Microglia-selective drug release through biodegradable polymeric nanoparticles administered in the cerebrospinal fluid.

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Abstract

Cell-selective drug release ~~Modulation of neuroinflammation~~ in the central nervous system (CNS) holds great promise for the treatment of ~~several neurodegenerative diseases~~many CNS disorders but it is still challenging. ~~However, selective cell targeting remains challenging.~~ We previously ~~exploited~~ demonstrated that polymeric nanoparticles (NPs) injected intra-parenchyma in the brain can be internalized specifically in ~~to obtain cell specific uptake in~~ microglia/macrophages surrounding the injection site~~, upon intra-parenchyma administration in mice~~. Here, we explored NPs administration in the cerebrospinal fluid (CSF) to achieve a wider spreading and increased cell targeting throughout the CNS, generated new NPs variants and studied the effect of modifying size and surface charge on NPs biodistribution and cellular uptake. As hypothesized, intra-cerebroventricular administration resulted in ~~a~~ widespread ~~NPs~~ brain distribution ~~throughout the CNS~~ with prevalent localization in proximity to stem-cell niches, such as around the lateral ventricles, the subventricular zone and the rostral migratory stream.

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NPs dimensions of 37-39 nm allowed extensive penetration in the brain parenchyma and targeting of up to 15% of microglia. Transient ~~D~~disruption of the blood-brain barrier with mannitol improved NPs brain penetration leading to their internalization in up to 25% of microglia cells. A fraction of the targeted cells was positive for markers of proliferation or stained positive for stemness / progenitor-cell markers such as Nestin, c-kit, or NG2. Interestingly, through these newly formulated NPs we obtained controlled and selective release of chemotherapeutics otherwise difficult to formulate (such as busulfan and etoposide) to~~loaded drugs (such as the chemotherapeutics busulfan or etoposide) efficiently and could release them selectivity in~~ the target cells, preventing unwanted side effects and the toxicity obtained by direct brain delivery of the same not encapsulated drugs. Overall, these data provide proof of concept of the applicability of these novel NP-based drug formulations~~s~~ for targeting not only mature microglia but also possibly progenitor cells in the brain and pave~~s~~ the way for brain-restricted microglia-targeted drug delivery regimens.

Keywords

Microglia, nanoparticle, drug delivery, intracerebroventricular administration, chemotherapeutics

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Introduction

~~Accumulating evidence suggests that neuroinflammation, i.e. the activation of glial cells in the central nervous system (CNS), is not just the result of reaction to neuronal death or damage, but it is a key pathological process that actively contributes to worsening and progression of symptoms in several neurodegenerative diseases, such as acute brain or spinal cord injury, Alzheimer's disease or Amyotrophic Lateral Sclerosis [1]. Microglia cells, the innate immune cells in the CNS [2], have recently gained great interest as potential targets for several therapeutic applications, as fine tuning of microglia/macrophage reactivity could contribute to modulating neuroinflammatory processes, with potential therapeutic effects [3]. The major challenge in the development of such therapeutic applications is the achievement of a selective targeting of reactive microglia/macrophages in the CNS.~~

Recent advances in nanomaterial science have opened the way for exploiting polymeric nanoparticles (NPs) as innovative pharmacological platforms allowing cell specific uptake and controlled drug release. This could be particularly critical for obtaining cell-selective and/or improved drug targeting in the Central Nervous System (CNS), with important implications for development of efficacious therapeutic approaches for several CNS disorders, such as Alzheimer's disease, Amyotrophic Lateral Sclerosis or brain tumors. Several efforts have been spent to generate NPs formulations capable of penetrating in the brain upon systemic administration [1,2] and/or to achieve brain-selective drug release with some encouraging results [3,4]. However, cell-selectivity and widespread CNS biodistribution remain challenging. We and others showed that microglia cells are a CNS cell type that can be targeted efficiently with different types of NPs [5–8], thanks to their intrinsic nature of professional phagocytes of the CNS. We took advantage of this feature for targeted release of minocycline (an anti-inflammatory drug) to activated microglia (a major driver of neuroinflammation) in a mouse model of spinal cord injury, by exploiting ~~A~~ a ~~new~~ drug delivery biodegradable and biocompatible nanocarrier tool based on poly-caprolactone (PCL) NPs ~~, which is mainly taken up by microglia/macrophages after intra-parenchyma administration in a spinal cord injury mouse model, was recently described~~ [7]~~.~~ This tool ~~was used to obtain selective and controlled release of minocycline (an anti-inflammatory drug) in activated microglia/macrophages in the spinal cord soon after injury,~~allowed to maximi~~ze~~zing drug efficacy at doses lower than those needed with systemic administration of the not-encapsulated drug [7]. ~~Thus, NPs able to target microglia/macrophages may provide a strategy to potentiate drug efficacy and reduce side effects.~~ The clinical transferability of a NPs platform ~~designed for treatment of CNS related disorders,~~ like the one described [7], would greatly benefit from some additional improvements. Firstly, drug loaded NPs should be easy to formulate and to be stored, have a high batch-to-batch reproducibility and colloidal stability. Moreover, their targeting ability and bioavailability to a great portion of CNS

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microglia/macrophages should be guaranteed also when using a poorly invasive route of administration such as the intrathecal one. To address these aspects, we here tested recently developed NPs made of amphiphilic block-copolymers that can self-assemble once ~~solved~~dissolved in a buffered aqueous solution such as PBS (self-assembly NPs) [9]. In this work we demonstrate that ~~W~~when the newly formulated NPs are administered through a single injection in the CSF in the presence of a transient blood brain barrier (BBB) disruption, they ~~the newly formulated NPs~~ distribute~~d~~ effectively in the CNS and ~~were~~are taken up by microglia and macrophages, including cells displaying features of progenitors (i.e. positivity for stemness markers and proliferation potential). Moreover, we highlight that the~~with~~ biodistribution ~~being~~is influenced by NP dimensions and surface charge. Selective killing of bona fide microglia progenitors (through exposure to chemotherapeutics such as busulfan [19]) is a critical step to induce and foster cell engraftment in the CNS after hematopoietic stem cell (HSC) transplantation. It has been demonstrated that HSC-derived cells can become a long-lasting source for delivery of therapeutics to the CNS[10–12], however patients could benefit from the use of less toxic, brain-targeted conditioning regimens. As first step towards the development of a brain-restricted cell-specific and less toxic chemotherapy conditioning, here ~~W~~we demonstrate~~d~~ that with ~~these~~ the self-assembly NPs developed in this work ~~are~~ we are able to achieve very high drug payload and to obtain controlled and selective release of chemotherapeutics otherwise difficult to formulate (such as busulfan and etoposide here adopted) in a significant percentage of brain microglia cells.~~release drug in a controlled fashion over time with the therapeutics here adopted (busulfan and etoposide). When administered through a single injection in the CSF in the presence of a transient blood brain barrier (BBB) disruption, the newly formulated NPs distributed effectively in the CNS and were taken up by microglia and macrophages, with biodistribution being influenced by NP dimensions and surface charge. Importantly, when administering drug loaded self assembly NPs with this optimized protocol, a controlled and selective drug release was achieved in a significant percentage of brain microglia cells.~~

Results

Synthesis of the nanoparticles

We recently described biodegradable NPs based on PCL, displaying preferential uptake in microglia upon direct injection in the spinal cord parenchyma [7]. These NPs were synthesized via emulsion polymerization (EP) of a biodegradable macromonomer that bears three units of caprolactone adopting a

polyethylene glycol methacrylate as colloidal stabilizer. This procedure allowed to obtain NPs directly in water without the need of organic solvents [7]. These NPs, here identified as EP NPs (Table 1), are very well suited for the efficient loading of lipophilic drugs. However, the harsh conditions of the emulsion polymerization do not allow the direct loading of the lipophilic drug during the NP formation [13]. For this reason, the lipophilic drug is generally dissolved in a water-miscible organic solvent and then nanoprecipitated on the pre-formed NPs. Thus, subsequent dialysis and ~~a lyophiliza~~lyophiliza~~e~~

tion steps are necessary to remove the unloaded drug and the potentially toxic organic solvent, and to remove water that otherwise would affect the shelf-life of the final drug-NPs preparation. Overall these post-processes impact negatively on the potential clinical transferability of these NPs formulations [14]. To overcome these issues, NPs with a similar comb-like structure were synthesized from the same class of oligo-caprolactone macromonomers via the combination of the reversible addition-fragmentation chain transfer (RAFT) polymerization and the ring opening polymerization (ROP) [9]. A polyehtyleneglycol(PEG)~~-~~-based ~~PEGylated~~ macro RAFT agent was produced via the RAFT solution polymerization of the same class of PEGylated methacrylates used as surfmers in the emulsion polymerization of the PCL-based macromonomers. This latter step was substituted by another RAFT solution polymerization to produce a well-defined biocompatible and biodegradable block copolymer. The polymeric surfactant thus formed was co-nanoprecipitated with the drug directly into PBS to form NPs of two different dimensions via self-assembly (SA-N2 and SA-N1~~N~~ NPs in Table 1). This method, which allows for control over the characteristics of the final copolymer and~~,~~ in turn~~,~~ of the final NPs, was adopted to incorporate also a negatively charged monomer (3-sulfopropyl methacrylate potassium salt, SPMAK) during the RAFT polymerization, as shown in the supplementary Figure S1, in order to control the surface charge of the final NPs accordingly (SA-SP NPs in Table 1). In this work, we compared the behavior of the EP NPs with the ones obtained via self-assembly of block-copolymers to study the impact of the main NPs characteristics on their efficacy and biodistribution once injected in the CNS. In particular, we tested NPs of different size (*Dn* = 37-39 nm or 108 nm for the SA NPs; *Dn* = 138 for the EP NPs) and surface charge (ζ= -20 mV for the SA-SP NPs or neutral for SA-N1 and SA-N2 ~~NPs~~ and EP NPs). A fluorescent dye (i.e Rhodamine B) was covalently attached to all the synthesized nanoparticles via the adoption of a methacrylate derivative [15], to allow traceability ~~(~~by fluorescent microscopy or flow cytometry~~)~~ in vitro or after administration in vivo (the physicochemical features of the NPs are reported in Table 1).

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Effect of size and surface charges on NPs biodistribution after administration in the CSF

Previous experiments demonstrated preferential uptake of NPs by microglia cells upon injection in the CNS parenchyma [7]. Through this route of administration, NPs were localized only around the injection site with limited diffusion beyond the site of administration. We decided to explore administration of NPs in the cerebrospinal fluid, to aim at wider distribution across different CNS regions. Five µl of rhodaminated EP NPs (2% w/v in PBS) were injected bilaterally in the cerebral lateral ventricles (intra-cerebroventricular, ICV), then NPs biodistribution and cellular uptake were investigated by ~~histology and~~ flow cytometry and histology 3-5 days post-administration. Overall, a single ICV injection targeted about 2.9±0.4% of total $CD45^{low}CD11b^{high}$ microglia cells (assessed by flow cytometry) (Fig. 1~~D~~A, ~~E~~B). ~~Rhodamine (Rh+) signal (corresponding to the NPs) was spread across the entire brain (Fig. 1A) with higher signal intensity in the corpus callosum (cc) and regions surrounding the lateral ventricles (LV), and in the subventricular zone (SVZ) and rostral-migratory stream (RMS) (Fig. 1B, C). Overall, a single ICV injection targeted 2.9±0.4% of total $CD45^{low}CD11b^{high}$ microglia cells (assessed by flow cytometry) (Fig. 1D, E).~~ One method for increasing brain penetration for compounds or macromolecules administered in the bloodstream or in the CSF is to inject systemically a bolus of mannitol[16–18], a hyperosmotic agent that allows to transiently loosen the tight junctions between the cells forming the BBB and the brain-CSF barrier [19]. Accordingly, when we ~~To increase the brain penetration of ICV-administered compounds by inducing a transient BBB disruption we~~ administered mannitol 10 minutes before NPs injection ~~[9–11]. ~~, In this setting,~~ the percentage of targeted microglia increased to 8.8±0.7% (Fig. 1E). Reduction of NP size (from 138 to about 108 nm, by adopting SA-N2 NPs) did not modify the extent of microglia targeting significantly, thus SA-N2 NPs were not characterized further. On the other hand, reduction of dimensions to 37-39 nm, by adopting SA-N1 or SA-SP NPs instead of EP-NPs or SA-N2) ~~was sufficient to~~determined an increase of about three-fold in the percentage of targeted cells (Fig. 1E). Mannitol-mediated BBB disruption was able to increase the overall uptake of the 37-39 nm NPs (irrespectively of surface charge) to 25%, on average, suggesting an improved distribution within CNS parenchyma for smaller sized NPs. When we analyzed brain slices under the microscope to evaluate NPs bio-distribution, we observed for EP NPs ~~R~~rhodamine (Rh+) signal (corresponding to the NPs) ~~was~~ spread across the ~~entire~~ brain (Fig. 1~~A~~C) with higher signal intensity in the areas surrounding the injection site, i.e. corpus callosum (cc) and regions surrounding the lateral ventricles (LV), and in the subventricular zone (SVZ) and rostral-migratory stream (RMS) (Fig. 1~~B~~D, ~~C~~E). For 37-39 nm sized NPs (namely SA-N1 and SA-SP) ~~w~~we observed a more widespread biodistribution ~~of 37-39 nm sized NPs~~ as compared to the 138 nm NPs (i.e. EP NPs), with negatively charged NPs (SA-SP) displaying the most reproducible results across different experiments. This result is consistent with a better penetration of smaller size NPs in the ~~with~~

~~presence of rhodamine positive signal belonging to the smaller NPs all over the~~ brain parenchyma, including CNS regions far from the injection site such as the midbrain, hindbrain and cerebellum ~~-~~(Fig. S2A, C and quantification in Fig. S2E). The degradation rate of the NPs studied in this work (based on PCL polymers) was pretty fast in vivo, with a 60% drop in the amount of Rh+ microglia from 3 to 7 dpi, with further 50% drop at 14dpi (Fig. 1F).

