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Stimuli-responsive star polyethylene glycol conjugates for improved intracellular delivery of N-acetyl cysteine in neuroinflammation

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Abstract

N-Acetyl cysteine (NAC) is an important drug currently under clinical trials for the treatment of neuroinflammation in maternal-fetal applications. The free sulfhydryl groups in NAC lead to high plasma protein binding, resulting in low bioavailability. Preparation and activity of conjugates of NAC with thiol terminated multi-arm (6 and 8) poly(ethylene-glycol) (PEG) with disulfide linkages involving sulfhydryls of NAC are reported. Multiple copies (5 and 7) of NAC were conjugated on 6 and 8-arm PEG respectively. Both the conjugates released 74% of NAC within 2h by thiol exchange reactions in the redox environment provided by glutathione (GSH) intracellularly (2–10 mM). At physiological extracellular glutathione concentration (2μM) both the conjugates were stable and did not release NAC. MTT assay showed comparable cell viability for unmodified PEGs and both the PEG-S-S-NAC conjugates. The conjugates were readily endocytosed by cells, as confirmed by flow cytometry and confocal microscopy. Efficacy of 6 and 8-arm PEG-S-S-NAC conjugates was evaluated on activated microglial cells (the target cells, *in vivo*) by monitoring cytokine release in lipopolysaccharide (LPS) induced inflammatory response in microglial cells using the reactive oxygen species (ROS), free radical nitrile (NO), anti-inflammatory activity and GSH depletion. The conjugates showed significant increase in antioxidant activity (more than a factor of 2) compared to free drug as seen from the inhibition of LPS induced ROS, NO, GSH and tumor necrosis factor- α (TNF- α) release in microglial cells.

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Supporting Information Available: FTIR spectrum, ¹H NMR, HPLC chromatogram, MALDI-TOF analysis and cytotoxicity graph of the compounds. This material is available free of charge via the Internet.

1. Introduction

In the last few decades PEGylation of drugs has been extensively studied for drug targeting and site specific delivery.¹ PEGylation of drugs offers several advantages, including increased water solubility, plasma circulation time, improved tumor targeting by the enhanced permeability and retention (EPR) effect, and reduced immunogenic response.² PEGylation of drug involves covalent linking of the drug to polyethylene glycol (PEG) which yields the 'prodrug'. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug, and has improved delivery properties over the parent molecule.⁴⁻⁷

PEG is non-toxic and it can be eliminated by a combination of renal and hepatic pathways thus making it ideal carrier in pharmaceutical applications. PEG has the lowest level of protein or cellular absorption of any known polymer.⁸ PEG has been approved for human use by FDA in dosage forms such as intravenous (IV), oral and dermal applications. PEGylation has been reported for several drugs such as PEG-Paclitaxel,⁹ PEG-Camptothecin,¹⁰ PEG-Ara-C,¹¹ PEG-Doxorubicin,¹² PEG-Adriamycin,¹³ PEG-Daunomycin,¹⁴ and anti HIV PEG-Saquinavir.¹⁵ The examples of commercialized PEGylated products are PEG-INTRON®, PEGASYS®, ADAGEN® and ONCASPAR® and few in clinical trial include: PEG- Paclitaxel, PEG-Camptothecin and PEG-Aspartic acid.¹⁶

Drugs are often linked to PEG *via* hydrolyzable or enzymatically cleavable bonds such as esters, carbonates, carbamates and hydrazones. In certain selective cases amide linkages which can be broken down in plasma as well as in the lysosomal compartment by peptidases or cathepsins have been explored.¹⁷ Rapid breakdown of the conjugate can lead to dumping of the drug cargo, while too slow a release rate will compromise the efficacy of the drug. The rate of drug release is governed by the nature of the 'linker molecule', and for optimal drug release linkages should be chosen such that either pH or enzymatic degradation mediates drug release. In the case of PEG conjugates it is clear that the solubility of the prodrug will almost always exceed that of the original drug, usually overcoming any existing aqueous insolubility and thus increasing the possibility of more effective drug delivery.

Relatively few drugs are approved for maternal-fetal applications, due to the potential for additional side effects associated with the baby. N-acetyl cysteine (NAC) is one of the few drugs currently under clinical trials for treating neuroinflammation associated with maternal fetal infections.¹⁸⁻¹⁹ However, the use of NAC requires higher and repeated dosing due to the poor bioavailability and blood stability. NAC has free sulfhydryl groups which are capable of spontaneous oxidation, and forming disulfide bonds with plasma proteins.²⁰ The low blood concentrations and low oral bioavailability of NAC (6-10%) can be attributed to its plasma protein binding.²¹⁻²² The higher and frequent dosing of NAC can lead to cytotoxicity and side effects including increased blood pressure.²³⁻²⁸ Hence there is a need to develop a prodrug of NAC which eliminates its plasma protein binding. Recent studies with disulfide-linked polyamidoamine (PAMAM) dendrimer-NAC conjugates showed that the conjugates released the drug effectively at intracellular glutathione levels, showing superior efficacy compared to NAC in activated microglial cells.²⁹⁻³⁰ The disulfide bonds are sufficiently stable in the circulation and in the extracellular milieu, and are prone to rapid cleavage under a reductive environment through thiol-disulfide exchange reactions found intracellularly.³¹⁻³² However, dendrimers are still not approved for human use, therefore, star PEG is explored in this study and are known to have longer circulation times.¹ Yet another significant advantage of PEG is that it does not invoke an immunogenic response.¹ We use a thiol terminated multi-arm PEG scaffold (6 and 8-arm-PEG) to conjugate to NAC. The branched PEGs offer the advantage of multivalency over the linear PEGs and hence were chosen to attain higher drug

payloads. The PEGylation of NAC to achieve targeted release in the treatment of neuroinflammation in perinatal applications is being explored for the first time.

