Title:

EGF-conjugated Luteolin tethered Bio-Safe Gold Nanosystems in treatment of Triple Negative Breast Cancer using targeting approach.

Introduction:

Triple negative breast cancer (TNBC) is an extremely intrusive variant of BC and is prominent in approximately 20% of all BC cases [1]. Moreover, the disease offers poor prognosis with increased risk of relapse after conventional chemotherapy. TNBC also leads to metastasis of the lung, liver and brain and presents leads the highest death rate among all other breast cancer types [2]. Prevalent treatment strategy for TNBC is combination of chemotherapy, surgery and radiation depending on the patient condition [3]. Adding to the complexity, clinical strategy for TNBC management often involves non-targeted chemotherapeutic regimens leading to toxic outcomes, as the cancer variant lacks suitable drug targets like Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor (HER2) [4].

Luteolin (Lu) is a natural 3′,4′,5,7-tetrahydroxyflavone linked to management of diverse cancers like liver, skin, stomach, prostrate, pancreas, ovary and colon [5]. It is reported that Lu helps in blocking metastasis of TNBC cells to the brain by reversing EMT along with down regulating the Wnt/β-catenin pathway [6]. Lu is also reported to be effective for management of both early-stage and late-stage TNBC by suppressing angiogenesis and metastasis [7]. PEGylated gold nanoparticles (Aunps), on the other hand, is reported to be cytotoxic to basal-like TNBC cells in a dose-dependent manner [8]. In a study with bifunctional theranostic gold nanoprobes, selective cytotoxicity to TNBC cells by alterations of ROS levels was observed keeping the normal cells remained unaffected [9].

One of the main reasons for the failure in drug development strategy in order to overcome TNBC is the absence of molecular targets *viz.* ER, PR, and HER2, which instigates further mining for novel therapies and strategies. However, EGFR overexpression has been found in 45–70% of cases of TNBC, which is the highest among other breast cancer subtypes [10]. Though HER2 remains absent in TNBC, the gene amplification and specific translation of this gene (HER-2) by RNA is linked with the overexpression of EGFR (a HER family subtype, HER1) [11]. Elevated occurrence of EGFR in TNBC makes it a promising target in the clinical management of TNBC. Keeping this fact in mind, we conjugated EGF with flavonoid derived

nano-scale gold to achieve the site specificity to TNBC's. Present work marks the first report of Lu conjugated gold nanoparticles (LuAunp) as targeted therapeutics in TNBC management.

Objectives:

1. Development of EGFR targeted LuAunp nanosystems for control of TNBC cells.
2. Characterization of the developed LuAunp.
3. Cytotoxicity evaluations of LuAunp, free Lu & EGF-LuAunp against TNBC and normal cells.
4. Analysis of cell proliferation and apoptosis assay in EGF-LuAunp sensitive cell lines.
5. Cellular uptake studies in EGF-LuAunp sensitive cell lines.

Materials and Methods:

Materials:

Chloroauric acid (HAuCl4, $3H_2O$), and 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were procured from Alfa Aesar (Haverhill, Massachusetts, United States). 3′,4′,5,7-tetrahydroxyflavone (Luteolin, Lu), N-hydroxysuccinimide (NHS) were purchased from TCI (Tokyo, Japan). FTIR grade KBr was purchased from Merck (Burlington, Massachusetts, United States). Human triple negative breast cancer (TNBC) cell line, MDA-MB-231 and mouse fibroblast cell line, NIH/3T3 were procured from the National Centre for Cell Sciences (Pune, India). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), and antibiotic-antimycotic mix were obtained from Himedia (India). EGF (epidermal growth factor-human), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Annexin V-FITC Early Apoptosis Detection Kit, 4′ ,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and Triton X-100 were procured from Sigma Aldrich (St. Louis, MO, USA). Paraformaldehyde, phosphate buffer saline (PBS) and RNaseA were acquired from MERCK (USA). ProLong™ antifade reagent was procured from Thermo Fisher (USA). HPLC grade solvents procured from Spectrochem (Mumbai, India) was used for the entire experimentation. Chemicals for the study were used as received without further purification or modification. For the synthesis and analytical experiments, standard glasswares (Borosil®, Mumbai, India) were used. All other reagents used were of analytical grade.