NPs are ~~preferentially~~ internalized in ~~hematopoietic, myeloid cells and~~ microglia and in myeloid hematopoietic cells expressing stemness markers localized in CNS neurogenic niches

To study the cell-type specificity of NPs uptake we first performed a cytofluorimetric phenotypic characterization of different cell types displaying positivity for NPs (supplementary Fig. S3). We focused our attention on hematopoietic myeloid cells ($CD45^{\pm}$; either $CD11b^{-}$ or $CD11b^{\pm}$) and on some of the $CD45^{-}$ cells, ~~by focusing our attention on~~ i.e. astrocytes - identified by positivity for ACSA2 marker [20], oligodendrocytes - identified through O4 antigen [21], vascular endothelial $CD31^{\pm}$ cells and $CD45^{-}$ $Thy1^{\pm}$ neurons [22] (Fig. 1~~H~~G). The $CD45^{+}CD11b^{\pm}$ fraction (microglia) within NPs~~-~~containing cells moved from 34-37%~~40%~~ in the case of EP and SA-SP NPs to about 50~~60~~% in the case of ~~smaller sized~~ SA-N1 NPs~~, namely SA-N and SA-SP~~ (Fig.1~~F~~G). ~~Overall, the majority of $CD45^{+}$ NPs^{+} cells were also positive for $CD11b^{+}$ (about 75-80% for all three different NPs types), confirming that these NP^{+} cells are indeed CNS myeloid elements, including *bona fide* microglia (Fig. 1G). We then decided to investigate the phenotype of the NP^{+} $CD45^{-}$ cells, by focusing our attention on astrocytes – identified by positivity for ACSA2 marker [16], oligodendrocytes – identified through O4 antigen [17], vascular endothelial $CD31^{-}$ cells and $CD45^{-}$ $Thy1^{+}$ neurons [18] (Fig. 1H). All the three tested NPs were internalized in these cells.~~ However, as opposed to EP NPs, the self-assembly smaller sized NPs displayed a tendency to higher uptake by astrocytes, endothelial cells and oligodendrocytes. Strikingly, SA-SP NPs were the only NPs internalized also by a small fraction of Thy1+ neurons and showed a significantly higher uptake by endothelial cells and by $O4^{+}$ oligodendrocytes.

~~**Myeloid hematopoietic cells expressing stemness markers in CNS neurogenic niches can selectively uptake NPs**~~

Since the cytofluorimetric immunophenotypic characterization of the NPs^{+} cell types did not account for the totality of the $rhodamine^{+}$ population, we decided to complement this information by using other known markers of glial cells and immunohistochemistry. In this setting, NPs signal was predominantly associated with $Iba1^{+}$ microglia and $CD68^{+}$ macrophages scattered throughout the brain parenchyma for all the tested formulations (Fig. 2A-B) ~~with no co-localization detected with the GFAP astrocyte marker (Fig. 2C-D)~~. Interestingly, we detected NPs in a subset of $Iba1^{+}$ cells that was also positive for Nestin, a

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stemness marker (Fig. 2~~E~~C-~~F~~D), and in NG2$^+$ glia progenitor cells (Fig. 2~~G~~E-~~H~~F). These cells were mainly localized in the SVZ and in the regions lining the lateral ventricles. Specificity of the rhodamine+ signal was confirmed on sections from mice not injected with NPs (Fig. S2F).

Based on these findings, we investigated whether the cells capable of internalizing the NPs were also proliferating. Edu nucleoside analog was administered to mice (100 µg/g bw; one single injection) daily for 2 days before NPs ICV administration, and then daily up to the time of sacrifice (3 days post NPs administration). The biodistribution of NPs in different Edu$^+$ or Edu$^-$ brain myeloid populations was assessed by flow cytometry (Fig. 3A-C). About 20% of brain myeloid cells was positive for Edu (Fig. 3B). About 40% of NP-containing CD45$^+$CD11b$^+$ microglia/macrophage cells were also positive for Edu signal (Fig. 3D). Consistently, the analysis of proliferating Edu$^+$ cells revealed that the majority were also Rh$^+$ (Fig. 3E), suggesting a preferential uptake of NPs by cells that are actively proliferating. This finding was confirmed at immunohistochemistry through detection of NPs in Iba1$^+$ cells that were also positive for Edu (Fig. 3F). On the same brain tissue sections from NPs and Edu injected mice, we then performed a quantitative characterization of the proliferating (Edu$^+$) cells (Fig. 3G) in the regions mainly targeted by NPs (area lining the lateral ventricles). This analysis revealed that Edu$^+$ cells that were also positive for NPs (Fig. 3H), were either doubly labeled by the microglia marker Iba1 and the macrophage marker F4/80 (about 3% of tot Edu$^+$), or positive for F4/80 (about 3%) or Iba1 (about 13%) alone, or negative for either of the two markers (about 20% of total Edu$^+$ cells, Fig.3H and arrowheads in Fig.3F). We hypothesize that the fraction of Edu$^+$ cells that are negative for markers of differentiated microglia or macrophages (Iba1; F4/80) may be immature myeloid brain-associated cells not yet expressing markers of mature microglia. Accumulating evidence suggests that microglia progenitors in the brain may belong to a subpopulation of cells expressing the c-kit surface marker [23]. By flow cytometry, we confirmed that NPs can be internalized in CD45$^+$/c-kit$^+$ cells upon ICV administration and mannitol-mediated BBB disruption (in a range spanning between 45% and 75% of total CD45$^+$/c-kit$^+$ population, depending on NPs size and surface charge; Fig. 3I).

In vitro uptake of NPs does not predict the in vivo distribution and uptake profile

To interpret the differential CNS bio-distribution of the tested NPs of different size and surface charge, we studied the kinetics and the overall efficiency of their uptake by microglia like cells (BV2 cells, which recapitulate several functional features of murine microglia/macrophages [24]) in vitro. Almost 100% of the cells turned out to be Rh+ short-term (30 min) after incubation with NPs, suggesting that BV2 microglia-like cell line is very efficient in internalizing the NPs. However, when we measured the Rh

mean fluorescence intensity (MFI) of the cells (as readout of the extent of NPs internalization), we highlighted a time-dependent increase of rhodamine MFI in BV2 cells treated with 138 nm (EP) NPs suggesting a progressive accumulation of NPs within the cells over time (Fig. 4A). The extent of internalization for 37-39 nm sized NPs (SA-N1 and SA-SP) in BV2 cells was significantly higher than the uptake of the larger EP NPs in the early phases of NP-cell contact and overall more variable, with a greater uptake of neutrally charged SA-N1 NPs than of negatively charged SA-SP NPs (Fig. 4A).~~A time-dependent increase in 138 nm (NE) NPs uptake by BV2 cells, measured as Rh mean fluorescence intensity (MFI) of the cells, was observed (Fig. 4A). The uptake of 37-39 nm sized NPs (SA-N and SA-SP) by BV2 cells was significantly higher than the uptake of the larger EP NPs in the early phases of NP-cell contact and overall more variable, with a greater uptake of neutrally charged SA-N NPs than of negatively charged SA-SP NPs (Fig. 4A).~~ However, the level of NP uptake stabilized to values similar for all the tested NPs at 24h post-treatment indicating that size and surface charge have a short-term influence on BV2 cell uptake, at least in vitro. The lack of correlation between the percentage of Rh+ cells and the MFI of Rh that we observed in vitro was confirmed also in vivo, on the NP+ microglia cells retrieved from the brain 3-5 days post-NPs administration (Fig. S2G).

The uptake of NPs could also be influenced by the differential interaction of different NPs with receptors on the cell surface. Since the surface of the NPs tested in this work was not functionalized with any specific cell-receptor ligand, we argued that scavenger receptors, expressed on several cell types including immune system cells and microglia/macrophages and involved in recognition and uptake of several moieties, could mediate NPs uptake by cells. When we exposed BV-2 cells to NPs in the presence of fucoidan, a scavenger receptor pan-inhibitor, the uptake of all the tested NPs formulations, irrespectively of size and surface charge, was significantly reduced of about 60%. However, the uptake was not fully inhibited by fucoidan, which may be due to some level of receptor-independent uptake that is equally relevant for all the tested NPs.

Chemotherapeutic drug loading in NPs benefits their pharmacodynamics

We next tested the capability of the NPs obtained via the self-assembly method (that have a greater translational potential) to uptake and release drugs. The neutral 39 nm (SA-N) NP are highly suited for uptake of lipophilic drugs, whereas the negatively charged 37 nm NPs (SA-SP) are more suited for the loading of molecules with an amphiphilic nature. Given these NPs features, we tested two chemotherapeutic compounds, etoposide and busulfan, as prototypic drugs (the former being highly liposoluble, the latter being more amphiphilic). We first measured the loading efficiency of etoposide and busulfan in SA-N and SA-SP, respectively, confirming a loading efficiency of 86% for etoposide and

95% for busulfan. We exploited a functional in vitro assay to discriminate, on BV2 cells, the effects of the drugs when released from NPs. Busulfan and etoposide loaded in SA-SP and SA-N NPs were prepared at a nominal drug concentration of 1 mg/ml or 275 µg/ml, respectively. Busulfan- or etoposide-loaded NPs, or the not encapsulated drugs were added to BV2 cells and cellular viability was measured by the CellTiter cell proliferation MTS assay after 24 or 72 hours of incubation at 37°C. Different drug concentrations were tested (Busulfan at 3.0, 6.0 or 30 µg/ml; Etoposide at 1.6, 6.25 and 25 µg/ml). As control, empty NPs (without drug) were tested at a final concentration matched to the drug-loaded NPs. At 24 hours post-administration, busulfan tested as free drug was slightly toxic on cells, leading to a 25% reduction of cellular viability, on average (Fig. 4C). Busulfan-loaded NPs did not exert any specific effect as the empty SA-SP NPs resulted partially toxic *per se* when used at concentrations of 0.06 and 0.30 µg/ml w/v (Fig. 4C). However, when the viability test was performed after 72 hours of incubation, i) busulfan tested as free drug at 30 µg/ml determined about 50% reduction of cellular viability, ii) cells incubated with empty NPs fully recovered, resulting in a viability superimposable to untreated controls, whereas iii) busulfan loaded in NPs at the a nominal final concentration of 30 µg/ml (NPs at 0.30 µg/ml v/w) determined a significantly extensive and drug specific reduction of cellular viability of almost 75% on average (Fig. 4C). To confirm these~~is~~ findings we employed phosphorylated H2AX (γH2AX) that is a sensitive and early pharmacodynamic marker of busulfan-related toxicity. Busulfan is an alkylating agent, leading to cell death through the formation of double strand breaks in cellular DNA. Phosphorylation of H2AX is one of the first responses occurring in cells upon exposure to agents causing double-strand breakes (DSBs) [25] and treatment of cells with busulfan leads to the formation of γH2AX+ foci in cells nuclei that are detectable by histology and flow cytometry. Exposure to both busulfan as free drug at 30 µg/ml and to busulfan-loaded NPs resulted in an increased percentage of cells displaying positivity for γH2AX (Fig. 4D and S4A). A significantly higher amount of $\gamma H2AX^{+}$ cells was detected when busulfan-loaded NPs were employed with a dose dependent effect as shown by both flow cytometry (Fig. 4D) and histology, as $\gamma H2AX^{+}$ nuclear foci (Fig. S4B). Incubation with scavenger receptor inhibitors was able to prevent the effect exerted by busulfan encapsulated in NPs (Fig 4E), further confirming that the observed improved drug efficacy of the NP formulation was due to the encapsulation of the drugs within the NPs.

Similar results were obtained by loading etoposide in SA-N NPs. As shown in Fig. 4F, with SA-N empty NPs we did not retrieve significant toxicity at any time-point of analysis and even at the highest tested concentrations. When etoposide was loaded in NPs, it determined cell death already after 24 hours of incubation at a level similar to the one of the not encapsulated drug. However, after 72 hours of incubation, the toxicity observed with etoposide-loaded NPs tested at the lowest concentration was greater than the one observed with the not encapsulated drug, suggesting that also the encapsulation of etoposide

in NPs enhances its efficacy, determining an improvement of the cytotoxicity of the drug already at very low concentrations.

