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Enhancement of neuroprotective and anti-edema action in mice ischemic stroke model using T3 loaded nanoparticles

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Abstract

Background: cerebral ischemia still represents one of the most common causes of death and disability worldwide. A prompt treatment using strong neuroprotective medications is one potential method of pharmacological therapy for brain ischemic stroke patients. Thyroid hormone (T3) has been demonstrated to protect against ischemic damage. Despite the fact that thyroid hormone may pass across the blood-brain barrier (BBB).

Objective: we hypothesized that the effectiveness of thyroid hormone in ischemic brain stroke can be improved by encapsulation in nanoparticulate delivery vehicles.

Methods: We tested our hypothesis by generating thyroid hormone encapsulated in nanoparticles or brain-targeted nanoparticles using biodegradable polymers by utilizing an environment-friendly Supercritical Assisted Atomization (SAA) process as an alternative to a thyroid hormone solution in the setting of the MCAO stroke model. The biggest benefit of our proposed exploit of thyroid hormones in ischemic stroke is the fact that this strategy uses the body's endogenous hormones at sub-toxic levels to afford significant improvement in a life-endangering situation. According to our preliminary studied considerations, some tests were performed setting the saturator operating conditions in a pressure range between 5 and 15MPa and a temperature range between 70 and 90°C.

Results: The best results in terms of stability of the process and morphology of thyroid hormone nanoparticles were observed operating at 10MPa and 80°C. Our preliminary investigations also show that treatment with T3 significantly decreased infarct area (~36%) and analysis of hemispheric areas for edema formation showed that the edema formation induced by transient-MCAO was reduced by ~60% upon T3 treatment.

Conclusion: Thus, innovation in our proposal lies in our hypothesis, and our novel approaches directed at tackling edema in stroke.

Keywords: Anti-edema activity, blood–brain barrier, brain targeted nanoparticles, ischemic brain stroke, supercritical fluid technique, thyroid hormone.

INTRODUCTION

Ischemic stroke is one of the health challenges in India and the rest of the world. The absence of effective neuroprotective techniques in humans leaves stroke victims with permanent impairments brought on by the irreversible death of brain neurons [1]. One of the main causes of sudden and substantial death following a stroke is the development of cerebral edema [2]. The studies outlined in this proposal seek to establish the anti-edema and neuroprotective properties of thyroid hormones in ischemic stroke. In the literature, over 1000 candidate compounds for stroke have been reportedly tested with the hope of eventual clinical use [3]. However, not a single compound has been successfully translated into a viable clinically useful drug. There is considerable interest in the neuroprotective properties of endogenous hormones (estrogen, progesterone) or proteins (superoxide dismutase, erythropoietin) [4-8]. Our research adds to this concept of using endogenous neuroprotectants rather than xenobiotic compounds. The underlying tenet of our proposal is that thyroid hormone possesses anti-edema activity in brain stroke.

Development of brain edema in ischemic stroke

Brain ischemia can result from a variety of etiologies, including arterial blockade by atherosclerosis, embolism, vasospasm, intracranial hemorrhage, or loss of perfusion secondary to cardiac arrest. Risk factors for stroke include old age, hypertension, cardiac arrhythmias and myocardial infarction, diabetes, smoking, hyperlipidemia, and chronic alcoholism. Brain ischemia causes an insufficient supply of oxygen and glucose and a loss of ATP. When perfusion levels drop below 20-25% of normal, the tissue becomes electrically silent, which leads to loss of potassium from cells. Increased potassium is partially buffered by glial uptake. While potassium is lost from the cells, sodium and chloride enter the cells, together with passive water, causing cellular edema. The influx of sodium and chloride is much larger than the efflux of potassium, and early edema formation negatively affects the perfusion of the surrounding tissue. Water accumulation is an early process of neuronal degeneration. One of the most serious effects of ischemic brain injury is the production of cerebral edema, which is seen after a massive hemisphere stroke and is a critical factor in determining survival following traumatic brain injury [9]. While a number of processes maintain the homeostatic regulation of water content in healthy brains, ischemia leads to the breakdown of these systems, which includes severe dysregulation of ionic distribution, which increases intracranial pressure and causes swelling of the brain [9-11]. The CA1 region's pyramidal cells are the most sensitive to ischemia among all brain areas, with the hippocampus being the most vulnerable [12]. Furthermore, it is well known that astrocytes grow in response to excitotoxic glutamate concentrations [13, 14], and this swelling may have a significant impact on changes in intracranial pressure. The increased intracranial pressure associated with brain edema is a major determinant of patient survival beyond the first hours after stroke. Edema formation is strongly promoted by excitotoxic mechanisms. Calcium enters

