Supporting Information

Chlorogenic acid-conjugated nanomicelle attenuates disease severity in experimental arthritis

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Material and methods

Materials

Methotrexate was purchased from sigma Aldrich. Chlorogenic acid (CGA), DCC-(dicyclohexyl carbodiimide), DMAP (4-Dimethylaminopyridine) was purchased from Tokyo Chemical Industry, India. PLGA (Poly D L-Lactide-co-glycolide, (50:50)) was purchased from Ott scientific, Polysciences. Collagen type II was purchased from Elastin products INC. Himedia, India. Complete freunds adjuvant (CFA) and Pyrene were purchased from Sigma Aldrich.

Formulation of PLGA conjugated chlorogenic acid (PLGA-*b*-CGA) copolymer

Synthesis of PLGA conjugated chlorogenic acid copolymer

PLGA was conjugated with chlorogenic acid by esterification in presence of DCC and DMAP, briefly chlorogenic acid (CGA) (300mg) was dissolved in DMF (20ml) in presence of DCC (20mg), and the reaction was stirred on 0°c for 15 min. Then we added PLGA (200mg) and DMAP (25mg) simultaneously and allowed to stir for 24 hr to complete the reaction. The reaction product was purified by solvent extraction method. The solvent was evaporated by rotary evaporator and the product was then lyophilized.

Preparation of Methotrexate loaded-chlorogenic acid conjugated PLGA (MTX-PLGA-*b*-CGA) copolymer

The incorporation of MTX into PLGA-*b*-CGA was performed by solvent evaporation method.[1,2] Briefly, 20% w/w of MTX added with PLGA-*b*-CGA in 4ml of acetone to disperse all constituents and allowed to stir for 15min, further the self-assembly was initiated by addition of water dropwise. Due to the self-assembly the constituents were arranged as nanomicelle entrapping the MTX. The un-entrapped MTX was then removed by the centrifugation at speed of 15000 rpm for 30min.The supernatant was removed and the entrapped drug was calculated by using following formulas.

Characterization ofPLGA conjugated chlorogenic acid copolymer (PLGA-*b*-CGA) copolymer

Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR spectra of PLGA, Chlorogenic acid (CGA), MTX, PLGA-*b*-CGA and MTX-PLGA-*b*-CGA were recorded on Cary Agilent 660 IR spectrophotometer from Agilent Technologies

USA. Each sample was scanned for 128 scans and 4 cm^{-1} resolution kept for wavelength scan ranging from 4000-400 cm^{-1}[2].

^{1}H Nuclear Magnetic Resonance (NMR)

PLGA-*b*-CGA conjugation was evaluated by ^{1}H NMR. The sample was dissolved in DMSO-d6 and scanned for 64 scans on Bruker Advance-II spectrometer at 400 MHz[2].

Size and surface potential determination

Size of self-assembled PLGA-*b*-CGA and MTX-PLGA-*b*-CGA was determined by (DLS Zetasizer Nano ZSP; Model-ZEN5600; Malvern Instruments LTD, Worcestershire, UK), and Transmission electron microscopy (TEM) by dropping a 4µl solution on carbon coated copper grid (Ted Pella Inc.) JEOL JEM-2100 (Tokyo, Japan).The samples were placed in (disposable polystyrene cuvettes SARSTEDT AG & Co. D-51588 Numbrecht). For determination surface potential the samples were analysed by(Zetasizer Nano ZSP; Model-ZEN5600; Malvern Instruments LTD, Worcestershire, UK).The values were obtained in n=3 and represented as mean for Size and surface potential.[2]

Ultraviolet Visible (UV) spectroscopy

The concentrations of MTX released from the nanomicelle was calculated by interpolating on standard curve of MTX by UV visible spectroscopy (Shimadzu UV-2600, quartz cuvette, path length 1 cm) .[2]

