# Aptamer functionalization of nanosystems for Glioblastoma targeting through the Blood-Brain-Barrier.

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ABSTRACT: Polymeric nanoparticles (PNPs) offer unique possibility for *in vivo* delivery of diagnostics and/or therapeutics to tumor tissues, when conjugated to specific targeting agents. Among those, aptamers are able to cross the Blood Brain Barrier (BBB) and the Gint4.T aptamer specifically recognizes the platelet-derived growth factor receptor β. Here, we describe the preparation of Gint4.T-conjugated PNPs which, *in vitro*, are able of high uptake into U87MG glioblastoma (GBM) cells and kill them with an astonishing EC50 value of 38 pM, when loaded with a PI3K-mTOR inhibitor. Ultimately, *in vivo*, we also demonstrate BBB passage and tumor accumulation in an orthotopic model of human GBM.

## INTRODUCTION

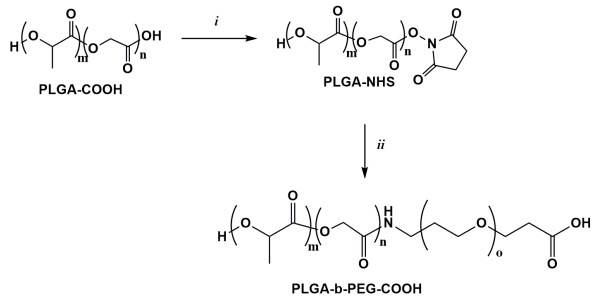
Glioblastoma (GBM) is the most common and most aggressive type of primary brain tumor and the astrocytoma of the highest grade.[1] Surgery, radiotherapy and chemotherapy are the current standard of care for its treatment, but the prognosis remains unforgiving, partly due to impediment of the blood-brain barrier (BBB).[2,3,4] Thanks to targeted drug delivery nanovectors, nanotechnology represents an attractive strategy, and is receiving increasing attention for its ability of transporting drugs within the cerebral tissue with minimal off-target toxicity.[5,6] Because of its ability of creating biodegradable polymeric nanoparticles (PNPs) suitable for targeted drug delivery approaches, poly(lactic-co-glycolic)-*block*-poly ethylene glycol (PLGA-*b*-PEG) copolymer received great attention in the past decade as main component for nanovectors.[7,8] Polymeric nanoparticles (PNPs) administered systemically may reach the cancerous site by passive targeting, due to the so-called "enhanced permeability and retention" (EPR) effect, which is the natural consequence of the defective vascularization and reduced lymphatic drainage in the tumor environment.[9] However, the mere EPR effect cannot accomplish a significant improvement in intracellular uptake, thus it is not sufficient to ensure a high accumulation of PNPs in cancerous tissues. A more promising strategy is the introduction, onto the surface of PNPs, of targeting agents for tumor specific recognition and internalization, which lead to selective accumulation of the drug in cell compartments.[10] Potential agents for targeting tumor cells are small molecules, peptides or monoclonal antibodies but lately aptamers arose as the most promising ones. Aptamers, generated by the Systematic Evolution of Ligands by EXponential enrichment (SELEX) method, are short, artificial, single-stranded oligonucleotides that, similarly to antibodies, interact at high affinity with their targets by recognizing a specific three-dimensional structure. Thanks to their unique properties (small size, high stability, convenient synthesis and modification with minimal inter-batch variability, lack of immunogenicity), aptamers have been proved as a valid alternative to antibodies as targeted cancer therapeutic agents on their own or as carriers of chemotherapeutic agents, small interfering RNAs or drug-loaded nanoparticles.[11,12,13] Specifically, proof-of-concept studies have suggested aptamers as efficacious escort agents to deliver PNPs to tumor in intracranial GBM-bearing mice. [14,15] In

choosing aptamers for targeting GBM cells, it is essential that they recognize receptors that are preferentially expressed on the surface of cancer cells. Several studies demonstrated the expression and activation of multiple growth factor receptors in GBM that dictate the tumor response to targeted therapies. Among them, platelet-derived growth factor receptors (PDGFRs), both α and β forms, are amplified and/or overexpressed in human glial tumors with PDGFRα strongly associated with the proneural subtype and PDGFRβ more commonly expressed in different GBM subtypes and preferentially expressed by self-renewing tumorigenic GBM stem cells.[16] Further, PDGFRβ upregulation has been recently shown as a cause of acquired resistance of GBMs to epidermal growth factor receptor inhibitors,[17] highlighting the importance of specifically targeting this receptor for GBM treatment. Here, we investigated the possibility to associate the anti-PDGFRβ aptamer with PNPs acting as nanovectors for the delivery of a promising chemotherapeutic drug, NVP-BEZ235, a potent dual PI3K-mTOR inhibitor that is currently under investigation for the treatment of solid tumors and has recently proved to be a radio- and chemosensitizer in preclinical mouse GBM models.[18] When tested for functionality, the final targeted drug-loaded nanovectors were strongly effective to specifically induce cytotoxicity in U87MG GBM cells. Importantly, fluorescence imaging approaches demonstrated that, upon intravenous injection in nude mice bearing intracranial U87MG tumors, the aptamer-PNPs conjugates cross the BBB and accumulate at the tumor site. Accordingly, the assembled nanovectors were successful in delivering NVP-BEZ235 to the tumors, as evidenced by their ability to inhibit mTOR activity *in vivo*, therefore encouraging their further investigation as active drug delivery systems in patient-specific orthotopic GBM xenograft models.