cells particularly through N-methyl-D-aspartate (NMDA) channels, an event that is considered of special neurotoxic significance. Calcium that cannot be sequestered by neurons is toxic and initiates the breakdown of proteins, nucleic acids, and membrane phospholipids, causing cell death by necrosis or apoptosis. High levels of glutamate are observed after stroke, and the excitotoxic actions of glutamate on NMDA receptors and calcium influx are central to the concept of ischemia-induced neuronal cell death [15, 16]. Reactive oxygen radicals causing lipid peroxidation and the formation of nitric oxide may aggravate cellular damage at this point. Major gateways for calcium entering cells are ligand-operated calcium channels, such as the NMDA receptor complex, and voltage-sensitive calcium channels, such as L-type channels [17-19]. NMDA receptor antagonists have been tested in many experimental and clinical studies. Whereas NMDA blockers were promising in animal models of stroke, clinical trials of these compounds failed, partly due to limited efficacy but mainly due to their psychotomimetic and cardiovascular side effects. Due to the complexity of the cellular response to glutamate excitotoxicity future therapies for stroke and stroke associated consequences (brain edema) have been proposed to include multiple drugs to block multiple cellular responses [20].

As was mentioned above, cellular swelling (brain edema) during brain ischemia is due to influx of sodium and chloride ions with water accumulation and is intimately involved in the regulation of necrotic and apoptotic death of cells [21]. Our proposal rests on the hypothesis that thyroid hormones inhibit neurotoxicity by inhibiting water influx into the cell.

Nanoparticles as a vehicle for the delivery of therapeutic agents to the brain

Nanoparticles are polymeric particles made of natural or artificial polymers ranging in size between 10 and 1000 nm. The polymeric nature of these particles greatly facilitates controlled or sustained delivery of therapeutic drugs adsorbed or encapsulated on these particles. Further, surface modifications of these particles allow them to be tagged with specific sequences or moiety tags for tissue targeting. Researchers have developed and validated such a drug delivery vehicle wherein they use glutathione coating of poly-(lactide-co-glycolide)-polyethylene glycol (PLGA-b-PEG) nanoparticles (NPs) (21). Glutathione coated on the surface of these NPs facilitates greater brain uptake of these nanoparticles and the drugs therein via the glutathione transporters expressed at the blood-brain barrier (BBB) [21, 22]. In this application, we proposed to use thyroid hormone encapsulated in nanoparticles (T3- BTNP or brain-targeted nanoparticles) as an alternative to a thyroid hormone solution in the setting of the MCAO stroke model. Our hypothesis that nanoparticles can permeate more profound into the ischemic core even in conditions of ischemia and low perfusion is supported by a recent publication highlighting this finding [23]. Moreover, there are multiple reports wherein drug encapsulation in NPs has been reported to diminish the metabolic degradation of drugs [24, 25].

MATERIAL AND METHODS:

Preparation of nanoparticles:

Preparation of polymeric nanoparticles: Initially, 30 mL of DMSO was used to dissolve 100 mg of T3 and 200 mg of a copolymer or fatty acid, which included stearic acid (SA), glyceryl monostearate (GMS), and PLGA. The mixture was then put into a syringe. Drop by drop, while stirring constantly, this DMSO solution was added to a beaker that had 100 mL of phosphate buffered solution (PBS) at pH 7. 4 and 2 mL of Tween-80 at 60°C. For one hour, the resulting dispersion was sonicated. Whatman filter paper (0.22µm) was utilized to filter the sonicated dispersion. The polymeric micelles that were produced were refrigerated until they were needed [25].

