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PEGylated squalenoyl-gemcitabine nanoparticles for the treatment of glioblastoma

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Abstract

New treatments for glioblastoma multiforme (GBM) are desperately needed, as GBM prognosis remains poor, mainly due to treatment resistance, poor distribution of therapeutics in the tumor tissue, and fast metabolism of chemotherapeutic drugs in the brain extracellular space.

Convection-enhanced delivery (CED) of nanoparticles (NPs) has been shown to improve the delivery of chemotherapeutic drugs to the tumor bed, providing sustained release, and enhancing survival of animals with intracranial tumors. Here we administered gemcitabine, a nucleoside analog used as a first line treatment for a wide variety of extracranial solid tumors, within squalene-based NPs using CED, to overcome the above-mentioned challenges of GBM treatment. Small percentages of poly(ethylene) glycol (PEG) dramatically enhanced the distribution of squalene-gemcitabine nanoparticles (SQ-Gem NPs) in healthy animals and tumor-bearing animals after administration by CED. When tested in an orthotopic model of GBM, SQ-Gem-PEG NPs demonstrated significantly improved therapeutic efficacy compared to free gemcitabine, both as a chemotherapeutic drug and as a radiosensitizer. Furthermore, MR contrast agents were incorporated into the SQ-Gem NP formulation, providing a way to non-invasively track the NPs during infusion.

GRAPHICAL ABSTRACT

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Supporting Information Available. Movies of 3D reconstructions of the distributions in the healthy brain and the tumor bearing brain. This material is available free of charge via Internet at <http://pubs.acs.org>.

Keywords

glioblastoma; convection-enhanced delivery; nanoparticles; squalene; gemcitabine

Of the ~70,000 people diagnosed with primary brain tumors each year, it is estimated that 17% suffer from glioblastoma multiforme (GBM), the most aggressive form of glioma [1]. Despite major progress in the development of chemotherapeutic drugs and improved techniques for surgery and radiotherapy, GBM prognosis remains poor, with a median survival of 15 months [2]. Current standard of care includes surgical resection, when possible, followed by radiation therapy (RT) and chemotherapy, using the DNA methylating agent temozolomide (TMZ) [3]. However, despite this multi-modal aggressive treatment, few patients survive beyond 5 years. Resistance to TMZ arises in a significant number of patients, for the most part mediated by O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein [4, 5]. Moreover, TMZ has been shown to be a relatively poor radiosensitizer, compared to other cytotoxic agents [6], so current approaches do not take full advantage of synergistic capabilities between chemotherapy and radiotherapy.

Gemcitabine (Gem) is a nucleoside analog used as a first line treatment for a wide variety of extracranial solid tumors [7]. Gem induces the irreversible inhibition of DNA synthesis, and one of its active metabolites - gemcitabine triphosphate - is able to incorporate into DNA, competing with the natural nucleotide deoxycytidine triphosphate. Since its mechanism of action is MGMT independent, Gem has the potential to overcome resistance to conventional TMZ-based treatments. Gem is also a potent radiosensitizer, targeting homologous DNA recombinant repair via its interference with Rad51 foci formation [8–10]. Gem has demonstrated efficacy against human glioma cell lines in vitro [11] and has been tested in clinical trials for the treatment of glioma as a chemotherapy drug [11, 12] and as a radiosensitizer [13]. Although Gem has been shown to cross the blood-brain barrier (BBB) to a certain extent, its clinical effectiveness in GBM treatment has been limited by a very short half-life after systemic delivery due to rapid metabolism by blood deaminases, which limits drug exposure to brain tumor cells. Due to those limitations, high drug doses are needed to reach therapeutic drug concentrations within brain tumors after intravenous delivery, resulting in systemic side effects. Bioconjugation of gemcitabine with the natural and biocompatible lipid squalene (“squalenylation” [14]) produces a squalenoylated prodrug—squalenoyl-gemcitabine (SQ-Gem)—that spontaneously forms nanoparticles (NPs), able to overcome several of the limitations of free Gem mentioned above. In previous studies, SQ-Gem NPs were found to improve anticancer activity in tumors outside the brain

[15], likely due to better pharmacokinetics and biodistribution compared to free Gem [16], as well as improved intracellular penetration [17]. However, it has been shown that squalene-based NPs fail to cross the BBB [16, 18], and thus are not appropriate for the treatment of GBM after systemic delivery.