## RESULTS AND DISCUSSION

**Synthesis and Characterization of NVP-BEZ235@PNPs Nanovectors.** To demonstrate the improved efficiency of targeted nanovectors in delivering drugs into the brain tumor environment, we prepared PNPs based on poly(lactic-co-glycolic)-*block*-poly ethylene glycol (PLGA-*b*-PEG) copolymer and loaded them with a lipophilic dye, BODIPY®505-515 (BODIPY), for visualization purposes or with a PI3K-mTOR inhibitor, NVP-BEZ235, to evaluate their efficacy for cancer cell killing. Specifically, we loaded PNPs with NVP-BEZ235 and conjugated them with an anti-PDGFRβ aptamer,[19] named Gint4.T, onto the external surface for selective targeting against GBM cells. For this purpose, the copolymer PLGA-b-PEG-COOH was synthetized as the previously reported method showed in the **scheme 1**.[20] NVP-BEZ235 has the severe limitation of being poorly water soluble, thus its bioavailability is reduced and huge administration doses are necessary for ascertaining a significant effect.

**Scheme 1 – Synthesis of copolymer (PLGA-b-PEG-COOH)**

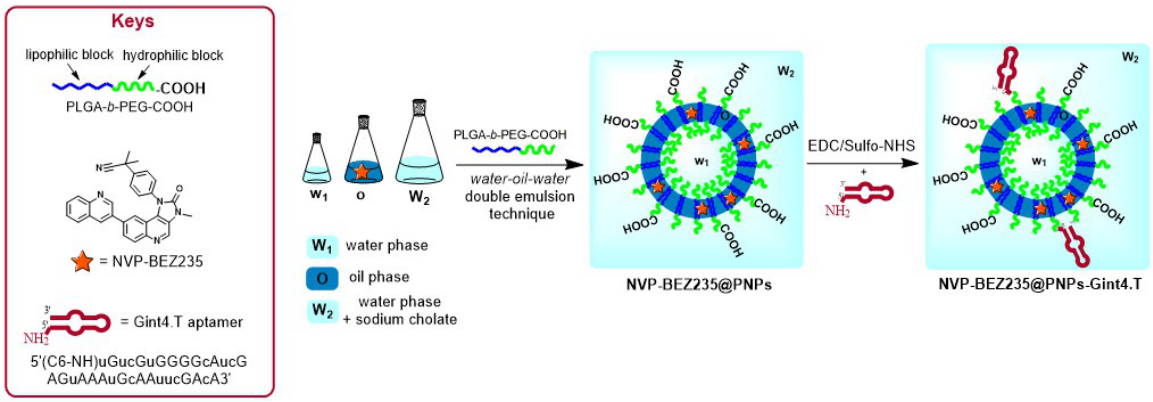



Reagents and conditions: (i) DCC/NHS, $CH_2Cl_2$, room temperature, 12 hours; (ii) $NH_2$-PEG-COOH, DIPEA, room temperature, 12 hours.

In our case, by using the *water-in-oil-in-water* double-emulsion sonication method,[21] we entrapped the drug in the lipophilic portion of polymeric nanoparticles obtaining a final water dispersible formulation.[22] After purification of the obtained NVP-BEZ235@PNPs (**Scheme 2**), conjugation of amino-terminated Gint4.T was performed exploiting the superficial residual carboxylic groups onto the PNPs surface, derived from PEG chains. NVP-BEZ235@PNPs-Gint4.T were then purified and characterized by Dynamic light scattering (DLS), which revealed particles with diameter equal to 52 ± 1 nm, a low polydispersity index (PDI = 0.169) and negative ζ-potential value of -13.1 mV due to unreacted carboxylic acid groups. NVP-BEZ235 concentration was determined to be 25.2 μM by fluorimetric analysis. The amount of aptamer conjugated to the PNPs was evaluated by RT-qPCR analysis on NVP-BEZ235@PNPs-Gint4.T. The concentration of Gint4.T was 1.4 nM and the conjugation efficiency was 5.4%. The overall NVP-BEZ235@PNPs-Gint4.T concentration in solution was established by gravimetric analysis to be 18.4 mg/mL. In order to prove a direct correlation between results obtained and the presence of a specific aptamer onto the surface of the nanoparticles, NVP-BEZ235@PNPs were also conjugated with a 2′F-Py RNA with no affinity for PDGFRβ receptor, consisting in the scrambled sequence of a previously generated anti-EGFR aptamer and herein used as negative control (indicated as NVP-BEZ235@PNPs-SCR, see Supporting information and **Figure S1**).[19,23] Conversely, for fluorescence-based studies (internalization studies by confocal laser microscopy and *in vivo* tumor targeting), we substituted NVP-BEZ235 with a lipophilic dye, BODIPY®505-515, allowing for visualization of the nanoparticles (see Supporting information and **Figure S2**).