Standard tissue culture plates and cell strainers from HIMEDIA were used in determining biological activities. All glassware were cleaned with aqua-regia prior to experimentation.

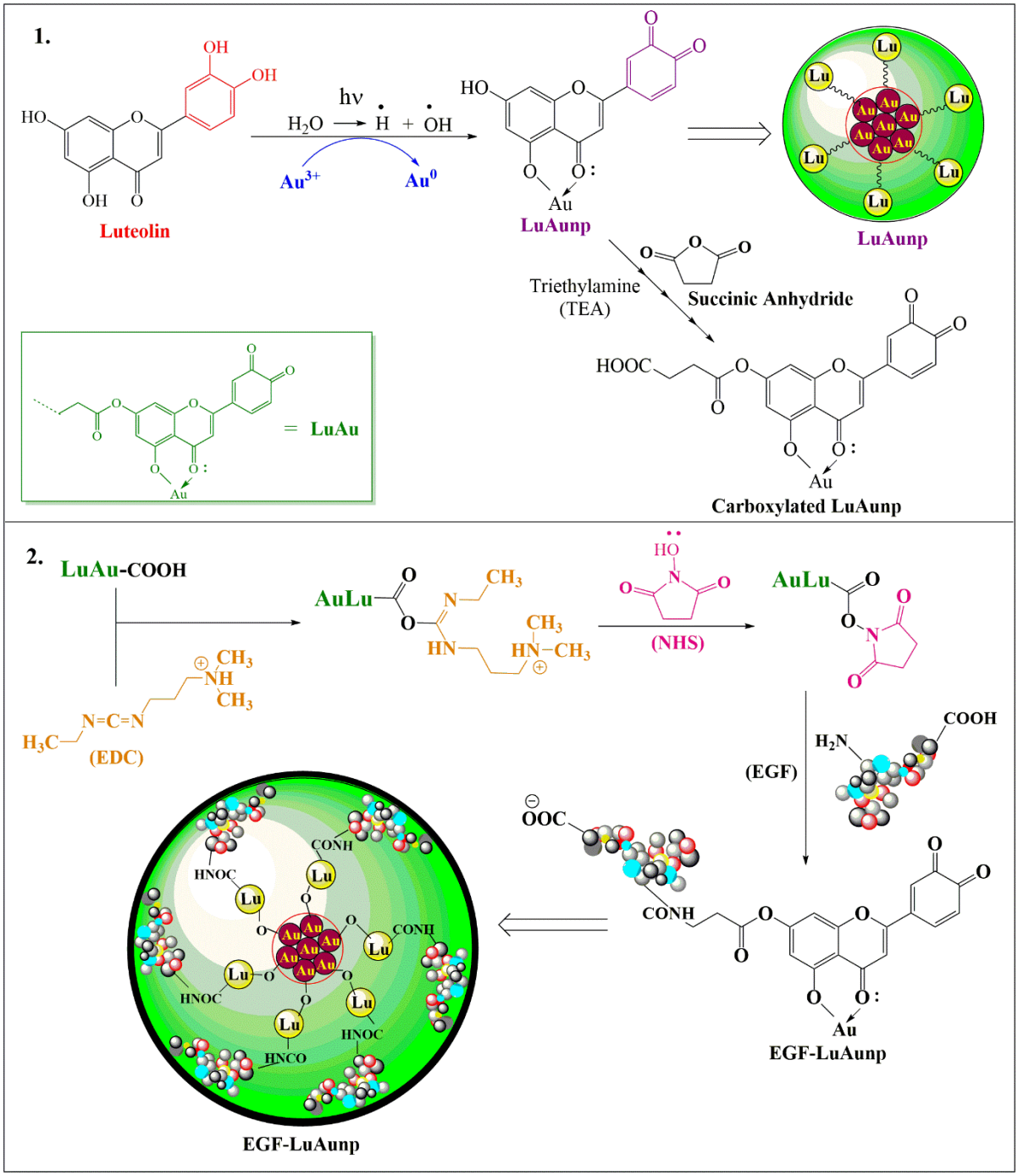
Methods:

1. **Development of EGFR targeted LuAunp nanosystems for control of TNBC cells.**

Facile, ultrasonically driven green synthesis of AuNPs was carried out using phyto-constituent 'Lu' performing dual role of reducer as well as stabilizer. Synthesis optimization is done varying different molar ratio and reaction time points. Progression of the reduction process was evidenced from incessant colour changes initiating from pale yellow to stable purple on reaction completion.