Local delivery methods, such as intracerebral infusions [19] or the placement of drug-loaded wafers in the resection cavity during brain surgery [20], have been developed to overcome the presence of the BBB. However, those techniques can be limited by poor distribution of the drug through the brain parenchyma, since they rely on diffusion [21]. In contrast, convection-enhanced delivery (CED) relies on the infusion of therapeutic molecules directly into the brain under a positive pressure gradient to create bulk fluid movement in the brain interstitium [22]. Recent clinical trials showed that CED is safe and feasible; however, current techniques fail to improve patient outcomes [23, 24]. This failure has been attributed to the short half-lives of most drugs in the brain, which leads to rapid disappearance of drugs after the end of the infusion period [25], as well as difficulties in controlling the drug volume of distribution. It has been previously shown that nanoparticles can be introduced into the brain by CED [26], allowing for molecule protection from metabolism and for a more precise control of drug distribution, providing an effective approach to treating intracranial tumors [27, 28]. Size and surface chemistry of nanoparticles have been shown to be critical parameters to ensure effective distribution in the brain interstitium, by limiting steric hindrance, preventing particle aggregation, and reducing interactions with the extracellular matrix. Different strategies have been developed to control the diameter and the surface properties of nanoparticles in order to provide enhanced distribution after CED, such as size selection by ultracentrifugation [28], or surface modification using poly(ethylene)glycol (PEG) [29] or hyperbranched polyglycerol (HPG) [Song et al., *Nat Com*, *in preparation*].

Non-invasive monitoring of NPs delivery into the brain facilitates clinical translation, as it allows for the observation of particle distribution from the injection site through the brain parenchyma, and the long-term evaluation of NP persistence. Magnetic resonance imaging (MRI) is a particularly attractive approach, as it is routinely used to examine brain tumors before and after surgical resection, and has been implemented in clinical CED set-ups. NPs can be engineered to carry both therapeutic agents and diagnostic agents, such as an MRI contrast agent. Ultrasmall superparamagnetic iron oxide (USPIO) particles are an excellent T₂ MR-contrast agent that has been used for brain tumor imaging in mice [30], rats [31] and dogs [32]. Unlike gadolinium-based contrast agents, MRI contrast from USPIO does not rely on the entry of water in the immediate hydration sphere of the agent [33], which can be encapsulated inside the hydrophobic core of nanoparticles without losing its contrast properties. It has also been demonstrated that the incorporation of USPIO does not modify the physico-chemical properties of polymeric NPs formulation [34]. Moreover, although a thick hydrophobic coating can reduce the contrast enhancing properties of USPIO [35], it has been shown that encapsulation of USPIO in biodegradable polymeric NPs can induce the formation of USPIO aggregates and result in increased contrast properties [36]. USPIO particles have been successfully encapsulated in SQ-Gem NPs, allowing magnetic guidance of particles to a sub-cutaneous tumor site after systemic delivery, and imaging of a targeted tumoral nodule [37].

In this study, we show the effectiveness of SQ-Gem NPs for the treatment of GBM using CED. To accomplish effective delivery by CED, SQ-Gem NPs were surface-modified using a small amount of PEG, which provided a wide and uniform distribution of the NPs throughout the brain parenchyma. SQ-Gem-PEG NPs were found to improve significantly the pharmacological activity of Gem in an orthotopic model of GBM, both as a chemotherapeutic and a radiosensitizing agent. Finally, USPIO were successfully encapsulated into the SQ-Gem-PEG NPs and did not alter their distribution after CED, ensuring the ability to track NPs after their infusion into the brain parenchyma.

MATERIALS AND METHODS

SQ-Gem NPs and SQ-Gem-PEG NPs formulation

SQ-Gem and SQ-PEG bioconjugates were synthesized as previously described ([14] and [38], respectively). The size of the PEG chain used to synthesize the SQ-PEG bioconjugate was 2 kDa. SQ-Gem NPs were prepared as previously described by nanoprecipitation [14]. 4 mg of SQ-Gem were dissolved in 0.5 mL of absolute ethanol and added drop-wise under strong mechanical stirring to 1 mL of DI water. The ethanol was then completely evaporated using a Rotavapor® to obtain an aqueous suspension of pure NPs. The evaporation step was used to obtain different concentrations of SQ-Gem (4 mg/mL, 10 mg/mL or 20 mg/mL) depending on their further use. SQ-Gem-PEG NPs were prepared by co-nanoprecipitation of SQ-Gem and SQ-PEG, with different percentages of SQ-PEG (5%, 10%, 30%, 50%, 70%, w/w) added to the ethanolic phase containing SQ-Gem, before drop-wise addition to the aqueous phase. Finally, fluorescent nanoparticles were also obtained using the same procedure, with 1% w/w of the fluorescent probe CholEsteryl BODIPY® 542/563 C₁₁ (λ_{em} = 542 nm and λ_{exc} = 563 nm) dissolved in the ethanolic phase before drop-wise addition to the aqueous phase.