***In vitro* internalization studies**. Targeting efficiency of aptamer-decorated nanovectors is a crucial factor for optimal delivery of drugs to cancer cells. BODIPY@PNPs, conjugated with Gint4.T or scrambled (SCR) aptamers, were incubated with U87MG cells, a widely used GBM cell line, and nanovectors were visualized by confocal microscopy taking advantage of a fluorophore incorporated in

**Scheme 2. Representation of the water-in-oil-in-water procedure for the obtainment of the final nanovectors**




the oleic phase of PNPs, namely BODIPY. PNPs conjugated with Gint4.T aptamer resulted easily internalized in U87MG cells, becoming clearly visible 10 minutes after the beginning of the treatment; conversely, SCR decorated nanovectors were undetectable until 50 minutes (**Figure 1 and S3**). These data indicated that the conjugation of PNPs with the anti-PDGFRβ aptamer strongly enhanced the internalization of the nanovectors in GBM cells. To additionally control that specificity of targeting depended on the recognition of the PDGFRβ by the Gint4.T aptamer, we next produced U87MG clonal cells depleted from this receptor by an shRNA approach. PDGFRβ-depleted cells were, therefore, evaluated by immunoblot analysis, revealing significantly reduced protein levels of this receptor compared with control cells silenced with scrambled shRNA and with parental cells (**Figure S4A**). PNPs uptake was therefore monitored in PDGFRβ positive cells (parental and non silencing shRNA U87MG) compared to different PDGFRβ-depleted cell clones (PDGFRβ shRNA U87MG clones 3 and 6). After a 40 minutes incubation, fluorescent BODIPY@PNPs-Gint4.T were clearly internalized exclusively in PDGFRβ positive cells (**Figure S4B and S4C**) while, as an additional control, untargeted, fluorescent BODIPY@PNPs-SCR were not internalized in any of the cell clones. Altogether, these data clearly indicate that the Gint4.T aptamer specifically targets PNPs to GBM cells, strongly enhancing their intracellular uptake.

***In vitro* cytotoxicity studies.**

Based on our results demonstrating efficient and specific internalization of PDGFRβ-targeted PNPs in GBM cells, we next studied their ability to efficiently release drugs loaded into the nanovectors. For this purpose, we selected the lipophilic drug NVP-BEZ235, a novel dual kinase inhibitor directed against PI3K and MTOR, which is currently under investigation as a therapeutic tool against multiple solid tumors.[24] Taking advantage of the lipophilic behavior of this compound, NVP-BEZ235 entrapment in PNPs was obtained by using the water-in-oil-in-water double-emulsion sonication method,[21] ultimately allowing us to obtain a final water dispersible formulation.[22] Pairwise, in order to check the potential toxicity of the nanoparticle carrier itself, unloaded PNPs targeted with either SCR or Gint4.T aptamer were tested for cell viability on U87MG glioblastoma cell line. Unloaded carriers resulted in a very limited toxicity even at the maximal concentration (**Figure S5**). Furthermore, NVP-BEZ235 loaded PNPs were used in cytotoxicity studies at carrier concentrations lower than 0.020 mg/ml, at which unloaded carrier is extremely safe and completely atoxic. First, we confirmed the ability of NVP-BEZ235 to inhibit the PI3K/MTOR pathway, by evaluating *in vitro* phosphorylation of the 4EBP1 protein, a well-established substrate of MTOR.[25] Specifically, 4EBP1 phosphorylation was monitored after 40 minutes incubation of U87MG cells with NVP-BEZ235@PNPs-SCR and NVP-BEZ235@PNPs-Gint4.T or after 40 minutes incubation followed by washing and 6 hours recovery period. In both conditions, NVP-BEZ235@PNPs-Gint4.T efficiently inhibited 4EBP1 phosphorylation at levels comparable to "free" NVP-BEZ235 and, interestingly, more efficiently than NVP-BEZ235@PNPs-SCR (**Figure S6A**). Interestingly, NVP-BEZ235@PNPs-Gint4.T were very less efficient in MTOR pathway down-regulation when compared to NVP-BEZ235@PNPs-SCR, in shPDGFRβ U87MG cells in which the expression of Gint4.T aptamer receptor, PDGFRβ is strongly decreased (**Figure S6B-C**). Overall, these data therefore established the ability of the drug to be effective on its specific biochemical targets also when delivered inside our PNPs, and suggested improved efficacy for U87MG targeting for Gint4.T-coupled PNPs. Therefore, the ability of drug-loaded nanovectors of killing U87MG target cells was next measured by cytotoxicity assays, evaluating EC50 values for "free" NVP-BEZ235, NVP-BEZ235@PNPs-SCR and NVP-BEZ235@PNPs-Gint4.T. After 72 hours of continuous incubation with cells, free NVP-BEZ235 showed an EC50 of about 165 nM on the GBM cell line, while the same drug entrapped in PNPs resulted at least 1000-fold more cytotoxic, with PNPs-Gint4.T about 4-fold more effective than PNPs-SCR (**Ta-**