Nanoparticles prepared by Supercritical Assisted Atomization.

Supercritical CO2 processing offers a useful and "clean" substitute for traditional polymeric nanoparticle processing techniques. In the SCF process, a cooler brought the CO2 supplied from a CO2 cylinder down to around 0°C to guarantee the gas's liquefaction and avoid cavitations. The liquefied CO2 was then transferred to the high-pressure vessel using a high-pressure meter pump. A heat exchanger was used to pre-heat the liquid CO2 to the required operating temperature after it left the pump head. When the desired pressure of the high-pressure vessel was reached, a steady flow of CO2 was maintained, and the system pressure was controlled by adjusting a downstream valve and monitored by a pressure gauge to keep the pressure constant. Supercritical CO2's moderate critical conditions (Tc=31.1°C, Pc=7.38MPa), non-toxicity, non-flammability, and inexpensive cost have made it an excellent candidate for use in particle engineering for biological purposes. The effects of temperature (70–90°C) and pressure (5–18 MPa) were investigated in the preliminary trials. Since ethanol exhibits high solubility for PLGA/polymer, it was used for SAA micro/nanonization studies in this study. Because of the high affinity that ethanol has for CO2, a significant amount of CO2 can dissolve in the ethanolic solution, leading to a significant decrease in viscosity. Depending on the weight ratio of the polymer to the medication, the mean diameter of the produced composite particles ranged from around 0.8 to 1 nm and had a regular, spherical form. Eighteen batches were prepared in the form of variations in the temperature or pressure represented in Table 1.

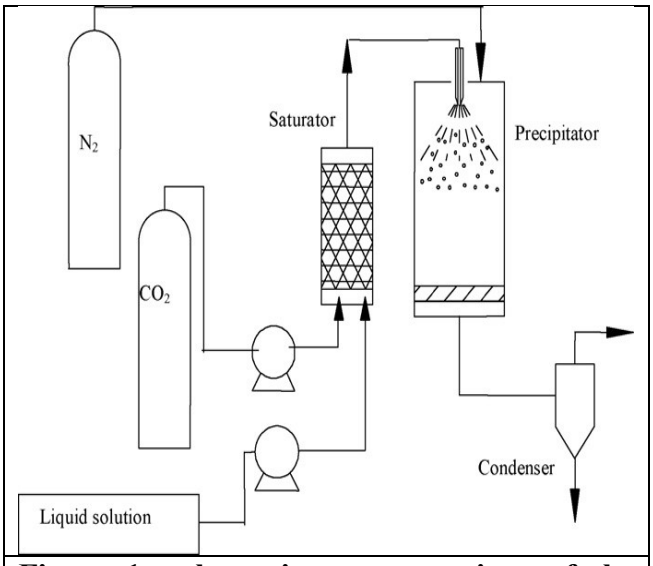


Figure 1: schematic representations of the SAA apparatus

Morphology study (FE-SEM)

The morphology of produced nanoparticles was observed by Field emission scanning electron microscopy (FE-SEM). The FE-SEM image for nanoparticles is shown in Figure 2.

Micromeritics, entrapment efficiency (EE) and drug loading (DL) studies

The obtained data pertaining to micromeritics, surface charge, drug entrapment, and drug loading of the prepared nanoparticles with different polymersis presented in Table 2. The particle size of prepared nanoparticles was measured as a suspension via dynamic light scattering using a Zetasizer Nano (Malvern Instruments, Malvern, United Kingdom), and results are shown in Figure 4.

In-vitro Drug Release Studies

Dialysis Cassettes (10000 MW CO, Thermo Scientific) were used for the *in-vitro* release study of T3 from the nanoparticulate formulation. For the release study, firstly, the dialysis cassettes were soaked for the night in the diffusion medium, and then, with the help of a syringe, a definite quantity of T3-loaded nanoparticles equivalent to 0. 1mg of drug was precisely inserted into the dialysis cassettes. After that, the dialysis cassettes loaded with the nanoparticulate formulation were kept hanging in the glass beaker containing 100 mL of PBS (Phosphate Buffer Saline) having pH 7.4 at (37±0.5°C). The assembly formed was set aside on a magnetic stirrer at a speed of 50 rpm, and at pre-determined intervals of time 3 mL samples were collected over a period of 24 hrs. and were replaced by fresh dissolution medium after every withdrawal. The results are shown in Figure 5.

