Synthesis of sevoflurane loaded reduced graphene oxide nanoparticles system for neuroprotective effects for preconditioning against focal cerebral ischaemia

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ABSTRACT
In this study, sevoflurane (SF) loaded, Fas ligand conjugated reduced graphene oxide (rGO) system is fabricated as a therapeutic agent to target brain ischaemic region. The fluorescence investigation of mice brain denoted that the encapsulated SF in rGOs adsorbed with Fas ligand antibody could be significantly distributed to the ipsilateral side of the ischaemic brain. In addition, the immune-histochemical assay presented that the specific nanoparticles especially deposited in the ischaemic part of the tested mice model. Furthermore, SF encapsulated rGO system exhibited noticeable progress in the brain damage along with neurological deficit post ischaemia with limited dosages in contrast to regular SF. Additionally, Rhodamine labelled nanoparticles were used to find whether Fas ligand antibody has the ability to lead the SF-encapsulated nano rGO to enter the ischaemic part of brain as well as carry out neuro-protection. Overall, these experimental findings suggested that rGOs conjugated Fas ligand system could be treated as an ideal brain targeting drug for cerebral ischemia.

Introduction
Ischaemia is the shortage of oxygen and glucose supply required for cellular metabolism while ischaemic stroke is a serious medical condition that results from occluded cerebral vessel caused by the obstruction of blood supply to a part of the brain. Nowadays, tissue plasminogen activator (tPA) is a barely available healing remedy against acute ischaemic stroke which targets on thrombus in the blood vessels [1]. Although thrombolytic therapy is in use, ischaemic stroke has instigated adult disability in the contemporary period. In order to reduce the hazardous consequences of stroke, researchers persistently continued to explore rather effective recovery ways that could limit the damage in ischaemic patients. In this current scenario, a potent therapeutic remedy should be intended to prevail over the existing drawbacks in the conventional drug delivery system by targeting the brain to reduce the neural tissue damage. Substantial research on neuro-therapeutic agents has focused on the blood–brain barrier (BBB), as most of the molecules possess restricted permeability across the blood-brain barrier system [2,3]. However, competent BBB penetration ability and brain circulation serve as fundamental criteria for brain levelling agents [4,5]. As a consequence, the latest drug design approaches significantly concentrate on the bioavailability and BBB infiltration capability of the drugs [6]. Most effective neurovascular ischaemic therapeutic agents principally target on the ischaemic tissue, thus we estimated that Fas ligand conjugated system could be an appropriate contender for the treatment of brain-targeted cerebral ischaemia [7].

In the recent times, graphene has grabbed the immense interest of researchers because of its wide range of physico-chemical properties such as tremendous mechanical strength, strong electron mobility, amazing electronic transport as well as rich electrical conductivity [8–10]. In addition, graphene demonstrated significant applications as nanomaterials [11], bactericidal [12–14], antiviral [15], targeting and therapy [16–18], cancer cell imaging [19], disease diagnosis and drug delivery [11,20,21]. Nanomaterial science has spotted a sparkling path in the advancement of therapeutics, in particular, graphene oxide (GO) was established as a nano-carrier for drug transport in the field of medicine. In comparison with the rest of the carbon materials like carbon nanotubes and fullerences, GO has the ability to accommodate greater surface area and bear enhanced solvent dispersal potential [22]. In addition, stable colloidal GO suspensions can be prepared due to the presence of hydrogen bonds among H2O molecules and polar functional groups on the GO surface [23]. Interestingly a number of nanoparticles were developed on the surface of GO or GO was reduced to enhance its optical properties. In particular, some literature denoted that reduced...
graphene oxide (rGO) exhibited considerably improved NIR absorption in contrast to that of GO.

For the preparation of rGO sheets, a great deal of research has endeavored through different synthetic approaches like chemical vapor deposition [24], chemical reduction of GO [25–27] along with micromechanical cleavage. Among them, chemical reduction of GO is regarded to possess the paramount potential because of its economic availability for large-scale synthesis. Moreover, chemically synthesized graphene is found to exhibit hydrophobic nature as well as can readily accumulate through effective pi–pi stacking as well as van der Waals interactions existing between the graphene sheets (GS) [28,29].

Therefore, it is apparent that the evasion of graphene sheets aggregation would be of immense significance in order to uphold the characteristic properties in distinctive graphene sheets. Consequently, it is believed that functionalization would be an appropriate approach to seize graphene agglomeration, by making use of both covalent and non-covalent methods. In the recent times, a unique, non-toxic, and environmentally benign method for the synthesis of graphene sheets has been developed by making use of L-ascorbic acid (AA), casein, and glucose as reducing and functionalizing agents [30]. However, usage of some reducing sugars and numerous plant extracts has been earlier reported in the preparation of various nanomaterials [31–34]. In particular, plant extracts of pomegranate [35], Terminalia bellirica [31], and Terminalia chebula [36] have been also employed in the preparation of graphene sheets.