**ble 1**), demonstrating that our nanovectors strongly improved bioavailability of the drug and that PNP-coating aptamers additionally increased killing efficacy, by enhancing nanovectors internalization.

**Table 1 EC50 values on GBM cell line**

| | EC50 (72h) | EC50 (40 min + WO + 72h) |
|---|---|---|
| NVP-BEZ235 | 165693±1822 pM | 1017585±8774 pM |
| NVP-BEZ235@PNPs-SCR | 156±18 pM | 10443±632 pM |
| NVP-BEZ235@PNPs-Gint4.T | 38±6 pM | 160±11 pM |

**Table 1**. EC50 ± SD for free NVP-BEZ235, NVP-BEZ235@PNPs-SCR and NVP-BEZ235@PNPs-Gint4.T were evaluated after continuous incubation (EC50 at 72h) and after 40 minutes incubation followed by washes (WO = washout) and 72 hours recovery. EC50 were assessed by automatic cell counting and estimated on the basis of three different experiments (n=3).

**Table 1 EC50 values on shSCR and shPDGFRβ U87MG cells**

| | EC50 on U87 shSCR | EC50 on U87 shPDGFRβ |
|---|---|---|
| NVP-BEZ235 | 143237±1542 pM | 134527±1288 pM |
| NVP-BEZ235@PNPs-SCR | 448±51 pM | 441±43 pM |
| NVP-BEZ235@PNPs-Gint4.T | 141±36 pM | 486±38 pM |

**Table 2.** EC50 ± SD for free NVP-BEZ235, NVP-BEZ235@PNPs-SCR and NVP-BEZ235@PNPs-Gint4.T were evaluated after continuous incubation (EC50 at 72h). EC50 were assessed by Cell Titer Glo® and estimated on the basis of three different experiments (n=3).

Moreover, Gint4.T aptamer specificity was confirmed also in this system, taking advantage of our shSCR and shPDGFRβ U87MG cells for evaluating EC50 of "free" NVP-BEZ235, NVP-BEZ235@PNPs-Gint4.T and NVP-BEZ235@PNPs-SCR upon 72 hours of continuous incubation with cells (**Table 2** and **Figure S7**). In U87MG cells with downregulated PDGFRβ, the uptake advantage offered by Gint4.T aptamer was strongly reduced, as expected, as evidenced by highly similar EC50 for SCR and Gint4.T decorated NVP-BEZ235@PNPs, which resulted 441±43 nM and 486±38 nM, respectively. To further confirm quick PNPs internalization driven by Gint4.T aptamer, we next evaluated EC50 of "free" NVP-BEZ235, NVP-BEZ235@PNPs-Gint4.T and NVP-BEZ235@PNPs-SCR, upon a short 40 minutes incubation time followed by washing and a 72 hours recovery period. In these settings, EC50s of NVP-BEZ235@PNPs-Gint4.T was 6500-fold better than free drug and 65-fold better than NVP-BEZ235@PNPs-SCR (**Table 1**), demonstrating faster upload and more efficient GBM cells killing of targeted as compared to untargeted nanovectors. Overall, our data therefore suggest that our PDGFRβ targeted, drug-loaded nanovectors already represent more efficient alternatives to water-insoluble "free" drugs such as NVP-BEZ235. EC50 for free NVP-BEZ235, NVP-BEZ235@PNPs-SCR and NVP-BEZ235@PNPs-Gint4.T were evaluated after continuous incubation (EC50 at 72h) and after 40 minutes incubation followed by washes (WO = washout) and 72 hours recovery.

