Targeted nano-drug delivery system for glioblastoma therapy: In vitro and in vivo study

Targeted Nano-drug Delivery System for Glioblastoma Therapy: *In Vitro* and *In Vivo* Study

Running title: nano-drug delivery system for glioblastoma therapy

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Abstract:

This study developed polymeric nanoparticles (NPs) conjugated with monoclonal antibody (mAb) for glioblastoma treatment. In this study, the physicochemical properties of poly(butyl cyanoacrylate) (PBCA) NPs were characterized. The cytotoxicity of NPs conjugated with mAb (NPs+mAb) and *in vitro* drug release evaluation was tested on glioblastoma cell lines. Their therapeutic efficiency and side effects were then assessed *in vivo* in a rat model of glioblastoma. The conjugation of NPs to mAb revealed an average particle size of 365nm and an encapsulation efficiency of 41.95%. The findings also showed that cytotoxicity was augmented by 40% compared to the free form of carboplatin. Moreover, *in vivo* studies showed that body weight remained relatively stable in rats treated by NPs+mAb and their survival time was longer (23.5 days) compared to rats treated with free carboplatin (19.5 days). In addition, *ex vivo* investigation showed that rats administered with NPs+mAb exhibited less side effects in the brain, kidney and liver compared to other groups.

Keywords: Glioblastoma; nanoparticle; targeted drug delivery; monoclonal antibody

1. Introduction

The treatment of glioblastoma tumors is a challenging issue, with survival rates for patients, averaging only 14.6 months [1-3]. Currently, there is a lack of effective therapy for almost all brain tumors, and the development of a new therapeutic system for brain tumors is both difficult and challenging [4]. Chemotherapy is applied as an adjuvant treatment after or before surgery for different types of cancers, including head, neck and brain. However, chemotherapy causes significant side effects, including vomiting, constipation, diarrhea, weight loss and nausea [5]. These side effects are associated with the high drug dosage required to ensure the drug reaches the tumor site in sufficient concentration [6]. For this reason, developing a novel method to enhance the efficiency of treatment and reduce side effects is vital or paramount. Furthermore, the main reason for the low efficacy of brain tumor treatments is related to the role of the blood brain barrier (BBB). The BBB provides a unique chemical, immunologic and functional environment in the CNS which restricts the entrance of leukocytes and neuro-toxic macromolecules [4, 7, 8]. In the same way, the BBB affects the delivery of chemotherapeutic agents to the tumor site [9]. Therefore, developing new therapeutic strategies to overcome these challenges is a growing focus for successful treatment of brain tumors.

Recently, three types of transport mechanisms have been found to be effective for CNS drug delivery: transport or carrier-mediated transcytosis, receptor-mediated transcytosis and adsorptive-mediated transcytosis. [10, 11]. Nano-drug delivery is a novel technique with great potential to enhance standard chemotherapy systems. The accumulation of NPs at the cancer tissue site is higher than that of the free form of standard drugs. Previous studies reveal that the accumulation of nano-drugs in most tumors is 200–500% greater than free anti-cancer drugs [12]. This is due to the improved permeation and retention (EPR) of nano-drugs. Nano-particulate systems can significantly affect drug bio-distribution, thus increasing drug concentration at the tumor site [13, 14]. One significant breakthrough for nano-drug delivery to the brain is that the modified nano carriers can effectively penetrate the BBB and CNS [8]. Nano-particulate drug delivery systems, by using surface modifications techniques including attachment of receptors or polymer coating, are enhancing drug delivery to the brain [15, 16]. Therefore, developing a biodegradable carrier with the ability to permeate the BBB and with high encapsulation efficiency is a key focus for developing NPs that can target brain diseases.

Different polymeric NPs have been proposed to optimize anti-cancer treatment since NPs were nominated as highly potent drug delivery methods. Poly (butyl cyanoacrylate) (PBCA) NPs may be of significant interest given their ability to enhance the plasma half-life of the drug while reducing their unspecific cytotoxicity, when coupled with their biocompatible and

biodegradable characteristics [17, 18]. In addition, these NPs have useful properties for drug delivery in the tumor, including the ease of synthesis and bio distribution of drugs in the tissue [19, 20]. Surface modification has been studied to enhance the efficiency of the nano delivery system. The modification of the NP surface by polyethylene glycol (PEG), known as PEGylation, decreases reticuloendothelial system (RES) uptake and enhances circulation time compared to NPs without surface modification [21]. PEG is a versatile, FDA approved and inexpensive compound for various applications [22]. Solubility and EPR effect is increased by PEGylation due to the hydrophilic ethylene glycol repeats [23]. Moreover, the use of targeting agents may increase the intracellular concentration of drugs in cancerous cells while avoiding toxicity in normal cells [24, 25]. A number of different targeting molecules have been studied in combination with NPs, including antibodies. Traditionally, the binding of antibodies to NPs has been achieved covalently through various linker chemistries. Engineered NPs, similar to antibody-drug conjugates (ADCs), can "link" pharmaceutical drugs to targeting monoclonal antibodies to generate highly specific therapeutics [26].