For target specific biological activity, EGF protein was tagged with prepared LuAunps in two steps (Scheme 1). Carboxyl groups were introduced to the LuAunps in the first step using succinic anhydride and TEA under continuous stirring [12]. In the second step, EGF was conjugated to carboxylated LuAunp via amide bond formation through EDC/NHS coupling reaction [13]. Prepared EGF-LuAunps were purified by centrifugation, re-dispersed in HPLC water and was stored at 4°C until further use.

For uptake study, FITC tagged nanoparticles were also prepared by simply adding freshly prepared FITC solution to EGF-LuAuNP under stirring condition continued for 45-60 min in dark condition [14]. Prepared FITC-EGF-LuAunps were purified by centrifugation, re-dispersed in HPLC water and was stored at 4°C with protection from light until further use.



Scheme 1: Scheme for synthesizing EGF conjugated LuAunp.

2. Characterization of the developed LuAunp.

LuAunps and EGF-LuAunps were characterized in UV-Vis spectrophotometer, DLS, HR-TEM, XRD, EDX spectroscopy, FTIR studies. Hydrodynamic diameter, polydispersity index (PDI) and Zeta potentials (ζ) of nanoparticles were analysed by Zetasizer Nano ZS. Association

of Lu, EGF with gold was confirmed by Fourier Transform Infra-Red spectroscopy (FTIR). Gold sol molar concentration in optimized LuAunp was determined using high resolution inductively coupled plasma mass spectrometry (HR-ICPMS). Morphological features were analyzed in high resolution transmission electron microscope (HRTEM). Elemental composition of the optimized nanoparticles was determined using energy dispersive X-ray (EDX) analysis and X-ray diffraction (XRD) analysis was performed.

3. **Cytotoxicity evaluations of LuAunp, free Lu & EGF-LuAunp against TNBC and normal cells.**

The anti-cancer activities of Lu, LuAuNP and EGF-LuAuNP were assessed via MTT assay against TNBC (MDA-MB-231) cell line and non-malignant fibroblast (NIH-3T3) cell line. The absorbance was quantified at 570 nm in a Microplate Absorbance Reader. Cell viability was recorded as the ratio of mean absorbance from triplicate findings and calculated as ($A_{test}/A_{control}$) [15].

4. **Analysis of cell proliferation and apoptosis assay in EGF-LuAunp sensitive cell lines.**

Cell cycle analysis & apoptosis assay were conducted with tagged and untagged nanosystems against MDA-MB-231 cell line using Annexin V-FITC and PI assay method. The DNA content, percentages of apoptotic and necrotic cells was determined exploring flow cytometer and analyzed with BD FACS Diva software [15].

5. **Cellular uptake studies in EGF-LuAunp sensitive cell lines.**

Time dependant qualitative cellular uptake study of both EGFR-targeted and non-targeted nanoparticles has been evaluated against TNBC cells (MDA-MB-231) and was observed in confocal microscope [16].

Results:

1. **Development of EGFR targeted LuAunp nanosystems for control of TNBC cells**

Current method reports *in-situ* synthesis of nano-gold using Lu under ultrasonic conditions. Completion of reaction was marked by the transient change of colour from pale yellow to deep purple (Fig. 1A). To optimize the reaction route, the molar ratio of Lu with fixed gold precursor was varied (1:0.4, 1:0.5, 1:0.6, 1:0.7, 1:0.8, 1:0.9, 1:1, 1:1.1) and sonication time was varied (6

min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min). Increasing the molar concentration of Lu up to 3 mM (molar proportion of $HAuCl_4$ to Lu 1:1) and sonication time to 12 min resulted in the maximum intensity of UV-peak with λmax of 541 nm (Fig. 1B and 1C).