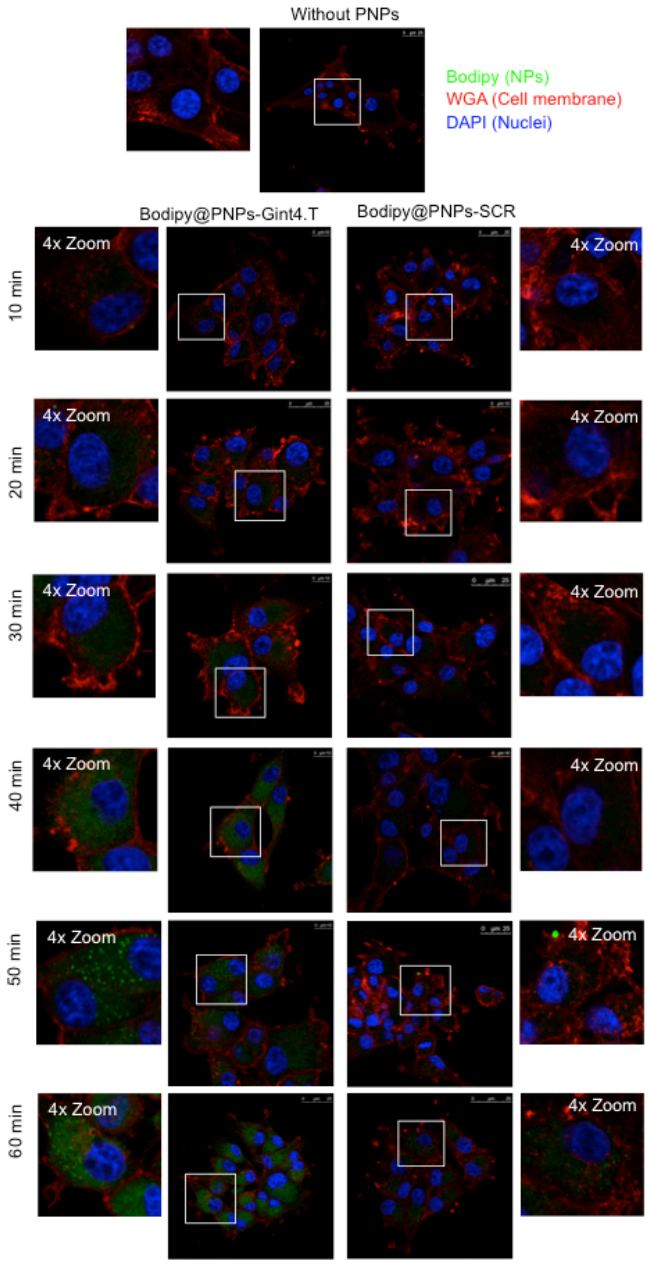



**Figure 1** U87MG cells were incubated for different period (from 10 to 60 minutes) with BODIPY@PNPs-Gint4.T or BODIPY@PNPs-SCR or left untreated. After, washing and fixation U87MG cells were labeled with WGA-647 to visualize cell membrane (red) and with DAPI to stain nuclei (blu). BODIPY@PNPs were displayed in green. White square indicate the area showed in insets.

**Specific tumor targeting of aptamer-based nanovectors**. The existence of the BBB is a major impediment for the delivery of drugs to brain tumors, through the blood. It is, therefore, of uppermost importance also for any novel systemic drug delivery system to prove its ability to pass this barrier to specifically accumulate into targeted