Plant extracts comprise of several polyphenols, like tannic acid and gallic acid, which play a crucial role as capping and reducing agents throughout the synthesis of silver and gold nanoparticles. Consequently, these polyphenols are expected to serve as reducing agents for GO reduction and switch to quinone forms. Similarly, SF is used in the present work to convert GO to rGO during the preparation of Fas ligand-conjugated rGO loaded with SF (SF/Fas/rGO).

According to our hypothesis, rGO nanoparticles could sustain the shielding effect of drug candidate in in vivo treatment of cerebral ischaemia. As an enhancement to this concept, SF was rGO encapsulated and Fas ligand antibody conjugated in order to carry out extended exploration about its efficiency against focal cerebral ischaemic reperfusion injury in animal models.

**Experimental section**

**Chemicals and animals**

All regents and used chemicals were bought from “Sigma-Aldrich Chemical Co”. In this current study, adult wild C57BL/6J male mice models were employed. The rhodamine, which is taken as control along with rhodamine–labelled rGOs, SF loaded Fas ligand conjugated antibody (30 mg per each gram body weight) were administered intravenously into the animal models. The mice were accommodated at 12 h (light and dark cycle) and water and food was made accessible ad libitum. In addition, all animal handling protocols, as well as experimental procedures, were done in compliance as per the guidelines of National Institute of Health (NIH, USA).

**Fabrication of Fas ligand-conjugated rGO loaded with SF**

To prepare Fas modified SF loaded rGO, the following experiments were performed. Graphene oxide (1 mg) was dispersed ultrasonically in (5 mmol/L) 2–(N–Morpholino) ethane sulfonic acid buffer solution, whose pH should be about 4.0. To activate the GO, MES buffer with (4 mg/mL) EDC and (6 mg/mL) NHS was further mixed with GO–dispersed MES suspension. Initially, the mixture was allowed to stir at 15°C for about 30 min, followed by centrifugation and washing thrice with (20 mmol/L) phosphate buffer with the intent to wash out excess reagents. Activated GO was redispersed in PBS to interact with (50 μg) Fas ligand for GO modification. An electronic shaker was used to mix the samples for 2 h at 15°C. Leftover GO active sites were obstructed using 2% BSA in PBS buffer for about 30 min. Later, the obtained solution was subjected to centrifugation at 16,000 rpm for about 10 min to eliminate any unreacted molecules. 20 mg of Fas/rGO was diffused in 16 mL of deionized water, followed by the addition of 10 mg SF with constant stirring for 24 h at room temperature in the absence of light. Thus, formed product (SF/Fas/rGO) was allowed to centrifuge followed by thorough washing by making use of deionized water and then vacuum dried. All supernatants were collected together.

**Loading of SF on Fas conjugated rGO**

The content of SF in Fas/rGO was measured by recording absorbance at nearly 228 nm by means of a UV spectrophotometer. The drug loading efficiency was determined by using a centrifugal-ultrafiltration technique. 0.5 ml of SF/Fas/rGO suspension was inoculated into centrifugal–ultrafiltration tube and subjected to centrifugation at 10,000 rpm for about 20 min. The concentration of SF in the obtained ultra-filtrate was calculated. Fas/rGO drug loading could be determined by means of the below equation:

\[
\text{Drug loading} = \left( \frac{\text{Charged amount of SF in Fas/rGO} - \text{mass of SF in ultrafiltrate}}{\text{Charged amount of SF in Fas/rGO} + \text{charged amount of Fas/rGO in mg}} \right) \times 100\%
\]