Controlled chemotherapeutic delivery and selective targeting of CNS resident microglia

Hematopoietic stem cell (HSC) progeny can represent a vehicle for therapeutic molecule delivery to the CNS upon their transplantation in myeloablated hosts [11]. CNS delivery of therapeutics by means of HSC-derived cells depends on the engraftment in the recipients' CNS of donor myeloid cells producing the molecule of therapeutic relevance. Instrumental for inducing and fostering this cell engraftment in the CNS after HSC transplantation is the use of busulfan, that is likely affecting a fraction of microglia and/or their endogenous progenitors [26]. We reasoned that the NPs characterized in this work could be exploited to obtain controlled busulfan delivery in the CNS. As first step towards this goal, we evaluated the brain penetration and pharmacodynamics of busulfan upon systemic administration at a myeloablative dose (i.e. 25 mg/kg/day, usually administered for 4 consecutive days [26]) to obtain a reference exposure for NPs-mediated busulfan delivery. Busulfan was administered i.p. (single administration), then animals were sacrificed at different time-points post-dosing to collect plasma and brain samples for a pharmacokinetic analysis. Busulfan reached maximal concentration in the plasma and in the brain at 15 and 30 minutes post-administration, respectively (Fig. 5A). Afterwards, it was rapidly washed out from both compartments with a very similar rate of clearance, resulting in half-life of 137 minutes in plasma and of 133 minutes in the brain (see Table 2). This rapid partitioning of busulfan between plasma and brain may reflect the amphiphilic features of the compound, which is known to have also good blood brain barrier permeability. Based on these data, considering that the actual dose of administered busulfan for a mouse of 20 g of body weight would be 500 µg, the total brain exposure to the drug (during the 480 minutes of observation for this experiment) is about 31.6 µg (the weight of the brain for a 20 g mouse is about 400-500 mg). This corresponds to only about 6.3% of the dose administered systemically reaching the brain. We next identified in the brain the areas where busulfan exerted its effect by γH2AX staining in animals exposed to a fully myeloablative dosing [26] and analyzed 1 and 5 days after the last busulfan dose. γH2AX was hardly detectable in untreated control mice (Fig. 5B), except for few $\gamma H2AX^+$ foci found in neuronal cells, mainly localized in the hippocampal region, in line with literature evidences demonstrating a correlation between physiological neuronal activity and H2AX phosphorylation in the hippocampus of healthy mice [27]. On the contrary, in busulfan-treated mice $\gamma H2AX^+$ signal was present in nuclei of cells lining the lateral ventricles or located in the SVZ and rostral-migratory stream (Fig. 5B, inset). γH2AX immunoreactivity was already present one day after the last dosing and became more pronounced and diffuse at day 5 after the last administration, with positive cells spread throughout the brain, including hippocampus, cortex, hypothalamus and cerebellar nuclei (not shown). $\gamma H2AX^+$ foci

were present both in neurons and glial cells, i.e. microglia and astrocytes (Fig. 5C). We next explored the possibility to reproduce the same pharmacodynamics and pharmacokinetic effects by administering busulfan in its clinical-grade formulation (i.e. Busilvex) directly in the brain ventricles at a dose of 30µg/mouse equivalent to brain exposure after systemic drug delivery. The drug administered monolaterally (Fig. S5A) rapidly distributed throughout the brain within 5 minutes post administration, but its total concentration dropped of almost 70% during the following 15 minutes (Fig. S5B). Moreover, despite the drug was administered only in the brain, it could be detected in the plasma already 5 minutes after ICV administration, suggesting a leakage of this drug formulation out of the brain. Notably, daily ICV administration of busulfan for four days, to mimic the overall total brain exposure achieved by systemic busulfan myeloablative regimens, resulted in very few γH2AX$^+$ foci localized only in the areas lining the lateral ventricles (Fig. S5C). Moreover, it led to profound brain damage, with enlargement of the ventricles at the injection site (data not shown) and strong neuroinflammatory reaction, as demonstrated by the extensive astrocytosis (GFAP staining in Fig. S5C) and microgliosis (Iba1 staining in Fig. S5C) in the areas lining the lateral ventricles. On the contrary, when we administered busulfan loaded into Rh$^+$ SA-SP NPs in the lateral ventricles for four days at the maximum feasible nominal dose of 5 µg/mouse/day, a substantial increase of γH2AX signal over the basal was observed with robust γH2AX staining found within microglia of the treated mice (Fig.6A). This is remarkable, as the busulfan dose that we could test with the NPs formulation (5µl injected bilaterally of a NPs suspension containing busulfan at ~0.3µg/µl, corresponding to ~3µg busulfan/mouse) was lower than the reference brain exposure achieved with systemic administration (31.6 µg), due to technical limitations. The γH2AX signal was spread throughout the brain, with high intensity in the subventricular zone, cortex and areas lining the lateral and fourth ventricles (Fig. 6B). Signal distribution was superimposed to the bio-distribution of the rhodaminated NPs that were spread throughout the brain parenchyma, with high density of signal in the areas surrounding the lateral ventricles and the fourth ventricle (Fig. S2B). Unexpectedly, by immunohistochemistry we could detect Iba1$^+$ microglia cells that stained positive for NPs but were negative for γH2AX (arrows in Fig. 6C-D), as well as cells that were negative for NPs but had γH2AX signal in the nucleus (arrowheads in Fig. 6C-D), suggesting potential rapid release of the drug by the NPs, i.e. before internalization into the cells. Of note, no acute toxicity nor neuroinflammation were detected after 4 busulfan-loaded NPs (SA-SP-BUS NPs, **Table 1**) administrations.

The physicochemical properties of the compound loaded in NPs influence dramatically the loading efficiency and the release profile; thus, the loading of a highly lipophilic drug is predicted to be more efficient and the release to be slower, as compared to an amphiphilic compound. In order to achieve a better and more specific killing of cells targeted by NPs, we replaced the NPs formulation containing

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busulfan (that is an amphiphilic compound) with NPs loaded with etoposide (SA-N1-ETO NPs, **Table 1**), a highly lipophilic molecule. Busulfan (an alkylating agent) covalently modifies cellular DNA causing the accumulation of double-strand breaks and inducing cell death. Similarly, etoposide (a podophyllotoxin) induces cell death by causing the accumulation of unrepaired nicks in the DNA helix through inhibition of topoisomerase type II. ~~-~~Annexin positivity, an early marker of cell death, was used here as readout of NPs efficacy in this case. A significant increase of Annexin$^+$ signal was detected in CD45$^+$ cells (Fig. 6E), in microglia (Fig. 6F) identified by flow cytometry for double positivity for CD45(low) and CD11b(high) surface antigens, and in CD45$^+$/c-kit$^+$ cells (Fig. 6G), from animals injected with etoposide-loaded NPs (SA-N1 NPs ~~ETO~~). The Annexin$^+$ cell fraction in ~~NP-ETO~~SA-N1 NPs treated mice was greater than the one measured in control untreated mice, ~~,~~ mice receiving empty NPs or animals receiving a systemic busulfan myeloablative regimen (BUS). Notably, the observed effect was specific for: i)~~; i)~~ microglia and c-kit$^+$ cells~~,~~ and ii) cells containing NPs. In fact,~~since~~ no significant changes in Annexin staining were detected in CD45 negative cells (Fig. 6H) and in CD45 negative Rh$^+$ cells (Fig. 6L) in line with the negligible NPs uptake reported in these cell populations (not shown); whereas,~~; and ii) cells containing NPs~~ on the other hand, ~~, since~~ a striking increase of Annexin could be detected in Rh$^+$ cells (Fig. 6I-K), that was significant in the CD45$^+$/c-kit$^+$ population.

Discussion

~~Microglia are often termed the immune cells of the CNS as they constantly monitor the environment in a resting state and become activated upon acute or chronic neuronal damage, eliciting a strong pro-inflammatory response [2]. They can also actively participate in CNS remodeling in physiological conditions, by interacting with astrocytes and sensing the functionality of neuronal synapses, or by removing the degenerating neurons through phagocytosis [21]. Activation and proliferation of microglia at sites of neuronal demise in the CNS represents a pathological hallmark shared by many neurodegenerative diseases [22]. Strategies targeted at restoring a neuro-supportive microglia phenotype, either by delivery of anti-inflammatory stimuli or replacement of resident aberrant microglia with a healthier population, could contribute to neuronal damage attenuation. Exploiting NPs as a cell selective drug delivery tool could help reaching this goal. Biodegradable PCL-based NPs were previously adopted to target microglia/macrophages after intra-parenchyma administration in a spinal cord injury mouse model. This tool was used to obtain selective and controlled release of minocycline in activated microglia/macrophages in the spinal cord soon after injury, maximizing drug efficacy at doses lower than those needed with systemic administration [4]. In this work we aimed at expanding the potential applicability and clinical transferability of these initial investigations. We first adopted a method that~~

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~~generates via self-assembly drug-loaded NPs by means of a syringe and a small amount of organic solvent [5]. This allows preparing drug-loaded fully functional NPs starting from the native dry block-copolymer, with the possibility to avoid all the common post-processing steps that hamper the transferability of the nanotherapeutics to clinics.~~ In this work we ~~We then~~ investigated the bio-distribution and drug release profile of ~~self-assembly~~different NPs formulations administered ICV in the CSF in the mouse, as strategy to improve widespread targeting of microglia/macrophages in the CNS. As the surface properties of the NPs could play a role in the stability and bio-distribution of the NPs once injected in the CSF, we tested NPs with different features in terms of dimension and surface charge. Upon administration in the CSF, we observed a widespread distribution of NPs in different CNS districts, consistent with the CSF circulation, with major signal localized in the areas surrounding the ventricles and the SVZ and rostral migratory stream. Among the NPs formulations tested in this work, NPs of lower dimension were more efficiently internalized in microglia, suggesting a better penetration of small NPs into the parenchyma. Disrupting the BBB with mannitol improved the brain penetration of all tested NPs [17]. Irrespectively of dimensions or surface charge, NPs uptake occurred mainly in CNS microglia/macrophages. Indeed, microglia/macrophage cells are professional phagocytic cells of the brain; they are expected to be able to internalize NPs (as demonstrated also by our in vitro experiments performed on microglia cell lines). The surface of the NPs exploited in this work is not functionalized with any receptor-specific ligands, and it is composed mainly of PEGylated (neutrally or negatively charged) moieties meant to improve shielding versus unspecific binding to cell membranes. Thus, it can be deduced that it is the phagocytic nature of microglia/macrophage cells that dictate the preferential uptake of NPs in these cells, rather than the presence of specific surface features. The high efficiency of NPs internalization observed on cell cultures and the lack of correlation that we found between Rh MFI and the percentage of Rh+ cells (assessed by flow cytometry both in vitro and in vivo) suggests that once microglia gets in contact with the NPs, the latter are internalized in the cells rapidly and keep accumulating intracellularly as long as available NPs are present in the extracellular environment. This may have important implication for microglia-selective drug targeting, since high accumulation of NPs in microglia cells would allow to expose preferentially this cell type to the drug released by the NPs.

However, our flow cytometric analysis highlighted NPs uptake also in a sub-fraction of cells displaying positivity for astrocytes, oligodendrocytes and endothelial markers (though to a lesser extent as compared to what observed in microglia). This phenomenon was relatively more prominent for smaller size self-assembly NPs, namely SA-NE1 and SA-SP, with SA-SP displaying the highest uptake in endothelial cells and oligodendrocytes. Overall, this suggests that NPs size and surface charge (especially small dimensions and negative charge among the tested conditions) could determine a drift of the uptake to

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target also a few macroglia cells, in addition to microglia. ~~Irrespectively of dimensions or surface charge, NPs uptake occurred mainly in CNS microglia/macrophages; however, NPs were also detected in a sub-fraction of cells displaying positivity for astrocytes, oligodendrocytes and endothelial markers. This phenomenon was relatively more prominent for smaller size self assembly NPs, namely SA-N and SA-SP, with SA-SP displaying the highest uptake in oligodendrocytes. Overall, this suggests that NPs size and surface charge (especially small dimensions and negative charge among the tested conditions) can determine a drift of the uptake to target also macroglia cells, in addition to microglia.~~

However, the total number of NP+ positive endothelium and oligodendrocytes represents less than 1% of the respective total population (data not shown) so NPs-mediated drug release, through the formulations tested in this work, is expected to affect preferentially only microglia/macrophage populations.