Synthesis and characterization of PBCA NPs loaded with carboplatin was evaluated in this study. Carboplatin is a crystalline powder with the molecular formula of $C_6H_{12}N_2O_4Pt$ and a molecular weight of 371.25. It is soluble in water at a rate of approximately 14 mg/mL, and the pH of a 1% solution is 5 to 7. Carboplatin is a platinum based chemotherapeutic agent with a similar mechanisms to Cisplatin, however it differs in terms of the toxicity and structure that is applied for cancer treatment [27]. A new therapeutic technique which enhances the therapeutic efficiency of this drug could provide extensive opportunities to treat different cancers [28, 29]. It is hypothesized that the targeting agent helps NPs to pass through the BBB more efficiently, delivering a higher dose of carboplatin to tumor tissue. By using PEG, NP aggregation decreases due to non-adhesive surfaces.

Epidermal growth factor receptors (EGFR) induce proliferation of cancer cells and have been implicated in glioblastoma pathogenesis and resistance to treatment. Mutations and amplifications in EGFR were detected in 45–57% of glioblastoma cases studied [30, 31], indicating their substantial role in the pathogenesis of glioblastoma. Therefore, in this study, monoclonal antibodies specific to the EGFR were used as a targeting agent conjugated to PEGylated NPs. Furthermore, to investigate the efficacy of the prepared NP performance, an *in vitro* cytotoxicity and drug release evaluation were carried out. Then, antitumor and side effects were assayed *in vivo* to investigate the therapeutic efficacy of NPs using several parameters, including survival time and body weight change compared to free drug treatment.

Finally, an *ex vivo* investigation was performed to compare the side effects of prepared NPs on the brain, liver and kidney, in comparison with free drug treatment.

2. Material and methods

2.1 Materials

The monomer of BCA, dextran 70000, carboplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) (0.5 mg/mL), polyethylene glycol (PEG 4000), polysorbate 80, Cetuximab (IMC-C225) and Nimotuzumab monoclonal antibodies (mAb) were obtained from Sigma-Aldrich (USA). Sodium hydroxide and hydrochloric acid were purchased from Merck (Germany). N-Hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) cross-linker was obtained from SolTechBioScience (USA). C6 rat (CCL-107™) and A172 human (CRL-1620™) glioma cell lines were provided from the American Type Culture Collection (ATCC).

2.2 Preparation of PBCA NPs

The formulation was prepared using a total of 220 µL poly(butylcyanoacrylate) monomers which were added to the mixture of 150 µg PEG-4000 and 150 µL HCl (0.01 N). Then, 50 mg carboplatin and 100 mg of dextran 70000 were added. Following on from this, 30 mL of cold distilled water was added during two steps and stirred at 240 rpm for 15 min to obtain a pre-emulsion solution. The emulsion was again placed on the stirrer after 24hrs maintenance in 4°C, and slowly stirred for 3.5 hrs at 140 rpm. Subsequently, the pH of the mixture was neutralized using NaOH. The freeze dryer was used for 24 hrs to dry NPs at vacuum condition (FD 300, Dynavac). Lypophilized powder of NPs was added to PBS (10 M).

2.3 Conjugation process of PBCA NPs and mAb

Sulfo–HSAB was applied to cross-link the C-H bonds of the dextran-coated PBCA NPs to covalent amine groups from the mAb. The resulting suspension was mixed with 250 µL of sulfo–HSAB (2 mg/mL) solution and 25 mM PBS. Then, 50 µL mAb was added to the mixture. The solution was irradiated for 30 min by UV light. Lastly, to remove any physically adsorbed component, the solution was mixed with 0.1 wt% polysorbate 80 and incubated for 20 min. Suspension was centrifuged at 10,000 rpm for 1 h, and unconjugated mAb was removed. NPs were suspended in PBS (1M) which included 1% polysorbate 80. Suspension was vortexed for

30 min. Finally, the flask was placed in cold water and then sonication was performed for 2 min (50 W) by a probe sonicator (Bandelin Sonopuls HD 2070, Germany).

2.4 Characterization

The morphological analysis of NPs was performed by SEM (XL30 scanning electron microscope, Philips, Netherlands). To perform this, after the addition of 3% mannitol, suspension was lyophilized. The zeta potential and size distribution were determined by Zeta sizer (Nano-ZS Zen 3600, Malvern Instruments Ltd, UK). Figure 1A outlines the conjugation process of NPs and mAb. Figure 1B shows the schematic action between the cancer cell and NPs. In order to determine encapsulation efficiency (E.E) and drug loading efficiency (D.L.E), the non-PEGylated NPs and non-conjugated PEGylated as well as PEGylated NPs conjugated with mAb were centrifuged for 10 min at 4°C (10000 rpm and 2 times iterate) (Beckman type 90Ti, USA). Concentration of carboplatin in supernatant was investigated by inductively coupled plasma optical emission spectrometry elemental analysis (730-OES, ICP-EOS, USA). E.E was calculated by equation (1) (all in mg/mL).