Fluorescence Spectroscopy

The critical micelle concentration of (CMC) PLGA-b-CGA was analysed by pyrene fluorescence assay and measured on (FS5, Module SC-5, Standard cuvette holder from Edinburgh Instruments, UK). A Number of concentrations from lowest range to highest range were incubated with 100µl of pyrene for 4hr and then measured for the quenching by excitation at 343 nm. The slit width for excitation and emission kept 3.0 nm. Logarithmic concentration (log C) was plotted against fluorescence intensity ratio of (I383/I373) to calculate the CMC.[2]

***In-vitro* drug loading and encapsulation efficacy**

The drug loading was performed by the conventional solvent evaporation method.[3] Twenty milli grams of MTX drug along with 100 mg of nanomicelles was dissolved in 2 mL of acetone, and then it was stirred to form a homogeneous solution. Furthermore, with the help of a syringe, an equivalent amount of water was mixed and the solution was kept for self-assembly in the

presence of water. The traces of acetone were then evaporated by heating the solution. The sample was centrifuged at 15000rpmfor 30 min to determine the entrapped drug. These diment and supernatant were separated, and the concentration of drug in the supernatant was evaluated by UV analysis. The calibration curve of MTX was plotted with its maximum absorption waveleng that 303 nm. The entrapped drug was calculated with the help of the standard curve of MTX.

Total entrapped drug = Total drug taken - Drug in supernatant (1)

Drug loading (%) = Entrapped drug in micelle (μg) /Amount of polymer (μg) ×100 (2)

Encapsulation efficiency (%) = Entrapped drug in micelle (μg) / Amount of drug (μg) ×100 (3)

***In-vitro* drug release**

In vitro drug release was performed by following dialysis bag method in 250ml volume phosphate buffer saline (PBS ~ pH7.4, pH 5.8) Briefly, a known amount of MTX loaded PLGA-*b*-CGA was suspended in 2ml of PBS and dispersed in activated dialysis bag tied/clipped at one end, after incorporating the MTX loaded PLGA-*b*-CGA another end was tied/clipped and stirred in PBS 250ml in beaker. Then a 1ml of sample was taken at predetermined interval from 0hr to 48hr to interpret the release at that particular time which is further determined by UV-Visible spectrophotometer.[4]

Cell viability test

The cell viability assessment of PLGA-*b*-CGA nanomicelle was performed to determine the cytocompatibility on normal or healthy cells by XTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) [5]. The assay was performed against normal human foreskin fibroblasts (hTERT-BJ) cells. The cells were grown on 96 well cell culture plates at concentration of 1×10^4cells per well, a range of concentrations of PLGA-*b*-CGA were chosen to incubate on the cells for the 24hr. Further, XTT (70μl/well) along with electron coupling reagent (60:10) was incubated at 37°C for 4hr. The absorbance was taken at 590 nm using ELISA plate reader to determine the % cell viability. The normalization was performed by taking control cells as 100% viable.

% cell viability = [100 × (sample absorbance)/ (control absorbance)]

***In-vivo* therapeutic study for the assessment of potential of MTX loaded PLGA-*b*-CGA on collagen induced arthritis in wistar rats.**

Induction of arthritis in female wistar rats

The arthritis induction was started by injecting the Collagen Type-II (C-II) emulsion with complete freunds adjuvant (CFA). The emulsion was prepared night before the injection or induction. Briefly, the 2mg/ml of C-II dissolved in 10mM of glacial acetic acid, further the equal volume of CFA was added in mortar and pestle to homogenise and form milky slurry.[6] The emulsion was store in freeze and 100µl of it administered intra-dermally 1.5cm away from the base of the tail. Animals were observed for the swelling and redness of the paw daily. After 7 days the booster dose 50µl was administered to enhance the severity of the symptoms of the disease.

Therapeutic protocol and grouping of animals

To evaluate the therapeutic potential of methotrexate loaded CGA-*b*-PLGA nano-micelle against collagen induced arthritis in female wistar rats, rats were distributed accordingly in 6 groups containing 6 rats each by keeping the average weight of every group as close as possible. The treatment was started after development of arthritis symptoms (after 14 days). The treatment was given by three times keeping minimum gap of 2 days among each dose.