The present study discusses the preparation, characterization and efficacy of disulfide-linked star PEG-NAC conjugates that are tailored to release the drug under intracellular GSH levels. The PEGylation of NAC was confirmed by ^1H -NMR and MALDI-TOF, and the stability and the drug release from conjugates in the presence of GSH was measured using HPLC. The cellular uptake of these conjugates was assayed using flow cytometry and confocal microscopy. The anti-oxidative properties of the PEG-S-S-NAC conjugates (6 and 8-arm) were tested in activated BV-2 microglial cells by measuring the reactive oxygen species (ROS), free radical NO, anti-inflammatory activity and GSH depletion. To our knowledge these are the first such studies on PEG-NAC conjugates for neuroinflammation, where the conjugates show significantly better efficacy than free drug in cells.

2. Experimental procedures

2.1. Materials

The 6-arm-PEG-SH (10 kDa) was purchased from Sunbio, USA and 8-arm-PEG-SH (20 kDa) was purchased from NOF America Corporation, USA. Other reagents were obtained from assorted vendors in the highest quality available. Of these, 2, 2'-dipyridyldisulfide (Aldrithiol), N-acetyl cysteine (NAC), glutathione (GSH), phosphate buffer saline (PBS, pH=7.4), 2, 5 dihydroxybenzoic acid, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, monochlobimane and lipopolysaccharide (LPS), and HPLC-grade solvents were obtained from Sigma-Aldrich. Fluorescein 5-maleimide (FITC), dulbecco's modified eagle medium, fetal bovine serum, penicillin-streptomycin and 0.05% trypsin-EDTA were purchased from Invitrogen USA. The assay kits; Amplex-Red hydrogen peroxide/peroxidase, nitrate/nitrite determination and Mouse TNF- α ELISA kit were purchased from Invitrogen, Cayman Chemical and BD Biosciences, respectively.

2.2. Methods

All ^1H NMR spectra were recorded on Varian mercury spectrometer 400MHz in CHCl_3 . MALDI-TOF/MS spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and a matrix of 2, 5 dihydroxybenzoic acid.

2.2.1. Reverse phase-HPLC—HPLC characterization of conjugates was carried out with Waters HPLC instrument equipped with two pumps, an autosampler and dual UV detector interfaced to Breeze software. The mobile phase used was acetonitrile / water (pH=2.25) both containing 0.14% TFA. The water phase was freshly prepared and both the phases were filtered and degassed prior to use. Supelco discovery BIO Wide pore C5 HPLC column (5 μm particle size, 25 cm length, 4.6 mm I.D.) equipped with two C5 supelguard cartridges (5 μm particle size, 2 cm length, 4.0 mm I.D.) was used for characterization of the conjugates as well as release and stability studies. Gradient method was used for analysis (100:0) water: acetonitrile to (60:40) water-acetonitrile in 25 min followed by returning to initial conditions in 5 min. The flow rate was 1 mL/min. The dual UV absorbance detector was used at wavelengths 210 nm and 280 nm simultaneously. Standard calibration curves were plotted for NAC, GSH and their oxidized forms (NAC-S-S-GSH, GS-SG and NAC-S-S-NAC) based on peak area obtained at 210nm for release.

2.2.2. Stability study of conjugates—The conjugate stability was evaluated for a period of 3 days at 37°C in a phosphate buffer saline (PBS) at physiological pH (7.4). 1 mg/mL of conjugate (**1** or **3**) dissolved in PBS was kept at 37°C and stirred continuously. At specific time

intervals, 20 μ L of sample was withdrawn and analyzed by HPLC. All experiments were run in triplicate for statistical analysis.

2.2.3. Preparation of S-(2-thiopyridyl) N-acetyl cysteine (NAC-TP)(6)—S-(2-thiopyridyl) N-acetyl cysteine (**6**) was prepared by the reaction of 2, 2¹-dithiodipyridine (TP-TP) (5.398g, 0.0245mol) with NAC (**4**) (2g, 0.0122mol) in a mixture of methanol and water (1:1) stirred for 15h at room temperature (r.t). Upon completion of the reaction (monitored by TLC), most of the methanol was removed *in vacuo* and the residue was dissolved in water extracted into dichloromethane and concentrated on rota-evaporator under reduced pressure to get crude product. Crude product was purified by silicagel column chromatography by elution with dichloromethane/methanol (8:2) to pure NAC-TP (**6**) as a light yellow solid in 80% yield (2.66 g, 0.098 mole). Calculated mass: ESI m/z (M+H) 273, ¹H-NMR (400 MHz, CD₃OD) δ , 1.99 (s, 3H), 3.10-3.20 (m, 1H), 2.30-2.38 (m, 1H), 4.65-4.70 (m, 1H) 7.20-7.27 (m, 1H, Ar), 7.80-7.85 (m, 2H Ar), 8.40-8.45 (m, 1H). ¹³C-NMR (100 MHz, CD₃OD), 21.22, 39.91, 52.05, 120.26, 121.37, 122.08, 137.98, 149.00, 159.56, 172.14.

2.2.4. Preparation of 6-arm-PEG-S-S-NAC conjugate (1)—For the preparation of 6-arm-PEG-S-S-NAC (**1**), NAC-TP (**6**) (0.245 g, 0.897 mmole) in ethanol (10 mL) was added to a solution of 6-arm-PEG-SH (**7**) (1.0 g, 0.1 mmole) in a PBS pH 7.4 (20 mL) and reaction was stirred at room temperature for 4h. The reaction was monitored with HPLC. After completion of the reaction, the reaction mixture was purified by size exclusion chromatography using sephadex LH-20 column (Amersham Pharmacia Biotech, 3.8 $\times$ 45cm) with water as mobile phase. The fractions containing (**1**) were lyophilized to remove water and to get pure compound (**1**) in 95% yield (0.102 g, 0.0094 mmole). ¹H-NMR (400 MHz, CD₃OD) δ , 2.00 (s, 3H), 2.95-3.10 (m, 1H), 3.30-2.38 (m, 1H), 3.58-3.80 (br, m, 4H, -OCH₂-CH₂O-) 4.40-4.50 (m, 1H), 6.95 (br, s 1H, NH amide)

2.2.5. Preparation of 6-arm-PEG-FITC conjugate (2)—To a stirred solution of 6-arm-PEG-SH (**7**) (0.05 g, 0.005 mmole) in PBS (2 mL, pH 7.4) was added fluorescein 5-maleimide (**8**) (0.0064 g, 0.0149 mmole), the reaction was continued for 2h at room temperature in dark. Reaction was analyzed using RP-HPLC to determine the extent to which fluorescein 5-maleimide had reacted with 6-arm-PEG-SH (**7**) polymer. After completion of reaction, the reaction mixture was purified by size exclusion chromatography using sephadex LH-20 column (Amersham Pharmacia Biotech, 3.8 $\times$ 45cm) with water as mobile phase. The fractions containing (**2**) were lyophilized to remove water and to get pure compound (**2**) in 96% yield. ¹H-NMR (400 MHz, DMSO-d₆) δ , 3.25-3.70 (br, m, 4H, -OCH₂-CH₂O-) 4.20-4.30 (m, 1H), 6.20-6.30 (br, s 3H), 6.60-6.67 (d, 3H), 7.20-7.26 (br, s, 2H), 7.35-7.43 (br, s, 2H), 7.87-7.92 (s, 1H).