$$\text{EE \%} = \frac{\text{The actual amount of carboplatin encapsulate in NPs } (\frac{mg}{ml})}{\text{Initial drug concentration } (\frac{mg}{ml})} * 100 \quad (1)$$

Loading capacity assists in evaluating the separation of NPs from the medium in order to determine their drug content. D.L.E was calculated by equation (2) (all in mg/mL).

$$\text{D.L.E \%} = \frac{\text{The amount of drug } (\frac{mg}{ml})}{\text{Weight of NPs } (\frac{mg}{ml})} * 100 \quad (2)$$

In addition, the stability of NPs, at 4°C, including D.L.E, Zeta potential E.E and size distribution were evaluated after 1-2 months. Dialysis method was used to investigate the profile of drug release. In our drug release experiments we used analytical method to evaluate the drug release taking into account sink condition. One mL of each formulation was poured in a dialysis bag (cut off 12000 Dalton, Sigma), transferred in 20 mL of PBS (pH 7.4) and placed on a magnetic stirrer (48 h, 120 rpm, 37^{o}C). ICP-EOS was performed to evaluate the concentration of drug in supernatant from 1hr up to 48 hr.

2.5 Cytotoxicity study

Cytotoxicity of free drug, non-PEGylated NPs and non-conjugated PEGylated as well as PEGylated NPs conjugated with mAb, was evaluated by MTT assay using a human A172 and rat C6 glioma cell lines. Cells were seeded at a density of 1×10^4 (10,000) cells, and were then cultured in RPMI-1640 medium containing fetal calf serum (FCS) (10%), sodium pyruvate

(1%), glutamine (0.5%), and antibiotic penicillin, and was incubated at 5% CO2 at 37 °C. The medium was replaced twice after 24 hrs. MTT solution (4 mM) was added to each well for three hrs. Cells were treated with NPs formulations and carboplatin at different concentrations (0, 5, 10, 30, 50, 70, 90, 110, 120, 140 and 160 µM). IC50 of non-PEGylated NPs and non-conjugated PEGylated as well as PEGylated NPs conjugated with mAb and carboplatin were determined after 24, 48, 72 and 96 hrs incubation. The absorbance (540 nm and 570 nm) was detected by a plate reader (Synergy Multi-Mode Elisa Reader, BioTek, USA).

2.6 In-vivo Study

Fifteen healthy male albino Wistar rats weighing 250-300 g were housed under standard conditions (25°C, 12h dark/light). All animal experiments were approved by the Institutional Animal Ethics Committee (IR. PII. REC. 1395.19; 27 (February 2017)) [32, 33] and they adhered to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals in compliance with "The Basis of Laboratory Animal Science" [34]. Schematic representation of the in vivo experimental design is shown in Figure 1C. Animal body weights were recorded daily. Animal survival was monitored up to the point where the animals lost more than 30% of their initial body weight prior to surgery.

Animals were divided into 5 groups randomly. Group 0 without induced glioblastoma did not receive interventions or treatments (control group). Groups 1-4 underwent sterile surgery under anesthesia with 25 mg/kg ketamine and 8 mg/kg xylene. Briefly, the head of the animal was fixed into the stereotaxic frame, shaved and 1 cm of the skin was cut to open the skull. To induce glioblastoma, $1x10^4$ of C6 primary rat glioblastoma cells in 10 µL PBS were injected into the frontal lobe 2 mm to the right and 2 mm forward from the bregma as previously described by Miura et al [35]. A 25 µL a Hamilton syringe was used to slowly inject cells at 3 mm depth of brain in 1 min. The syringe was withdrawn slowly after the injection and ethicon suture was used to stitch up the skin. Seventy two hours after the cancer cell implantation, animals in groups 1, 2 and 3 received intraperitoneal (*i.p.*) injections of 0.8 mg/kg of carboplatin products in 100 µL of sterile 1X PBS with the following formulation: group 1 free form of carboplatin, group 2 unconjugated NPs loaded with carboplatin, group 3 NPs conjugated to mAb loaded with carboplatin every 48 hrs. Group 4 received 0.1 mL PBS injections (vehicle-treated group).

Following the completion of treatments, animal were euthanized by *i.p.* injection of a ketamine overdose. Then, fresh brains were evaluated macroscopically. Brain weight was measured by laboratory balances after tissue collection, and brain size was determined using a ruler. For

histology experiments, brain, kidney and liver samples were fixed in 10% buffered formalin solution and stored in 70% ethanol until embedding [36].

2.7 Tissue collection

Brains, livers and kidneys of the animals were collected to investigate histologically. Tissues were embedded in paraffin and cut, then deparaffinized, cleared and rehydrated in graded ethanol. Cross-sections of the tissue were stained with eosin and hematoxylin and mounted on glass slides with distrene plasticizer xylene (DPX) mountant. Assessment of organs was performed by a semi-quantitative scoring system [37]. The toxicity of organs was reported as 0 where no changes were found, 1 for any morphological changes and 2 for significant morphological changes in the collected organs.

