Supporting Information

Biomaterials based combinatorial approach of aescin comprised zein coated gelatin nanoparticles alleviates synovial inflammation in experimental inflammatory arthritis

Chandrashekhar Jori[1#], Md. Meraj Ansari[1#], Anas Ahmad[2], Nemat Ali[3], Syed Shadab Raza[4], Rehan Khan[1*]

[1]Chemical Biology Unit, Institute of Nano Science and Technology, Sector 81, Knowledge city, Sahibzada Ajit Singh Nagar, Mohali, Punjab 140306, India.

[2]Julia McFarlane Diabetes Research Centre (JMDRC) and Department of Microbiology, Immunology and Infectious Diseases, Snyder Institute for Chronic Diseases and Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada.

[3]Department of Pharmacology & Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia.

[4]Laboratory for Stem Cell and Restorative Neurology, Department of Biotechnology, Era's Lucknow Medical College Hospital, Sarfarazganj, Lucknow226003, India.

[#]These authors contributed equally to this work.

***Corresponding Author:**

Dr Rehan Khan,

Chemical Biology Unit, Institute of Nano Science and Technology, Knowledge City, Sector-81, SahibzadaAjit Singh Nagar, Mohali, Punjab140306, India

*Email: rehankhan@inst.ac.in.

Tel: +91-172-2210075.

1. Materials, methods and experimental section

1.1. Chemicals and reagents

Zein (CAS no. 9010666) and Aescin was purchase from TCI and Griess Reagent system was purchased from Alfa Aesar.Gelatin were purchased from TCI Chemicals Pvt. Ltd. Acetone (Me2CO) was obtained from Rankem. Haematoxylin (Harris) and eosin (2% w/v) were purchased from Sigma-Aldrich (USA).Sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na2HPO4), potassium dihydrogen phosphate (KH2PO4) and paraformaldehyde etc. were purchased from Himedia Laboratories. Primary antibodies (anti-COX2, anti-iNOS, anti-NF-κB, and anti-BAX) and HRP-conjugated secondary antibody were purchased from invitrogen. Carbon tetrachloride and all other routine chemicals used in this investigation were of research grade.

1.2. Formulation and characterization of AES loaded gelatin nanoparticles

1.2.1. Formulation of nanoparticles

GNPs (gelatin nanoparticles) were formulated by completely dissolving gelatin in water with completely dissolved Aescin (Aes), followed by dropwise addition of equal volume of acetone. Acetone addition causes precipitation of GNPs which were kept overnight on gentle stirring for solvent evaporation. Nanoparticles so formed were collected by centrifugation at 13000 rpm for 20 minutes and lyophilized.

1.3. Characterization techniques

1.3.1. Fourier Transform Infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectra of free aescin, gelatin, blank gelatin nanoparticles (Ze@GNPs), zein coated aescin loaded gelatinnanoparticles (Ze@Aes-GNPs) were measured on Cary Agilent 660 IR spectrophotometer. For each spectrum, 256 scans and 4 cm-1 resolution was applied over the range of 400–4000 cm-1.

1.3.2. Dynamic light scattering (DLS)

Mean hydrodynamic diameter (particle size and size distribution), zeta potential (ZP) and polydispersity index (PDI) of Ze@Aes-GNPs was measured by dual angle dynamic light scattering (DLS) technique also known as photon correlation spectroscopy (DLS Zetasizer Nano ZSP; Model-ZEN5600; Malvern Instruments LTD., Worcestershire, UK). Experiments were recorded in triplicate and mean of 3 readings was reported as actual particle size. Temperature was maintained at 25 ºC during measurements.

1.3.3. Zeta potential (ZETA SIZER)

Zeta potential of Ze@Aes-GNPs was determined by Zetasizer Nano ZSP; Model-ZEN5600; Malvern Instruments Ltd, Malvern, UK, in disposable folded capillary cells (Model-DTS1070; Malvern) by Phase Analysis Light scattering (PALS) technique, using palladium electrodes at 25°C. Average of three independent readings was represented as actual zeta potential.

1.3.4. Transmission electron microscopy (TEM)

For TEM analysis, one drop of Ze@Aes-GNPs aqueous dispersion was drop casted onto a carbon-coated 300 mesh copper grid (Ted Pella Inc.) and incubated for 20–30 min. for proper adsorption. Samples loaded onto grids were then placed inside desiccator under vacuum for overnight drying and were viewed under a tungsten filament at an accelerating voltage of 120 kV using a JEOL JEM-2100 transmission electron microscope (Tokyo, Japan).TEM micrographs were digitally recorded and processed using camera software Gatan.

