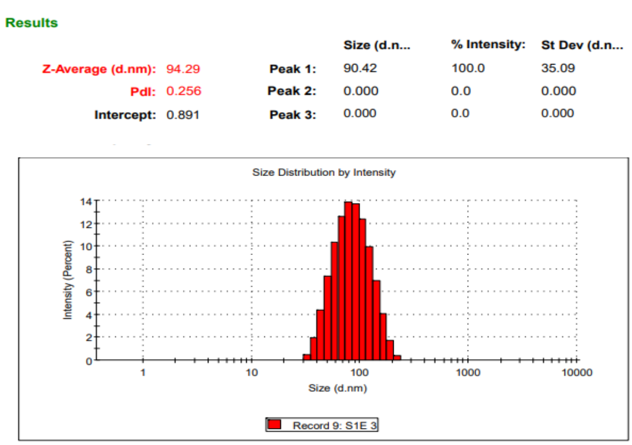


Fig. 8. DLS analysis of ESNP exhibiting high stability and size distribution of particle size

3.3.7 Analysis of time dependent stability of ESNP exhibited relatively high stability

UV-visible spectroscopy revealed distinct absorbance spectra of Silver-17-beta estradiol nanoparticles at different time intervals: one week, 3 weeks, 1 month, 2 months, 3 months and 4 months. Notably, absorbance values within the 400-500 nm range increased progressively with longer UV exposure times.

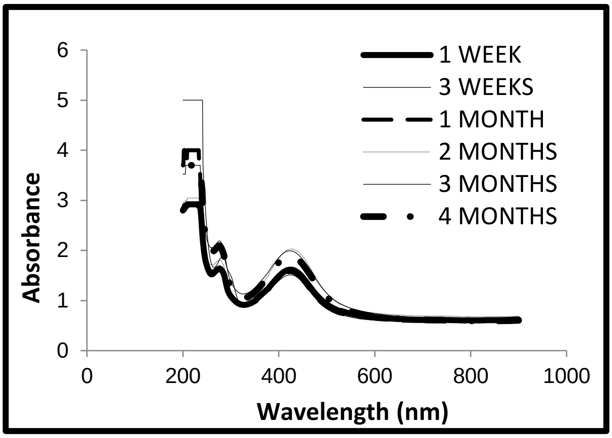


Fig. 9. UV -Visible spectra exhibiting stable peaks at varying time after synthesis exhibiting a relatively high stability of ESNP

4 Conclusion

17-beta estradiol-reduced AgNPs, ESNP is synthesized and characterized using various methods. Optimization studies of ESNP indicated that moderate alkalinity and elevated temperature with a 1:3 reductant: precursor ratio favoured optimum ESNP formation and stability, evidenced by consistent plasmon bands in the 430–437 nm range. The resulting nanoparticles were characterized using UV spectroscopy, FTIR, X-Ray Diffraction, Scanning Electron Microscopy, DLS and TEM to confirm their properties. SEM images confirmed nanoscale dimensions (~30-40 nm) and morphology, TEM images confirms the sherical shape and size distribution of the silver nanoparticles (~30-40 nm), and FT-IR supported strong 17β-estradiol association that likely contributes to colloidal stabilization. Together, these findings support the potential utility of ESNP as a wound-healing nano-drug candidate (Fig 10).

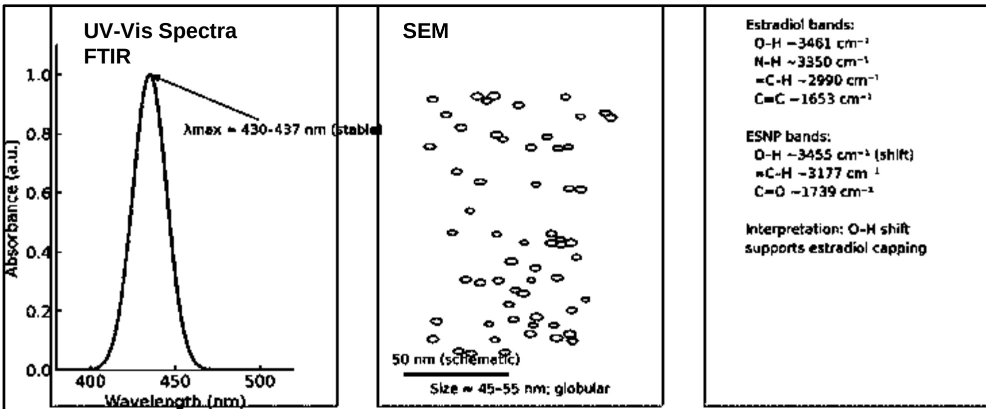


Fig.10. Optimized ESNP synthesis : 17β Estradiol : $AgNO_3$ 1:3 Molar Concentration ; pH10.7; and 20 Minutes incubation at $60^{o}C$

Fig. 10. Showing the summary of optimized conditions of ESNP synthesis and characterization; A 17β-estradiol-reduced and capped silver nanoparticle (ESNP) system was synthesized and optimized at 1:3 (17β-estradiol:$AgNO_3$), pH 10.7, 60 °C, 20 min. The product exhibits a stable plasmon band (~430–437 nm), and nanoscale size (~30-40 nm)

Stability studies exhibited relatively high stability even after 4 months of synthesis demonstrating their potential to be used as a drug in promoting wound healing. Notably, these compounds may exhibit antimicrobial, anti-inflammatory, and wound healing

properties as the components are strongly antimicrobial and regeneratve in nature; making them promising candidates for controlling bacterial growth and facilitating wound repair. Optimization indicated that moderate alkalinity and elevated temperature with a 1:3 reductant:precursor ratio favored ESNP formation and stability, evidenced by consistent plasmon bands in the 430–437 nm range. SEM and TEM confirmed nanoscale dimensions (~30–40 nm), TEM confirmed spherical shape and particle size distribution and DLS exhibiting the strong surface charge and high stability of the nanoparticles and FT-IR supported strong 17β-estradiol association that likely contributes to colloidal stabilization. The progressive increase in fluorescence after 12–24 h points to enhanced BSA association with time, while SDS-PAGE corroborated protein presence in ESNP samples. Together, these findings support the potential efficacy of ESNP as a wound-healing nano-drug candidate.

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