2.8 Statistical analysis

SPSS software version 15 was used for statistical analysis of results. Statistically, *P* values less than 0.05 were considered significant. Results are expressed as a mean ± standard deviation (SD).

3. Results

The analysis of physicochemical characteristics of carboplatin-loaded NPs demonstrated that using PEG in formulations reduces size and improves the D.L.E and E.E of NPs (Table 1). Consistent with our previously published results [47], in this study PEGylation remarkably reduced the diameter of NPs. However, no significant changes in the surface charge were observed in PEGylated NPs compared to non-PEGylated NPs. Moreover, our results show that conjugating mAb to NPs does not demonstrate significant change in NP characteristics. Furthermore, the morphological analysis carried out by SEM demonstrates that PEGylated NPs have a spherical shape compared to non-PEGylated PBCA NPs and they do not aggregate together (Figure 2).

The physicochemical characteristics for all formulations of NPs which were evaluated after 1 and 2 months of storage are summarized in Tables 2 and 3, respectively. We found that PEGylated NPs maintain their smaller size, lower Zeta potential, and higher E.E compared to non-PEGylated NPs throughout and after this time. Furthermore, morphological evaluation was repeated after 2 months, which showed low aggregation of PEGylated NPs. Our results

demonstrate that non-PEGylated NPs display significant changes in their characteristics such as increase in their size, reduction of D.L.E, Zeta potential and E.E. Results show that PEGylated NPs are more stable compared to non-PEGylated NPs. In addition, results revealed that PEGylated NPs+mAb remained in a similar way to PEGylated NPs throughout this period of time.

The dialysis method was used to evaluate the impact of mAb conjugation and PEGylation on the drug release profile for NPs. The profile of drug release from PBCA NPs is presented in Figure 3. Drug release from NPs is slow compared to free drug. The slow drug release continues for 48 hrs. Indeed cumulative drug release from non-PEGylated NPs at 48 hrs was reported at 34% which was significantly different compared to 19.4% of carboplatin released from PEGylated NPs at these time points. Furthermore, results illustrate that conjugation of mAb to NPs cannot lead to significant change in release rate profile compared to PEGylated NPs. Overall, our results reveal that PEGylated NPs conjugated to mAb have a higher drug retention capability, with 15.4% (W/W) of the carboplatin released after 48 hrs compared to other formulations.

Table 1. Characteristics of PBCA NPs loaded immediately after preparation

	Size (nm)	**Zeta potential (mV)**	**E.E[1] (%)**	**D.L.E[2] (%)**	**PDI**
Non-PEGylated NPs	479±34	-11.6±0.2	39.9±0.9	3.1±0.1	0.27±0.04
PEGylated NPs	361±25*	-10.8±0.1	41.8±0.5*	3.7±0.2*	0.263±0.027
PEGylated NPs conjugated to mAb	365±23*	-10.7±0.1	41.9±0.3*	3.6±0.2*	0.264±0.008

[2]D.L.E: Drug loading efficiency; [1]E.E: Encapsulation efficiency; * $P < 0.05$ in comparison with non-PEGylated NPs.

Table 2 Characteristics of PBCA NPs after 1 month storing at 4°C fridge

	Size (nm)	**Zeta potential (mV)**	**E.E[1] (%)**	**D.L.E[2] (%)**
Non-PEGylated NPs	495±35	-11.2±0.1	36.9±0.4	3.2±0.2
PEGylated NPs	364±20*	-10.7±0.1	41.5±0.5*	3.6±0.1*

PEGylated NPs conjugated to mAb	366±20*	-10.6±0.1	41.9±0.5*	3.6±0.1*

[2]D.L.E: Drug loading efficiency; [1]E.E: Encapsulation efficiency; * $P < 0.05$ in comparison with non-PEGylated NPs.

Table 3 Characteristics of PBCA NPs after 2 months storing at 4°C fridge

	Size (nm)	**Zeta potential (mV)**	**E.E[1] (%)**	**D.L.E[2] (%)**
Non-PEGylated NPs	512±28	-10.5±0.1	35.5±0.9	3.0±0.1*
PEGylated NPs	364±20*	-10.7±0.1*	41.0±0.5*	3.5±0.1*
PEGylated NPs conjugated to mAb	364±20*	-10.6±0.1*	41.4±0.4*	3.6±0.1*

[2]D.L.E: Drug loading efficiency; [1]E.E: Encapsulation efficiency; * $P < 0.05$ in comparison with non-PEGylated NPs.

Cytotoxicity of free drug and NPs on C6 cell line was tested after 24, 48, 72 and 96 hrs incubation (Figure 4). Results illustrate that the cytotoxic effect of NPs was significantly higher at all-time points compared to free drug. Moreover, these results reveal that using PEGylation in NPs does not cause major changes in cytotoxicity compared to non-PEGylated NPs. However, adding PEG in the formulation of NPs increases cytotoxicity in comparison with non-PEG NPs in a time dependent manner. Acquired data also show that using mAb as a targeting agent improved the cytotoxic effects on C6 by more than 30% compared to other NPs formulations at 24, 48, 72 and 96 hrs.