1.3.5. Scanning electron microscopy (SEM)

For SEM measurements, Ze@Aes-GNPs were dispersed in Milli-Q water. Samples were drop-casted onto a silicon wafer and then dried overnight (slow evaporation). Morphology of nanoparticles was then explored using JEOL JSM-7600F scanning electron microscope, Tokyo, Japan. Accelerating voltage was kept around 5.0–10.0 kV.

1.3.6. Ultraviolet-visible (UV-Vis) spectroscopy

Shimadzu UV-2600 was used to calibrate standard curve of Aescin(AES) in order to assess amount of loaded drug into the Ze@Aes-GNPs, which was followed by estimation of loading capacity of nanocarriers and encapsulation efficiency of the drug. Various concentrations of AES were analyzed in a quartz cuvette having 1 cm path length by UV-Vis spectrophotometer at a specific wavelength.

1.3.7. Drug loading and release kinetics

Drug loading capacity of Ze@Aes-GNPs and entrapment or encapsulation efficiency of drug was assessed to demonstrate efficiency of nanoparticles as a drug delivery system to encapsulate mentioned drug. During preparation of NPs, drug was loaded and ultra-centrifugation was carried out. Then supernatant was taken and analyzed by UV-Visible

spectrophotometer for determination of free unloaded drug. Loading capacity of nanoparticles and encapsulation efficiency of drug was calculated as follows:

Amount of drug loaded in NPs=Total amount of drug added-drug present in supernatant ….. eq. (1)

Loading capacity of NPs (%)=(Amount of drug loaded in NPs)/(Total amount of NPs used)× 100 ….. eq. (2)

Entrapment efficiency of drug (%)=(Amount of drug loaded in NPs)/(Total amount of drug taken)× 100 ….. eq. (3)

Release kinetics study of the drug was performed at 37ºC in phosphate buffered saline (PBS) (pH 7.4) by dialysis bag method [1] with slight modification. Increasing release of drug from nanoparticles was examined in PBS. Drug loaded lyophilized nanoparticles were suspended in 2 mL of PBS and kept in previously activated 5 cm long dialysis bag (Dialysis Membrane-70, 12–14 kDa molecular weight cut off, HiMedia Laboratories Pvt. Ltd., India), suspended in 100 mL of PBS and kept for continuous stirring speed of 100 rpm at 37oC. At predetermined time points of 0.25, 0.5, 1, 2, 4, 8, 12, 24,36, 48,60,72,84 and 96h , 1 mL of receptor media was withdrawn from container and same volume of fresh PBS was added each time to maintain sink conditions. Amount of drug released from loaded nanoparticles in each sample was determined by UV-Vis spectrophotometer.

1.3.8. X-ray powder diffraction studies

X-ray powder diffraction was performed by Bruker D8 ADVANCE eco system (Bruker, USA) with Cu Kα radiation (λ =1.5406 Å). Scans of freeze-dried Ze@Aes-GNPs, Ze@GNPs and free Aes, and physical mixture were performed from 2 to 60 degree 2θ at 0.2 degree/min scan rate with 40 kV voltage and 25 mA current.

1.3.9. Cytocompatibility of nanoparticles

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay was performed to determine cytocompatibility of Ze@GNPs(without drug) against immortalized normal human foreskin fibroblasts (hTERT-BJ) cells as described earlier [2]. Cells were seeded at a density of 1×104 cells/well in 96-well culture plate. Cells were treated with Ze@GNPsat different concentrations for 24 h. Cell viability was assessed by means of EZcountTM MTT

Cell Assay Kit (Himedia) according to manufacturer's protocol. MTT solution was added and incubated for 3-4 h, and then formazan crystals were solubilized in 100 µL of solubilization buffer. Absorbance was taken at 590 nm using ELISA plate reader to calculate % cell viability. Normalization was performed by taking cell viability of control cells (without any treatment) as 100% viable.

2. Statistical analysis

Results were expressed as mean ± S.D. All data were analyzed using one way analysis of variance followed by Tukey's test. Values of $p<0.05$ were considered as significant. All statistical analyses were performed using graph pad prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA).