Group A (Control) - Healthy rats received basal diet and water ad libitum

Group B (CIA) – This group were served as collagen induced arthritis (CIA) group.

Group C (CIA+MTX-PLGA-*b*-CGA) - Rats were served as treatment group of CIA + Methotrexate loaded PLGA-*b*-CGA Nanomicelle (10mg/kg eq.wt. of micelle given i.a.)

Group D (CIA+MTX) - Rats were served as treatment group for naïve drug CIA+ Methotrexate (10mg/kg b.w. of wistar rats given i.a.)

Group E (CIA+PLGA-*b*-CGA) - Rats were served as treatment group for Blank PLGA-*b*-CGA Nanomicelle.

Group F (Control +PLGA-*b*-CGA) - Rats were served as safety group of Blank PLGA-*b*-CGA Nanomicelle in healthy rats.

All rats were anesthetized with 5% isoflurane anaesthesia and sacrificed by cervical dislocation.

Paw diameter change

The paw diameter measures the essential changes such as swelling and arthritis severity. The paw diameter was measured by digital Vernier calliper. [7] The paw diameter of each animals from group was measured every 2nd day and represented as mean.

Body Weight Changes

Body weight changes were assessed by weighing the rats on every alternate day for 21 days. The body weight is crucial parameter to assess the health of animals.[7,8]

X-ray visualization

The X-rays imaging was performed on last days of study before the sacrifice the animals were anaesthetized and placed on the radiographic plate. The X-ray machine was used to film the joint part (Siemens Klinoskop-H) with a 38-kvp exposition for 6.5 mAs. [2]

Hematoxylin and eosin staining

The hind limb of rats were subjected to decalcification and then embedded in paraffin wax. 5µm thick section was cut from the wax block of decalcified bone. The sections were subjected for hematoxylin and eosin staining by following protocol[2]. After successful staining the slides were imaged by Thermo-Fisher microscope at 10X magnification.

Safranin-O staining

The cartilage integrity was visualized microscopically by safranin-O staining. The sections deparaffinised by heating and xylene treatment, further dehydration in graded series of alcohol then water. The slides were stained with hematoxylin and excess stain was destained with acid ethanol. Further the sections were dipped in 0.001% fast green (FCF) and washed with acetic acid. The sections were the incubated with 0.1% safranin-O for15-25mins. Further these slides were dehydrated with increasing concentration of ethanol and then Xylene. The sections were mounted with DPX and observed under microscope. [2]

Toluidine blue staining for cartilage and mast cells

The mast cells and the cartilage were metachromatically stained with toluidine blue atpH 2.3 and pH 4.0 respectively. The sections were deparaffinised hydrated with various concentration of alcohol followed by water. The slides were then stained with toluidine blue stain (0.4%) for 20-30min and excess stain then washed by water. The sections were then back-stained with fast green (FCF) for 15 min. The slides were then incubated with alcohol and then xylene. Theslides were mounted with DPX and visualized by microscopy. The cartilage specifically indicated by bright purple blue colour lining over the joints whereas the mast cells were stained purple blue colour as well.[2]

Immunohistochemical staining

The localization of the upregulation or downregulation of proteins on the histological sections were identified by performing Immunohistochemistry.[2] The sections were heated and deparaffinised by xylene and subjected for the dehydration with graded series of alcohol. The sections then subjected for the antigen retrieval in 10mM sodium citrate buffer pH 6.0. The sections were treated with 0.3% hydrogen peroxide (H_2O_2) to remove the activity of the endogenous peroxidase enzyme. Then slides were washed 3 times with PBS and then incubated with 10% bovine serum albumin in ethanol for 45min, furthermore, the sections were washed with 5times PBST (PBS, 0.25% tween 20). Primary antibody against protein RANKL (receptor activator of nuclear factor kappa beta (NF-κB ligand) and NF-κB(nuclear factor kappa beta) were incubated on sections covered by boundary of hydrophobic pen. The sections were maintained with moist and humid environment and kept at 4°C overnight. The sections were then washed with the PBST 5 times. Then HRP- Conjugated secondary antibody was incubated for 2hours, and after washing with PBST the sections were stained with DAB (3,3′-Diaminobenzidine) which reacts with HRP and exerts brown colored product. Then the sections were stained with hematoxylin and then mounted with DPX and visualized under microscope for the expression of the proteins.