2.2.6. Preparation of 8-arm-PEG-S-S-NAC conjugate (3)—To a stirred solution of NAC-TP (**6**) (0.163 g, 0.599 mmole) in ethanol (2 mL), a solution of 8-arm-PEG-SH (**9**) (1.0 g, 0.05 mmol) in PBS buffered pH 7.4 (20 mL) was added, and the reaction was stirred at room temperature for 4h. The reaction was monitored with HPLC. After completion of reaction, the reaction mixture was purified by size exclusion chromatography using Sephadex LH-20 column (Amersham Pharmacia Biotech, 3.8 $\times$ 45cm) with water as mobile phase. The fractions containing (**3**) were lyophilized to remove water and to get pure compound (**3**) in 92% yields. ¹H-NMR (400 MHz, CD₃OD) δ , 2.00 (s, 3H), 2.95-3.10 (m, 1H), 3.30-2.38(m, 1H), 3.58-3.80 (br m 4H, -OCH₂-CH₂O-) 4.40-4.50 (m, 1H), 6.95 (br, s 1H, NH amide).

2.2.7. Dynamic light scattering and zeta potential—Dynamic light scattering (DLS) and Zeta Potential analyses were performed using a Malvern Instruments Zetasizer Nano ZEN3600 instrument (Westborough, MA) with reproducibility being verified by collection

and comparison of sequential measurements. PEG-S-S-NAC conjugate samples were prepared using PBS pH=7.4. DLS measurements were performed at a 90° scattering angle at 37°C. Z-average sizes of three sequential measurements were collected and analyzed. Zeta potential measurements were collected at 25°C, and the Z-average potentials following three sequential measurements were collected and analyzed.

2.2.8. In- vitro NAC release—The *in-vitro* release of NAC from the conjugates 6 and 8-arm-PEG-S-S-NAC (**1** and **3**) was performed in PBS (pH =7.4) at 37°C. 1 mg/mL solutions of 6 and 8-arm-PEG-S-S-NAC (**1** and **3**) conjugate in PBS (pH 7.4) were prepared respectively. To mimic intracellular conditions GSH was added to each of the conjugate solutions to form 10 mM and 2 mM solutions and NAC release was monitored. Further, to mimic the extracellular conditions (*eg* blood) 2 μ M GSH was added to another set of 1mg/mL solutions of 6 and 8-arm-PEG-S-S-NAC (**1** and **3**) conjugate respectively in PBS pH 7.4 and again NAC release was monitored. As a control, 1mg/mL of each conjugate in PBS pH 7.4 was analyzed for NAC release in the absence of reducing agent GSH. The solutions were kept at 37°C and stirred continuously. At predetermined time intervals, 30 μ L of samples were withdrawn and immediately analyzed to quantify the NAC and GS-NAC release using RP-HPLC. All samples were run as triplicates for statistical analysis.

2.2.9. Flow cytometry to determine uptake of 6-arm-PEG-FITC (2**) by microglial cells**—Mouse microglial cell line (BV-2) was obtained from Children's Hospital of Michigan cell culture facility. Cells were grown in 75 mm² culture flasks using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C with 5% CO₂ in an incubator. The cells were subcultured every 48 hours and harvested from subconfluent cultures (60–70%) using 0.05% trypsin-EDTA. BV-2 cells (passage 21) were grown overnight in 6-well plate using DMEM cell culture medium supplemented with 5% FBS and 1% penicillin-streptomycin. When the cells were 70% confluent, they were treated with 10 μ g/mL of PEG-FITC (**2**) for 15, 60, 120 and 240 min. The cells were washed with PBS, trypsinized and centrifuged at 1500 rpm for 5 min to obtain a cell pellet. The cells were washed three more times with PBS, and resuspended in 1% formaldehyde, and analyzed using a flow cytometer (FACS caliber, Becton Dickinson) by counting 10,000 events. The mean fluorescence intensity of cells was calculated using the histogram plot.

2.2.10. Confocal laser scanning microscopy—The procedure for cell culture and 6-arm-PEG-FITC (**2**) treatment was the same as described in previous section. After treating BV-2 cells (passage 21) for 2 h with 10 μ g/mL of 6-arm-PEG-FITC (**2**), the cells were washed with PBS 3 times and fixed with 4% para-formaldehyde for 20 min. Images were captured under the confocal microscope (Zeiss LSM 310) using a magnification of 400x. The emission and excitation wavelengths were 488 and 518 nm for FITC.

2.2.11. Cell cytotoxicity assay—MTT assay was performed to assess the cytotoxicity of 6- and 8-arm PEG-S-S-NAC conjugates (**1** and **3**). BV-2 cells (passage 21) were seeded into 96-well plate at 5×10^3 /well. Cell were incubated overnight at 37°C and the medium was removed, cells were exposed to 100 ng/mL of LPS and varying concentrations of both the conjugates in serum free medium for 24 h. Control treatments included varying concentrations of free NAC or PEG, positive control with 100 ng/mL of LPS induction, and negative control with medium alone. 10 μ L of MTT (5 mg/mL) was added to each well. After 4 hours of incubation at 37°C, the supernatant was removed, and 100 μ L of DMSO was added to dissolve precipitated formazan crystals. The plate was shaken for 10 min. OD value was measured with ELISA reader at a wavelength of 570 nm. The proportion of viable cells in the treated group was compared to that of the negative control.