Determination of the neuroprotective and anti-edema action of T3 in p-MCAO

We have conducted a pilot study to investigate the effects of thyroid hormones (T3 and T2) on brain stroke and edema formation after transient MCAO (1 h). Animals were treated intravenously with nanoparticle suspension of thyroid hormone. No restriction was placed on food and water intake. The dose of the drug is given according to the body weight of mg/kg. Mice were treated with T3 or T2 (500ng in 100ml PBS, i.v. through the jugular vein) 10-15 min after reperfusion (post-stroke treatment). HPLC method is used to analyze the concentration of thyroid hormone in the brain tissues and blood plasma samples. Following brain stroke to observe the neurological outcome in mouse behavior, a sensorimotor corner test was performed.

RESULTS AND DISCUSSION

According to our preliminary study consideration, some tests were performed, setting the saturator operating condition in a pressure range between 5 and 15 Mpa and in a temperature range between 70 to 90 °C. The best results in terms of stability of the process and morphology of thyroid hormone nanoparticles with PLGA were observed operating at 8 MPa pressure and 70°C temperature conditions, showing very good spherical-shaped nanoparticles of small size.

Table 1: Preliminary trails batches: effect of the pressure and temperature*

Sr. No	Pressure (Mpa)	Temperature (°C)	Observation
1	5	70	Irregular nanoparticles
2	10	70	Irregular nanoparticles
3	15	70	Irregular nanoparticles
4	5	75	Aggregated nanoparticles
5	10	75	Non-spherical nanoparticles
6	15	75	Aggregated nanoparticles
7	5	80	Non-spherical nanoparticles
8	10	80	Spherical nanoparticles
9	15	80	Non-spherical nanoparticles
10	5	85	Non-spherical nanoparticles
11	10	85	Non-spherical nanoparticles
12	15	85	Aggregated nanoparticles
13	5	90	Non-spherical nanoparticles
14	10	90	Non-spherical nanoparticles
15	15	90	Aggregated nanoparticles
16	**8**	**70**	**Spherical nanoparticles with less size**
17	12	85	Spherical nanoparticles
18	18	90	Non-spherical nanoparticles

*at thyroid hormone: PLGA ratio1:1, mass feed ratio(*R*) of 1.8 between CO2 and the liquid solution, preheated N2 at a flow rate of 0.8Nm^3/h.

Figure 2 shows the FE-SEM microphotograph of three different types of polymeric nanoparticles. It is vivid that the nanoparticles were present in segregation, devoid of any evidence of agglomeration. The developed systems were spherical in shape, but size is not accepted in nm; it is more than 800 nm.

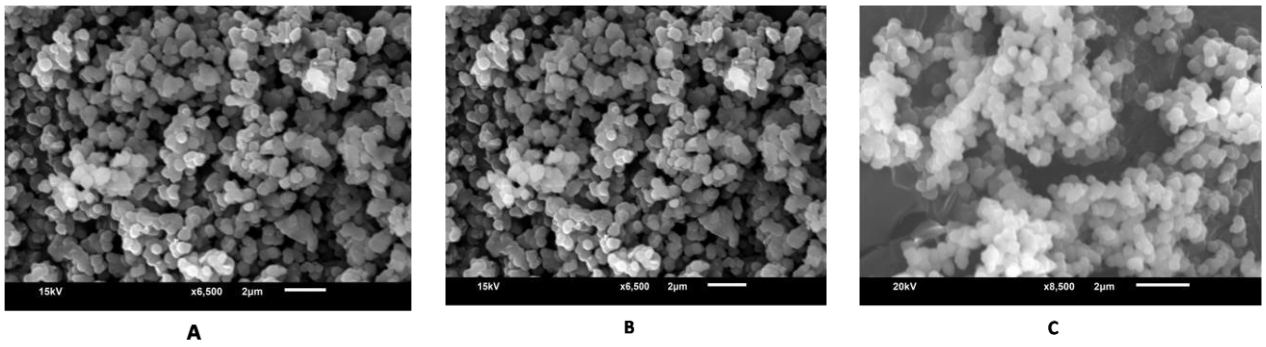


Figure 2: FE-SEM images of (A) SA nanoparticles, (b) GMS nanoparticles, (C) PLGA nanoparticles