Statistical Analysis

All the statistical analysis were performed by software graph pad prism 5(Graph Pad Software, Inc., San Diego, CA, USA). The values are mentioned are mean± s.d.The level of significance and comparison were performed by ANOVA and followed by Tukey's test. The value of $p \leq 0.05$ mentioned as significant.

Results

Mast cells staining

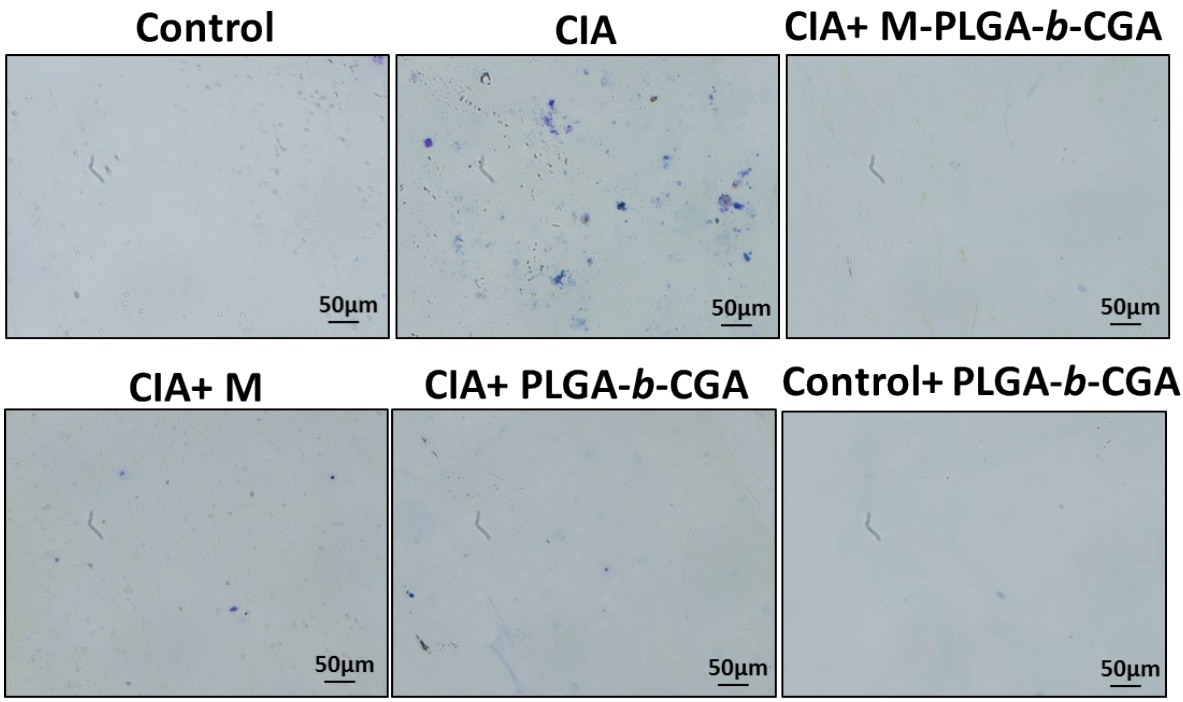

Figure S1. Microscopic images of mast cells staining by toluidine blue in animals treated with different treatment.(N=3 number of animals) All images are taken at 40X magnification