SQ-Gem NPs and SQ-Gem-PEG NPs characterization

SQ-Gem NPs were characterized by measuring their size (hydrodynamic diameter) and surface charge (zeta potential) using a Malvern Nano-ZS (Malvern Instruments, UK). For size measurements, 1 µg of NPs were dispersed in 1 mL of DI water to obtain a good attenuator value (6 to 9). For zeta potential measurements, 1 µg of NPs were suspended in 1 mL of NaCl 10 mM before filling the measurement cell. Particle stability was measured after dilution of the NPs in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA) at 37°C with a standard operating procedure taking size measurements every minute

Cellular uptake

The ability of the SQ-Gem NPs to be internalized by U87 cells was evaluated by confocal microscopy and flow cytometry. Briefly, 75,000 cells/well were seeded in 24-well plates and grown over-night, before being incubated with 0.5 µM of fluorescently labeled Gem-SQ NPs or Gem-SQ-PEG 70% NPs for 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h of incubation. At the end of the incubation period, for flow cytometry analysis, the cells were washed thoroughly three times with a warm 1% BSA solution before adding trypsin and re-suspending cells in a cold 1% BSA solution on ice. Flow cytometry was performed using an Attune NxT (Invitrogen). At least 5,000 iterations were acquired and the data were analyzed

using FlowJo v.10.0.8r1. For confocal imaging, the cells were washed thoroughly three times with PBS. Cells were fixed with PFA 4% for 10 min, and membranes were stained with 10 µg/mL WGA AlexaFluor 555 (Life Technologies) for 20 min at 37°C, and mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Images were taken using a Leica TCS SP5 confocal microscope (Leica).

In vitro cytotoxicity

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was used to test the cytotoxicity of SQ-Gem NPs and SQ-Gem-PEG 70% NPs in comparison to free gemcitabine. Briefly, 5,000 cells/well (U87 or RG2 cells) were seeded in 96-well plates and grow over-night, before being incubated with increasing gemcitabine concentrations (from 1 nM to 500 µM, N = 6 wells per concentrations) under the form of Gem-SQ NPs, Gem-SQ-PEG 70% NPs or free gemcitabine for 72h. At the end of the incubation period, MTT was added to the wells at a final concentration of 0.5 mg/mL, and further incubated for 3h. DMSO was added to each well to dissolve the formazan crystals and the absorbance was measured at 550 nm. The percentage of surviving cells were calculated as the absorbance ratio of treated to untreated cells, after subtraction of blank. The experiment was repeated three times, independently.

In vivo volumes of distribution

All animal studies were run according to the YALE protocol #2013-11149 approved by the Institutional Animal Care and Use Committee (IACUC) and in accordance with the guidelines and policies of the Yale Animal Resource Center (YARC). All animals were kept in the Yale Animal Resource Center and given free access to food and water.

Convection enhanced delivery in the healthy brain—Animals were anesthetized using a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg), injected intraperitoneally. Rats' heads were shaved and then placed in a stereotaxic frame. After sterilization of the scalp with alcohol and betadine, a midline scalp incision was made to expose the coronal and sagittal sutures, and a burr hole was drilled 3 mm lateral to the sagittal suture and 0.5 mm anterior to the bregma. A 50 µL Hamilton syringe with a polyamide-tipped tubing, loaded with the NPs, was inserted into the burr hole at a depth of 5 mm from the surface of the brain and left to equilibrate for 7 min before infusion. Those stereotaxic coordinates correspond to an infusion site in the caudate putamen, which is one of the largest grey matter structures of the rat brain, and thus allows for the widest and the most reproducible distribution of particles after CED. A micro-infusion pump (World Precision Instruments, Sarasota, FL, USA) was used to infuse 20 µL of 10 mg/mL fluorescently labeled Gem-SQ NPs or Gem-SQ-PEG NPs at a rate of 0.667 µL/min. Once the infusion was finished, the syringe was left in place for another 7 min before removal of the syringe.