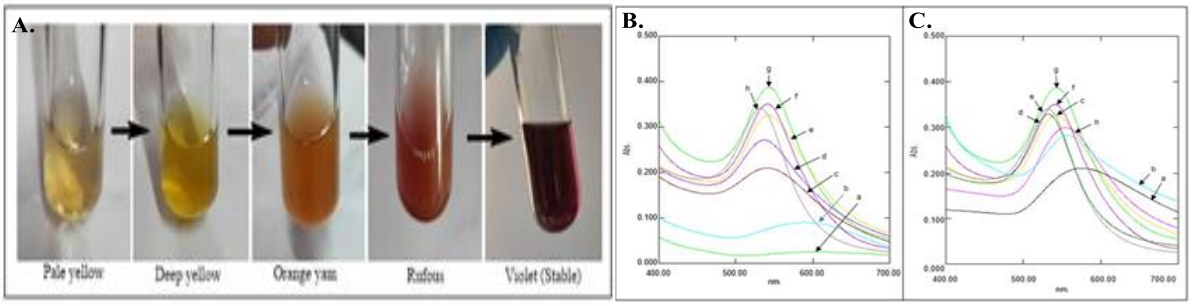


Fig. 1: [A] Gold nanoparticle synthesis was visually monitored by the colour change from pale yellow to violet; UV Spectrum of gold-nanoparticles with different **[B]** molar ratio; **[C]** sonication time points; Route optimized with molar ratio of 1:1 ($HAuCl_4$:Luteolin) and sonication time of 12 min. Maximum absorbance recorded at 541 nm. (Abs = 0.392).

2. Characterization of the developed LuAunp.

Hydrodynamic diameter was measured as 65.12±4.54 nm, PDI of 0.257±0.01, zeta potential of -24 ± 1.73 mV for LuAunp (Fig. 2A) and 97.3±2.54 nm, PDI of 0.215±0.01, zeta potential of -38 ± 2.68 mV for EGF-LuAunp (Fig. 2B). The average particle diameter of 25.12±10.35 nm for LuAunp (Fig. 2C) and 30.23±9.96 nm for EGF-LuAunp (Fig. 2D) was recorded in HRTEM. The clear lattice fringes were observed in HRTEM image with fringe spacing of 0.173 nm (Fig. 2E). The EDX spectrum indicated the presence of gold (Au) with oxygen (O) and carbon (C) atom (Fig. 2F). HR-ICPMS analysis of gold (Au) concentration in LuAunp dispersed in aqueous medium confirmed Au^0 concentration at 156 µg/mL Au. From XRD analysis, Bragg's diffraction peaks of purified LuAunp was indexed for (1 1 1), (2 0 0), (2 2 0), (3 1 1) sets of lattice plane at 38.00°, 44.62°, 64.72° and 77.68° respectively (Fig. 2H). FTIR spectra of LuAunp showed characteristic peaks at 3160, 1612, 1502, and 1165 cm^{-1} for hydroxyl, ketone, aromatic alkene and etheric stretching, whereas EGF-LuAunp showed main characteristic peak at 1653 cm^{-1} for amide linkage in FTIR spectrum suggesting the association of Lu with gold and also the tagging of protein with LuAunp (Fig 2I).