The cytotoxic effects of free drug and NPs after 24, 48, 72 and 96 hrs incubation are represented in Figure 5. Results show an increased cytotoxicity of NPs in comparison with free carboplatin. The cytotoxic performance of NPs improved compared with free drug. Moreover, PEGylation improved the cytotoxicity of NPs. In addition, a ~ 40% improvement of cytotoxic effects was reported for NPs conjugated with mAb on A172 cell compared to free drug at 24, 48, 72 and 96 hrs.

Figure 6 shows IC_{50} of free drug and NPs on A172 and C6 cells. Results reveal that the cytotoxicity of free drug had not changed significantly after 48 hrs. PEGylated NPs demonstrate higher cytotoxic effect compared to free carboplatin and non-PEGylated NPs after 24, 48, 72 and 96 hrs with a 15-17% decrease every 24 hrs. Significant change in the cytotoxic

effect of non-PEGylated NPs was revealed from 24 to 48 hrs (14%). Furthermore, the NPs+mAb had less IC_{50} compared to other groups. An almost 30% IC_{50} decrease within 96 hrs was observed for NPs+mAb.

Animal weight was monitored after surgery for 16 days and results are displayed in Figure 7A. Groups 1, 2 and 3 received 0.8 mg/kg/dose of carboplatin formulations on the 3rd, 5th, 7th, 9th and 11th days after cancer was induced. Group 4 received equal 0.1mL PBS injections throughout the time period. Animals were culled immediately after losing more than 30% of their initial body weight during the monitoring period. Results show that all animals lost at least 10% of their body weight after 72 hrs following cancer cell injection. Group 1, treated by a free form of carboplatin, showed an increased body weight until day 10, and a 20% body weight loss until day 16, after which the animals were culled. Group 2 animals treated with unconjugated NPs displayed a 10% change in body weight until day 16. The body weights of Group 3, treated by PEGylated NPs conjugated to Cetuximab (IMC-C225) mAb, increased until day 16. Finally, body weights of Group 3 showed less than 2% change compared to their initial body weights. During this period, Group 4 animals that received PBS for treatment showed a remarkable reduction in body weight, with a decrease of 30% before day 15.

Survival time for animals with tumors induced with C6 cells was also investigated. Results as represented in the Kaplan-Meier survival plot (Figure 7B) demonstrate that the use of unconjugated PEGylated NPs did not cause substantial change in animal survival time. Results revealed that using PEGylated PBCA NPs+mAb significantly enhanced survival time by 40% compared to the free form of carboplatin. Conversely, it was discovered that unconjugated NPs did not deliver a sufficient dose of the drug to the tumor site. This phenomenon demonstrates that the use of mAb as a targeting agent has significant impact on the delivery of higher doses of NPs specifically to the brain tumor site.

Morphometric studies, including tumor weight and size, were conducted after tissue collection; results are shown in Table 4. Results indicated that the brain weight of animals treated with free form carboplatin (Group 1) was 5.6% lower compared to brains from healthy animals (Group 0). In addition, brain size of Group 1 animals was reduced by 16.5%. Animals in Group 2 revealed significant changes in comparison with Group 0, with almost 35% and 40% increase in weight and size of brain, respectively. Brain weight in Group 3 animals treated by PEGylated NPs+mAb showed a 1% change compared to group 0. Moreover, Group 3 reported a 6%

increase of brain size compared to Group 0. Group 4 animals showed a 45% increase in weight along with a 75% increase in brain size compared to Group 0. Images of collected tissues are displayed in Figure 8A.

Table 4 Morphometric analysis of the brains

Group	Weight (g)	Size (mm)
Group 0 Healthy (no glioblastoma, no treatments)	1.8±0.20	15 x 9.5 x 23
Group 1 Glioblastoma + Free carboplatin	1.7±0.09	16 x 9 x 19
Group 2 Glioblastoma + Unconjugated NPs loaded with carboplatin	2.5±0.22	17 x 11 x 25
Group 3 Glioblastoma + Conjugated NPs loaded with carboplatin	1.7±0.28	15.5 x 10 x 22.5
Group 4 Glioblastoma + PBS	2.6±0.38	18 x 12 x 27

Results are from 3 iterations (n=3) expressed as a mean ± standard deviation (SD).

Histological studies performed by eosin and hematoxylin staining of brain tissue are displayed in Figure 8B. Results demonstrate that the rates of bleeding in Groups 2 and 4 were increased compared to the other groups. In addition, results for Group 1, treated with the free form of carboplatin, demonstrated higher bleeding than Group 0. Group 3 indicated the most significant reduction in brain damage compared with other groups.

Histological studies of kidney and liver tissues are presented in Figure 9 (A and B respectively). The results of quantitative assessment histological data for the kidney and liver are summarized in Table 5. Results demonstrate that acute tubular necrosis (ATN) and liver necrosis are more prevalent in free carboplatin-treated animals. Moreover, results of the histological studies confirmed that animals treated with all forms (unconjugated PEGylated NPs and NPs+mAb) caused the least damage to the liver and kidney compared with other groups.