Convection enhanced delivery in the tumor bearing brain—Orthotopic RG2 tumors were inoculated as previously described [39]. Briefly, 3 µL containing 2.5×10^5 RG2 cells suspended in PBS were administered over 3 min using the same procedure and the same coordinates as CED. Post-infusion, the burr hole was filled with bone wax, the wound was closed with surgical staples, and the animal was placed in a recovery cage until sternal.

The animals' weight, grooming, and general health were monitored daily. Tumors were grown for 4 days before administration of particles. CED in tumor bearing rats was conducted following the exact same procedure as for the healthy rats, by reopening the burr hole used for tumor implantation.

Volume of distribution—Brains were harvested immediately after the end of the infusion and flash frozen using dry ice. Brains were sliced in 100 μm slices using a Leica Cryostat CM3000 (Leica, Germany). Slides were imaged using a Zeiss Lumar. V12 stereoscope (Carl Zeiss AG, Germany) and images were analyzed using a MATLAB code setting a threshold using the Otsu's method.

Survival study

Chemotherapeutic study—RG2 tumors were implanted as previously described for the evaluation of the volume of distribution in tumor bearing rats, and 20 μL of dextrose 5%, Gem-SQ-PEG 5% NPs diluted in dextrose 5% at 20 mg/mL (0.16 mg of gemcitabine/animal) or free gemcitabine diluted in dextrose 5% at 8 mg/mL (0.16 mg of gemcitabine/animal) were administered by CED (N = 9 animals/group). Animals health status were monitored daily, and decision to euthanize was made after either a 15% loss in body weight, when it was humanely necessary due to clinical symptoms from tumor progression, or at 60 days after tumor induction.

Radiosensitization study—RG2 tumors were implanted as previously described for the volume of distribution in tumor bearing rats, and 20 μL of dextrose 5%, Gem-SQ-PEG 5% NPs diluted in dextrose 5% at 20 mg/mL (0.16 mg of gemcitabine/animal) or free gemcitabine diluted in dextrose 5% at 8 mg/mL (0.16 mg of gemcitabine/animal) were administered by CED (N = 9 animals/group). 24 h and 48 h after treatment, the animals were re-anesthetized using ketamine/xylazine and placed in a homemade lead handler with an opening at the head to allow selective cranial radiation. The animals in the handler were placed in a XRAD 320 cabinet Y-ray irradiator (Precision Xray, CT, USA). The window was set to 5 $\times$ 5 cm and the animals were radiated to receive a total of 5 Gy of radiation therapy daily, as previously calculated using known dosimetry for the cabinet. As previously described, animals health status were monitored daily, and decision to euthanize was made after either a 15% loss in body weight, when it was humanely necessary due to clinical symptoms from tumor progression, or at 60 days after tumor induction.

SPIO loaded SQ-Gem-PEG NPs formulation, characterization and distribution

SPIO loaded SQ-Gem-PEG NPs were prepared as previously described [37] using 4 mg of SQ-Gem, 70% w/w SQ-Gem and 10% w/w of SPIO dissolved together in 0.5 mL of ethanol. The mixture SQ-Gem/SQ-PEG/SPIO was vortexed and sonicated for few seconds, and then added dropwise under magnetic stirring to 1 mL of DI water. The ethanol was then completely evaporated using a Rotavapor® to obtain an aqueous suspension of SQ-Gem-PEG 70% SPIO NPs. The NPs were characterized using exactly the same procedures as for unloaded particles, and distribution in the healthy brain was evaluated as previously described.