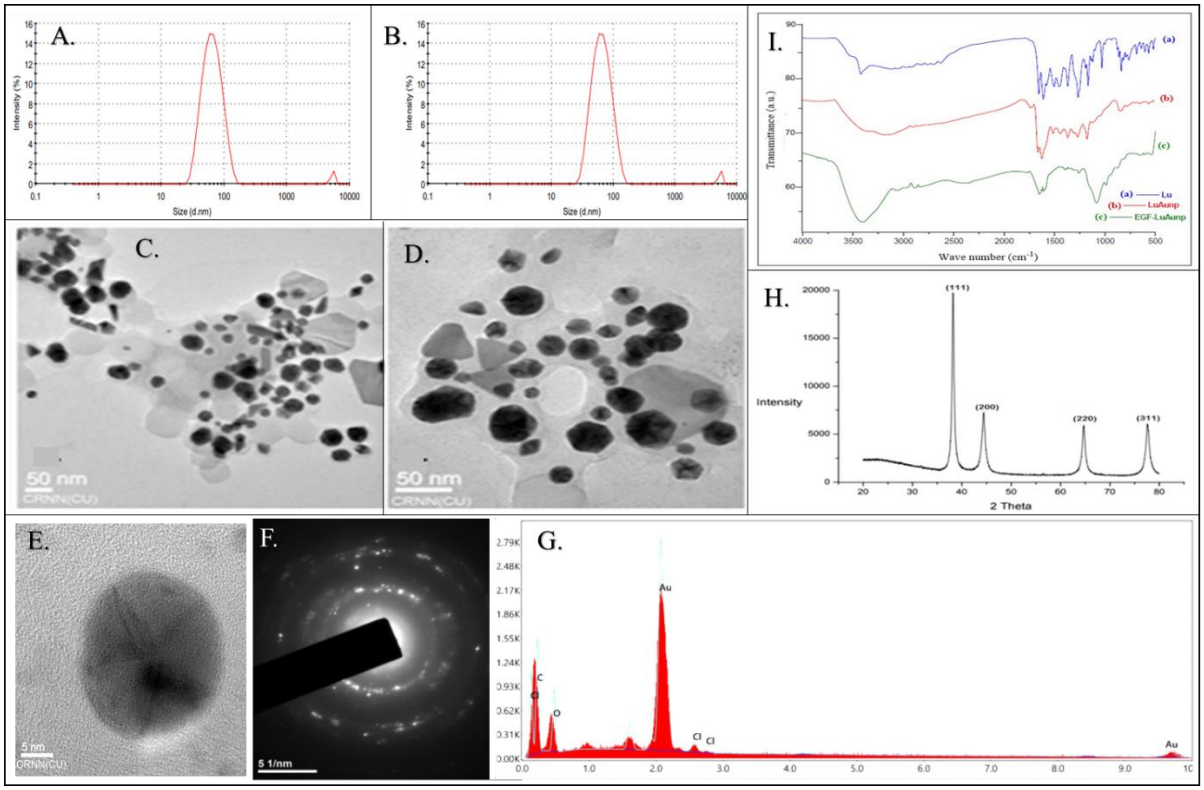


Fig. 2: Particle size intensity curve of **[A]** LuAunp and **[B]** EGF-LuAunp; HR-TEM particle population of **[C]** LuAunp and **[D]** EGF-LuAunp; **[E]** Single particle monograph of EGF-LuAunp and **[F]** SAED pattern of gold nano; **[G]** EDX spectrum **[H]** XRD pattern of conjugated nanoparticles; **[I]** FTIR Spectrum further confirms the association of Lu & EGF with nanoparticles.

3. **Cytotoxicity evaluations of LuAunp, free Lu & EGF-LuAunp against TNBC and normal cells.**

The IC_{50} of Lu was assessed to be 12 µg/mL, whereas that for LuAunp was 4 µg/mL and for EGF-LuAunp was 2 µg/mL. EGF-LuAunps showed significantly pronounced anti-proliferative and cytotoxic effects on MDA-MB-231 cells than LuAunps (by 6-fold) and free Lu (by 3-fold) (Fig. 3A). In case of non-malignant NIH/3T3 cells, neither Lu nor the conjugated nanoparticle showed any significant cytotoxicity up to 12 µg/mL conc. confirming the bio-compatibility of the nanosystem (Fig. 3B).

4. **Analysis of cell proliferation and apoptosis assay in EGF-LuAunp sensitive cell lines.**

MDA-MB-231 cells exhibited a sharp decline in G2/M phase following Lu, LuAunp and EGF-LuAunp treatment, along with a rise in cell population in the sub-G1 suggesting the occurrence of apoptosis (Fig. 3C and 3D).

5. Cellular uptake studies in EGF-LuAunp sensitive cell lines.

Time-dependent increase in FITC green fluorescence was observed due to cytoplasmic accumulation of nanoparticles in MDA-MB-231 cells. Cellular uptake of EGF-LuAunp in MDA-MB-231 cells was quite perceivable within 1 h of incubation suggesting an efficient uptake which was not observed in case of non-targeted particles, thus spotting the significance of tagging EGF to the nanoparticles (Fig. 3E).