A deeper investigation on the cells targeted by NPs revealed that about 50% of them are positive for a marker of proliferation (Edu). Interestingly, a fraction of the cells containing NPs were also positive for Nestin or c-kit, which are known stemness markers, and negative for markers of mature microglia/macrophages (Cd11b, Iba1 or F4/80); these cells were also positive for NG2, a marker of oligodendroglia progenitors. Given the fact that the CNS areas mainly targeted by NPs bio-distribution are stem-cell niches (namely, the SVZ and rostral migratory stream), we hypothesized that a fraction of cells targeted by the NPs could be microglia with progenitor features. Microglia cells originate from the yolk sac and colonize the CNS during development, before birth [28]. Soon after birth, once the CNS has formed and the BBB comes in place, resident microglia cells become long-lived and display the capacity to divide and self-renew, with little/no contribution from blood-derived circulating monocytes [29–31]. Recently, Elmore and colleagues demonstrated the existence in the brain parenchyma of a fraction of microglia that could behave as progenitors [23]. These might be the cells targeted by busulfan regimens employed before HSC transplantation and allowing for a robust engraftment of transplant-derived cells and possibly turnover of endogenous microglia with the transplant-cell progeny [26]. Here we reasoned that a direct administration of the chemotherapeutic drug in the brain parenchyma could exert a similar effect, possibly sparing the marrow hematopoietic niche from busulfan exposure. However, our pharmacokinetic studies highlighted a leakage of the drug out of the brain into the blood circulation. Moreover, busulfan administered directly in the brain as clinical-grade formulation determined a dramatic damage to the brain parenchyma, as demonstrated by the enlargement of the lateral ventricles (site of injection) and the profound neuroinflammatory reaction documented a few days after administration. This toxicity may be due not only to busulfan *per se*, but also to dimethylacetamide, which is the major component of the clinical grade formulation. We thus assessed whether the NPs here described could safely and effectively target busulfan to microglia and/or to their functional progenitors upon ICV

administration. Similar approaches have in fact been exploited successfully for other chemotherapeutic drugs. Doxorubicin encapsulated in paramagnetic NPs was used to improve tumor targeting while reducing the cardiotoxicity associated with systemic administration of the drug [4]. Other groups demonstrated that encapsulation of camptothecin within NPs strikingly improves drug stability in vivo and allows efficient brain tumor targeting after systemic intravenous administration or when delivered directly to intracranial tumors [32–34]. Here, we exploited the microglia-targeted NPs as nanovectors for selective release of busulfan upon intra-brain administration. Notably, despite several endeavors, stable NPs-based formulation of busulfan with sufficiently high drug loading are not available or have not yet been tested for the efficiency of releasing pharmacologically active amount of the drug in vivo, in the brain [35–39]. In our work, not only we managed to develop a new busulfan formulation based on the new self-assembly NPs, but we could verify the efficacy of these NPs upon CNS administration by using γH2AX as a validated pharmacodynamic marker of busulfan efficacy. We also tested etoposide (a compound already used for treatment of intracranial tumors and successfully encapsulated in NPs, including PCL-based [40,41]) as alternative molecule for NPs encapsulation and delivery to microglia and/or their progenitors. In vitro tests on BV2 microglia cell line highlighted that the encapsulation of either busulfan or etoposide determined an increase of drug efficacy as compared to the free drug. This effect was mediated by the loading of drugs into NPs, since the efficacy of drugs was partially ablated upon incubation with inhibitors of scavenger receptors, which reduced NPs uptake. Encapsulation of the drugs within NPs determined an improved drug release profile also in vivo, upon administration in the lateral ventricles in mice. In fact, both busulfan and etoposide loaded in NPs respectively induced DSBs (i.e. γH2AX) or annexin positivity in the cells targeted by NPs. However, while etoposide efficacy was mainly limited to the cells that were positive for NPs, suggesting a selective drug release upon uptake of NPs in the target cells, for busulfan the effect was widespread throughout the brain parenchyma (as highlighted by γH2AX staining) and it was detected also in cells that were negative for NPs. The NPs developed by our group are very well suited for uptake and release of lipophilic compounds. Since busulfan is an amphiphilic molecule, it can be hypothesized that the release from NPs in vivo occurs more rapidly than etoposide, as observed also by other authors in vitro in PBS [42], which may explain the more widespread effect observed upon CSF administration of busulfan loaded NPs. Nevertheless, the encapsulation of busulfan into SA-SP NPs increased its pharmacodynamic profile, since we could retrieve a significant increase of γH2AX+ signal in microglia cells at a lower dose as compared to the one needed to achieve a similar effect by systemic administration. This is in line with our previous observations showing an improved pharmacological profile of an anti-inflammatory drug (i.e. minocycline) loaded in PCL-based NPs [7]. Moreover, busulfan loaded into NPs displayed an improved safety profile, as compared to the toxicity observed by employing the free clinical grade drug formulation (i.e. Busilvex).

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2)*Ukawala, Mukesh, et al. "Investigation on design of stable etoposide-loaded PEG-PCL micelles: effect of molecular weight of PEG-PCL diblock copolymer on the in vitro and in vivo performance of micelles." Drug delivery 19.3 (2012): 155-167.*

In fact, dimethylacetamide (DMA), used to allow the solubility and stability of the free drug in the clinical grade formulation[43], was replaced in our NPs formulations with dimethyl sulfoxide (DMSO) at a final concentration lower than 10%. This allowed maintaining the solubility and stability of the chemotherapeutic in the NPs formulations while resulting in a much lower or negligible overall toxicity, as demonstrated by the tests performed with empty NPs both in vitro (Fig. 4) and in vivo (Fig. 6).

Overall, in this study we describe novel NPs formulations that can safely render bio-available cytotoxic drug to specific cell populations in the brain by ICV delivery, with administration in the CSF being an effective strategy to obtain widespread NPs bio-distribution in the CNS parenchyma. Modulation of NPs dimension and surface charge could improve the extent of cell targeting. The NPs developed by our group not only target mature microglia/macrophages ~~(which may represent an interesting target for therapeutic approaches aimed at modulating neuroinflammatory responses in neurodegenerative diseases or in cancer),~~ but also a fraction of immature cells, which are proliferating and express stemness markers, possibly comprising functional microglia progenitors in the brain. These results could set the ground for a number of potential future innovative applications: i) NPs-mediated selective targeting of reactive microglia cells, could represent a promising therapeutic approach for several neurodegenerative diseases, including Alzheimer's disease or Amyotrophic Lateral Sclerosis [44], where fine tuning of microglia/macrophage reactivity could contribute to modulating neuroinflammatory processes, with potential therapeutic effects [45]; ii) ~~T~~the possibility to target chemotherapeutic drugs to microglia progenitors ~~would pave the way for~~could pave the way for ~~the~~ the development of brain-specific conditioning regimens allowing for a selective brain engraftment upon hematopoietic stem cell transplantation. This would represent a key step toward the development of cell and gene therapy approaches aiming to treat ~~neurological~~ pathologies of the nervous system; iii) NPs-mediated controlled release of chemotherapeutics only in specific cells could pave the way for more efficient and safer therapeutic approaches for brain tumors. Work is in progress to optimize the NPs platform here described, to allow surface functionalization with targeting molecules favoring selective internalization in different cell types[46].~~.~~

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Materials and methods

Materials

Hydroxyethyl methacrylate (HEMA, 97%, Sigma Aldrich), ε-caprolactone (CL, 97%, Sigma Aldrich), 2-ethylhexanoic acid tin(II) salt ($Sn(Oct)_2$), ~95%, Sigma-Aldrich), poly(ethylene glycol)methyl ether methacrylate (PEGMA950, Mn = 950 g/mol, Sigma Aldrich), 3-sulfopropyl methacrylate potassium salt (SPMAK, 98%, Sigma Aldrich), 4-cyano-4-(phenylcarbonothioylthio)-pentanoic acid (CPA, >97%, Sigma Aldrich), 4-4' azobis(cyanovaleric acid) (ACVA, 98%, Sigma Aldrich), potassium persulfate (KPS; >99% purity, ACS reagent), Rhodamine B (RhB, Sb sensitivity < 0.1 μg mL^{-1}, Carlo Erba reagents), dicyclohexylcarbodiimide (DCC; 99% purity, Sigma Aldrich), 4-(dimethylamino)-pyridine (DMAP; >99% purity, Sigma Aldrich) were used as received except when specifically noted. Poly(ethylene glycol)methyl ether methacrylate (PEGMA2000, Mn = 2000 g/mol, 50 wt.% in H_2O, Sigma Aldrich) was extracted with DCM and dried under reduced pressure. All the solvents used were of analytical-grade purity and were purchased from Sigma Aldrich.

PCL-based macromonomers (HEMA-CL_n) and HEMA-RhB synthesis and characterization

Poly ε-caprolactone-based macromonomers were produced via ring opening polymerization (ROP) of CL in bulk with HEMA as initiator and $St(Oct)_2$ as catalyst according to a previous protocol [47]. The initiator to catalyst ratio was kept constant to 200 while the monomer to initiator molar ratio was set to 3 and 5 in order to obtain a macromonomer with 3 (HEMA-CL_3) and 5 (HEMA-CL_5) caprolactone units, respectively. For HEMA-CL_5, 4.5 g of HEMA and 71 mg of $St(Oct)_2$ were mixed in a 10 ml vial at room temperature until complete dissolution. 20 g of caprolactone and 82.6 mg of Na_2SO_4 were mixed in a septum sealed flask and placed at 130°C under stirring in a controlled temperature oil bath. The HEMA and $St(Oct)_2$ mixture was then added into the flask and the reaction was left to react for 3h. After cooling, the macromonomers were characterized via ^{1}H-NMR (400 MHz, Bruker, Switzerland). A Fluorescent monomer based on Rhodamine B was synthesized via Steglich esterification of RhB with HEMA in the presence of DCC and DMAP according to a protocol reported in literature [48].

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Block copolymer synthesis and characterization

Two PCL-based block copolymers were synthesized via two subsequent RAFT solution polymerizations slightly modifying a previously published protocol [9], as shown in Figure S1. In the first step, a PEGylated macro RAFT agent (5PEGMA2000) was synthesized via the RAFT polymerization of PEGMA2000 with a monomer to CPA and ACVA to CPA molar ratio equal to 5. Briefly, 14.8 g of

PEGMA2000, 425 mg of CPA and 85 mg of ACVA were dissolved in 75 ml of ethanol and poured in a septum sealed flask. After purging with nitrogen for 30 min, the mixture was heated to 65°C in a controlled temperature oil bath under stirring. After 24 h, other 85 mg of ACVA were dissolved in 2.5 ml of ethanol and injected into the reactor with a syringe. The reaction was stopped after other 24 h and the mixture was dried under nitrogen. The final macro RAFT agent was washed 3 times with diethyl ether to remove the unreacted PEGMA2000.

In the second step, two different block copolymers were synthesized with and without the addition of SPMAK in order to produce a neutral and negatively charged polymeric surfactant, respectively. For the neutral one, named 510 (5 for the number of PEGMA2000 units and 10 for the number of HEMA-CL_5 repeating units), the RAFT solution polymerization of HEMA-CL_5 was carried out at a monomer to RAFT agent and initiator to RAFT agent molar ratio equal to 10 and 3, respectively. 5.8 g of 5PEGMA2000, 4 g of HEMA-CL_5, 54 mg of ACVA and 13.8 mg of HEMA-RhB were dissolved in 50 ml of ethanol and poured in a round bottom flask. After purging with nitrogen for 30 min, the mixture was heated to 65°C and left to react for 24 h under stirring. The final polymer was dried under nitrogen and washed three times with diethyl ether. Another neutral block copolymer was synthesized with the same protocol, but with a monomer to RAFT agent ratio equal to 30 (named 530).

For the production of the negatively charged block copolymer (510-SP), the same amount of initiator, macro-RAFT agent, HEMA-RhB and 5PEGMA2000 of the 510 protocol synthesis were dissolved with 0.28 g of SPMAK into 39 g of an acetic buffer/ethanol (20/80 wt.%) mixture. The same reaction conditions and purification protocol of the 510 were applied. Conversion, number average molecular weight (*Mn*), and dispersity (*Đ*) of the final copolymers obtained were determined via gel permeation chromatography (GPC) with a Jasco (Series) apparatus according to a previously published protocol. [9,48].

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NPs production via nanoprecipitation of the block copolymers

The two fluorescent block copolymers were used to produce NPs directly into PBS via self-assembly according to a previously published protocol [49]. Briefly, 60 mg of the polymeric surfactant (ei.ge. 510 for the synthesis of SA-N NPs, 530 for the SA-N2 NPs, and 510-SP for the SA-SP NPs) was dissolved in 0.3 g of DMSO and then aspired with a 5 ml syringe pre-loaded with 3 ml of PBS. After three cycles of aspiration and ejection, the mixture was filtered through a 0.2 µm polyethersulfone (PES) pore-size filter syringe (Millex). The NP size (*Dn*), polydispersity index (PDI), and ζ-potential (ζ) were determined by dynamic laser light scattering analysis (DLLS, Zetasizer Nano Series, Malvern Instruments).