RESULTS AND DISCUSSION

Preparation and characterization of SQ-Gem NPs and SQ-Gem-PEG NPs

SQ-Gem and SQ-PEG bioconjugates were synthesized as previously described [14, 38]. SQ-Gem NPs were prepared by nanoprecipitation of an ethanolic solution of SQ-Gem to distilled water. One of the main advantages of forming nanoassemblies from a prodrug is that drug loading is dictated by the molecular weight ratio of the drug versus the prodrug, ensuring a high drug loading: a loading of 41% was achieved in the case of SQ-Gem NPs. It has been previously described that those particles present an inverse hexagonal structure [40], and slowly release gemcitabine in the presence of cathepsin B enzyme, one of the proteases present in the lysosomal compartment of cells [14]. Finally, the SQ-Gem NPs can be easily lyophilized, ensuring long-term storage for further use in clinical settings [41]. Adding different amounts of SQ-PEG in the organic phase permitted incorporation of SQ-PEG into the nanoparticles—to obtain SQ-Gem-PEG NPs—while keeping the final concentration of SQ-Gem constant. Stable colloidal suspensions were obtained for SQ-Gem:SQ-PEG weight ratios varying from 1:0 to 1:0.7, with all formulations presenting monodisperse populations ($PdI < 0.2$), with hydrodynamic diameters ranging from 150 to 90 nm (Fig. 1A). As previously described, increasing the amount of SQ-PEG in the formulation decreased the diameter of the particles, likely due to a reduced surface tension between the aqueous phase and the organic phase [38]. The addition of SQ-PEG also decreased the surface charge of the nanoparticles, rendering them nearly neutral (Fig. 1B). Finally, the addition of SQ-PEG improved the nanoparticles stability in artificial cerebrospinal fluid (aCSF) for up to 5 h at 37°C (Fig. 1C). Given the limited size of the pores forming the brain extracellular space (ECS) [42], it has been suggested that nanoparticles should be very small (< 64 nm) to be able to distribute through the brain parenchyma after administration by CED. However, poor distribution may also result from interactions of nanoparticles with the extracellular matrix [43], depending on NPs surface properties. Recent studies have demonstrated that larger nanoparticles, with hydrodynamic diameters of up to 150 nm [28, 44], were able to distribute through the brain parenchyma after CED, as long as they were presenting a nearly neutral surface charge, and were not aggregating in the interstitial fluid. Hence, the physico-chemical characterization of SQ-Gem and SQ-Gem-PEG NPs suggest that all formulations containing SQ-PEG should be able to penetrate into the brain parenchyma after CED, while unmodified SQ-Gem NPs will likely coalesce and remain at the injection site.

Distribution of SQ-Gem and SQ-Gem-PEG NPs in the brain of healthy rats after CED

Fluorescently labeled SQ-Gem and SQ-Gem-PEG NPs were infused into the caudate putamen (caude) of healthy Fischer 344 rats via CED. Volumes of distribution (V_d) were calculated using fluorescent based volumetric image reconstruction; all formulations incorporating SQ-PEG yielded V_d ranging from 50 to 60 mm³ (Fig. 2A). Given a volume of injection (V_i) of 20 μ L, those values correspond to V_d/V_i ratios ranging from 2.5 to 3. These values were lower than the theoretical V_d/V_i of 5 that has been estimated for agents that freely distribute through the brain extracellular space [45] - such as most small molecules or non-binding macromolecules - but in the range of previously reported values obtained after the infusion of NPs by CED [28]. As expected, given its instability in aCSF, the unmodified

SQ-Gem NP formulation did not distribute throughout the brain, likely due to aggregation at the injection site (Fig. 2B). The addition of SQ-PEG provided a uniform distribution throughout the caude (Fig. 2C–G), while increasing the percentage of SQ-PEG from 5% to 70% did not significantly increase V_d . Previous studies suggested that, to improve distribution of polymeric NPs into the brain tissue, surface modification should be performed with high densities of PEG [29, 44]. The squalene molecule itself presents tensioactive properties and has been used as an emulsion adjuvant for vaccines [46], and the squalenoyl prodrugs have been shown to retain these properties [47]. Accordingly, we observed that low PEG densities could be sufficient to confer stability of NPs in interstitial fluid (Fig. 1), also improving distribution in the brain (Fig. 2). This result strongly suggests that the amount of PEG necessary to stabilize the structure and ensure distribution is dependent on the properties of the material used to form the nanostructure.