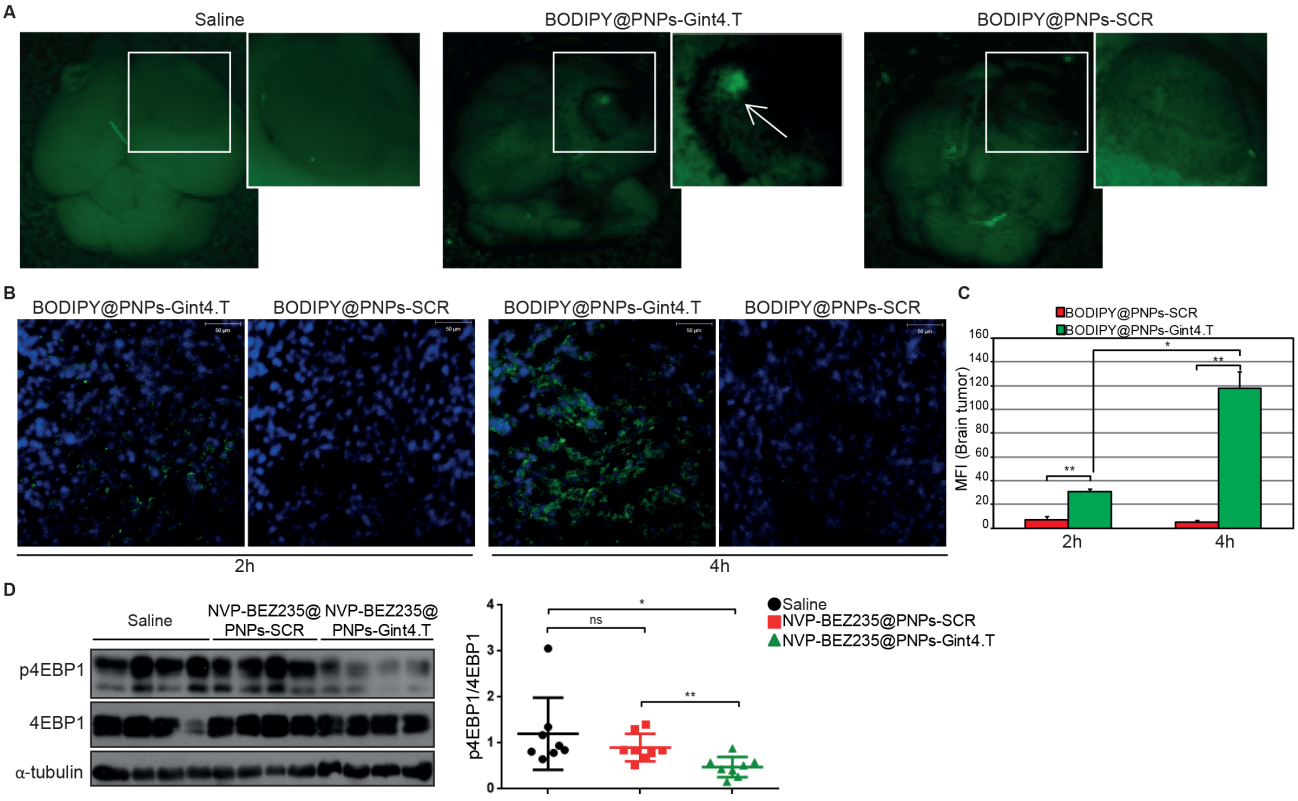



**Figure 2.** (A) *Ex vivo* fluorescence imaging of entire brains explanted from mice bearing U87MG orthotopic xenografts 2h after injection of PBS, BODIPY@PNPs-Gint4.T or BODIPY@PNPs-SCR. Magnified views of the tumor area (white square) are indicated in the insets. White arrows indicate fluorescent signals from Gint4.T-PNPs. (B) Tumor targeting of aptamer-PNPs conjugates in brain cryosections via confocal microscopy. Representative confocal microscopy images of tumors at 2h and 4h post injection of BODIPY@PNPs-Gint4.T or BODIPY@PNPs-SCR. All digital images were captured at the same setting to allow direct comparison of staining patterns. Blue: nuclei; green: aptamer-PNPs. (C) Aptamer-PNPs fluorescence in the tumor was quantified on 6 separate slices for each experimental group and expressed as mean fluorescence intensity (MFI) ± SD of 10 microscopy fields randomly taken from each slice.(D) Western blot of p4EBP1 and total 4EBP1 in intracranial U87MG xenografts after completion of the 5-day treatment with saline, NVP-BEZ235@PNPs-SCR or NVP-BEZ235@PNPs-Gint4.T. Left, 4 representative tumors per group are shown. α-tubulin was used as internal control. Right, quantitation of p4EBP1/4EBP1. Each data point represents the sample from an individual mouse (n = 8). One-way ANOVA followed by Tukey's multiple comparison test (D) or student's *t*-test (C). **P < 0.01; *P < 0.05; ns, no significance.

tumors. The intracranial U87MG (PTEN null) tumor model was used to test, *in vivo,* the BEZ235-loaded nanovectors, since these tumors share, with a large proportion of GBM, the deregulation of the PI3K pathway. Further, even though U87MG tumor xenografts are not representative of the infiltrative behaviour of human GBM, their fast grow with delineated boundaries (**Figure S8**),[26] allows easy identification and dissection, thus representing the most frequently used glioma model. First, to evaluate passage through the BBB and specific brain tumor accumulation, BODIPY@PNPs-Gint4.T or BODIPY@PNPs-SCR nanovectors were systemically administered in nude mice bearing intracranial U87MG tumor xenografts. High-resolution fluorescence imaging of whole brains, explanted from mice sacrificed 2 hours after injection, showed a clear signal, distinct from noises of autofluorescence background, in tumor from BODIPY@PNPs-Gint4.T-treated animals, while no specific signal was observed in tumor from mice treated with the negative control nanovectors (**Figure 2A**). To further corroborate these findings, indicating the accumulation into the tumor of the nanoparticles conjugated with Gint4.T, but not with the scrambled aptamer, sagittal sections of the brains were analyzed by confocal microscopy (**Figure 2B**) or H&E stained to confirm the location and the boundary of the tumor (**Figure S8**). Consistently with the entire brain imaging results, the fluorescence intensity, distributed in the tumor, from the Gint4.T-conjugated PNPs was significantly higher than from the SCR-conjugated PNPs. Importantly, the fluorescence from the control nanoparticles was almost undetectable also at 4 hours after injection while the fluorescence from the Gint4.T-nanovectors was significantly higher at 4 hours than at the 2 hours-time point (**Figure 2B and 2C**).