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~~Busulfan~~ Loading of chemotherapeutics in NPs and ~~Stability~~ stability tests

The loading of busulfan or etoposide was performed during the self-assembly of the block-copolymer into NPs directly in PBS. Briefly, for the preparation of busulfan-loaded NPs (SA-SP NPs), 60 mg of the ~~polymeric surfactant (i.e.~~ 510-SP~~)~~ was dissolved in 0.3 g of DMSO, 6 mg of ~~Busulfan~~ busulfan and then aspired with a 5 ml syringe pre-loaded with 3 ml of PBS. In the case of etoposide-loaded nanoparticles (SA-N NPs), 10 mg of 510 and 550 µg of etoposide were dissolved in 50 mg of DMSO and then aspired with a 5 ml syringe pre-loaded with 2 ml of PBS. After three cycles of aspiration and ejection, the mixture was filtered through a 0.2 µm PES pore-size filter syringe (Millex). The NP Dn and PDI were determined by dynamic laser light scattering analysis (DLLS, Zetasizer Nano Series, Malvern Instruments). In the case of SA-SP NPs, ~~A~~an aliquot of the NPs ~~has been~~was taken after 0 h, 1 and 2 hours, filtered with a syringe filter to eliminate possible busulfan aggregates, and diluted 1:500 in methanol followed by a 1:10 dilution in acetonitrile containing an internal standard (100 ng/mL). The samples were analyzed via a LC-MS/MS (UFLC Shimadzu AC20 coupled with a API 45000) equipped with a precolumn UPLC C18*4.6mm and a column Kinetex 2.6u C18 100A (75*3.00mm). 5 ul of each sample were eluted at 35°C at a flow rate of 300 uL/min (Phase A: 2mM Ammonium acetate, 0.1% formic acid in H_2O; Phase B: 2mM ammonium acetate, 0.1% formic acid in methanol). The busulfan concentration detected in the ~~510~~SA-SP formulation was equal to 1020.2 ± 1.1 µg immediately after NP production, 999.9 ± 1.1 µg after 1 hours, and 1079.6 ± 26.8 µg after 2 hours. 6 mg of Busulfan were not soluble when mixed with 3 ml of PBS and 0.3 g of DMSO and large aggregates were visible. In the case of SA-N NPs, 200 µl of the NPs were dissolved in 800 µl of DMSO, filtered and then analyzed via HPLC-UV (Waters Associates, Milford, MA, USA, model 2487 Variable Wavelength Detector) with a RP C18 analytical reverse phase column (50 mm×4 mm, 5 µm, Merck Millipore®) at 40 °C, with isocratic mode. The mobile phase consisted of a mixture of acetonitrile:phosphate buffer (31:69 v/v, pH = 5.0). 50 µL of the sample were injected at a flow rate of 1 mL min^{-1} and analyzed at 240 nm. The evaluated etoposide concentration in the mixture was equal to 221 µg/ml for a loading efficiency of 86± 4%.

~~**Etoposide Loading**~~

~~The loading of etoposide was performed during the self-assembly of the 510 polymeric surfactant into PBS. Briefly, 10 mg of 510 and 550 µg of etoposide were dissolved in 50 mg of DMSO and then aspired with a 5 ml syringe pre-loaded with 2 ml of PBS. After three cycles of aspiration and ejection, the mixture was filtered through a 0.2 PES pore size filter syringe (Millex). The NP Dn and PDI were determined by dynamic laser light scattering analysis (DLLS, Zetasizer Nano Series, Malvern Instruments). 200 µl of the NPs were dissolved in 800 µl of DMSO, filtered and then analyzed via HPLC-UV (Waters Associates,~~

~~Milford, MA, USA, model 2487 Variable Wavelength Detector) with a RP-C18 analytical reverse-phase column (50 mm×4 mm, 5 µm, Merk-Millipore®) at 40 °C, with isocratic mode. The mobile phase consisted of a mixture of acetonitrile:phosphate buffer, 31:69 (v/v) The pH of the mobile phase was adjusted to 5.0 using 5 M sodium hydroxide. 50 µL of the sample were injected at a flow rate of 1 mL min^{-1} and analyzed at 240 nm. The evaluated etoposide concentration in the mixture was equal to 221 µg/ml for a loading efficiency of 86 %.~~

NPs produced via emulsion polymerization

PEGylated PCL-based NPs (EP NPs in Table 1) were synthesized via monomer starved semi-batch emulsion polymerization (MSSEP) of HEMA-CL_3 as previously described [50]. Briefly, 0.4 g of PEGMA950 were dissolved in 45 ml of deionised water in a three-neck round-bottom flask and heated to 80°C. After three nitrogen/vacuum cycles, 2.1 g of HEMA-CL_3 were mixed with 2.1 mg of HEMA-Rh and added into the reactor at a feeding rate of 2 mL h^{-1}. 0.02 g of KPS were dissolved in 2.5 mL of deionized water and injected with a syringe at the beginning of the lipophilic monomer feeding. After 3 h, the reaction was stopped, and the final latex was characterized via DLLS.

In vitro experiments

BV-2 immortalized microglia cell line was used to assess the kinetics of NPs uptake and the efficacy of different drug formulations on cells. BV-2 cells were cultured at 37°C, 5% CO2 with complete Dulbecco`s modified eagle`s medium (DMEM) ~~(DMEM medium~~ supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM glutamine and 1% penicillin/streptomycin). The medium was changed two or three times a week. For the assays, cells were detached from the culture dishes, centrifuged at 150g for 5 min and plated in 96-well tissue culture plates (5000 cells/well) and/or onto 48-well tissue-culture plates (15000 cells/well). Twenty-four hours after plating, the medium was replaced with complete DMEM with or without the tested compounds: different NPs formulations, the scavenger receptor inhibitor, busulfan or etoposide dissolved in DMSO or encapsulated in NPs. NPs uptake was analysed at different time-points after NPs administration, by checking for live rhodamine+ cells by flow cytometry. Cell survival was analyzed on 96-well plates at 24h and 72h after treatment by the Cell Titer MTS assay (Promega). For cells treated with busulfan or busulfan encapsulated in NPs, γH2AX marker was also analysed. For these experiments, cells were collected by trypsinization and fixed with ice-cold 70% Ethanol in PBS and stored at -20°C up to the analysis. Cells were incubated with rabbit anti- γH2AX antibody (Cell Signaling technology) at 1:500 dilution in 1% bovine serum albumin (BSA)~~1% ~~, 0.1% Triton X-100 ~~0.1%~~ in PBS for 2h at 4C. Afterwards, cells were washed with PBS and incubated with anti-rabbit Alexa.488 antibody at 1:500 dilution in BSA1% Triton X-100 0.1% in PBS for 45' at 4°C, then

washed and analysed by flow cytometry. In order to measure the number of nuclear γH2AX+ foci, cells plated on glass coverslips were fixed with 4% PFA in PBS for 20 min at room temperature (RT), then they were stained with anti- γH2AX antibody (Cell Signaling technology) following the same protocol used for flow cytometry. After the staining, nuclei were stained with DAPI, then cells were washed and mounted on slides with Mowiol.

In vivo experiments

8 weeks old C57BL6/J from Jackson lab were used in the experiments. For intra-cerebroventricular injection, animals were anesthetized by Ketamine/Xylazine mixture and mounted on a stereotaxic apparatus. Rhodaminated NPs (5 µl/injection site) were injected bilaterally in the lateral ventricles through 30G injectors with a flow rate of 0.35 µl/min. Stereotaxic coordinates, referred to Bregma, were AP: -0.5, L: ±1.0; DV: -2.5. BBB disruption was achieved by administration of Mannitol 23% in PBS, 30 µl/g i.p., performed 10 min before NPs injection. For administration of busulfan (clinical grade formulation: Busilvex, Pierre-Fabre) or busulfan loaded in SA-SP NPs, or etoposide loaded in SA-N NPs, animals were implanted with a 30G cannula in one lateral ventricles (Stereotaxic coordinates, referred to Bregma, were AP: -0.5, L: +1.0; DV: -2.5). The compounds (5 µl/injection site) were administered once daily for four consecutive days (flow rate of 0.35 µl/min). For assessment of cell proliferation, the nucleoside analogue Edu (Life Technologies) was administered at 100 µg/g dose i.p. Animals were sacrificed at 3-5 days post-NPs/drugs administration to measure NPs uptake or drug efficacy. On the day of sacrifice, animals were irreversibly anesthetized by overdose of ketamine/xylazine mixture, then intracardiacally perfused with 10 ml of PBS. Then brain was dissected and longitudinally sectioned in two halves. One half was processed for flow analysis as previously described [26], the contralateral side was post-fixed for 24h in 4% buffered PFA, cryopreserved in 30% sucrose until it sank and then frozen in TissueTek optimal cutting temperature (OCT) compound and stored at -80°C until cryosectioning and histology.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines according to the international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). The specific protocols covering the studies described in this paper were approved by the Italian Ministry of Health, an internal ethical committee at Ospedale San Raffaele and the Dana Farber Cancer Institute Institutional Animal Care and Use Committee.

Flow cytometry

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Cells from brain were analyzed by flow cytometry upon resuspension in blocking solution (PBS; 5% FBS and 1% BSA) and labeling at 4 °C for 15 min with: rat APC.Cy7 anti-mouse CD45 (BD Pharmingen) 1:100; rat APC or APC.780 anti-mouse CD11b (eBioscience) 1:100; rat PE.Cy7 anti-mouse CD117/c-kit (BD Pharmingen) 1:100; rat BV510 anti-mouse CD45 (Biolegend) dil.1:100; rat BV421 anti-mouse CD31 (BD Biosciences) dil.1:100; rat biotinylated anti-mouse O4 (Mylteni) dil.1:100; rat APC anti-mouse ACSA2 (Mylteni) dil.1:100; rat PE.Cy7 anti-mouse Thy1 (Biolegend) dil.1:100; fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Pharmingen). Rhodamine signal was detected in the PE channel. For γH2AX staining cells were first stained for surface antigens and then fixed in 4% buffered PFA for 20 min at 4°C. Then cells were permeabilized by incubation in Saponin 1x in BSA1% in PBS for 30' and then incubated with rabbit anti- γH2AX antibody (Cell Signaling technology) at 1:500 dilution in Saponin 1X BSA1% in PBS for 2h at 4°C. Afterwards, cells were washed with PBS and incubated with anti-rabbit Alexa.488 antibody at 1:500 dilution in Saponin 1x BSA1% PBS for 45 min at 4°C, then washed and analysed by flow cytometry. Determination of Edu+ cells was done by the Flow-cytometry Pacific-Blue Edu kit (Life Technologies) according to manufacturer instructions. Specificity of the staining was verified by use of isotype controls.

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Immunohistochemistry

20 μm sagittal brain cryostat sections were first incubated for 1h in blocking solution (NGS 10% Triton 0.1% in PBS) at room temperature. Then they were incubated O/N at 4°C with blocking solution containing one of the following primary antibodies: rabbit anti-GFAP (Dako, 1:2500 dilution); mouse anti-Nestin (Millipore, 1:100 dilution); rabbit anti-Iba1 (Wako, 1:250 dilution); rat anti-CD68 or rat anti-F4/80 (Abd Serotec, 1:250 dilution); mouse anti-NG2 (Millipore, 1:100 dilution); rabbit anti- γH2AX (Cell Signaling, 1:750 dilution). After extensive washings, sliced were incubated with appropriate secondary antibodies conjugated to Alexa 488 or Alexa 647 fluorophores (Molecular Probes, 1:1000 dilution) for 1h at room temperature in blocking solution. In the case of γH2AX an anti-rabbit biotinylated antibody (Vector Labs, 1:200 dilution) was used, followed by ~~TSA~~ Cy5-tyramide amplification step (Perkin Elmer), performed according to manufacturer instructions and as previously described [51]. After the staining, slices were extensively washed in PBS and then counterstained for nuclei (with DAPI at 0.5 μg/ml for 10 min). Edu proliferation marker was highlighted by using a Pacific-blue Edu proliferation kit (Life technologies) according to manufacturer instructions. Finally, slices were mounted on glass coverslips with Mowiol and acquired in sequential scanning mode with a Leica TCP2 or TCP5 microscope, equipped with laser lines: Ar-Kr (488 nm), He-Ne green (532 nm) and a UV diode, at the San Raffaele Alembic and DFCI microscope imaging cores. Images were processed and slightly adjusted

for background, illumination and contrast by using Fiji public domain software [52]; 3D reconstructions were performed with Imaris 6.0 (Bitplane) available through the DFCI microscopy core.

Quantification of markers of proliferation, microglia, rhodaminated nanoparticles and macrophages on brain slices

The analysis was performed in Edu-treated mice sacrificed at 3-5 days post NPs ICV administration, on 4 sagittal brain slices. Sections were stained with anti-Iba1 or anti-F4/80 antibodies and with Edu kit, as described above. 4-5 z-stack confocal images for each animal were acquired at 40x magnification over a 10 μm z-axis with a 0.45 μm step size in relevant brain regions including cortex, hippocampus, the subventricular zone and the lateral ventricles. Image segmentation and classification of cells according to marker positivity was performed through Volocity software (Perkin Elmer). The pipeline developed for image segmentation and analysis is available on demand.