USPIO loaded SQ-Gem-PEG NPs for particle tracking during CED

Optimization of the NPs localization and distribution during clinical procedures requires the ability to non-invasively track the NPs during the infusion. MRI is now routinely used clinically when performing CED, and USPIO are a promising contrast agent for nanoparticle encapsulation. Since the incorporation of USPIO may provide destabilization of the nanoparticle suspension, we used the SQ-Gem-PEG 70% formulation, in order to ensure a stable formation of nanoparticles with suitable physico-chemical properties. The co-nanoprecipitation of USPIO, SQ-Gem, SQ-PEG and a fluorescent dye yielded a monodisperse population of nanoparticles with similar size to unloaded SQ-Gem-PEG 70% NPs (Fig. 3A), with a slightly more negative surface charge (Fig. 3B), likely due to the adsorption of hydrophobic USPIO particles at their surface. These USPIO loaded NPs displayed excellent stability in aCSF (Fig. 3C) and distributed efficiently throughout the brain parenchyma after administration by CED (Fig. 3D). Distribution of USPIO loaded NPs was uniform throughout the caude (Fig. 3H), similar to SQ-Gem-PEG 5% NPs (Fig. 3F) and SQ-Gem-PEG 70% NPs (Fig. 3G). These data suggest that USPIO-loaded SQ-Gem-PEG 70% NPs can be used as surrogate to evaluate the distribution of both SQ-Gem-PEG 5% NPs and SQ-Gem-PEG 70% NPs after CED. Similar nanoparticle formulations were successfully used to perform MRI particle tracking in the past [37]; still, a complete characterization of these particles should be performed to ensure definitive proof-of-concept.

Cellular uptake and *in vitro* cytotoxicity of SQ-Gem and SQ-Gem-PEG NPs

There are a significant number of FDA-approved medicines using PEG and PEG-conjugates, making PEG a popular polymer for the modification of particle surfaces [48]. However, such modification may have the following disadvantages: (1) PEG is a non-biodegradable polymer, and (2) anti-PEG immune responses can be induced when PEGylated materials are injected repeatedly [49–51]. Moreover, several studies suggest that surface modification with PEG decreases cell uptake [52], including after local administration by CED [Song et al., *Nat Com*, *in preparation*]. Thus, we investigated if the presence of PEG at the surface of SQ-Gem NPs may reduce SQ-Gem NPs uptake as previously described for other nanoparticle systems. To investigate the extremes, we compared unmodified SQ-Gem NPs with SQ-Gem-PEG 70% NPs, the formulation incorporating the maximal amount of SQ-PEG. Cellular uptake of both formulations was evaluated by flow cytometry (Fig. 4A) and confocal

microscopy (Fig. 4B–C) in U87 cells, a human glioma cell line. Flow cytometry analysis demonstrated a significantly lower uptake of PEGylated NPs compared to the un-PEGylated formulation, and this result was confirmed by confocal microscopy after 24 h of incubation. However, both SQ-Gem and SQ-Gem-PEG 70% NPs demonstrated similar cytotoxicity in U87 (Fig. 4D) and RG2 (Fig. 4E) cells after 72 h of incubation, suggesting that the reduced uptake of SQ-Gem-PEG NPs during the first hours of incubation did not result in a reduced pharmacological efficacy. Since SQ-Gem and SQ-Gem-PEG NPs incorporate the same amount of drug, this result suggests that, over the course of 72 h, the SQ-Gem-PEG NPs are sufficiently internalized to induce cytotoxicity. This hypothesis is strongly supported by the saturation of the SQ-Gem internalization process after 6 h of incubation: SQ-Gem NP stop entering the cells after 6 h, whereas SQ-Gem-PEG NPs keep entering the cells over the course of 72 h, likely reaching the same level of internalization as SQ-Gem NPs. Finally, squalene-based nanoparticles have been previously shown to enter cells via different pathways, depending on the therapeutic agent incorporated and the cell line characteristics. In particular, SQ-Gem NPs have been shown to interact with low-density lipoproteins (LDL), leading to NPs disassembly and LDL mediated cell internalization of SQ-Gem molecular bioconjugates [17]. Altogether, these results suggest that SQ-Gem-PEG NPs may use different pathways to enter the cells, which allows them to retain cytotoxic activity comparable to SQ-Gem NPs.

Brain distribution of SQ-Gem and SQ-Gem-PEG NPs after CED in rats with intracranial RG2 tumor