Eighteen batches were prepared in the form of variations in the temperature or pressure. Batch 16 (8 MPa pressure and 70°C temperature condition showing very good spherical shaped nanoparticles with small size. The FE-SEM image for nanoparticles prepared with supercritical-assisted atomization is shown in Figure 3

The obtained data on micromeritics, surface charge, drug entrapment, and drug loading of the prepared nanoparticles with different polymerase are presented in Table 2. The particle size of prepared nanoparticles was measured as a suspension via dynamic light scattering using a Zetasizer Nano (Malvern Instruments, Malvern, United Kingdom), and the results are shown in Figure 4.

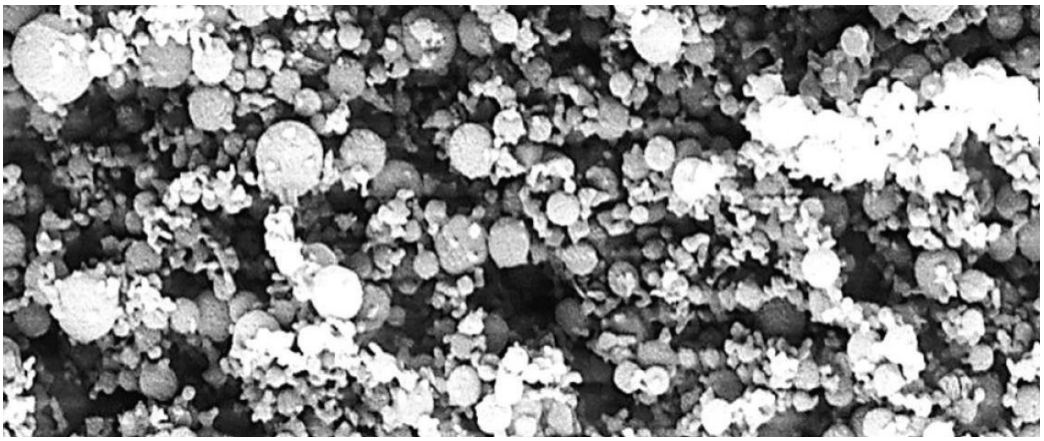


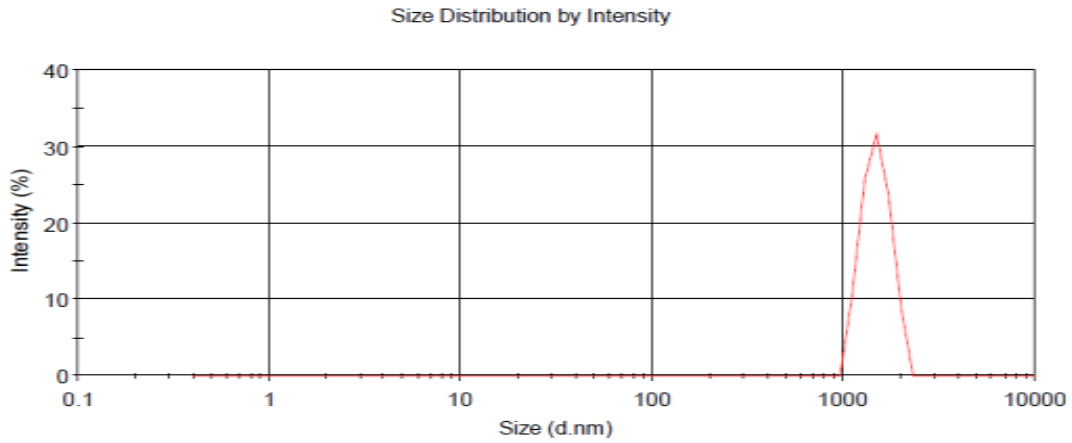
Figure 3: Nanoparticles obtained by Supercritical Assisted Atomization (FE-SEM image)