Quantification of γH2AX+ nuclear foci in BV2 cells

The analysis was performed on images acquired at 20× magnification through a CCD Hamamatsu camera connected to a Zeiss Axio Observer epifluorescence microscope. Cells nuclei were identified by DAPI nuclear stain whereas γH2AX was stained as described above. Image segmentation and classification of cells according to γH2AX positivity was performed through CellProfiler public domain software [53]. The pipeline developed for image segmentation and analysis is available on demand.

TABLES

Table 1. Characterization of the block copolymers and of the corresponding NPs.

NPs preparation	Name	Mn,Theory [Da]	Mn,GPC [Da]	Đ [-]	*Dn* [nm]	*PDI* [nm]	ζ [mV]	Drug -	η [%]
Emulsion precipitation	EP NPs	-	-	-	138±24	0.034± 0.023	-	-	-
Self-assembly	SA-N2 NPs	31305~~SA-N2 NPs~~	25323± 17093~~1305~~	1.06± 0.03~~25~~ ~~323±1709~~	108±11 ~~1.06±0.03~~	0.164± 0.036~~1~~ ~~08±11~~	- ~~0.164± 0.036~~	--	--
	SA-N1 NPs	17288	15981± 1961	1.13± 0.01	39±3	0.156± 0.028	-	ETO	86±4
	SA-SP NPs	17781	11633± 1591	1.08± 0.01	37±4	0.230± 0.050	-20±5	BUS	95±3

Mn,Theory: Theoretical block copolymer molecular weight evaluated according to a previously published protocol[48]~~; (Ref. Capasso JCR);~~ Mn,GPC: Block copolymer number averaged molecular weight obtained via GPC and relative to polystyrene standards~~.~~; *Đ:……; Dn:……; PDI: polydispersion index; ζ: z-potential;* η: drug loading efficiency; ETO: etoposide; BUS: busulfan.

Table 2. Pharmacokinetic parameters in brain and plasma after IP busulfan administration in the mouse.

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Tabella formattata

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Parameter	Unit	Brain	Plasma
t ½	Min	133	137
MRT 0-inf_obs	Min	173	172
Tmax	Min	30	15
Cmax	ng/g or ng/ml	18847	30600
AUC 0-t	ng/g(ml)*min	3091248	4174875
AUC 0-inf_obs	ng/g(ml)*min	3344123	4528221
Brain/plasma (%)		74	

FIGURE LEGENDS

Figure 1. Biodistribution of NPs in brain parenchyma upon intra-cerebroventricular (ICV) administration.

A. Representative flow cytometry plots showing NPs uptake by $CD45^{\pm}$ $CD11b^{\pm}$ microglia, in vivo. **B.** Improved NPs biodistribution in mouse CNS upon reduction of NPs size and BBB disruption (EP = neutral NPs, 138 nm; SA-N2 = self-assembly neutral 108 nm; SA-N1 = self-assembly neutral 39 nm NPs; SA-SP = self-assembly negatively charged 37 nm NPs). *** = p < 0.001, ** = p < 0.01, * = p < 0.05 Kruskal Wallis followed by Dunn's test versus EP; #### = p < 0.0001 Mann-Whitney ~~versus no-BBB-disruption; 2-way ANOVA followed by Tukey's test.~~ **C.** Representative epifluorescence microscope microphotograph of the distribution of Rhodamine^{+} EP-NPs in ~~in~~ the brain of mice, at 3~~-5~~ days post ICV-administration (arrows

highlight the injection site); mannitol was co-administered to induced transient BBB disruption). **D.** ~~**B.**~~ Higher magnification of the region highlighted by the dashed square in ~~A~~C, showing localization of NPs in the subventricular zone (SVZ) and rostral-migratory stream (RMS). **E.** ~~**C.**~~ Quantification of Rh~~odamine~~+ (NPs) signal in different CNS regions (ob, olfactory bulb; Cx, cortex; cc; corpus callosum; Hipp, hippocampus; Fimbria; LV, lateral ventricle; Th, thalamus; Hypo, hypotalamus; Cnu, striatum; MidB, midbrain; HindB, hindbrain; Cb, cerebellum; 4V, fourth ventricle). The box-and-whiskers plot represents, for each CNS region, the percentage of total area occupied by NPs (n=4 different animals). ~~**D.** Representative flow cytometry plots showing NPs uptake by CD45$^{\pm}$CD11b$^{\pm}$ microglia, in vivo. **E.** Improved NPs biodistribution in mouse CNS upon reduction of NPs size and BBB disruption (EP = neutral NPs, 138 nm; SA-N = self-assembly neutral 39 nm NPs; SA-SP = self-assembly negatively charged 37 nm NPs). *** = p < 0.001, ** = p < 0.01, * = p < 0.05 versus EP; #### = p < 0.0001 versus no-BBB disruption; 2-way ANOVA followed by Tukey's test.~~ **F-~~H~~.** Wash-out of NPs in vivo over time, evaluated as percentage of NPs$^{\pm}$ CD45$^{\pm}$CD11b$^{\pm}$ microglia still detectable at different time-points post-administration, i.e. 3-7-14 days post-injection (dpi). **G.** Cytofluorimetric quantification of the composition of the NP+ cells (in terms of percentage of different cell subtypes within the total NP+ population): ~~biodistribution of EP, SA-N or SA-SP NPs within different CNS cell subtypes, namely CD45+ versus CD45- (F),~~ microglia (CD45^{+}CD11b^{+}) versus lymphocytes (CD45^{+}CD11b^{-}), ~~within CD45+ fraction (G),~~ O4^{+} oligodendrocytes versus ACSA2^{+} astrocytes or CD31^{+} vascular endothelial cells and neurons (Thy1^{+}) within CD45^{-} fraction (**H**). *~~*~~ = p < 0.0~~01~~5 ~~versus EP and SA-N O4+ olygodendrocytes; 2-way ANOVA followed by Tukey's test~~Kruskal Wallis. Scale bar in C = 400 µm; scale bar in D = 200 µm.

Figure 2. Co-localization of NPs with microglia/macrophage and progenitor cells markers.

Laser scanning confocal microphotographs (A, C, E~~, G~~) and corresponding 3D reconstructions (B, D, F~~, H~~) of Rhodamine^{+} nanoparticles (NPs) in cells stained for different ~~glial~~ markers. **A-B.** Co-localization of NPs with microglia/macrophages, identified by Iba1 (blue) or CD68 (green). Arrowheads highlight internalization of NPs in the cytoplasm of amoeboid-like Iba1^{+}/CD68^{+} cells; arrow highlights NPs internalization in Iba1^{+}/CD68^{+} ramified microglia. **C-D.** ~~Lack of co-localization of NPs with GFAP astrocyte marker (green). Arrows highlight internalization of NPs in Iba1^{+} microglia cells (blue) but not in astrocytes.~~ ~~**E-F.**~~ Arrows highlight the internalization of NPs in some Iba1+ cells (blue) that stain positive also for Nestin stemness marker (green). ~~**G-H**~~**E-F**. Arrows highlight internalization of NPs in some cells positive for NG2 oligodendrocytes progenitor~~s~~ marker (green). Scale bars in A, C, E~~, G~~ = 20 µm.

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Figure 3. Internalization of NPs in brain proliferating cells.

A. Representative plots showing the gating strategy to discriminate microglia/macrophage brain cell populations according to CD45 and CD11b stain: mature resting microglia is $CD45^{+}CD11b^{high}$; immature transiently amplifying microglia (TaM) is $CD45^{+}CD11b^{low}$; CNS resident macrophages (CNSm) are $CD45^{high}CD11b^{+}$. **B.** Flow cytometric quantification of Edu+ proliferating brain myeloid cell populations. **C.** Representative plots showing the gating strategy to quantify the percentage of Edu+ cells shown in B. **D.** Flow cytometric quantification of the percentage of Edu^{+} proliferating cells that are positive for NPs (Rh^{+}) within total $CD45^{+}$ compartment, or among different $CD45^{+}CD11b^{+}$ subpopulations, namely CNSm, microglia or TaM. **E.** Flow cytometric quantification of the percentage of Rh^{+} cells within the populations of cells that are either proliferating (Edu^{+}, blue histograms) or not (Edu^{-}, red histograms). **F.** Laser scanning confocal microphotographs of $Rhodamine^{+}$ nanoparticles (NPs) in Edu^{+} proliferating cells (blue) either negative or positive for Iba1 (green) microglia marker (arrowheads and arrows, respectively). **G.** Representative microphotographs showing the segmentation strategy to quantify markers of proliferation (Edu, light blue), microglia (Iba1, green), rhodaminated nanoparticles (NPs, red), macrophages (F4/80, dark blue) on brain slices. **H.** Quantification of NPs, Edu, Iba1 and F4/80 staining in rhodamine negative or positive cells (blue and red histograms, respectively). **I.** Flow cytometric quantification of NPs uptake (Rh^{+}) in $CD45^{+}$/c-kit^{+} hematopoietic progenitor cell subpopulation upon NPs administration ICV and BBB disruption. Scale bar in F = 20 μm.

Figure 4. NPs-mediated drug delivery, in microglia cell lines.

A. Extent of uptake of $Rhodamine^{+}$ NPs with different size and surface charge, in BV2 microglia cell line, assessed over-time by flow cytometry. **B.** Effect of the scavenger receptor inhibitor fucoidan on NPs uptake in BV2 microglia cell line. *=$p < 0.05$; **=$p < 0.01$ versus untreated; 2-way ANOVA followed by Bonferroni's post-hoc test. **C.** MTS cell viability assay performed on BV2 microglia cell lines upon incubation (for 24 or 72h) with different concentrations of busulfan. The drug was encapsulated in 38 nm negatively charged NPs (SA-SP, blue filled bars) or tested alone (green histograms); empty NPs were tested as controls (blue dashed histograms). The cellular viability is expressed as percentage of the untreated control value (histograms are the mean + SEM of n=3 independent experiments). *=$p < 0.05$; ***=$p < 0.001$; ****=$p < 0.0001$; 1-way ANOVA followed by Tukey's post-hoc test. **D.** Flow cytometric quantification of the percentage of $\gamma H2AX^{+}$ BV2 microglia-like cells upon exposure to busulfan or to different concentrations of NPs loaded with busulfan or not (histograms represent the mean + SEM of n>= 3 independent experiments). *=$p < 0.05$; 1-way ANOVA followed by Tukey's post-hoc test. **E.**

Effect of scavenger receptor inhibitor on the percentage of γH2AX+ cells (determined by flow cytometry) after exposure to NPs loaded with busulfan or not (box and whiskers plots of n>= 3 independent experiments). * = $p < 0.05$; Mann-Whitney not parametric test. **F.** MTS cell viability assay performed on BV2 microglia cell lines upon incubation (for 24 or 72h) with different concentrations of etoposide. The drug was encapsulated in 39 nm neutrally charged NPs (SA-N, blue filled histograms) or tested alone (orange filled histograms); empty NPs (blue dashed bars). The cellular viability is expressed as percentage of the untreated control value (histograms are the mean + SEM of n=3 replicates). ****=$p < 0.0001$; 1-way ANOVA followed by Tukey's post-hoc test.

Figure 5. Brain and plasma levels of busulfan upon systemic administration in vivo.

A. Brain penetration and plasma levels of busulfan, at different time-points post-systemic administration. **B.** Representative epifluorescence microscope photomicrographs of γH2AX– immunostaining (red) and DAPI nuclear stain (green) in sagittal brain slices from control untreated mice (CTR) or from mice analyzed 1 day after systemic busulfan conditioning (BUS). Inset shows higher magnification of the subventricular zone (SVZ) highlighted by the dashed box. **C.** Laser scanning confocal microphotographs of co-immunostaining for γH2AX (red), neurons (NeuN), microglia (Iba1) or astrocytes (GFAP), green, and– DAPI nuclear stain (blu). Arrows highlight co-localization of γH2AX with neuronal cells, microglia and astrocytes. Scale bar = 25 μm.

Figure 6. Effect of NPs mediated drug delivery in the CNS upon ICV administration.