2.2.12. Efficacy study of PEG-S-S-NAC conjugates—BV-2 cells (passage 22) were seeded in 24 well plates at 10^5 /mL/well and incubated for 24 hours. The medium was removed and cells were exposed to 100 ng/mL of lipopolysaccharide (LPS) and varying concentrations of 6 and 8-arm-PEG-S-S-NAC conjugates (**1** and **3**) in 500 μ L of fresh serum free medium for 72 h. Control treatments included varying concentrations of free NAC or PEG, positive control with 100 ng/mL of LPS induction, and negative control without any LPS induction and treatment. The supernatant was collected at specific time intervals of 72 h, and spun at 1500 rpm for 5 min. The supernatant was stored at -80°C for further assays.

2.2.13 Intracellular GSH measurement—The reduction in intracellular GSH level was assessed by spectrofluorometer using monochlobimane staining. The procedure for culture and conjugate treatment was the same as described in previous section. BV-2 cells (passage 22) were seeded in collagen I coated 96-well plates and washed once with PBS and incubated with 50 μ M monochlobimane diluted in phenol red free medium. The fluorescence intensity was measured after 15 min at 37°C . Excitation and emission wavelengths were 390 nm and 478 nm, respectively. Intracellular GSH reduction rate was calculated according to formula: [GSH depletion rate (%) = (fluorescence intensity of negative control – fluorescence intensity of treatment group)/ fluorescence intensity of negative control $\times$ 100%].

2.2.14 Measurement of ROS (Reactive oxygen species)— H_2O_2 released from BV-2 cells was measured using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), followed the manufacturer's instructions. The procedure for cell culture and conjugate treatment was the same as described above. The supernatant was mixed with 0.05 μ M of horseradish peroxidase and 1 μ M of Amplex Red in 96-well plate. After 30 min incubation, the fluorescence intensity was measured using spectrofluorometry. Excitation and emission wavelengths were 530 nm and 590 nm.

2.2.15. NO release assay—Production of NO was assayed by measuring the levels of nitrite and the stable NO metabolite in the supernatant. Accumulation of nitrite in the supernatant was determined by colorimetric assay with Griess reagent system, which uses sulfanilamide and N-(1-Naphthyl)-ethylene diamine. The procedure for cell culture and conjugate treatment was the same as described in previous section. 100 μ L of the supernatant was incubated with 50 μ L of Griess reagent 1 (sulfanilamide) and 50 μ L of Griess reagent 2; N-(1-Naphthyl)-ethylenediamine for 10 min at room temperature. The absorbance at 540 nm was then measured, and nitrite concentration was determined using a curve calibrated with nitrite standards.

2.2.16. Detection of TNF- α —The procedure for cell culture and conjugate treatment was the same as described above. TNF- α secretion was measured using an ELISA kit according to the manufacturer's instruction. In brief, 50 μ L of supernatant from each sample was added in 96-well ELISA plate. Biotinylated antibody reagent was applied to each well and the plate was incubated at room temperature for 2 h. After washing the plate with PBS-Tween 20, diluted streptavidin-HRP was added, and the plate was incubated at room temperature for 30 min. After washing the plate, premixed TMB substrate solution was added. The plate was developed in the dark for 30 min, and read at 450 nm using a microplate reader. The concentration of TNF- α was calculated using murine TNF- α as standard.

3. Results and Discussion

3.1. Preparation of 6-arm-PEG-S-S-NAC conjugate (**1**) and 6-arm-PEG-FITC (**2**)

S-(2-thiopyridyl) N-acetyl cysteine (NAC-TP) (**6**) was prepared from the reaction of 2, 2¹-dithiodipyridine (**5**) in excess and N-acetyl cysteine (**4**) in a mixture of methanol and water at room temperature (r.t.) (Scheme 1). Formation of NAC-TP (**6**) was confirmed by ¹H-NMR

from the appearance of aromatic protons at 7.20-7.27 (m, 1H, Ar), 7.80-7.85 (m, 2H Ar), 8.40-8.45 (m, 1H Ar) corresponding to the protons from thiopyridine. The ESI-MS of NAC-TP (**6**) shows a mass of 273 which further confirms the protection of 'SH' groups in NAC (M. wt = 163) by thiopyridine (Mol. wt = 110). Further, the formation of NAC-TP (**6**) was confirmed by Fourier transform infrared spectroscopy (FTIR) analysis. Free thiol group (SH) of NAC shows characteristic absorption at 2550 cm⁻¹ which disappears on formation of disulfide bond in the product NAC-TP (**6**) (Fig. S1, Supporting Information). Further, the formation of NAC-TP (**6**) was confirmed by HPLC and it shows a retention time of 11.25 min (Fig. S2, Supporting Information). NAC-TP (**6**) was reacted with 6-arm-PEG-SH (**7**) in PBS (pH = 7.4) to obtain the 6-arm-PEG-S-S-NAC conjugate (**1**) (Scheme 1) by the formation of disulfide bond. The formation of 6-arm-PEG-S-S-NAC conjugate (**1**) was confirmed by HPLC, ¹H-NMR, and MALDI-TOF/MS (Fig. S2, S3 and S4). The appearance of NAC protons in ¹H-NMR of 6-arm-PEG-S-S-NAC conjugate (**1**) at 2.00 (s, 3H), 2.95-3.10 (m, 1H), 3.30-2.38 (m, 1H), 4.40-4.50 (m, 1H) and 6.95 (br, s 1H, NH amide) and the disappearance of protons corresponding to thiopyridine of the NAC-TP (**6**) in the 6-arm-PEG-S-S-NAC conjugate (**1**) indicates the formation of the disulfide bond between the 6-arm-PEG-SH (**7**) and NAC-TP (**6**). The MALDI-TOF/MS analysis of the unmodified 6-arm-PEG-SH gave a broad M⁺ peak at Mw 9.5 kDa, which closely corresponds to the theoretical molecular mass of the 6-arm-PEG-SH (**7**) which has molecular weight (Mw) of 10 kDa. Coupling of thiol terminations in the 6-arm-PEG with NAC resulted in a shift of the peak from Mw 9.5 kDa to Mw 10.33 kDa. Since the molecular weight of NAC is 163 Da, this increase of Mw 830 corresponds to an average of 5 molecules of NAC appended on the 6-arm-PEG-SH scaffold. The purity of the conjugate was further confirmed by HPLC chromatogram. The HPLC chromatogram shows the retention time for NAC (**4**) as 5.5 min and NAC-TP (**6**) as 11.25 min whereas 6-arm-PEG-S-S-NAC (**1**) eluted at 21.5 min (Fig. S2, Supporting Information). The 6-arm-PEG-SH was tagged with fluorescent dye FITC (**8**) to form 6-arm-PEG-FITC (**2**) (Scheme 2). 6-arm-PEG-SH has a molecular wt of 9.5 kDa while on attachment of FITC (**8**) the corresponding mass increased to 10.35 kDa. The molecular weight of FITC (**8**) is 427 Da, so the increase in mass by 850 Da indicates the attachment of 2 copies of FITC (Fig. S5, Supporting Information). The molecular weights of conjugates and the solubility are summarized in Table 1.