3. Efficacy of Ze@Aes-GNPson collagen induced arthritis in rats

3.1. Ethical statement for use of experimental animals

Animal experiments were performed as approved by Institutional Animal Ethical Committee and according to the guidelines of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) and National Institutes of Health guide for the care and use of Laboratory animals. Female Wistar rats (140−160 g), 6–8 weeks old, obtained from animal house and maintained under standard laboratory condition following guidelines. Female rats were chosen as the effects of RA are more prominent due to hormonal imbalance. Rats were housed under an ambient temperature of 25 ± 1 °C with 12 h light /dark cycles after initial acclimatization for about 1 week. They had free access to standard rodent pellet diet and water ad libitum.

3.2. Protocol for *in-vivo* therapeutic efficacy study

To study effects of treatment of Ze@Aes-GNPs against collagen-induced arthritis, 30 female Wistar rats were randomly allocated to 5 groups of 6 rats each.

Group I (Control): Healthy Rats that received basal diet.

Group II (CIA): Rats served as collagen-induced arthritis (CIA) group.

Group III (CIA+ Free Aes): Rats served as CIA group, Aes 30mg/kg b.wt. *(i.a)*from onset of disease once daily, *i.e.* from 12th day of immunization upto day of sacrifice i.e. day 21.

Group IV (CIA +Ze@Aes-GNPs): rats served as CIA group, treated with Ze@Aes-GNPs in doses equivalent to 30 mg/kg body weight of AES from onset of disease once daily*(i.a)*, i.e. from 12th day of immunization upto day of sacrifice i.e. day 21.

Group V (Ze@GNPs): served as blank nanoparticle treated rats.

All rats were anaesthetized with mild anaesthesia and sacrificed by cervical dislocation on 21st day.

3.3. Induction of collagen-induced arthritis (CIA)

Arthritis was induced in Wistar rats as described previously[2]. Briefly, collagen type-II from bovine nasal septum was dissolved in 0.05 M acetic acid at a concentration of 2 mg/mL and emulsified with an equal volume of Complete Freund's Adjuvant (CFA) containing 1 mg/mL *Mycobacterium tuberculosis* H37 RA and stored on ice before use. Rats were immunized with prepared emulsion intradermally at about 1.5 cm distal from base of tail. From previous findings and during standardization of collagen type II-induced arthritis animal model, it was established that rats immunized with nasal bovine collagen type-II emulsified in CFA containing mycobacterium tuberculosis strain H37RA developed foot pad and ankle swelling indicative of arthritis, while rats immunized with nasal bovine collagen type-II emulsified in incomplete Freund's adjuvant showed no significant increase in foot pad and ankle width. Hence, CFA was chosen over incomplete adjuvant as CFA sensitized rats to develop Rheumatoid arthritis.

3.4. Measurement of clinical severity of arthritis (disease activity index and mean joint diameter)

To examine severity of disease, measurements of rat's joints (paw) diameter were carried out with digital Vernier's callipers (Yamayo, Japan) before and after onset of disease every 7th day. Evaluation of joint inflammation was performed by an observer having no knowledge of treatment protocol. Severity of arthritis was determined on day of sacrifice by a clinical score measurement as described earlier[3]i.e. from 0 to 4 as follows: 0, no signs of arthritis; 1, swelling of one group of joints (namely, wrist or ankle joints); 2, two groups of swollen joints; 3, three groups of swollen joints; 4; swelling of entire paw. Respective observations from different limbs of each rat were recorded and mean of these readings was reported as follows

3.5. Preparation of cell-free extract of ankle Joints

At the end of study, rats were lightly anesthetized and sacrificed by cervical dislocation. Rat joints were removed and cut into small pieces and homogenized in 5 vol. of 50 mM Tris HCl buffer, pH 7.4

containing 0.1 M NaCl and 0.1 % Triton X-100 and 1 vol. of fine glass powder by using a mortar and pestle. Crude extract was then sonicated for 20 s. Homogenate was centrifuged at 3,000 × *g* for 5 min, and resulting supernatant was then used for estimation of neutrophil elastase and nitrite level.