Table 5. Histological evaluation of organ toxicity after treatment

Group	Organ	Score
Group 1 Free carboplatin	Liver	2
	Kidney	1
	Brain	1
Group 2 Unconjugated NPs loaded with carboplatin	Liver	1
	Kidney	0-1
	Brain	2

	Liver	0-1
Group 3 Conjugated NPs loaded with carboplatin	Kidney	0-1
	Brain	0-1

4. Discussion

There is a common misconception that small molecules readily cross the BBB. However, >98% of all small molecules do not cross the BBB [38]. Due to the presence of the BBB, brain tumor treatment has long posed challenges. Moreover, the BBB is negatively affected by the chemotherapy treatment used for brain cancer [39, 40]. Thus, it is crucial to evaluate the therapeutic efficacy of drug delivery for brain disease treatment. A possible solution to this problem is the employment of receptor-mediated transport (RMT) or carrier through the BBB [41]. Using an appropriate nano-drug delivery system helps to increase drug concentration at a level sufficient to eradicate the tumor [42, 43]. Therefore, in this study a nano-particle delivery system for glioblastoma therapy was designed and tested in *in vitro* and *in vivo* experiments.

A significant requirement for the development of a suitable nano drug delivery system to the brain is that NPs must be biodegradable, over a short period of time [44, 45]. Due to its fast biodegradability properties, PBCA is the best option among other poly(alky cyanoacrylates) such as (lactide-co-glycolide), poly(lactic acid) or PBC [44]. PBCA NP application in drug delivery has been evaluated due to its biodegradability to reduce the toxicity of chemo drugs [46]. Therefore, NPs containing a high drug concentration were prepared. The previous finding of Hassanzadeganroudsari et al. (2019) confirmed that mini-emulsion polymerization is an appropriate method for preparing PBCA NP [47]. Our results in this study showed that the synthesis method and conjugation procedure are reliable. There are several parameters, such as surface properties, which impact the yield of drug delivery by NP systems. The surface properties of NPs play an imprtant role in the effective delivery of the chemotherapeutic drugs to the brain [48, 49]. Previous studies have shown that PEGylation increases the stability of NPs [50, 51]. In addition, PEGylation of NPs leads to the stealth effect that is characterized by a significant reduction in distribution into other healthy organs [48, 52]. PEG was utilised in the NPs formulation to enhance pharmacokinetic properties and improve the solubility of drugs. Previously, Calvo et al. (2001) showed that polysorbate 80 enhanced the ability of particles to deliver drugs to the brain [53]. Previous studies also showed that polysorbate 80

helped NPs to enhance drug permeability through the BBB [54]. Moreover, it has been previously reported that PBCA NPs coated with polysorbate 80 cause an unfolding of the strong ties of endothelial cells [55]. Also, Petri et al. (2006) showed that NPs coated with apolipoprotein E are absorbed into the brain [56]. Therefore, polysorbate 80 was added to the formulation, leading to absorption of apolipoprotein E in plasma. Using the transferrin receptor as a targeting ligand is one possibility for delivering drugs by NPs to the brain [57, 58]. There are many receptors that transport large molecules across the BBB [8, 59]. In this study, a mAb was conjugated to NPs to target EGFR in cancer cells. Previous studies indicated that cross-linker reactions can be utilized to conjugate protein to PBCA NPs [60]. Therefore, we hypothesized that using a targeting agent with biodegradable NPs is more suitable than other materials. Thus, sulfo–HSAB was used to cross-link the amine groups from the mAb to covalent C-H bonds of the dextran-coated PBCA NPs [61].

The size of our PBCA NPs was approximately 20% smaller compared to previously reported NPs loaded with cisplatin [32]. The size of NPs corrolate with surface area. By decreasing the size of NPs the provided surface area will increase which then enhance mass transfer properties. Moreover, results show that the preparation method and conditions have a remarkable impact on NPs characteristics [62, 63]. We demonstrated that many factors influence the quality of NPs, including sonication, pH, temperature and the application of dextran in NPs formulation. In our study, 1% dextran was used for prepration of NPs and pH was maintained at 4. Zeta potential of colloidal systems is another significant parameter with a positive impact. Zeta potential affects the stability of the loaded drugs in NPs and the rate of drug release from NPs. In addition, Zeta potential has a significant effect on the surface modification of the particulate system, thus surface modification is an important factor in efficient drug delivery. Surface modification is a common method for enhancing the sustainability of NPs in the blood for a longer period [64]. The results of this study showed -10.7 mV Zeta potential in PEGylated NPs, while -20 mV Zeta potential was reported in a previous study [32]. Changes in Zeta potential level is directly linked to improved drug release rate and enhanced stability of NPs.