Micromeritics, entrapment efficiency(EE)and drug loading (DL)studies

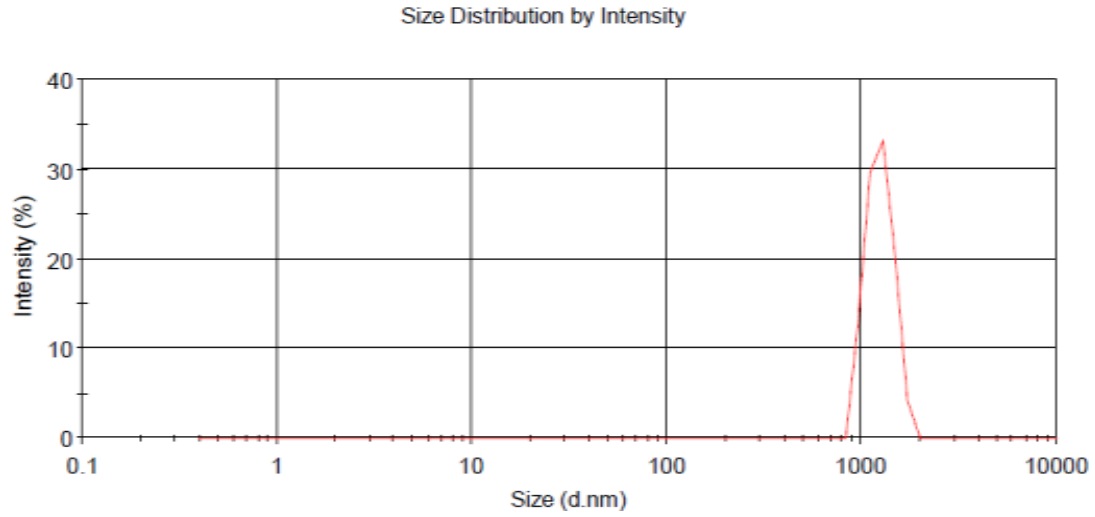
Table 2: Results of Micromeritics, entrapment efficiency and drug loading

Sr. No.	Name of sample	Particle Size (nm)	PDI	%EE	%DL
1	SA	1494.80±15.01	0.695	65.16±0.35	13.04±0.08
2	GMS	1247.78±18.84	0.527	71.89±0.81	10.47±0.20
3	PLGA	1243.00±15.39	0.800	81.70±0.17	14.67±0.04
4	SAA nanoparticles	236.07±19.52	0.310	94.33±0.57	23.58±0.14