Incorporation of SQ-PEG significantly improved distribution in the healthy brain after CED (Fig. 2A) and did not compromise cytotoxic activity (Fig. 4D and E). Because of the above mentioned limitations of PEG regarding biodegradability and immunogenicity, we chose to pursue our studies using the SQ-Gem-PEG 5% NPs formulation, which incorporated the lowest percentage of SQ-PEG but still provided widespread distribution in the brain (Fig. 2C). We first evaluated the distribution of both unmodified SQ-Gem NPs and SQ-Gem-PEG 5% NPs after administration by CED to rats with intracranial RG2 tumors. Inoculation of RG2 cells into the brain of syngeneic, immunocompetent Fischer 344 rats produces a simple, reproducible glioma model [53], that is well characterized and recapitulates several features of human malignant glioma. When administered by CED, the addition of SQ-PEG significantly increased the distribution of the NPs in rat brains bearing RG2 tumors (Fig. 5A), and yielded comparable volumes of distributions to those observed in rat brains without tumors. This is in accordance with previous results showing that the presence of a tumor, whatever its size, does not modify the particle volume of distribution [39]. However, it has also been shown that NPs heterogeneously distribute throughout the tumor mass, likely due to a high cellular density, a compromised BBB inducing elevated interstitial pressures [54], and increased tortuosity of flow pathways in the local environment [55]. We calculated the percentage of tumor coverage (Fig 5B), to evaluate if this heterogeneous environment also influenced SQ-Gem and SQ-Gem-PEG NPs distribution. Because they aggregated at the injection site and did not readily distribute, unmodified SQ-Gem NPs presented a small coverage percentage (Fig. 5B and C), while SQ-Gem-PEG 5% NPs covered up to 70% of the tumor (Fig. 5B and D). Penetration of SQ-Gem-PEG 5% NPs was deep, as expected

after administration by CED, but was not uniform over the tumor mass, as previously described [39].

Therapeutic efficacy of SQ-Gem-PEG NPs in rats bearing intracranial RG2 tumor

Since gemcitabine has been shown to be both a potent chemotherapeutic agent and a radiosensitizer, we evaluated the therapeutic efficacy of the SQ-Gem-PEG NPs in two treatment schedules: (1) vehicle (dextrose 5%), free gemcitabine or SQ-Gem-PEG 5% NPs were administered by CED four days after tumor implantation (chemotherapy treatment, Fig. 6A), and (2) identical CED treatments were followed by two doses of ionizing RT (5 Gy each), at 24 h and 48 h after CED (radiosensitizer treatment, Fig 6B). The mean survival times of the control groups were 11 and 12 days after treatment with and without RT respectively, demonstrating that in this setting, RT alone had no beneficial effect on survival. When used as a chemotherapeutic agent alone, free gemcitabine did not significantly enhance rats' survival in comparison to controls (Fig 6C), likely due to rapid metabolism in the extracellular space that prevented gemcitabine cytotoxic action [56]. However, a significant increase in survival as compared to the control group was observed when RT was added (Fig 6D, $p < 0.005$). The radiosensitization effect of gemcitabine has been shown to be mediated by gemcitabine itself, and by its main metabolite, difluorodeoxyuridine (dFdU) [57]. Thus, metabolism of gemcitabine into inactive dFdU in the extracellular space likely decreases drug cytotoxic activity, but still ensures some efficacy in the presence of RT. Administering an equivalent dose of gemcitabine using SQ-Gem-PEG 5% NPs significantly increased the mean survival time compared to the free drug, used as chemotherapeutic alone (Fig. 6C, $p < 0.0001$) or as radiosensitizer (Fig. 6D, $p < 0.0005$), which was attributed to improved drug stability, as previously described [16]. Despite their widespread distribution in the brain after CED, SQ-Gem-PEG NPs were unable to completely cure tumor bearing rats, possibly because of an only partial coverage of the tumor mass (Fig 5B). Noteworthy, tumor coverage can be improved in clinical settings, by adjusting catheter placement according to each tumor characteristic to fully optimize NPs volume of distribution.

CONCLUSIONS

This study demonstrates the ability of a PEGylated squalene-based nanoformulation to extend the use of gemcitabine for the treatment of GBM. More specifically, we demonstrate that the addition of small percentages of PEG were sufficient to ensure a widespread distribution both in the healthy brain and the tumor-bearing brain after administration by CED. Administration of the PEGylated formulation via CED resulted in increased survival of animals bearing orthotopic RG2 tumors, both when gemcitabine loaded NPs were used as a chemotherapeutic or as a radiosensitizer. Finally, the incorporation of USPIO into the nanostructure did not prevent the particles from distributing throughout the brain after CED, providing the potential for a clinical surrogate to non-invasively track the NPs during their infusion. Altogether, these results provide a new promising therapeutic alternative for the treatment of GBM using CED of gemcitabine-containing nanomaterials, able to overcome the limitations of current clinical approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Physico-chemical characterization of SQ-Gem NPs and SQ-Gem-PEG NPs

(A) The hydrodynamic diameter of the different formulations was measured using dynamic light scattering (DLS). All formulations presented a monodisperse population ($PdI < 0.2$), with a diameter below 150 nm, while the addition of SQ-PEG decreased the diameter of the particles. (B) The surface charge (zeta potential) was measured in 10 mM NaCl. The addition of SQ-PEG rendered the formulations nearly neutral ($ZP \approx -1$ mV). (C) Size stability was measured by DLS in aCSF at 37°C for 5 h. The diameter of the unmodified SQ-Gem NPs immediately increased upon dilution in aCSF, and kept dramatically increasing over time, while all formulations containing SQ-PEG were stable.