3.2. Preparation of 8-arm-PEG-S-S-NAC conjugate (**3**)

The influence of increase in molecular weight and size of the scaffold on biodistribution, possibility of enhanced permeation and retention effect (EPR) and long resident time in the systemic circulation is well known.¹ To determine the change in cellular uptake of the conjugates based on the size, the 8-arm-PEG-SH (Mw 20 kDa) with higher molecular weight as compared to 6-arm-PEG-SH (Mw 10 kDa) was chosen to conjugate NAC. The 8-arm-PEG-S-S-NAC (**3**) was synthesized in one step by reacting 8-arm-PEG-SH (**9**) and NAC-TP (**6**) in PBS/ethanol (4:1) (pH = 7.4) as shown in Scheme 3. The formation of disulfide bond was confirmed by HPLC and ¹H-NMR analysis. The results for the ¹H-NMR shifts in the protons of 8-arm-PEG-S-S-NAC (**3**) (Fig. S7 Supporting Information) are consistent to those observed for 6-arm-PEG-S-S-NAC (**1**). The MALDI analysis showed the peak corresponding to 8-arm-PEG-SH (**9**) at Mw 20.26 kDa and the 8-arm-PEG-S-S-NAC (**3**) showed an increment of Mw 1141 (Mw 21.41 kDa) (Fig. S6, Supporting Information) corresponding to an average of 7 molecules of NAC attached to the PEG scaffold. The MALDI data for the drug payload and the ¹H-NMR results correlate very well with each other. The yields of PEG-conjugates were highly reproducible.

3.3. Particle diameter and zeta potential of PEG-S-S-NAC conjugates

Particle size analysis by DLS showed that 8-arm-PEG-S-S-NAC (**3**) (Table 2) is larger in size than the 6-arm-PEG-S-S-NAC (**1**) as expected based on the molecular weights of these conjugates. The sizes of the conjugates are qualitatively consistent with previously reported

values for PEG polymer-drug conjugates.³⁵ The zeta potential for both the conjugates is negative, probably due to the surface modification with NAC which results in carboxylic acid end functionalities on surface. The carboxylic end functionalities on the conjugates (due to NAC) remain in ionized form at pH=7.4 and bear a negative surface charge. The 6-arm-PEG-S-S-NAC (**1**) has a total of 8.03% w/w of NAC as compared to 6.58% w/w of NAC in 8-arm-PEG-S-S-NAC (**3**), which is reflected in 6-arm-PEG-S-S-NAC (**1**) bearing a zeta potential of -12.68mV as compared to -10.75mV by 8-arm-PEG-S-S-NAC (**3**). Since the charge on both the conjugates does not differ appreciably they are expected to exhibit similar interactions with the cells. Further, the conjugation chemistry for 6 and 8-arm-PEG-S-S-NAC both involves disulfide bond, hence the release rates are identical as seen in Fig 2A and 2B.

3.4. In-vitro NAC release from conjugates

The *in-vitro* release profiles of PEG-S-S-NAC conjugates (**1** and **3**) were investigated in PBS at 37°C. Stability of conjugates was analyzed in physiological pH (PBS, pH=7.4) at 37°C, and both the conjugates were found to be stable for over 3 days. To mimic the intracellular and extracellular milieu, glutathione (GSH) was added to PBS and NAC release from PEG-S-S-NAC conjugates (**1** and **3**) was determined as a function of time (Fig. 1A, 1B and 1C). At the intracellular GSH concentration (2 and 10mM), 74% of NAC was released from both the conjugates within 2h. The HPLC analysis confirmed the release of NAC and the presence of its oxidized form GS-S-NAC. Of the 74% of NAC released, ~23% is in the oxidized form 'GS-S-NAC' as indicated by the HPLC results (see Figs. 2A and 2B). As seen from the HPLC chromatogram (Figs. 1B and 1C), a fraction of the released NAC is in the form of NAC-S-S-NAC (dimer) in addition to the total of 74% that is released. NAC in solution has a tendency to oxidize to NAC-S-S-NAC. However, in presence of GSH, the major components are NAC and NAC-S-S-GSH. At longer times, there is near complete release of NAC (well above 74%), but simultaneously a portion of it is converted to NAC-S-S-GSH and NAC-S-S-NAC, hence the plot appears as plateau on a NAC basis. There is a fraction of NAC that is slowly released and is converted to the other stabilized forms.