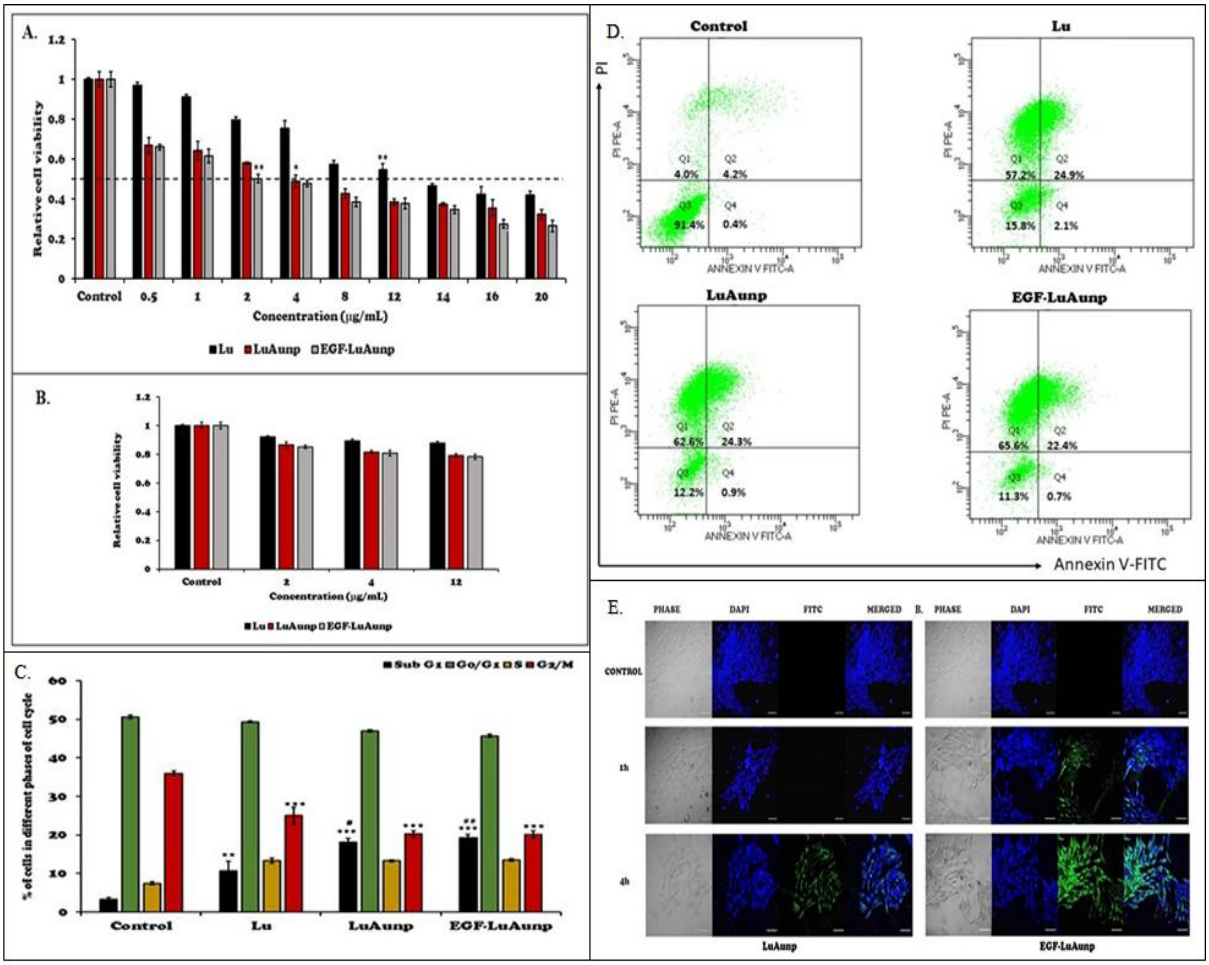


Fig. 3. [A] Cell viability assay of Lu conjugated particles against MDA-MB-231 TNBC Cell line; **[B]** Cell viability assay of Lu conjugated particles against NIH-3T3 fibroblast cell line; **[C]** Cell cycle analysis; **[D]** apoptosis assay **[E]** Time dependent cellular uptake of non-targeted and EGFR-targeted Lu gold nanoparticles in MDA-MB-231 after 1h and 4h of treatment.

Statistical Analysis:

Experimental results were analyzed by GraphPad Prism 5.0 and expressed as mean values ± standard deviation (SD) from at least three independent experimental sets. Student's t-test was performed for evaluating the comparison between groups. The statistical significance was denoted by *p-value <0.05, **p-value <0.01 and ***p-value <0.001 vs control group; #p-value <0.05, ##p-value <0.01 and ###p-value <0.001 vs Lu-treated group.