The cumulative release of the drugs from the carrier is an important parameter as it correlates with the efficency of the NPs [65, 66]. A burst drug release was prominent within the first hour of the study, which was due to the carboplatin release attached to the NPs surface. Then, profiles of continuous release and gradual increase were observed which confirmed the potential of NPs in drug entrapment. Profiles of drug release demonstrate the primary slow

phase and then a quick spread phases in non-PEGylated and PEGylated NPs which can be attributed to the inhibitory and coating impact of PEG. On the other hand, the profile release for NPs+mAb showed a mild ascending slope. This occurred because drugs that adhered physically to the surface of NPs were completely removed in the conjugation process. This indicates that only carboplatin capsulated in NPs was released during the experiment. Drug release results demonstrated the high retention capability of PEGylated NPs+mAb in that 15.4% of drug that was released from NPs over a period of 48 hrs. This profile of drug release is very high in comparison with the previous study (Ebrahimi et al, 2014), which reported only 3.18 % of drug release after 51 h from NPs loaded with cisplatin [32]. Furthermore, the E.E was increased from 25% reported in a previous study by Ebrahimi et al (2014) to 37% for non-PEGylated NPs and 41.45% for PEGylated NPs conjugated to mAb in our study. Therefore, the results of this study reported a small size of NPs that could carry a high dose of the drug. Cosco et al (2009) indicated that using PEG enhances drug loading efficiency [67]. It was demonstrated that applying PEG in NP formulation had a pivotal effect on the NP characteristics, which was confirmed by the higher D.L.E and E.E reported in PEGylated formulation compared to non-PEGylated formulation. This can be explained by the role of PEGylation in coating tight junctions on the surface of NPs. PEGylation clearly helped to decrease the release of the drug from the tight vesicles, and this correlated to the lower rate of drug release after PEGylation compared to non-PEGylated formulation. Therefore, the retention yield and load rate was increased by PEGylation.

The cytotoxic effects of NPs on C6 and A172 cell lines have been investigated by MTT assay. The results showed that PEGylated NPs+mAb had the lowest IC_{50} and, therefore, showed a higher cytotoxicity on cancer cells compared to other NPs and standard carboplatin. The standard form of carboplatin caused major damage to cells and the toxic effect continued after 24 hrs. After 48 hrs the IC_{50} remained stable for the standard form of carboplatin. The cytotoxicity effect of PEGylated NPs loaded with carboplatin was higher than standard carboplatin and non-PEGylated NPs. This finding correlated with our previously published study [47] which evaluated the cytotoxic effects of PBCA NPs on ovarian cancer cell lines. This could be due to the impact of using PEG in E.E. enhancement. Hassanzadegan et al. (2019) [49] proved that using PEG in liposomal NPs can also improve the cytotoxicity of carboplatin on A172 and C6 cell lines, which confirms the role of PEG in enhancing E.E in different types of NPs. In fact, our results confirmed that concentration of carboplatin close to targeted cancer cells was the highest when using NPs+mAb compared to other formulations.

This caused more damage to cancer cells. Such data concur with our results demonstrating the lowest IC_{50} and the increased cytotoxicity of carboplatin, which indicates enhanced drug efficiency in NPs+mAb formulation. The cytotoxicity results are consistent with the Arshad et al (2015) finding on PLGA loaded with carboplatin. Their work demonstrated that nano-encapsulation significantly increased the cytotoxic effects of carboplatin in both cell and animal model studies [68, 69]. Similarly, Hamelers et al (2006) reported that the cytotoxic effects of carboplatin loaded into a lipid formulation are multiplied by 1000 times compared to the standard drug [70-72].

Moreover, the survival study showed that unconjugated NPs are not able to efficiently deliver drugs to tumor tissue. Although PBCA NPs showed strong performance in cytotoxicity evaluation in both the present and the previously published studies [47], the survival study results revealed that PBCA NPs are not suitable for glioblastoma treatment. However, using surface modified PBCA NPs improved animal survival time by 40% compared to the free form of carboplatin. These results correlated with the findings previously reported by Gulyaev et al. [73] and Ambruosi et al. [74], which discovered that applying polysorbate 80 on NP formulation remarkably extended the biodistribution of NPs in brain tumors and consequently enhanced the survival time of the animal. This increase in survival time is longer compared to the study by Xin et al (2010) [75], which showed only 20% increase in survival time of animals treated by methoxy poly(ethylene glycol)-poly(ε-caprolactone) NPs. Our results reveal that the targeting agent plays a crucial role in delivering a higher drug dose to the targeted site. This significant enhancement occurred as a higher dose of the drug was delivered to the tumor. These results reveal that PEGylated PBCA NPs+mAb efficiently pass through the BBB. Furthermore, the anti-tumor efficacy of NPs was further substantiated by histological analysis of brain samples in *ex vivo* study. The morphological study showed no changes in the brain size and weight compared to the brains from other groups of rats. This reveals a remarkable effect of mAb as a targeting agent, which improved the efficiency of our drug delivery system. Lower toxicity of the NPs altered biodistribution of the drug mediated by the NPs, and this agrees with Wohlfart et al (2012) who showed that NPs reduce the toxicity of chemotherapeutic drugs and decrease damage to other organs [44].