The change in GSH concentration from 2 to 10mM in the release media did not significantly alter the NAC release though a small enhancement at 10mM GSH concentration was observed as expected. The molecular weight of the PEG scaffold did not affect the NAC release. The release profile was almost identical for both the conjugates and this can be attributed to the similar conjugation chemistry for 6 and 8-arm-PEG-S-S-NAC (**1** and **3**) which involves the disulfide bond, hence the release rates are identical. Neither of the conjugates release NAC in PBS containing 2μM GSH at 37°C (conditions mimicking plasma GSH concentration). From the above results it is evident that the rapid GSH-sensitive release of the NAC from the conjugate (**1** and **3**) occurs only when they are inside the cells. This is enabled by the use of a disulfide linker. The release profiles indicate that in the absence of GSH, or at low GSH levels (2 μM, the level found in blood circulation), the linker is very stable, and minimal drug release is seen (Fig. 2A and 2B). When the GSH concentration corresponds to intracellular levels (2–10 mM), rapid release is observed, due to exchange reactions between the disulfide linker in the conjugate and GSH. The relatively rapid release (within two hours) followed by a sustained release thereafter under intracellular conditions, may suggest that the efficacy of the conjugate may be better when measured against cells. As shown in previous results on HPMA-DOX conjugates, drug release from the conjugates is a key challenge for producing better efficacies both in cells and *in vivo*.³³

3.5. Uptake of 6-arm-PEG-FITC conjugate (2) by BV-2 microglial cells

Cellular entry of PEG-FITC conjugate (**2**) was evaluated using flow cytometry and confocal microscopy. The flow cytometry shows a significant increase (over two-orders of magnitude) in fluorescence intensity within 15 min indicative of rapid uptake of 6-arm-PEG-FITC

conjugate (**2**) by the microglial cells. The gradual increase in intensity was observed up to four hours as seen in Fig. 3A, suggesting the uptake to be time dependant and increased as time progressed. The cellular entry was also visualized using confocal microscopy (Fig. 3C). It is evident from the observed fluorescence that the 6-arm-PEG-FITC conjugate (**2**) localized mostly in the cytoplasm, while absence of fluorescence in nucleus suggests the absence of conjugate in nucleus (Fig. 3B). The cells without treatment with 6-arm-PEG-FITC (**2**) do not show any fluorescence.

3.6. Cytotoxicity of PEG-S-S-NAC conjugate

For cytotoxicity evaluations, the solutions of 6 and 8-arm PEG-S-S-NAC conjugates (0.05–5mM) and the solutions of free drug by itself (0.5–8 mM) were tested in the BV-2 microglial cells. Over the 0.05–5mM concentration range of the 6 and 8-arm-PEG-S-S-NAC conjugates (**1** and **3**), the concentrations of the PEG scaffolds correspond to 0.008–0.8 mM and 0.006–0.6 mM respectively. Therefore, the cytotoxicity was assessed at appropriate concentration ranges for free NAC, free PEGs, and the conjugates. The free drug by itself (NAC) did not show any toxicity at 0.5–8 mM in BV-2 cells (Fig S8, supporting information). Over the entire concentration range (0.008–0.8 mM), the 6-arm PEG was nontoxic to the microglial cells. The 8-arm PEG was nontoxic at 0.006–0.06 mM concentrations but exhibits some minor cytotoxicity at the higher concentration (0.6 mM, $p < 0.05$), where the cell viability was still close to 90%. The MTT assay showed that 6-arm-PEG-S-S-NAC conjugate (**1**) was nontoxic to BV-2 cells at 0.05–5 mM concentration after 24 h of treatment. The 8-arm-PEG-S-S-NAC was found to be nontoxic in the 0.05–0.5mM concentration range on treatment for 24 h (Fig. S8, Supporting Information). However at the highest concentration (5 mM, $p < 0.01$) some minor cytotoxicity was observed, with cell viability still better than 80%. These results point out that the free NAC, free PEG and the conjugates were mostly non-cytotoxic, and the efficacy results from the assays discussed next are not due to some gross cytotoxicity. The zeta potential for 6 and 8-arm-PEG-S-S-NAC (**1** and **3**) did not differ appreciably and hence they did not exhibit vast difference in the cytotoxicity towards BV-2 microglial cells. From the overall experiment it turns out that the size of PEG has no impact on release, zeta potential and cytotoxicity of the resultant conjugates.

3.7. Inhibitory effect of conjugates on intracellular GSH depletion

GSH depletion was induced in BV-2 cells by treatment with LPS. The inhibition of GSH depletion in the inflammation induced BV-2 cells on administration of PEG-S-S-NAC conjugates (**1** and **3**) and free NAC was monitored. Monochlobimane staining showed a decrease in GSH level by 16% after 18 h in BV-2 cells treated with solution of 100 ng/ml of LPS. At 5mM concentration, free NAC was able to inhibit GSH depletion by ~50%. The 6-arm-PEG-S-S-NAC conjugate (**1**) inhibited depletion of intracellular GSH at both 0.5 and 5 mM concentrations as compared to the positive control ($p < 0.05$) (treatment with LPS). Interestingly, at equimolar concentration of NAC (0.5 mM) the 6-arm-PEG-S-S-NAC (**1**) and 8-arm-PEG-S-S-NAC (**3**) were more efficient in inhibition of GSH depletion than the free NAC ($p < 0.05$). The efficacy of the conjugates (**1** and **3**) at 0.5 mM NAC concentration was comparable to that of free NAC at ten times higher concentration. However as the concentration of 6-arm-PEG-S-S-NAC (**1**) was increased to 5mM its ability to inhibit GSH depletion was comparable to equimolar concentration of free NAC. At high concentration (5mM) 8-arm-PEG-S-S-NAC conjugate (**3**) did not affect intracellular GSH depletion induced by LPS (Fig. 4.). Even though the PEG-S-S-NAC conjugates were more efficacious than free NAC at lower equivalent concentrations, at the highest concentration, the efficacy is comparable to free NAC. This may be because of the fact that too high a concentration of the drug is being delivered in the conjugate form. Some anti-inflammatory agents and steroids show a similar response, where high doses are less effective.³⁴

3.8. Anti-oxidative activity of PEG-S-S-NAC conjugate

ROS plays a key role in inducing oxidation and inflammation. The anti-oxidative properties of the NAC and PEG-S-S-NAC conjugates (**1** and **3**) were tested by monitoring the inhibition of ROS and free radical NO in BV-2 cells exposed to LPS and treated with NAC and the conjugates. At 5mM concentration, free NAC was found to inhibit ROS productions after 72h of treatment. Both the conjugates at 0.5 mM and 5 mM concentration contain 0.08 and 0.8mM of the PEG scaffold. The cells treated with 6 and 8-arm PEGs each at 0.08 and 0.8mM concentration did not affect the ROS production in BV-2 cells. However, the 8-arm PEG-SH at 0.6 mM concentration showed a very minor decrease in ROS production ($p<0.05$) (Fig. 5). Both 6 and 8-arm PEG-S-S-NAC conjugates (**1** and **3**), each at 0.5 mM and 5 mM concentration showed significant inhibition in ROS production when compared to free NAC at equimolar concentrations ($p<0.05$ and $p<0.01$, respectively). Further, the inhibition in ROS production by conjugates was found to be dose-dependant.