**Measurement of drug encapsulation efficiency of SF**

An ultraviolet spectrophotometer was used to determine the content of SF in the SF/Fas/rGO by recording the absorbance at 227 nm. Centrifugal-ultra filtration method was used to measure the drug encapsulation efficacy. ~0.5 ml of SF/Fas/rGO solution was inoculated into the centrifugal ultrafiltration tube followed by centrifugation at 10,000 rpm for about 20 min duration. The SF concentration in the ultrafiltrate suspension was estimated by using the following equation in order to calculate the drug loading percentage.
at the rate of 0.1 ml volume per 10 g of body weight. MCAO, the drugs were injected intravenously into the animal (rGOs, 1 mg/kg or 5 mg/kg). An hour prior to the inception of anterior or anterior to the bregma [37]. Later, the obtained microslicer (VT1000S; Leica, Wetzlar, Germany) is used to prepare 35 mm thickness coronal brain sections by cutting posterior or anterior to the bregma [37]. Later, the obtained brain sections were incubated for overnight at 4 °C with antibodies against OX-42, 1:300 and Fas ligand, 1:200 further were exposed to fluorescent secondary antibodies. Excluding the primary antibodies, negative staining controls for all the immuno-histochemical methods were carried out. Previously reported procedure was followed to prepare Fluoro-Jade B stained brain slices [38]. The slices were dipped in 0.06% KMNO₄ solution for almost 10 min followed by rinsing in double distilled water for about a minute. Later, these slices were later immersed in Fluoro-Jade B staining solution (0.0001%) for about 30 min. Subsequent to staining, the slices were rinsed thrice in distilled water for 1 min. Later, the slices were mounted on top of glass slides. Zeiss-LSM, 510 confocal microscope (Carl Zeiss, Jena, Germany) is used to detect the Immunofluorescence.

**Characterization**

A DU640, Beckman-Counter ultraviolet-visible spectrophotometer is used for optical absorption measurements. Zetasizer Nano-ZS9 was used to calculate the Zeta potential by means of dynamic light scattering measurements. GO samples were made to drip over freshly-cleaved mica by means of a pipette and air dried for calculating AFM. AFM (CSPM 4000), which is armed with silicon cantilever was used to scan the surface topographic aspects in contact mode. HR-TEM photographs were captured using a JEM-2100 microscope, which is functioned at 200 kV. UV–3600, UV–Vis–NIR spectrophotometer was used to obtain the absorption spectra. A Microcon YM-10, MWCO 3000 centrifuge was used for centrifugation of samples during experiments.

**Results and discussion**

Figure 1, showed the TEM image and DLS size distribution of fabricated graphene sheets, which showed the thin transparent graphene sheets with an average size of about 100 nm. AFM method was used to determine different stages of the GO surface modifications (Shown in Figure 2). Lee et al. [39] and Hosseini et al. [40] have earlier reported that an increase in the GO height will be recorded followed by SF treatment and antibody conjugation. However, these experimental studies were taken as reference to operate AFM in order to perform surface morphology characterization of simple GO, SF–activated GO, as well as FL–conjugated GO so as to confirm the occurrence of SF coupling process with the incidence of antibodies on GO surface (Figure 2(a–c)). Followed by SF activation, GO has recorded a significant increase in the thickness of ~4 nm. Subsequently, a 10 nm increase in the FL-conjugated GO height was observed as indicated by the height profile (Figure 2(d)). Additionally, few bright bands were recorded on the sample as reported by Hosseini et al. experiments [40]. These bright bands resulted because of the FL-antibody deposition on the GO surface (also confirmed from the zeta potential analysis as shown in Figure S1). From these surface morphology findings, it was obvious that the FL-antibody has been efficiently capped over the GO surface.
Encapsulation efficiency and drug loading percentages

In this experiment, the solvent diffusion method is used to prepare SF and SF loaded rGO (SF/rGOs). The SF/FL/rGO encapsulation efficiency percentage and drug loading percentage has presented not much variation between the SF and SF/FL/rGOs as shown in Table 1. However, no significant variations were observed between the *in vitro* results of the SF and SF/FL/rGOs (Figure 3).

Allocation of FL/SF/rGO in the ischaemic brain

The drug encapsulated rGo accumulation in the ischaemic brain is further assessed *in vivo*. *In vivo* rat model was already used to investigate the SF preconditioning, which has been employed for its time-course neuroprotection in the cerebral ischaemia [41]. Herein, specific accumulation of the drug in the brain ischaemic region with and without Fas ligand conjugation was compared. Intracellular fluorescence tracking imaging technique is employed to study the *in vivo* cooperative rGOs–Fas ligand specificity in the ischaemic region (Figure S1). The drug deposition after 24 h to intravenous injection endured significantly in brain regions in both cases. The image analysis after 24 h to rGO intravenous injection has presented fluorescence in both ipsilateral and contralateral sides of the brain in contrast to that of control mice (Figure S2). In particular, SF encapsulated rGO conjugated with Fas ligand (Fas/SF/rGO) recorded the greatest

| Table 1. Encapsulation efficiency percentage and drug loading percentage of SF/FL/rGO. |
|-------------------------------------------------------------|
| **SF/rGO** | **Antibody modified SF/rGO** |
| Number (nm) | 37.45 ± 2.5 | 70.13 ± 6.5 |
| EE (%) | 92.15 ± 0.45 | 94.25 ± 0.75 |
| DL (%) | 14.25 ± 0.07 | 14.39 ± 0.08 |