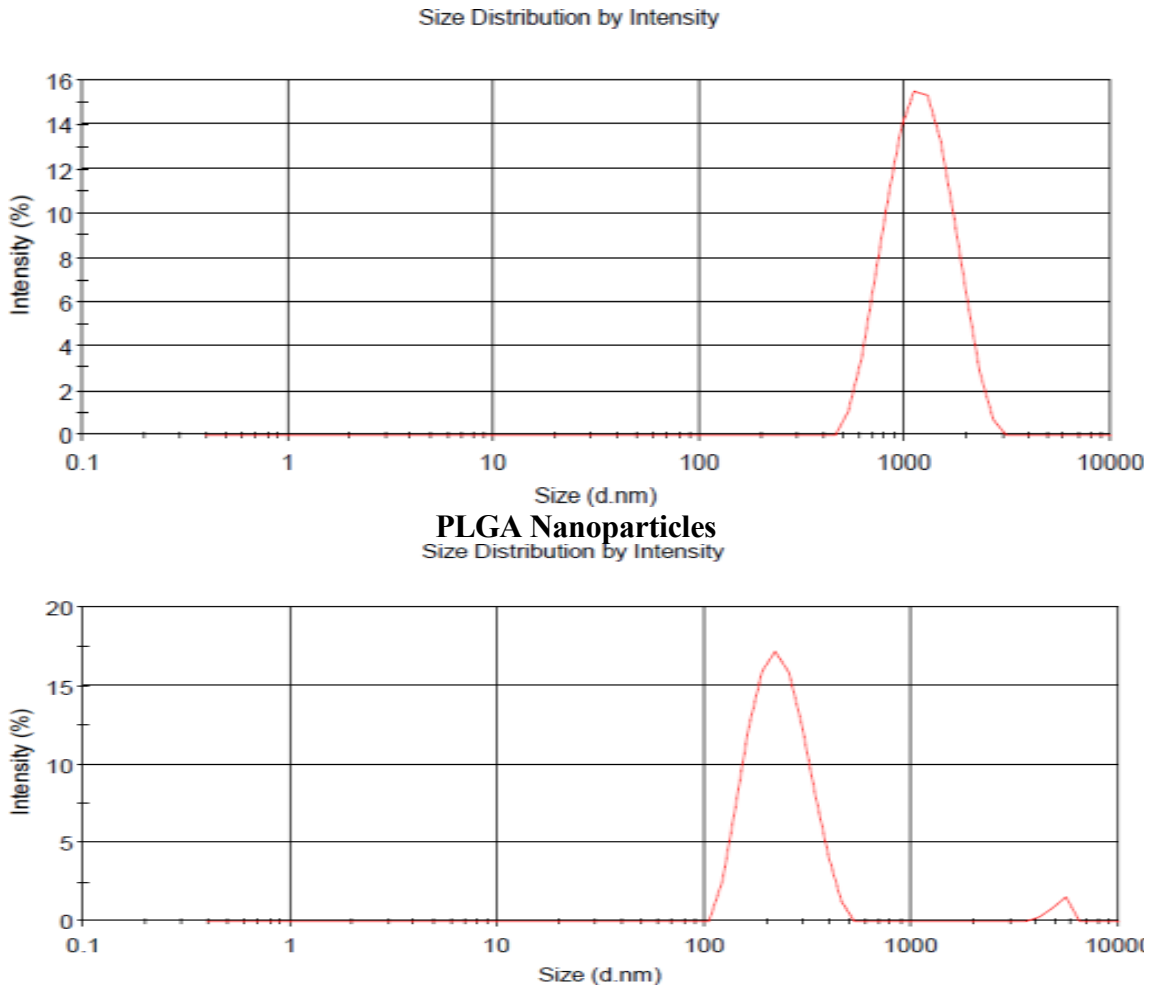
Where: SA- stearic acid; GMS- glyceryl mono stearate; PLGA- poly lactic-co-glycolic acid; SAA-Nanoparticles prepared by Supercritical Assisted Atomization Technique



SA Nanoparticles



GMS Nanoparticles



PLGA Nanoparticles

SAA Nanoparticles

Figure 4 Zetasizer result and Analysis of size

In-vitro drug release studies

The T3 release from nanoparticles was found to be between 20% and 30% within the first two hours in the stomach pH, indicating that the results were encouraging. On the other hand, normal T3 diffused to almost 100% in just 6 hours, while all of the nanocarriers released the medication up to 90% in the next 24 hours in the intestinal pH. The results show that the produced nanoparticles have the ability to regulate drug release, which is the most sought feature of drug delivery based on nano carriers. Figure 5 presents the drug release results, clearly illustrating the variations in the drug release characteristics. It was found that the medication release rate governing each nanoparticle's potential was almost similar throughout the release profiles of the synthesized nanoparticles.