Critical Micelle concentration

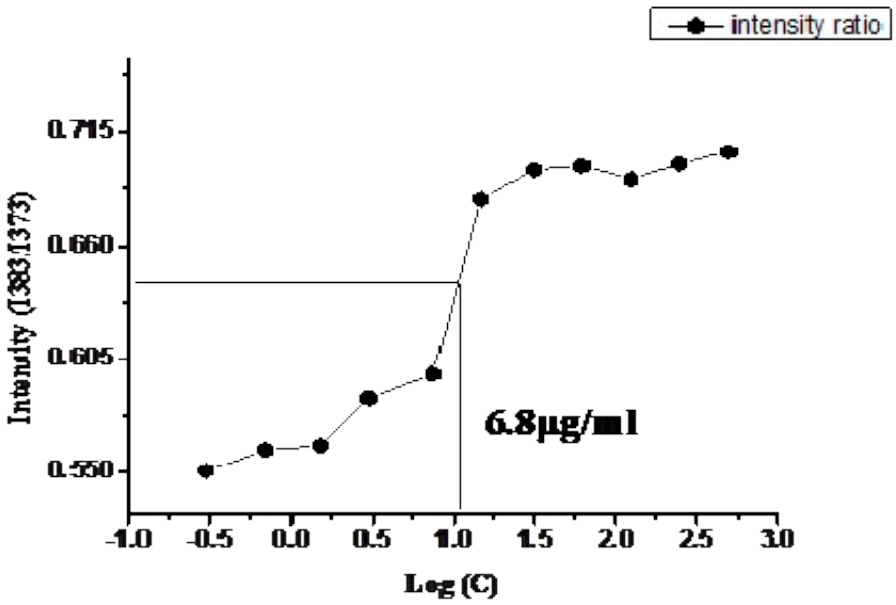

Figure S2. Graphical representation of the assessment of the critical micelle concentration of the PLGA-b-CGA nanomicelle. The values are represented as ration of I383/I375 vs Log concentration of the nanomicelle.

Estimation of H_2O_2 and Peroxidase activity

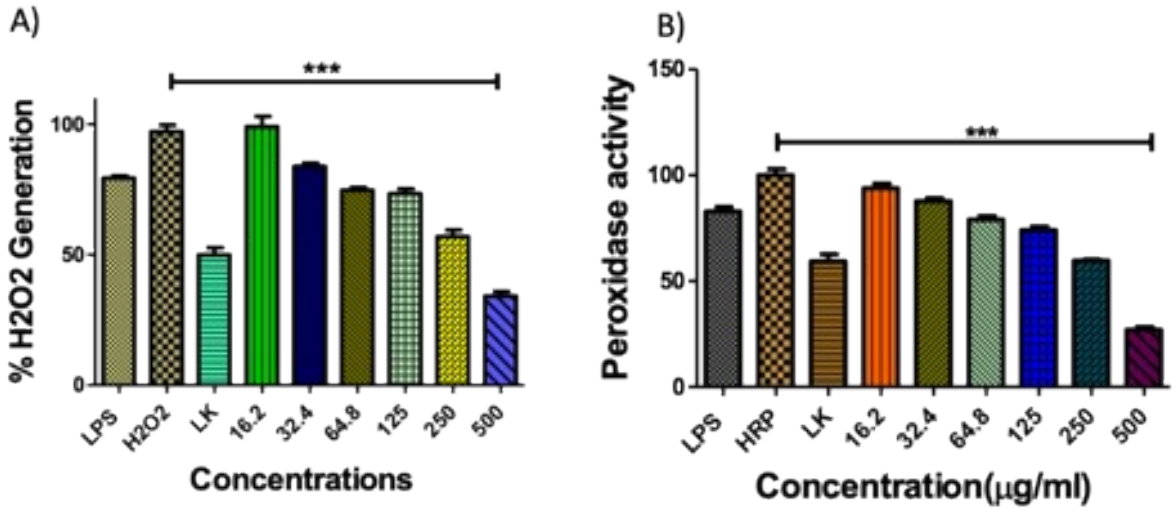

Figure S3: Representative graphs of the antioxidant and anti-inflammatory potential or the MTX-loaded PLGA-b-CGA. A) Represents the $\%H_2O_2$generation after treatment with various concentrations of the MTX-PLGA-b-CGA. B) Representative graphs of the Peroxidase activity on LPS activated RAW 264.7 cell lines. The values are mentioned as Mean ± S.D. The values are statistically different from each other whereas *** $p<0.001$.

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