5. Conclusion

Developing NPs incorporating retention power and high encapsulation efficiency aids in the designing of nano drug delivery. In this study we applied a mAb as a targeting agent to enhance the drug delivery efficiency of PBCA NPs, following on from our previously published study [47]. Our findings indicate that the mini-emulsion polymerization together with the conjugation method are reliable prepration procedures for designing PBCA NPs conjugated to mAb. Furthermore, the characteristics of the NPs loaded with carboplatin have been studied, demonstrating that NPs were stable over the two months. Also, sulfo–HSAB was used to cross-link the amine groups from the mAb to covalent C-H bonds of the dextran-coated PBCA NPs. The efficacy of the drug loaded onto NPs+mAb on glioblastoma cell lines was shown to enhance cytotoxicity compared to standard carboplatin. Furthermore, our results demonstrate that cytotoxicity correlates with the E.E and the drug concentration. Our findings showed that the use of PBCA NPs can significantly reduce the side effects of carboplatin on other organs, but does not improve survival time for treated animals compared to the free form of carboplatin. On the other hand, our study demonstrated that using a targeting agent in the NP structure significantly enhances the animals' survival rate. This demonstrates that NPs+mAb can significantly enhance the therapeutic effects of carboplatin with higher doses delivered to the tumor site with NPs+mAb compared to NPs non-conjugated NPs and free form drugs. The results also show that by using NPs+mAb, there was a reduction in carboplatin treatment-associated side effects, including changes in brain size and weight, body weight and effects on non-targeted organs (kidney and liver). Overall, our findings suggest that PEGylated NPs+mAb has extensive potential for enhancing efficiency of carboplatin-based therapy in glioblastoma.

Funding: This research has not received external funding.

Acknowledgments: The author would like to thank the Department of Pilot Nanobiotechnology, Pasteur Institute of Iran, Tehran, Iran and Dr. M. Chiani and Dr. H. Ebrahimi Shahmabadi for their significant contribution and support.

Conflicts of Interest: No conflicts of interest to declare.

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Figure Legends

Figure 1. A) Schematic conjugation of PBCA NPs to mAb. B) Schematic representation of targeted NPs drug delivery process. C) Scheme of the *in vivo* experimental design.

Figure 2. SEM micrographs of A - PEGylated NPs, B – non-PEGylated NPs and C - PEGylated NPs after 2 months.

Figure 3. Release profile of drug from PBCA NPs for non-PEGylated NPs and PEGylated NPs conjugated with mAb as well as non-conjugated PEGylated and free carboplatin within 48 hrs at 37°C. Results are from 3 iterations (n=3) expressed as a mean ± standard deviation (SD).

Figure 4. Cytotoxic effects of NPs and free carboplatin on the C6 cell line after 24, 48, 72 and 96 hrs of incubation (A, B, C and D respectively). Results are from 4 iterations (n=4) expressed as a mean ± standard deviation (SD). **P*<0.05 NPs compared to free carboplatin, *#P<0.05* PEGylated NPs conjugated to mAb compared to non-conjugated PEGylated and non-PEGylated NPs.

Figure 5. Cytotoxicity effects of NPs and free drug on the A172 cell line after 24, 48, 72 and 96 hrs of incubation (A, B, C and D respectively). Results are from 4 iterations (n=4) expressed as a mean ± standard deviation (SD). **P*<0.05 NPs compared to free drug, $^{\#}P$<0.05 PEGylated NPs conjugated to mAb compared to non-conjugated PEGylated and non-PEGylated NPs.

Figure 6. Effect of IC50 (μM) of non-PEGylated NPs and non-conjugated PEGylated as well as PEGylated NPs conjugated with mAb and free drug on A172 and C6 cell lines at 24, 48, 72 and 96 hrs time intervals. All results are from 4 iterations (n=4) expressed as a mean ± standard deviation (SD).

Figure 7. Changes in body weight and survival of animals after different treatments. A) Body weight change in groups received following treatments: Group 1 - Free form of carboplatin, Group 2 - Unconjugated NPs loaded with carboplatin, Group 3 - NPs loaded with carboplatin conjugated to mAb, Group 4 - PBS. B) Kaplan-Myer survival analysis of animals with tumors induced by C6 after different treatments: Group 1 - Free form of carboplatin, Group 2 - Unconjugated NPs loaded with carboplatin, Group 3 - NPs loaded with carboplatin conjugated to mAb, Group 4 - PBS.

Figure 8. Morphological and histological analysis of brains from different groups. A) The brains collected from the following groups: Group 0 - Healthy untreated animals without cancer induction, Group 1 - Tumor-bearing mice treated with free form of carboplatin, Group 2 - Tumor-bearing mice treated with unconjugated NPs loaded with carboplatin, Group 3 - Tumor-bearing mice treated with NPs loaded with carboplatin conjugated to mAb, Group 4 – PBS-treated tumor-bearing mice. B) Eosin and hematoxylin staining of brain tissue from corresponding groups of animals.

Figure 9. Hematoxylin and eosin staining of kidney (a) and liver (b) from the following groups: Group 1, Group 2, Group 3. Arrows indicate necrotic cells.