Fig. 2. Distribution of SQ-Gem NPs and SQ-Gem-PEG NPs after administration to healthy rats by CED

(A) Volumes of distribution (V_d) were calculated using fluorescent based volumetric reconstruction. All formulations incorporating SQ-PEG presented a V_d superior to 50 mm^3 .

(B–G) Representative images of the distribution of the different NPs formulations after administration by CED. Unmodified SQ-Gem NPs (B) aggregated at the injection site, while all SQ-Gem-PEG NPs formulations (C – 5%, D – 10%, E – 30%, F – 50%, G – 70%) distributed uniformly throughout the healthy striatum.

Fig. 3. USPIO loaded SQ-Gem-PEG NPs for particle tracking during CED

(A) The hydrodynamic diameter of USPIO loaded SQ-Gem-PEG 70% NPs was measured using DLS. The formulation presented a monodisperse population ($PdI < 0.2$), with similar diameter than SQ-Gem-PEG 5% NPs and SQ-Gem-PEG 70% NPs. (B) The surface charge (zeta potential) was measured in NaCl 10 mM. The addition of USPIO slightly decreased the surface charge compared to USPIO unloaded formulations. (C) Size stability was measured by DLS in aCSF at 37°C for 5 h, and it was observed that the incorporation of USPIO did not compromise the stability of the formulation. (D) V_d was calculated using fluorescent-based volumetric reconstruction following CED of fluorescently labeled, USPIO loaded NPs. The incorporation of USPIO did not prevent the NPs from distributing widely throughout the brain, yielding a V_d comparable to USPIO unloaded PEGylated formulations. (E–H) Representative images of the distribution of the different formulations after administration by CED. Unmodified SQ-Gem NPs (E) aggregated at the injection site, while all the SQ-Gem-PEG NPs formulations (F – 5%, G – 70%), including those incorporating USPIO (H), distributed uniformly throughout the healthy striatum.

Fig. 4. In vitro uptake and cytotoxicity of SQ-Gem and SQ-Gem-PEG NPs

(A) NP uptake by U87 cells was measured by flow cytometry for up to 24 h. After 24 h of incubation, internalization of unmodified SQ-Gem NPs (B) and SQ-Gem-PEG 70% (C) was visualized by confocal microscopy, confirming reduced uptake of PEGylated NPs (scale bar = 20 μ m). (D–E) Cytotoxicity of SQ-Gem NPs and SQ-Gem-PEG 70% NPs was evaluated in U87 (D) and RG2 (E) cells using an MTT assay, demonstrating that PEGylated NPs were as potent as unmodified NPs, and that they were both as cytotoxic as the free drug.

Fig. 5. Distribution of SQ-Gem NPs and SQ-Gem-PEG NPs after administration to tumor bearing rats by CED

(A) Volumes of distribution (V_d) after CED into brains with RG2 tumors of around 4 mm^3 were calculated using fluorescent-based volumetric reconstruction. SQ-Gem-PEG 5% NPs yielded a significantly higher V_d compared to unmodified SQ-Gem NPs. (B) Percentages of tumor coverage in small tumors were calculated for both formulations. (C–D) Representative images of the co-localization of the NPs with the tumor mass (C – unmodified SQ-Gem NPs, D – SQ-Gem-PEG 5% NPs). The tumor appears in green, the NPs in red, and the colocalized area in yellow.

Fig. 6. Anticancer activity of SQ-Gem-PEG NPs in rats bearing RG2 glioma

Anticancer activity of SQ-Gem-PEG 5% NPs when administered by CED 4 days after RG2 tumor implantation was evaluated without (**A**) and with (**B**) additional RT (two doses of 5 Gy, 24 h and 48 h after CED). (**C–D**) Survival of the animals, when treated without (**C**) and with (**D**) additional RT. In both settings, SQ-Gem-PEG 5% NPs were more potent than free Gem as they significantly increased the mean survival time.