3.5.1. Neutrophil elastase (NE) activity

Neutrophil elastase (NE) activity in articular joint were evaluated as an index of polymorphonuclear leukocyte (PMNs) accumulation and activation in inflamed tissue as described earlier[4]. Briefly, joint tissue samples were homogenized in a solution containing 20 mM potassium phosphate buffer pH 7.0 in a ratio of 1:10 (w/v) and centrifuged for 20 min at 10,000 x g at 4 °C. An aliquot of each sample was incubated for 24 h at 37 °C with 0.1 M Tris–HCl buffer, (pH 8.0), containing 0.5 M NaCl and 1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, a specific synthetic substrate for neutrophil elastase (NE). Amount of p-nitroanilide liberated was measured spectrophotometrically at 405 nm and was considered as an NE activity. NE activity was converted and expressed as nano gram/gram of protein using a molar extinction coefficient (9500) of substrate as per manufacturer recommendation.

3.5.2.Estimation of Thiobarbituric Acid Reactive Substances (TBARS) for LPO activity

The assay of TBARS was done according to an earlier method[5] and was adapted to the microplate reader by bringing the final volume to 150 µL. In brief, tissue homogenate was prepared in 0.15 M KCl (5% w/v homogenate) and aliquots of 30 µL were incubated at 0℃ and 37℃ for 1 h. Subsequently, 60 µL of 28% w/v TCA was added and the volume was made up to 150 µL by adding 60 µL of distilled water, followed by centrifugation at 3000×*g* for 10 min. The supernatant (125 µL) was taken and color was developed by the addition of 25 µL of 1% w/v TBA dissolved in 0.05 N NaOH and kept in a boiling water bath for 15 min. The absorbance was read at 532 nm in a plate reader (Bio-Rad, U.S.A.). The result was expressed in micromoles TBARS formed/hour/milligram of protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm.

3.5.3.Assay for Reduced Glutathione (GSH)

GSH was measured in joints as described earlier[6] and adapted to the microplate reader. Homogenized joint tissue (10% w/v in phosphate buffer pH 7.4) was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4 °C for 2 h. The contents were centrifuged at 2000×*g* for 15 min. Fifty microliters of supernatant was added to 200 µL of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA (pH 8.9) followed by the addition 20 µL of 0.01 M DTNB. The absorbance was read in a microplate reader at 412 nm and results were expressed as micromoles GSH/gram tissue using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

3.5.4. Assay for Superoxide Dismutase (SOD) Activity

SOD activity was measured in joints as described earlier[7,8] and adapted to microtiter plates by bringing the final volume to 100 µL. The reaction mixture consisted of 0.05 M phosphate buffer (pH 7.4), 1 mM xanthine, and 57 µM NBT. After incubation at room temperature for 15 min, reaction was initiated by the addition of 50 mU xanthine oxidase. The SOD activity is expressed in units/mg protein using a molar extinction coefficient of 4.02×10^3 M^{-1} cm^{-1}.

3.5.5. Assay for Catalase Activity

Catalase activity was assayed by the method of Claiborne[9].The reaction mixture consisted of 1.95 mL of joint buffer (0.1 M, pH 7.4), 1.0 mL of hydrogen peroxide (0.019 M), and 0.05 mL of 10% PMS in a final volume of 3 mL. Changes in absorbance were recorded kinetically by spectrophotometer at 240 nm. Catalase activity was calculated as nanomoles H_2O_2 consumed per minute per milligram protein by using a molar extinction coefficient of 39.6 M^{-1} cm^{-1}.

3.5.6. Nitrite Level: Griess Reaction

Joint tissue from the sacrificed animals were washed with PBS (pH 7.4) and placed on ice as described[10]. Briefly a 50 µL sample was added with 100 µL of Griess reagent and the reaction mixture was incubated for about 5–10 min at room temperature and protected from light; the optical density was measured at 540 nm in the microplate reader according to the manufacturer's protocol. Calculations were done after generating a standard curve from sodium nitrite in the same buffer which was used for preparation of homogenate.

3.5.7. Myeloperoxidase (MPO) Estimation

Myeloperoxidase activity was estimated as an index of neutrophils infiltration in the synovial tissues of the arthritic rat's joint. The estimation of MPO was carried out by the method as described earlier[11].The MPO activity was expressed as U/gram of protein.

3.5.8. Measurement of TNF-α and IL-1β

Tumor necrosis factor-alpha (TNF-α) and interleukin 1β is involved in pathogenesis of rheumatoid arthritis. Level of TNF-α and IL-1β was quantified in rat's serum by using an enzyme-linked immunosorbent assay-based kit (eBioscience, Inc., San Diego, USA) following instructions of manufacturer protocol.