Where, EE (%) and DL (%) denotes the encapsulation efficiency percentage and drug loading percentage, respectively.
deposition in the ipsilateral ischaemic brain region, while the highest fluorescence intensity was particularly recorded in the ischaemic region of the brain (Figure S3). Therefore, this study signified that the conjugation of rGOs and Fas ligand would comprise a perfect ischaemic targeting drug distribution model for cerebral ischaemia. Markedly, the (Fas/SF/rGO) ligand has been, particularly, transported to the ipsilateral ischaemic brain region, instead of being distributed to the entire brain region. This might be attributed to the targeting ability of the Fas ligand towards the specific injured molecules in the ischaemic brain, thus laying the foundation for more efficient injured region distribution and therapy against ischaemic brain damage [38,42]. Therefore, it is anticipated that the multifunctional nanomedicine advancements essentially be competent in treating diseases along with assisting imaging in the efficient targeted drug distribution [4,43].

Neurodegenerative effect of FL/rGO

Fluoro-Jade B is used to estimate the neuronal damage at 24 h followed by MCAO (Figure 4). The count of Fluoro-Jade B-positive cell lines in the vehicle group animals has progressively increased in the ipsilateral brain portion (Figure 5; Figure S4). While Fluoro-Jade B signal staining intensity evidently diminished after the intravenous injection of rGO/SF and FL/SF/rGOs (Figure S4). Above all, our experimental results denoted that the neuronal degenerative inhibitory efficacy of SF encapsulated FL/rGOs (5 mg/kg) was more significant than that of the regular SF (10 mg/kg) treatment.

Neuro-protection ability of Fas ligand/rGO-nanoconjugate

The activation of MMP or calpain induced deprivation of tight-junction proteins along with the subsequent proliferation in the BBB leakage play a fundamental role in the pathogenesis of several neurological diseases [44,45]. Here, a raise of metabolites of spectrin and MMP–9 in MCAO mice along with the ischaemia stimulated decline in the survival protein Akt (Ser 473) and ERK (Thr202/Tyr204) phosphorylation (Figure 6; Figure S4) were monitored. In comparison, we have observed that both SF (10 mg/kg) and Fas/SF/rGOs (SF 1, 5 mg/kg) treatment following MCAO. The data are expressed as the mean ± SEM (n = 8). *p < .05, **p < .01 versus sham mice; #p < .05, ##p < .01 versus vehicle mice; $p < .05 versus regular SF treated mice, FP-SF, and SF encapsulated Fas ligand/rGO nanoconjugate.

Figure 3. In vitro release profile of SF from rGO nanoparticles.

Figure 4. Immunoblot images representing corresponding signals of neurovascular damage measured 24 h after MCAO with nano conjugates treatment. Mice on Akt and ERK signalling.

Figure 5. Quantified data for the changes of protein expression after vehicle, regular SF (10 mg/kg), and FP-SF (1 or 5 mg/kg) treatment in MCAO mice (Right panel). Immunodetection of β-lactin was used as a loading control. The data values are expressed as the mean SEM (n = 4). **p < .01 versus sham mice; #p < .05, ##p < .01 versus vehicle mice; $p < .05 versus regular SF treated mice, FP-SF, and SF encapsulated Fas ligand/rGO nanoconjugate.

Figure 6. Effect of SF conjugated Fas ligand/rGO nanoconjugate on Fluoro-Jade B staining. The number of Fluoro-Jade B-stained cells was scored and quantified. The graph is the average values of brain sections after vehicle, regular SF (10 mg/kg), and FP-SF (1 or 5 mg/kg) treatment following MCAO. The data are expressed as the mean ± SEM (n = 8). *p < .05, **p < .01 versus sham mice; #p < .05 versus regular SF treated mice. FP-SF, SF encapsulated Fas ligand/rGO-nanoconjugate.
ligand/rGO encapsulated SF is associated with the defense of neurovascular components.

In the current study, rGOs encapsulated Fas ligand antibody presented huge deposition in the ischaemic part of the brain. In addition, SF encapsulated rGOs conjugated Fas ligand antibody depicted notable recovery from brain damage and in neurological dearth post ischaemia. In general, these experimental findings represent that rGOs encapsulated Fas ligand antibody possesses the promising potential for progressive applications in the treatment of brain diseases.

Conclusions

In conclusion, we successfully fabricated the Fas ligand conjugated SF loaded rGO system and characterized using microscopic techniques. Also, we showed that the conjugation of nanoparticles to antibodies or other ligands could significantly deliver the drug by targeting the ischaemic region, thus presenting a challenging path to explore novel ways in the treatment of cerebral ischaemia.

Disclosure statement

No potential conflict of interest was reported by the authors.

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