A. Flow cytometric quantification of the percentage of $\gamma H2AX^+$ $CD45^+CD11b^+$ microglia cells in the brain of mice 3-5 days after ICV administration of SA-SP NPs loaded (red histogram) or not (blue histogram) with busulfan. *** = $p<0.001$; ** =$p<0.01$; 1-way ANOVA followed by Tukey's post-hoc test. **B.** Representative epifluorescence microscope photomicrographs of γH2AX immunostaining (red) and DAPI nuclear stain (green) in sagittal brain slices from a mouse analyzed 3 days after ICV administration of SA-SP NPs loaded with busulfan. **C.** Laser scanning confocal microphotographs of co-immunostaining for $Rhodamine^+$ NPs (red), microglia (Iba1, green) and –DAPI nuclear stain (blu). **D.** Same acquisition shown in C, with γH2AX staining displayed in red. Scale bar = 20 μm. **E, F, G, H.** Flow cytometric quantification of the percentage of $Annexin^+$ (Ann^+) cells within the $CD45^+$ (E), $CD45^+CD11b^+$ microglia (F), $CD45^+$/$c\text{-}kit^+$ (G) or $CD45^-$ (H) compartment, upon administration of etoposide (ETO) loaded in SA-N NPs (red histograms). Untreated (UT) mice or mice injected with empty SA-N NPs (green histograms) are used as controls. Mice undergoing systemic busulfan conditioning (BUS, gray histograms) are used as reference. * = $p<0.05$; Kruskal Wallis not-parametric test. **I, J, K, L.**

Flow cytometric quantification of the percentage of Annexin$^+$ (Ann$^+$) cells within different CNS populations, namely CD45$^+$ (I), CD45$^+$CD11b$^+$ microglia (J), CD45$^+$/c-kit$^+$ (K) or CD45$^-$ (L), that display positivity also for NPs (Rhodamine$^+$). * =p<0.05; Mann-Whitney not-parametric test.

Figure S1. Synthesis of the block copolymers via a two-step polymerization.

i) RAFT polymerization of PEGMA2000 and ii) RAFT polymerization of $HEMACL_5$ with or without SPMAK in the presence of the PEGylated macro RAFT agent produced in the previous step.

Figure S2. Brain biodistribution of NPs with different dimensions and surface charge either loaded or not with drugs.

A-D, Representative epifluorescence microscope microphotographs of the distribution of Rhodamine$^+$ NPs in the brain of mice, at 3-5 days post ICV-administration. ~~of negatively charged~~ SA-SP NPs either empty (**A**) or loaded with busulfan (**B**), or neutrally charged SA-N1 NPs either empty (**C**) or loaded with etoposide (**D**) were tested. **E.** Comparison of the biodistributions of SA-N1, SA-SP or EP NPs in different brain regions (ob, olfactory bulb; Cx, cortex; cc; corpus callosum; Hipp, hippocampus; Fimbria; LV, lateral ventricle; Th, thalamus; Hypo, hypotalamus; Cnu, striatum; MidB, midbrain; HindB, hindbrain; Cb, cerebellum; 4V, fourth ventricle). The box-and-whiskers plot represents, for each CNS region, the percentage of total area occupied by NPs (n=4/5 different animals). * = p < 0.05; ** = p < 0.01; Kruskal Wallis followed by Dunn's post-hoc test. **F.** Representative laser scanning confocal microphotographs showing the control for the specificity of the Rhodamine signal detected in animals injected with NPs. Scale bar = 50 μm. **G.** Lack of correlation between rhodamine mean fluorescence intensity (MFI) and the percentage of Rhodamine$^+$ brain microglia cells with internalized NPs (Pearson's R^2 = 0.01816; n = 70 independent observations).

Figure S3. Gating strategy to identify different brain subpopulations by flow cytometry.

A. Representative flow cytometry plots showing the gating strategy to identify different cell subpopulation within single cell suspensions preparations obtained from brains of NPs injected mice: cell population is gated on FSC and SSC physical parameters, followed by selection for 7AAD- live cells; then cells are discriminated according to positivity for CD45 pan-leukocytes marker; finally, brain microglia/macrophages are identified as CD11b$^+$ cells within the CD45$^+$ fraction whereas astrocytes, oligodendrocytes, endothelial and neuronal cells are identified as ACSA2$^+$, O4$^+$, CD31$^+$ or Thy1$^+$ cells within CD45$^-$, respectively. **B.** Representative flow cytometry plots showing the gating strategy to

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identify different cell subpopulation within the fraction of cells displaying positivity for NPs. Cell population is gated on FSC and SSC physical parameters, followed by selection for $7AAD^-$ live cells; then cells are discriminated according to positivity for rhodaminated NPs and then furtherly subclassified into microglia/macrophages, astrocytes, oligodendrocytes, endothelial or neuronal cells according to the markers and gating strategy described in A.

Figure S4. Increased γH2AX staining upon exposure to busulfan in BV2 microglia-like cell lines.

A. Representative flow cytometry plot showing the increase in the percentage of cells displaying positivity for γH2AX staining (the mean fluorescence intensity of the staining is also increased) upon exposure to busulfan (BUS). **B.** Representative epifluorescence microscope photomicrographs and corresponding quantification $\gamma H2AX^+$ nuclear foci (green) upon exposure to busulfan either loaded in SA-SP NPs (NP BUS) or not (BUS). ** =p<0.01; **** = p<0.0001; 2-way ANOVA followed by Tukey's post-hoc test versus UT or NP (n=4 independent experiments). Scale bar = 5 μm.

Figure S5. Brain and plasma levels of busulfan upon ICV administration of the drug.

A. Scheme of the procedure for busulfan administration in the cerebral lateral ventricle, and brain sampling after administration. The brain was divided in four regions, named 1 through 4, with region 1 comprising the injection site. **B.** Concentration of busulfan measured in the brain or plasma at 5min (blue bars) or 20min (red bars) after the end of drug infusion. **C.** Representative laser scanning confocal microscope photomicrographs of co-immunostaining for γH2AX (red), microglia (Iba1, green) or astrocytes (GFAP, blue), and DAPI nuclear stain (cyan) in the brain of mice sacrificed at 1 or 5 days after four ICV injections of busulfan. Reactive astrocytosis and microgliosis are highlighted with asterisks and arrows, respectively. Scale bar in C = 50 μm.

Authors contribution

A.B. and M.P. contributed to the conception and design of the study. U.C.P. and D.M. contributed to the conception and optimization of the nanoparticles platform. M.P. performed the in vitro and in vivo biological experiments; ~~,~~ U.C.P. contributed to the synthesis and characterization of the nanoparticles formulations; M.P., U.C.P., R.M. and F.C. contributed to the acquisition and analysis of data. M.P., U.C.P. and A.B. contributed to drafting the text; M.P. ~~and preparing the~~ drafted the figures.

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Competing interests

M.P., U.C.P, D.M. and A.B. are authors on a patent application related to the use of nanoparticles in the CNS filed by Boston Children's Hospital, Ospedale San Raffaele, and Politecnico of Milano (application no. PCT/US17/56774, filed 16 October 2017). All other authors declare that they have no competing interests.

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Abbreviations

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BBB, blood brain barrier; BUS, busulfan; CNS, central nervous system; DSBs, double-strand brakes; ETO, etoposide; HSC, hematopoietic stem cell; ICV, intra-cerebroventricular; LV, lateral ventricles; NE, neutral nanoparticles; NP, nanoparticle; PCL, poly-caprolactone; RAFT, reversible addition-fragmentation chain transfer; RMS, rostral-migratory stream; ROP, ring opening polymerization; SA-N, self-assembly neutral NP; SA-SP, self-assembly negatively charged NP; SVZ, subventricular zone.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Bibliography

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[1] S.R. Hwang, K. Kim, Nano-enabled delivery systems across the blood-brain barrier., Arch. Pharm. Res. 37 (2014) 24–30. doi:10.1007/s12272-013-0272-6.

[2] J. V Georgieva, D. Hoekstra, I.S. Zuhorn, Smuggling Drugs into the Brain: An Overview of Ligands Targeting Transcytosis for Drug Delivery across the Blood-Brain Barrier., Pharmaceutics. 6 (2014) 557–83. doi:10.3390/pharmaceutics6040557.

[3] Z. Pang, W. Lu, H. Gao, K. Hu, J. Chen, C. Zhang, X. Gao, X. Jiang, C. Zhu, Preparation and brain delivery property of biodegradable polymersomes conjugated with OX26., J. Control. Release. 128 (2008) 120–127. doi:10.1016/j.jconrel.2008.03.007.

[4] P. Pouponneau, J.-C. Leroux, G. Soulez, L. Gaboury, S. Martel, Co-encapsulation of magnetic nanoparticles and doxorubicin into biodegradable microcarriers for deep tissue targeting by vascular MRI navigation., Biomaterials. 32 (2011) 3481–3486. doi:10.1016/j.biomaterials.2010.12.059.

[5] E. Nance, S.P. Kambhampati, E.S. Smith, Z. Zhang, F. Zhang, S. Singh, M. V. Johnston, R.M. Kannan, M.E. Blue, S. Kannan, Dendrimer-mediated delivery of N-acetyl cysteine to microglia in a mouse model of Rett syndrome, J. Neuroinflammation. 14 (2017) 252. doi:10.1186/s12974-017-1004-5.

[6] R. Sharma, S.-Y. Kim, A. Sharma, Z. Zhang, S.P. Kambhampati, S. Kannan, R.M. Kannan, Activated Microglia Targeting Dendrimer–Minocycline Conjugate as Therapeutics for Neuroinflammation, Bioconjug. Chem. 28 (2017) 2874–2886. doi:10.1021/acs.bioconjchem.7b00569.

[7] S. Papa, F. Rossi, R. Ferrari, A. Mariani, M. De Paola, I. Caron, F. Fiordaliso, C. Bisighini, E. Sammali, C. Colombo, M. Gobbi, M. Canovi, J. Lucchetti, M. Peviani, M. Morbidelli, G. Forloni, G. Perale, D. Moscatelli, P. Veglianese, Selective Nanovector Mediated Treatment of Activated Proinflammatory Microglia/Macrophages in Spinal Cord Injury., ACS Nano. (2013). doi:10.1021/nn4036014.

[8] S. Papa, R. Ferrari, M. De Paola, F. Rossi, A. Mariani, I. Caron, E. Sammali, M. Peviani, V. Dell'Oro, C. Colombo, M. Morbidelli, G. Forloni, G. Perale, D. Moscatelli, P. Veglianese, Polymeric nanoparticle system to target activated microglia/macrophages in spinal cord injury, J. Control. Release. 174 (2014) 15–26. doi:10.1016/j.jconrel.2013.11.001.

[9] U. Capasso Palmiero, L. Morosi, M. Lupi, M. Ponzo, R. Frapolli, M. Zucchetti, P. Ubezio, M. Morbidelli, M. D'Incalci, E. Bello, D. Moscatelli, Self-Assembling PCL-Based Nanoparticles as PTX Solubility Enhancer Excipients, Macromol. Biosci. (2018) 1800164. doi:10.1002/mabi.201800164.

[10] A. Biffi, E. Montini, L. Lorioli, M. Cesani, F. Fumagalli, T. Plati, C. Baldoli, S. Martino, A. Calabria, S. Canale, F. Benedicenti, G. Vallanti, L. Biasco, S. Leo, N. Kabbara, G. Zanetti, W.B.

Rizzo, N.A.L. Mehta, M.P. Cicalese, M. Casiraghi, J.J. Boelens, U. Del Carro, D.J. Dow, M. Schmidt, A. Assanelli, V. Neduva, C. Di Serio, E. Stupka, J. Gardner, C. von Kalle, C. Bordignon, F. Ciceri, A. Rovelli, M.G. Roncarolo, A. Aiuti, M. Sessa, L. Naldini, Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy., Science. 341 (2013) 1233158. doi:10.1126/science.1233158.

[11] M. Sessa, L. Lorioli, F. Fumagalli, S. Acquati, D. Redaelli, C. Baldoli, S. Canale, I.D. Lopez, F. Morena, A. Calabria, R. Fiori, P. Silvani, P.M. V Rancoita, M. Gabaldo, F. Benedicenti, G. Antonioli, A. Assanelli, M.P. Cicalese, U. del Carro, M.G.N. Sora, S. Martino, A. Quattrini, E. Montini, C. Di Serio, F. Ciceri, M.G. Roncarolo, A. Aiuti, L. Naldini, A. Biffi, Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial, Lancet. 388 (2016) 476–487. doi:10.1016/S0140-6736(16)30374-9.

[12] A. Capotondo, R. Milazzo, J.M. Garcia-Manteiga, E. Cavalca, A. Montepeloso, B.S. Garrison, M. Peviani, D.J. Rossi, A. Biffi, Intracerebroventricular delivery of hematopoietic progenitors results in rapid and robust engraftment of microglia-like cells, Sci. Adv. 3 (2017) e1701211. doi:10.1126/sciadv.1701211.

[13] C. Colombo, L. Morosi, E. Bello, R. Ferrari, S.A. Licandro, M. Lupi, P. Ubezio, M. Morbidelli, M. Zucchetti, M. D'Incalci, D. Moscatelli, R. Frapolli, PEGylated Nanoparticles Obtained through Emulsion Polymerization as Paclitaxel Carriers, Mol. Pharm. 13 (2016) 40–46. doi:10.1021/acs.molpharmaceut.5b00383.

[14] D. Moscatelli, M. Sponchioni, Bioresorbable polymer nanoparticles in the medical and pharmaceutical fields: A promising field, in: Bioresorbable Polym. Biomed. Appl. From Fundam. to Transl. Med., 2017: pp. 265–283.