3.5.9. Immunohistochemical staining

Immunohistochemistry for COX-2 and IL-6, was performed using paraffin embedded sections of 5 µm thickness. Sections were de-paraffinized three times in xylene followed by dehydration in graded

ethanol and finally rehydrated in running tap water. For antigen retrieval, sections were boiled in 10 mM citrate buffer (pH 6.0) for 10−15 min. Sections were incubated with hydrogen peroxide for 15 min to minimize non-specific staining by quenching endogenous peroxidase activity and then rinsed three times (5 min each) with 1X PBST (0.05 % Tween-20). Blocking solution was applied for 10 min, then sections were incubated with polyclonal anti-COX-2 antibody (dilution 1:400),and polyclonal anti-IL-6 antibody (dilution 1:200) overnight at 4 °C in a humid chamber and then sections were incubated with HRP-conjugated secondary antibody for 2 h at room temperature.Finally, sections were incubated with 3, 3`-diaminobenzidine (DAB), counterstained with haematoxylin, mounted with DPX and covered with cover slips. On the basis of diffuseness of DAB staining, sections were graded as 0 (no staining), 1 (staining, 25 %), 2 (staining between 25 % and 50 %), 3 (staining between 50 % and 75 %), or 4 (staining >75 %). According to staining intensity, sections were graded as follows: 0 (no staining), 1 (weak but detectable staining), 2 (distinct staining) or 3 (intense staining). Immunohistochemical staining scores were obtained by adding diffuseness and intensity scores. Analyses were carried out using an Olympus microsystems bright field Microscope using objectives with 40X magnifications.

3.5.10. Histological examinations

Rats were sacrificed on day 21st by using mild anaesthesia. Rat ankle joints were removed and fixed in 4% paraformaldehyde. After decalcification in 5% formic acid, samples were processed for paraffin embedding. Rat joint tissue sections (5 μm thick) were stained with hematoxylin & eosin (H&E) and observed under an Olympus microscope.

To assess safety of blank NPs, H&E staining was performed on liver and kidneys. Organs were removed after sacrifice and preserved in 10 % neutral buffered formalin for H&E staining. Organs were embedded in paraffin waxthen sectioned with a microtome. H&E-stained sections were observed under light microscope.

3.5.11. Protein content

Protein content was determined by Bradford method using bovine serum albumin (BSA) as a standard as described earlier, with slight modification[1].

References

1 Nanocarrier Composed of Magnetite Core Coated with Three Polymeric Shells Mediates LCS-1 Delivery for Synthetic Lethal Therapy of BLM-Defective Colorectal Cancer Cells, https://colab.ws/articles/10.1021/acs.biomac.7b01607, (accessed January 17, 2023).

2 Md. M. Ansari, A. Ahmad, R. K. Mishra, S. S. Raza and R. Khan, *ACS Biomater. Sci. Eng.*, 2019, **5**, 3380–3397.

3 T. M. Haqqi, D. D. Anthony, S. Gupta, N. Ahmad, M.-S. Lee, G. K. Kumar and H. Mukhtar, *Proceedings of the National Academy of Sciences*, 1999, **96**, 4524–4529.

4 G. M. Campo, A. Avenoso, S. Campo, A. Ferlazzo, D. Altavilla, C. Micali and A. Calatroni, *Free Radical Research*, 2003, **37**, 257–268.

5 D. Utley and H. M. Whyte, *Australas Ann Med*, 1967, **16**, 200–204.

6 J. Sedlák and null L' Hanus, *Endocrinol Exp*, 1982, **16**, 103–109.

7 Y. Bansal, R. Singh, P. Saroj, R. K. Sodhi and A. Kuhad, *Toxicol Appl Pharmacol*, 2018, **355**, 257–268.

8 C. Beauchamp and I. Fridovich, *Anal Biochem*, 1971, **44**, 276–287.

9 R. A. Greenwald, *Free Radical Biology and Medicine*, 1987, **3**, 161.

10 I. Guevara, J. Iwanejko, A. Dembińska-Kieć, J. Pankiewicz, A. Wanat, P. Anna, I. Gołabek, S. Bartuś, M. Malczewska-Malec and A. Szczudlik, *Clin Chim Acta*, 1998, **274**, 177–188.

11 D. L. Lefkowitz, M. P. Gelderman, S. R. Fuhrmann, S. Graham, J. D. Starnes, S. S. Lefkowitz, A. Bollen and N. Moguilevsky, *Clin Immunol*, 1999, **91**, 145–155.