In this context, it is worth to notice that, although the BBB is partially damaged at the tumor core of the intracranial U87MG model, it is conversely fully intact in the brain areas that are distant from the central tumor mass, such as the tumor rim and the contralateral normal hemisphere, where it prevents drug penetration.[26,27,28] Im-

portantly, such distribution mirrors what occurs in large part of human GBMs where it is essential to potentially treat not only the "core" of the tumor but also invasive peripheral areas and, even more so, sparsely invaded regions distant from the tumor bulk and protected by an intact BBB.[26,27,28] Therefore, to confirm that our delivery system was indeed able to cross an intact BBB, we analyzed the amount of fluorescence in the areas of normal brain distant from the tumor, *i.e.* the contralateral hemisphere, detecting a greater fluorescence in the brain from mice that received Gint4.T-PNPs injection compared to those treated with the scrambled nanovectors, again supporting that conjugation with the anti-PDGFRβ aptamer allows the nanoparticles to cross the BBB (**Figure S9**).

Next, to confirm the tumor-specific targeting of nanovectors, we assessed the effectiveness of the Gint4.T-PNPs in delivering the drug to mouse brain tumors. To this aim, NVP-BEZ235@PNPs-SCR and NVP-BEZ235@PNPs-Gint4.T were systemically administered daily over 5 consecutive days to tumor-bearing mice. At the end of the treatment, the tumors isolated from the brains were processed for inhibition of phospho-4EBP1. Following such a short and acute treatment, phospho-4EBP1 levels were significantly lower in Gint4.T-PNPs treated tumors than in the control tumors (**Figure 2D**). Overall, these results prove the efficacy of our specifically assembled nanosystem to overcome the BBB and accumulate in the tumor and suggest that both these processes are mediated by Gint4.T aptamer-dependent recognition of the PDGFRβ expressed on these cells. Regarding NP accumulation into GBM cells, we clearly prove that it is mediated by interaction of NP with the PDGFRβ expressed on tumor cells,[16,17] being abrogated by receptor downregulation obtained with an shRNA approach. Moreover, the high affinity and specificity of Gint4.T vs the receptor target allows a very low amount of aptamer to efficaciously drive GBM cells internalization of the nanoparticles and their tumor uptake upon *in vivo* administration. Regarding passage through the BBB, we also clearly demonstrate, *in vivo*, the requirement of the Gint4.T aptamer on the NP's surface as scrambled aptamers cannot induce such passage. In this regard, it should be pointed out that PDGFRβ is highly expressed on endothelial cells of vessels, which vascularize the tumor,[29] while it has been recently shown [30] that an *in vitro* reconstituted BBB model, consisting of endothelial cells, astrocytes and pericytes grown on a three-dimensional scaffold, resulted permeable to the Gint4.T aptamer, suggesting that the aptamer could cross the BBB model by transcytosis mediated by its target. Thus, the anti-PDGFRβ Gint4.T aptamer could plausibly allow both receptor-mediated transcytosis trough the BBB and GBM targeting, revealing a precious strategy to mediate highly specific and effective drug delivery to gliomas. Notably, despite an increasing number of cell-type specific aptamers have been integrated with nanocarriers for both imaging and therapeutic applications in different tumors, only few studies employed aptamers as targeting agent for glioma through the BBB.[14,15]Moreover, while Gao et al. needed to functionalize NP with both an anti-nucleolin AS1411 aptamer for tumor uptake and with the 12-aminoacid TGN peptide to cross the BBB in a C6 orthotopic glioma model,[14] the same group more recently demonstrated, in the U87MG orthototopic model, a better tumor accumulation of NPs functionalized with an aptamer, selected by glioma cell-SELEX, whose molecular target was not identified, limiting the possibility of deeper investigation of involved mechanisms.[15] Overall, our study indicates the Gint4.T-based nanosystems as a promising tool to efficiently deliver innovative drugs or conventional chemotherapeutics through the BBB for GBM treatment. Importantly, unconjugated Gint4.T aptamer has been shown to directly inhibit PDGFRβ function both *in vitro* and *in vivo*.[19] Thus, it will potentially act not only as a targeting moiety but also as PDGFRβ antagonist, thus further improving the therapeutic efficacy of the aptamer/drug-PNPs conjugates. Further, apart from therapeutic utility, the assembled nanovectors could be usefully applied for diagnostic purposes allowing the site-targeted controlled delivery of imaging agents to the brain tumor. Notably, given that the list of oligonucleotide aptamers interacting with biomarkers expressed on tumor cell surfaces is growing rapidly, the proposed approach could be generally applicable to different surface-decorating aptamers and/or entrapped drugs (or imaging agents) in order to test new therapeutic (or diagnostic) options for GBMs and other human cancers.