[15] L. Cova, P. Bigini, V. Diana, L. Sitia, R. Ferrari, R.M. Pesce, R. Khalaf, P. Bossolasco, P. Ubezio, M. Lupi, M. Tortarolo, L. Colombo, D. Giardino, V. Silani, M. Morbidelli, M. Salmona, D. Moscatelli, Biocompatible fluorescent nanoparticles for in vivo stem cell tracking., Nanotechnology. 24 (2013) 245603. doi:10.1088/0957-4484/24/24/245603.

[16] a Ghodsi, C. Stein, T. Derksen, I. Martins, R.D. Anderson, B.L. Davidson, Systemic hyperosmolality improves beta-glucuronidase distribution and pathology in murine MPS VII brain following intraventricular gene transfer., Exp. Neurol. 160 (1999) 109–116. doi:10.1006/exnr.1999.7205.

[17] C. Burger, F.N. Nguyen, J. Deng, R.J. Mandel, Systemic mannitol-induced hyperosmolality amplifies rAAV2-mediated striatal transduction to a greater extent than local co-infusion., Mol. Ther. 11 (2005) 327–331. doi:10.1016/j.ymthe.2004.08.031.

[18] D.M. McCarty, J. DiRosario, K. Gulaid, J. Muenzer, H. Fu, Mannitol-facilitated CNS entry of rAAV2 vector significantly delayed the neurological disease progression in MPS IIIB mice., Gene Ther. 16 (2009) 1340–1352. doi:10.1038/gt.2009.85.

[19] W.M. Pardridge, CSF, blood-brain barrier, and brain drug delivery., Expert Opin. Drug Deliv. 13 (2016) 963–75. doi:10.1517/17425247.2016.1171315.

[20] C.G. Kantzer, C. Boutin, I.D. Herzig, C. Wittwer, S. Reiß, M.C. Tiveron, J. Drewes, T.D. Rockel, S. Ohlig, J. Ninkovic, H. Cremer, S. Pennartz, M. Jungblut, A. Bosio, Anti-ACSA-2 defines a novel monoclonal antibody for prospective isolation of living neonatal and adult astrocytes, Glia. 65 (2017) 990–1004. doi:10.1002/glia.23140.

[21] L. Legroux, C.L. Pittet, D. Beauseigle, G. Deblois, A. Prat, N. Arbour, An optimized method to process mouse CNS to simultaneously analyze neural cells and leukocytes by flow cytometry, J. Neurosci. Methods. 247 (2015) 23–31. doi:10.1016/j.jneumeth.2015.03.021.

[22] J.M. Schwarz, S.H. Smith, S.D. Bilbo, FACS analysis of neuronal–glial interactions in the nucleus accumbens following morphine administration, Psychopharmacology (Berl). 230 (2013) 525–535. doi:10.1007/s00213-013-3180-z.

[23] M.R.P. Elmore, A.R. Najafi, M.A. Koike, N.N. Dagher, E.E. Spangenberg, R.A. Rice, M. Kitazawa, B. Matusow, H. Nguyen, B.L. West, K.N. Green, Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain., Neuron. 82 (2014) 380–97. doi:10.1016/j.neuron.2014.02.040.

[24] B. Stansley, J. Post, K. Hensley, A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease, J. Neuroinflammation. 9 (2012) 577. doi:10.1186/1742-2094-9-115.

[25] J.P. Banáth, P.L. Olive, Expression of phosphorylated histone H2AX as a surrogate of cell killing by drugs that create DNA double-strand breaks., Cancer Res. 63 (2003) 4347–50. http://www.ncbi.nlm.nih.gov/pubmed/12907603 (accessed January 16, 2016).

[26] A. Capotondo, R. Milazzo, L.S. Politi, A. Quattrini, A. Palini, T. Plati, S. Merella, A. Nonis, C. di

Serio, E. Montini, L. Naldini, A. Biffi, Brain conditioning is instrumental for successful microglia reconstitution following hematopoietic stem cell transplantation., Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 15018–23. doi:10.1073/pnas.1205858109.

[27] E. Suberbielle, P.E. Sanchez, A. V Kravitz, X. Wang, K. Ho, K. Eilertson, N. Devidze, A.C. Kreitzer, L. Mucke, Physiologic brain activity causes DNA double-strand breaks in neurons, with exacerbation by amyloid-β., Nat. Neurosci. 16 (2013) 613–21. doi:10.1038/nn.3356.

[28] G. Hoeffel, F. Ginhoux, Ontogeny of Tissue-Resident Macrophages, Front. Immunol. 6 (2015). doi:10.3389/fimmu.2015.00486.

[29] S.A. Wolf, H.W.G.M. Boddeke, H. Kettenmann, Microglia in Physiology and Disease, Annu. Rev. Physiol. 79 (2017) 619–643. doi:10.1146/annurev-physiol-022516-034406.

[30] E. Gomez Perdiguero, C. Schulz, F. Geissmann, Development and homeostasis of "resident" myeloid cells: The case of the microglia, Glia. 61 (2013) 112–120. doi:10.1002/glia.22393.

[31] G. Hoeffel, F. Ginhoux, Ontogeny of Tissue-Resident Macrophages., Front. Immunol. 6 (2015) 486. doi:10.3389/fimmu.2015.00486.

[32] Y. Cirpanli, E. Bilensoy, A.L. Dogan, S. Calis, Development of polymeric and cyclodextrin nanoparticles for camptothecin delivery., J. Control. Release. 148 (2010) e21--3. doi:10.1016/j.jconrel.2010.07.034.

[33] A.J. Sawyer, J.K. Saucier-Sawyer, C.J. Booth, J. Liu, T. Patel, J.M. Piepmeier, W.M. Saltzman, Convection-enhanced delivery of camptothecin-loaded polymer nanoparticles for treatment of intracranial tumors., Drug Deliv. Transl. Res. 1 (2011) 34–42. doi:10.1007/s13346-010-0001-3.

[34] K.T. Householder, D.M. DiPerna, E.P. Chung, G.M. Wohlleb, H.D. Dhruv, M.E. Berens, R.W. Sirianni, Intravenous delivery of camptothecin-loaded PLGA nanoparticles for the treatment of intracranial glioma., Int. J. Pharm. 479 (2015) 374–80. doi:10.1016/j.ijpharm.2015.01.002.

[35] A. Layre, P. Couvreur, C. Aymes-chodur, N. Ghermani, J. Poupaert, D. Requier, R. Gref, Busulfan Loading into Poly (alkyl cyanoacrylate) Nanoparticles : Physico-Chemistry and Molecular Modeling, (2005) 254–262. doi:10.1002/jbmb.

[36] T. Chalati, P. Horcajada, P. Couvreur, C. Serre, M. Ben Yahia, G. Maurin, R. Gref, Porous metal organic framework nanoparticles to address the challenges related to busulfan encapsulation., Nanomedicine (Lond). 6 (2011) 1683–1695. doi:10.2217/nnm.11.69.

[37] a Layre, P. Couvreur, H. Chacun, J. Richard, C. Passirani, D. Requier, J.P. Benoit, R. Gref, Novel composite core-shell nanoparticles as busulfan carriers., J. Control. Release. 111 (2006) 271–280. doi:10.1016/j.jconrel.2006.01.002.

[38] F. Ye, Å. Barrefelt, H. Asem, M. Abedi-Valugerdi, I. El-Serafi, M. Saghafian, K. Abu-Salah, S. Alrokayan, M. Muhammed, M. Hassan, Biodegradable polymeric vesicles containing magnetic nanoparticles, quantum dots and anticancer drugs for drug delivery and imaging, Biomaterials. 35 (2014) 3885–3894. doi:10.1016/j.biomaterials.2014.01.041.

[39] J. Bouligand, P. Couvreur, A.M. Layre, A. Deroussent, A. Paci, E. Delain, G. Vassal, R. Gref, Busulphan-loaded long-circulating nanospheres, a very attractive challenge for both galenists and pharmacologists., J. Microencapsul. 24 (2007) 715–730. doi:10.1080/02652040701500905.

[40] M. Snehalatha, K. Venugopal, R.N. Saha, A.K. Babbar, R.K. Sharma, Etoposide Loaded PLGA and PCL Nanoparticles II: Biodistribution and Pharmacokinetics after Radiolabeling with Tc-99m, Drug Deliv. 15 (2008) 277–287. doi:10.1080/10717540802006500.

[41] M. Ukawala, T. Rajyaguru, K. Chaudhari, A.S. Manjappa, S. Pimple, A.K. Babbar, R. Mathur, A.K. Mishra, R.S.R. Murthy, Investigation on design of stable etoposide-loaded PEG-PCL micelles: effect of molecular weight of PEG-PCL diblock copolymer on the *in vitro* and *in vivo* performance of micelles, Drug Deliv. 19 (2012) 155–167. doi:10.3109/10717544.2012.657721.

[42] A.-M. Layre, P. Couvreur, H. Chacun, C. Aymes-Chodur, N.-E. Ghermani, J. Poupaert, J. Richard, D. Requier, R. Gref, Busulfan loading into poly(alkyl cyanoacrylate) nanoparticles: Physico-chemistry and molecular modeling, J. Biomed. Mater. Res. Part B Appl. Biomater. 79B (2006) 254–262. doi:10.1002/jbm.b.30536.

[43] G. Hempel, D. Oechtering, C. Lanvers-Kaminsky, T. Klingebiel, J. Vormoor, B. Gruhn, J. Boos, Cytotoxicity of dimethylacetamide and pharmacokinetics in children receiving intravenous busulfan., J. Clin. Oncol. 25 (2007) 1772–8. doi:10.1200/JCO.2006.08.8807.

[44] U.B. Eyo, M.E. Dailey, Microglia: Key elements in neural development, plasticity, and pathology, J. Neuroimmune Pharmacol. 8 (2013) 494–509. doi:10.1007/s11481-013-9434-z.

[45] J.S. Henkel, D.R. Beers, W. Zhao, S.H. Appel, Microglia in ALS: the good, the bad, and the resting., J. Neuroimmune Pharmacol. 4 (2009) 389–398. doi:10.1007/s11481-009-9171-5.

[46] R. Auriemma, U. Capasso Palmiero, M. Sponchioni, T.D. Mathews, J.F. Molina Estevez, L.S.

Politi, D. Moscatelli, A. Biffi, M. Peviani, MRI/PET traceable microglia targeted nanovectors: a new potential theranostic platform for tracking and modulating neuroinflammation in amyotrophic lateral sclerosis, Amyotroph. Lateral Scler. Front. Degener. 19 (2018) 229.

[47] R.. Ferrari, Y.. Yu, M.. Morbidelli, R.. Hutchinson, D.. Moscatelli, ε-Caprolactone-Based Macromonomers Suitable for Biodegradable Nanoparticles Synthesis Through Free Radical Polymerization, Macromolecules. 44 (2011) 9205–9212.

[48] U. Capasso Palmiero, A. Agostini, E. Lattuada, S. Gatti, J. Singh, C.T. Canova, S. Buzzaccaro, D. Moscatelli, Use of RAFT macro-surfmers for the synthesis of transparent aqueous colloids with tunable interactions., Soft Matter. 13 (2017) 6439–6449. doi:10.1039/c7sm01084b.

[49] U. Capasso Palmiero, L. Morosi, E. Bello, M. Ponzo, R. Frapolli, C. Matteo, M. Ferrari, M. Zucchetti, L. Minoli, M. De Maglie, P. Romanelli, M. Morbidelli, M. D'Incalci, D. Moscatelli, Readily prepared biodegradable nanoparticles to formulate poorly water soluble drugs improving their pharmacological properties: The example of trabectedin, J. Control. Release. 276 (2018) 140–149. doi:10.1016/j.jconrel.2018.03.005.

[50] C.. Colombo, L.. Dragoni, S.. Gatti, R.M.. Pesce, T.R.. Rooney, E.. Mavroudakis, R.. Ferrari, M. D.;, Tunable Degradation Behaviour of PEGylated Polyester-Based Nanoparticles Obtained Through Emulsion Polymerization, I{&}ECR. 53 (2014) 9128–9135.

[51] M. Peviani, E. Salvaneschi, L. Bontempi, A. Petese, A. Manzo, D. Rossi, M. Salmona, S. Collina, P. Bigini, D. Curti, Neuroprotective effects of the Sigma-1 receptor (S1R) agonist PRE-084, in a mouse model of motor neuron disease not linked to SOD1 mutation., Neurobiol. Dis. 62 (2014) 218–32. doi:10.1016/j.nbd.2013.10.010.

[52] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis., Nat. Methods. 9 (2012) 676–82. doi:10.1038/nmeth.2019.

[53] A.E. Carpenter, T.R. Jones, M.R. Lamprecht, C. Clarke, I. Kang, O. Friman, D.A. Guertin, J. Chang, R.A. Lindquist, J. Moffat, P. Golland, D.M. Sabatini, CellProfiler: image analysis software for identifying and quantifying cell phenotypes., Genome Biol. 7 (2006) R100. doi:10.1186/gb-2006-7-10-r100.