Discussion:

Present work reflects the first report of Luteolin-conjugated bio-safe gold nanoparticles as targeted therapeutics against triple negative breast cancer. Flavonoid conjugated synthesis of gold nanoparticles was achieved by means of a facile and speedy technique with avoidance of hazardous chemicals. Gold nanoparticles majorly of spherical geometry with a diameter around 30 nm were confirmed through HRTEM. The stable nanoparticles were crystalline in nature in which EGF protein was tagged for intended site-specificity to EGFR of TNBC cells. Targeted nanoparticles synthesized exhibited remarkable cytotoxicity against triple negative breast cancer cells which far better than the free flavonoid and non-targeted nanoparticles. Apoptosis assay and cell cycle analysis confirmed significant reduction in cell viability at G2/M phase. Additionally, EGF tagged nanoparticles synthesized also proved its bio-safety by suggesting no cytotoxicity against non-malignant cells. Enhanced cellular uptake coupled with apoptosis of TNBC cells thus confer EGF-LuAunps to be an ideal candidate as anti-cancer therapeutics. Therefore, the present work envisages significant potential for further translation into pre-clinical and clinical evaluation stages.

Impact of the research in the advancement of knowledge or benefit to mankind:

The product proved successful in the pre-clinical stages in the present study which strengthened its potential for clinical applications as triple negative breast cancer management has limited therapeutic interventions.

- ✓ The team successfully developed, optimized and characterized EGFR targeted luteolin engineered gold nanosystems. Traditional Turkevich method, Fren's method, Brust-Schiffrin methods are widely being used but these chemical

synthesis generates hazardous chemical waste practically impacting the environmental sustainability. Green synthesis of gold nano using flavonoids (Luteolin) as reducer and stabilizer bypasses the toxic waste generation during synthesis. **This will encourage the researchers to apply green synthesis maintaining ecological behaviour**.

- ✓ Cytotoxicity evaluations of nanosystems designed against TNBC cells and normal cells. Lots of trials with gold nanoparticles have been reported to possess cytotoxicity against cancer as well as healthy cells questioning the biocompatibility of gold nanoparticles. Present work suggested potent cytotoxicity against TNBC cells and interestingly, our nanosystems exhibited excellent cyto-compatibility to normal cells **strengthening the point of using Luteolin-gold nanoparticles against cancer treatment**.
- ✓ Major edge of the project is the site-specific delivery to TNBC cells, which was confirmed from the results obtained by confocal microscopy. **Real-time analysis by confocal microscopy provided encouraging results which confirms immense scientific break though as drug targeting in TNBC is the most important clinical concern**.

Literature reference:

[1] T. Ovcaricek, S.G. Frkovic, E. Matos, B. Mozina, S. Borstnar, Triple negative breast cancer prognostic factors and survival, Radiol. Oncol. 45 (2011) 46, https://doi.org/10.2478/v10019-010-0054-4.

[2] E.C. Dietze, C. Sistrunk, G. Miranda-Carboni, R. O'regan, V.L. Seewaldt, Triple negative breast cancer in African-American women: disparities versus biology, Nat. Rev. Cancer 15 (2015) 248–254, https://doi.org/10.1038/nrc3896.

[3] M.A. Medina, G. Oza, A. Sharma, L.G. Arriaga, J.M. Hern´andez Hern´andez, V.M. Rotello, J.T. Ramirez, Triple-negative breast cancer: a review of conventional and advanced therapeutic strategies, Int. J. Environ. Res. Publ. Health 17 (2020) 2078, https://doi.org/10.3390/ijerph17062078.

[4] C.A. Hudis, L. Gianni, Triple-negative breast cancer: an unmet medical need, Oncol. 16 (2011) 1, https://doi.org/10.1634/theoncologist.2011-S1-01.

[5] M. Imran, A. Rauf, T. Abu-Izneid, M. Nadeem, M.A. Shariati, I.A. Khan, A. Imran, I.E. Orhan, M. Rizwan, M. Atif, T.A. Gondal, Luteolin, a flavonoid, as an anticancer

agent: a review, Biomed. Pharmacother. 112 (2019), 108612, https://doi.org/10.1016/j.biopha.2019.108612.