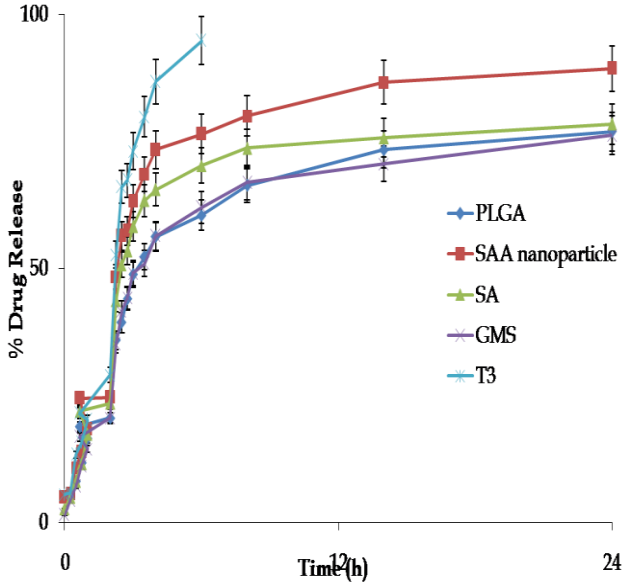


Figure 5: Drug release profile of T3 from the studied systems

Neuroprotective and anti-edema action of T3 in p-MCAO

Figure 5A shows representative brain slices of the control group as well as the T3-loaded nanoparticles group 24 h after MCAO. As seen in Fig. 5A, TTC staining is evident to be neuroprotective, with greater protection of the cortex. Treatment with T3 significantly decreased infarct area (~36%, Fig. 5B). Analysis of hemispheric areas for edema formation showed that the edema formation induced by t-MCAO was reduced by ~60% upon T3 treatment (Fig. 5C). Neuroprotection and resolution of edema were apparent from visible examination of TTC stained brain slices. It was clearly observed in Figure 7 that the improvement in neurological functions in mice treated with T3-loaded nanoparticles by corner test [25].

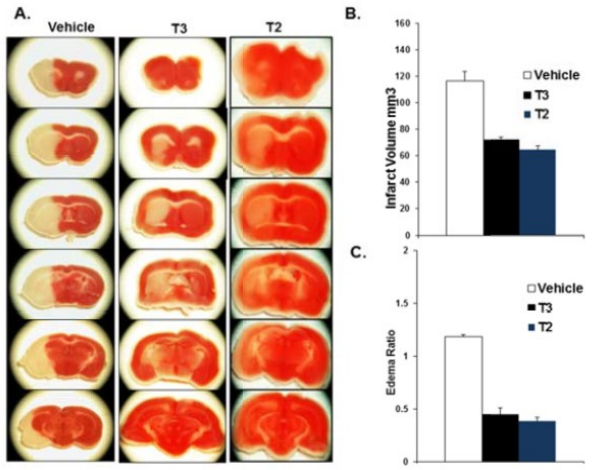


Figure 6 Reduction of tissue infraction and brain edema by T3 and T2 in t-MCAO

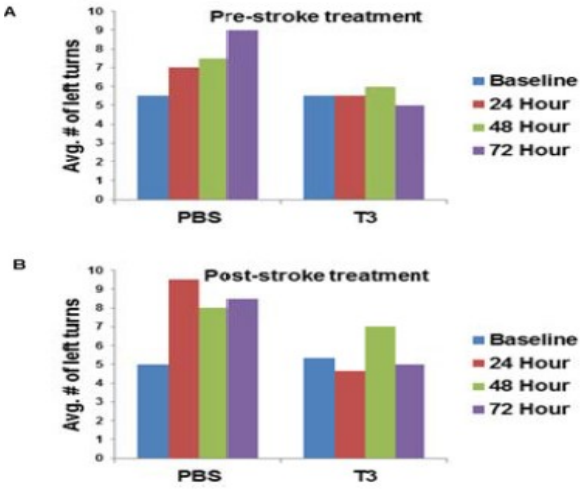


Figure 7 Improvement of neurological function in T3 treated mice as shown by a reduction of the number of left urns as measured by corner test

CONCLUSION

The SAA process is a good alternative to conventional processes and has also been demonstrated to be very efficient in the micro/nanonization of polymer-T3 composite particles. The amount of T3 in coprecipitates is the key factor in controlling the dissolution rate of polymer (PLGA). Moreover, SAA process induces the formation of dispersed and amorphous PLGA particles that show a higher dissolution rate in water. Our studies have shown Neuroprotection and anti-edema activity

for T3-loaded nanoparticles in a mouse model of ischemic brain stroke. A single dose of T3-loaded nanoparticles can attenuate in fraction and edema significantly.

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None

Conflict of interest

None

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