## CONCLUSIONS

In conclusion we have prepared a novel multifunctional nanosystem having specific PDGFRβ aptamer conjugated on the outer shell of the polymeric nanoparticle. This nanovector has been validated *in vitro* and proved to be extremely efficient for "active" targeting and internalization in glioblastoma cellular models. Importantly, entrapment of a very promising but low water soluble drug, NVP-BEZ235, for glioblastoma treatment strongly increased its bioavailability with a 1000-fold augmented cytotoxicity as compared to the free drug.

Ultimately, we have also demonstrated for the first time the *in vivo* specific GBM tumor uptake on orthotopic cancer-bearing mice after intravenously administration, thus demonstrating these nanovectors as very promising "carriers" for any drug through the BBB and to specifically target GBM tumors. Taken together, we show that the increased presence and activity of PDGFRβ aptamer can be used to specifically, effectively, and safely deliver a multifunctional nanosystem in orthotopic brain tumor. Through this approach important breakthroughs can be envisaged in biomedical sciences offering interesting potentialities for basic science and translational applications.

## EXPERIMENTAL SECTION

**Synthesis and Characterization of PNPs nanovectors** 100 mg of PLGA-b-PEG-COOH prepared as already reported

by us[31] and 6 mg (0.013 mmol) of NVP-BEZ235 or 0.5 mg (0.02 mmol) of BODIPY®505-515 were dissolved in 10 mL of dichloromethane and admixed to 1 mL of water. The two-phase mixture was emulsified, in an ice bath, with a tip-probe sonicator (600 W input, 50% ampl) for 45 seconds. 40 mL of 1.25% sodium cholate solution in water was slowly added to the obtained emulsion: the resulted two-phase mixture was further emulsified for 3 minutes, in an ice bath, at the above-reported amplitude. The solvent was evaporated under reduced pressure and the resulting particles washed and concentrated by using centrifugal filter devices (Amicon Ultra, Ultracell membrane with 100.000 NMWL, Millipore) to a final volume of 2 mL and finally filtered by using a syringe filters phenex-PES of polyether sulfone (26 mm, 0.20 µm, Phenomenex, Italy). NVP-BEZ235@PNPs were diluted in PBS (5 mL, 0.01 M) and a solution of N-hydroxysulfosuccinimide 2.3 mM (4.3 mL) and a solution of 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide 0.28 M (1.8 mL) were added. The reaction was carried out at room temperature for 15 min, then 102 pmoles of 2′F-Py RNA Gint4.T aptamer (5'-(C6-NH) UGUCGUGGGGCAUCGAGUAAAUGCAAUUCGACA-3') or the scrambled sequence of the anti-EGFR aptamers,[23] herein used as a negative control and indicated as scrambled (5'-(C6-NH)UUCGUACCGGGUAGGUUGGCUUGCACAUAGAACGU GUCA-3') (TriLink Biotechnologies), dissolved into 1 mL of water, was added and left to react for 24 h. After that the particles were purified and concentrated to a final volume of 4 mL with the same technology reported above. Dynamic light scattering (DLS) analysis and ζ-potential values were obtained with a Zetasizer Nano-S (Malvern) instrument, working with a 532 nm laser beam at 25 °C, using standard cuvettes or DTS1060C Clear Disposable zeta cells, and the results expressed as average of three measurements. Final concentration of the suspensions was determined by gravimetric analysis by drying 100 µL of solution at 120 °C for 24 h then accurately weighting the residual dry matter amount.

## ASSOCIATED CONTENT

**Supporting Information**. The supporting information is available free of charge via the Internet at http://pubs.acs.org.
Characterizations of the developed polymeric nanoparticles, quantitative determinations of the loaded BODIPY, NVP-BEZ235 and the conjugated aptamers. Cell culture and transfection, cell viability assays, protein extraction and immunoblot. *In vitro* internalization studies on PDGFRβ positive cells (parental and non silencing shRNA U87MG) compared to different PDGFRβ-depleted cell clones (PDGFRβ shRNA U87MG clones 3 and 6), studies of *in vitro* phosphorylation of the 4EBP1 protein. *In vivo* targeting studies**.** *Ex vivo* H&E-staining images of the brain tissue at 2h and 4h post-injection of aptamer-PNPs conjugates.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.
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