[6] D. Lin, G. Kuang, J. Wan, X. Zhang, H. Li, X. Gong, H. Li, Luteolin suppresses the metastasis of triple-negative breast cancer by reversing epithelial-to-mesenchymal transition via downregulation of β-catenin expression, Oncol. Rep. 37 (2017) 895–902, https://doi.org/10.3892/or.2016.5311.

[7] M.T. Cook, Y. Liang, C. Besch-Williford, S.M. Hyder, Luteolin inhibits lung metastasis, cell migration, and viability of triple-negative breast cancer cells, Breast Cancer 9 (2016) 9–19, https://doi.org/10.2147/BCTT.S124860.

[8] C. Massard, C. Dubois, V. Raspal, P. Daumar, Y. Sibaud, E. Mounetou, M. Bamdad, O. Awitor, Cytotoxicity study of gold nanoparticles on the basal-like triple-negative HCC-1937 breast cancer cell line, J. Biomaterials Nanobiotechnol. 9 (2018) 13–25, https://doi.org/10.4236/jbnb.2018.91002.

[9] M.L. Castilho, V.P.S. Jesus, P.F.A. Vieira, K.C. Hewitt, L. Raniero, Chlorin e6-EGF conjugated gold nanoparticles as a nanomedicine based therapeutic agent for triple negative breast cancer, Photodiagnosis Photodyn. Ther. 33 (2021), 102186, https://doi.org/10.1016/j.pdpdt.2021.102186.

[10] R. Dent, M. Trudeau, K.I. Pritchard, W.M. Hanna, H.K. Kahn, C.A. Sawka, L.A. Lickley, E. Rawlinson, P. Sun, S.A. Narod, Triple-negative breast cancer: clinical features and patterns of recurrence, Clin. Cancer Res. 13 (2007) 4429–4434, https://doi.org/10.1158/1078-0432.CCR-06-3045.

[11] Y. Tang, L. Zhu, Y. Li, J. Ji, J. Li, F. Yuan, D. Wang, W. Chen, O. Huang, X. Chen, J. Wu, Overexpression of epithelial growth factor receptor (EGFR) predicts better response to neo-adjuvant chemotherapy in patients with triple-negative breast cancer, in: J Translational Medicine, vol. 10, BioMed Central, 2012, https://doi.org/10.1186/1479-5876-10-S1-S4.

[12] F. Li, H. Xia, Dopamine-functionalized poly (vinyl alcohol) elastomer with melt processability and self-healing properties, J. Appl. Polym. Sci. 134 (2017), 45072, https://doi.org/10.1002/app.45072.

[13] K.A. Totaro, X. Liao, K. Bhattacharya, J.I. Finneman, J.B. Sperry, M.A. Massa, J. Thorn, S.V. Ho, B.L. Pentelute, Systematic investigation of EDC/sNHS-mediated bioconjugation reactions for carboxylated peptide substrates, Bioconjugate Chem. 27 (2016) 994–1004, https://doi.org/10.1021/acs.bioconjchem.6b00043.

bioconjugation reactions for carboxylated peptide substrates, Bioconjugate Chem.27 (2016) 994–1004, https://doi.org/10.1021/acs.bioconjchem.6b00043.

[14] C.Y. Lin, C.H. Liu, W.L. Tseng, Fluorescein isothiocyanate-capped gold nanoparticles for fluorescent detection of reactive oxygen species based on thiol oxidation and their application for sensing glucose in serum, Anal. Methods 2(2010) 1810–1815, https://doi.org/10.1039/C0AY00428F.

[15] A. Halder, M. Jethwa, P. Mukherjee, S. Ghosh, S. Das, A.B.M. Helal Uddin A. Mukherjee, U. Chatterji, P. Roy, Lactoferrin tethered betulinic acid nanoparticles promote rapid delivery and cell death in triple negative breast and laryngeal cancer cells. Artificial Cells, Nanomed. Biotech. 48 (2020) 1362–1371,https://doi.org/10.1080/21691401.2020.1850465.

[16] A. Halder, P. Mukherjee, S. Ghosh, S. Mandal, U. Chatterji, A. Mukherjee, Smart PLGA nanoparticles loaded with Quercetin: cellular uptake and in-vitro anticancer study, Mater. Today Proc. 5 (2018) 9698–9705, https://doi.org/10.1016/j.matpr.2017.10.156.

Swadeep Mal 30.8.23

Signature of the applicant