Free NAC inhibited nitrite production in a concentration-dependent manner after 72 h treatment. The 6-arm PEG did not affect the nitrite production over the entire concentration range, but at the highest concentration (0.6 mM) the 8-arm PEG caused a minor decrease in the nitrite production ($p<0.05$). Both the 6-arm and 8-arm PEG-S-S-NAC conjugates (**1** and **3**) showed higher inhibition of nitrite production at 0.5 mM and 5 mM concentration when compared to equimolar concentration of free NAC ($p<0.05$ respectively) (Fig.6). The studies demonstrate that the conjugates are superior in inhibition of the ROS and NO production as compared to the free NAC. At the highest concentration (5 mM), the free drug reduced the H_2O_2 levels and nitrite levels by ~30–40%, whereas the conjugates reduced the H_2O_2 and nitrite levels by more than 70%. This suggests that the conjugates are able to traffic the drug inside the cells, and release the drug in the free form, in over a time period to be significantly efficacious than the free drug. To our knowledge these are the first PEG conjugates that show superior activity compared to free drugs even in cells. Typically, the conjugates perform worse than free drugs in cells, but may perform better in vivo, due to EPR and other targeting.¹

3.9. Anti-inflammatory activity of PEG-S-S-NAC conjugates

BV-2 cells were treated with LPS to induce TNF- α release. Anti-inflammatory activity of NAC was compared with 6 and 8-arm-PEG-S-S-NAC conjugates (**1** and **3**) *in-vitro* by measuring the suppression of TNF- α release induced in BV-2 cells. Free NAC inhibited TNF- α production in a concentration dependent manner following 72 h treatment (Fig. 7), showing a ~40–45% reduction at the highest concentration. Even at the highest equivalent concentration in the conjugate (0.6 mM) free 6-arm-PEG-SH did not affect TNF- α release, whereas the 8-arm-PEG-SH suppressed the TNF- α release to a very minor extent ($p<0.05$). Both conjugates (**1** and **3**) showed a dose-dependant efficacy. At 5mM concentration 6-arm-PEG-S-S-NAC conjugate (**1**) showed significant inhibition (~70%) of TNF- α production when compared to equivalent concentration of NAC ($p<0.05$). 8-arm-PEG-S-S-NAC conjugate (**3**) showed significant inhibition of TNF- α production (~70%) at 5 mM when compared to equivalent concentration of NAC ($p<0.05$ and $p<0.01$). These results are quantitatively comparable to the anti-oxidative assays. The efficacies of the conjugates were superior to that of the free drug, and that the 6-arm and the 8-arm PEG-S-S-NAC conjugates (**1** and **3**) showed similar response. The improved efficacy is an indication of better intracellular transport and drug release from the conjugates.

4.0 Conclusions

The aim of the study was to design PEG-drug conjugates with the anti-inflammatory drug NAC, currently under clinical trial for maternal-fetal applications. NAC in the conjugate form is expected to be more plasma stable, since its free sulfhydryl groups are involved in forming the conjugate. This approach is expected to enhance the bioavailability. We synthesized two

conjugates 6-and 8-arm PEG-S-S-NAC and each had 5 and 7 molecules of NAC conjugated to PEG respectively, as characterized by NMR and MALDI. Since the disulfide linker is GSH sensitive, the conjugates rapidly released 74% of NAC in 2h at intracellular GSH concentrations (2 and 10mM). The zeta potential for 6 and 8-arm-PEG-S-S-NAC was comparable and did not seem to affect the cytotoxicity. At plasma GSH concentrations (2μM) the conjugates did not release NAC, further the conjugates exhibited stability for over 3 days in PBS buffer pH 7.4. Of course, the use of NAC, a drug with thiol functionality enabled us to form a disulfide bridge, which still releases the drug in the free form. Confocal microscopy revealed that the 6-arm PEG-FITC entered the cells readily and localized primarily in the cytoplasm. Both the PEG-S-S-NAC conjugates (**1** and **3**) showed significantly improved efficacy over free NAC as seen from the inhibition of ROS, NO and TNF-α release in LPS induced inflammatory response in microglial cells. This study demonstrates that despite the similar NAC release profiles from PEG-S-S-NAC and PAMAM-S-S-NAC conjugates, the change in scaffold affected the efficacy of the conjugates.^{31–32} To our knowledge these are the first such studies on PEG conjugates for neuroinflammation, where the conjugates show significantly better efficacy than free drug in cells. The NAC conjugates overcome the limitations of free NAC and exhibit site specific uptake, release and show enhanced efficacy over free NAC at lower dosing for the treatment of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. RP-HPLC UV absorbance chromatograms at 210 nm (arbitrary AU units) (A) chromatogram of 6-arm-PEG-S-S-NAC in absence of GSH (B) Chromatogram showing the release of NAC from 6-arm-PEG-S-S-NAC in 10mM GSH solution after 30 min (C) Chromatogram showing the release of NAC from 6-arm-PEG-S-S-NAC in 10mM GSH solution after 2h along with the formation of NAC-S-S-GSH, GS-SG and slight appearance of NAC-S-S-NAC.

Fig. 2.

Release of NAC from (A) 6-arm-PEG-S-S-NAC conjugate (1) and (B) 8-arm-PEG-S-S-NAC conjugate (3) in the medium comprising 2mM, 10mM and 2uM GSH solution respectively. NAC release occurs at 2mM and 10mM GSH concentration. At 2uM GSH concentration NAC release does not occur. The NAC-GSH represents the released NAC in presence of GSH solution (2mM, 10mM) which immediately forms disulfide bonds with the excess of GSH in the release medium.

Fig. 3.

(A) Flow cytometry results for the cell entry dynamics of 6-arm-PEG-FITC conjugate (**2**) in BV-2 microglial cell line. The log of FITC absorption intensity (FL1-H on x-axis) is plotted against the number of cells (counts on y-axis). The rapid increase in fluorescence intensity indicates cellular uptake of 6-arm-PEG-FITC within 15 min. The cellular uptake was found to be time dependant. Confocal microscopy images (400x) after 2h of treatment with (B) control, (C) 6-arm-PEG-FITC. The 6-arm-PEG-FITC conjugate appears to be mainly localized in the cytoplasm while the nucleus appears to be relatively free of the conjugate as seen by absence of fluorescence in nucleus.

Fig. 4.

GSH depletion assay. **(A)** BV-2 cells (passage 21) were co-treated with 100 ng/mL of LPS and NAC, 6-arm-PEG-S-S-NAC conjugate (**1**) and 6-arm-PEG-SH for 18 h at the concentrations indicated above. **(B)** BV-2 cells (passage 21) were co-treated with 100 ng/mL of LPS and NAC, 8-arm-PEG-S-S-NAC conjugate (**3**) and 8-arm-PEG-SH for 18 h. Four samples were collected for each group. The concentration of the 6 and 8-arm-PEG-SH corresponds to the concentration of PEG scaffold in the respective conjugates. Intracellular GSH level was assessed by monochlorobimane staining. The GSH depletion rate was calculated according to formula: GSH depletion rate (%) = (fluorescence intensity of negative control – fluorescence intensity of treatment group)/ fluorescence intensity of negative control × 100% (*P<0.05, VS group of LPS, ♦P<0.05, VS group of NAC in same concentration).

Fig. 5. ROS Assay to measure the anti-oxidative activity of PEG-S-S-NAC conjugates. **(A)** BV-2 cells (passage 22) were co-treated with 100 ng/mL of LPS and NAC, 6-arm-PEG-S-S-NAC conjugate (**1**) and 6-arm PEG-SH for 72 h. **(B)** BV-2 cells were co-treated with 100 ng/mL of LPS and NAC, 8-arm-PEG-S-S-NAC conjugate (**3**) and 8-arm PEG-SH for 72 h. The concentration of the 6 and 8-arm-PEG-SH corresponds to the concentration of PEG scaffold in the respective conjugates. The amount of ROS released into the media was measured using Amplex Red. (* $P < 0.05$, ** $P < 0.01$ VS group of LPS, ♦ $P < 0.05$, ♦♦ $P < 0.01$ VS group of NAC in same concentration)

Fig. 6. Inhibition of NO release in BV-2 cells by the conjugates. (A) BV-2 cells (passage 22) were co-treated with 100 ng/mL of LPS and NAC, 6-arm-PEG-S-S-NAC conjugate (1) and 6-arm PEG-SH for 72 h. (B) BV-2 cells were co-treated with 100 ng/mL of LPS and NAC, 8-arm-PEG-S-S-NAC conjugate (3) and 8-arm PEG-SH for 72 h. The concentration of the 6 and 8-arm-PEG-SH corresponds to the concentration of PEG scaffold in the respective conjugates. Nitrite in culture medium was measured using Griess reagent system. (*P<0.05, **P<0.01 VS group of LPS, ♦P<0.05, ♦♦ P <0.01 VS group of NAC in same concentration)

Fig. 7. Inhibition of TNF- α release by PEG-S-S-NAC conjugates. (A) BV-2 cells (passage 22) were co-treated with 100 ng/mL of LPS and NAC, 6-arm-PEG-S-S-NAC conjugate (1) and 6-arm-PEG-SH for 72 hours (B) BV-2 cells were co-treated with 100 ng/mL of LPS and NAC, 8-arm-PEG-S-S-NAC conjugate (3) and 8-arm PEG-SH for 72 hours. The concentration of the 6 and 8-arm-PEG-SH corresponds to the concentration of PEG scaffold in the respective conjugates. TNF- α in culture medium was measured using mouse TNF- α ELISA kit. (* $P < 0.05$, ** $P < 0.01$ VS group of LPS, ♦ $P < 0.05$, ♦♦ $P < 0.01$ VS group of NAC in same concentration).

Scheme 1.

Schematic for synthesis of 6-arm-PEG-S-S-NAC conjugate (1). NAC (4) was reacted with dithiopyridine (TP-TP) (5) to form NAC-S-S-TP (6) at room temperature (r.t.). The intermediate (6) (NAC-S-S-TP) was reacted with 6-arm - PEG-SH (Mw 9.5kDa) (7) to form the 6- arm-PEG-S-S-NAC conjugate (1) of Mw 10.33kDa.

Scheme 2.

Schematic for synthesis of 6-arm-PEG-FITC conjugate (**2**). 6-arm-PEG-SH (Mw =9.5kDa) (**7**) was reacted with fluorescein 5-maleimide (**8**) to form 6-arm-PEG-FITC conjugate (**2**) of Mw 10.35kDa.

Scheme 3.

Schematic for synthesis of 8-arm-PEG-S-S-NAC conjugate (**3**). 8-arm PEG-SH (Mw=20.26kDa) (**9**) was reacted with NAC-S-S-TP (**6**) to form 8-arm-PEG-S-S-NAC conjugate (**3**) of Mw 21.41kDa

Table-1

Molecular weight estimation of conjugates

Compound	Mol. wt	Molecules attached	Purity of compound	Solubility in PBS/H ₂ O
6-arm-PEG-SH	9.5 kDa [*]	-	100%	Highly Soluble
NAC-TP	272 Da [#]	-	100%	Not Soluble
8-arm-PEG-SH	20 kDa [*]	-	99.2%	Highly Soluble
6-arm-PEG-S-S-NAC	10.33 kDa [*]	5	99.5%	Highly Soluble
6-arm-PEG-FITC	10.35 kDa [*]	2	99.5%	Highly Soluble
8- arm-PEG-S-S-NAC	21.41 kDa [*]	7	99.5%	Highly Soluble

^{*} Molecular weight determined by MALDI-TOF

[#] Molecular weight determined by ESI-MS

Table 2

Size and Zeta potential for the PEG-S-S-NAC conjugates

Sample	Particle Diameter (nm)	Zeta Potential (mV)
6-armPEG-S-S-NAC	21–28	–12.68
8-armPEG-S-S-NAC	34–43	–10.75