Nanomedicine in Neurodegenerative Disorders: Understanding the journey

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Abstract

Nanocarriers can be useful tools for delivering drugs to the central nervous system (CNS). Their distribution within the brain and their interaction with CNS cells must be assessed accurately before they can be proposed for therapeutic use. We investigated these issues by employing poly-lactide-co-glycolide nanoparticles (NPs) specifically engineered with a glycopeptide (g7) conferring to NPs the ability to cross the blood brain barrier (BBB) at a concentration of up to 10% of the injected dose. g7-NPs display increased *in vitro* uptake in neurons and glial cells. *in vivo* administration of g7-NPs leads to a region- and cell type-specific enrichment of NPs within the brain. Moreover, g7-NPs are endocytosed in a clathrin-dependent manner and transported into a specific subset of early endosomes positive for Rab5 *in vitro* and *in vivo*. Moreover, in order to understand the journey of NPs, we demonstrated that g7-NPs can be transported intra- and intercellularly inside vesicles. Cell-to-cell transport is mediated by tunneling-nanotube (TNT)-like structures in cell lines and most interestingly in glial as well as neuronal cells in vitro. These in vitro findings were in part confirmed by in vivo evidence after i.p. administration in mice. We also tested Ab-modified g7-NPs both in vitro and in vivo to investigate the possibility of a specific targeting.

1. INTRODUCTION

The development of nanocarriers, such as liposomes and nanoparticles (NPs), able to reach brain cells after having crossed the blood brain barrier (BBB), is an important topic of the pharmaceutical research [1]. The crossing of the BBB has been widely investigated and approached by means of surface engineering of nanocarriers in healthy and diseased states allowing also to propose the pathway involved in BBB crossing (i.e. endocytosis/transcytosis) [2]. In this paper we would like to investigate in detail the fate of glycopeptide-enginereed poly(D,L-lactide-co-glycolide (PLGA) NPs (g7-NPs), that are able to cross the BBB after in vivo administration in rodents [3], by assessing their tropism for specific brain regions and cell types. In addition, we studied whether g7-NP uptake in neurons occurs through the same mechanisms in vivo and in vitro. Another topic, required for a more complete overview of the fate of NPs inside the CNS is their intra/inter-cellular trafficking and the possibility of governing and driving this trafficking.

2. MATERIALS and METHODS

2.1 Preparation of nanoparticles and characterization

Nanoparticles were prepared as described in literature with some modifications in the preparation procedure described before [3]. All the batches of NPs were characterized regarding their surface, chemico-physical, morphological properties, by photon correlation spectroscopy, scanning electron microscopy, atomic force microscopy and X-ray photoelectron spectroscopy.

2.2Animal procedures, immuno-histochemistry and in vivo endocytosis inhibition

g7-NPs or C-NPs suspended in saline solution were injected i.p. in C57Bl6 mice. After 10 min, 30 min, 1 hr, 2 hr, 6 hr, or 5 days, animals were sacrificed. For histological and histochemistry processing, we refer to established procedure [4,5]. A total of six C57Bl6 mice hippocampi/group were infused unilaterally with 1 uL of chlorpromazine hydrochloride (Sigma-Aldrich) (30 μM) or staurosporine (Sigma-Aldrich) (60 μM) to inhibit clathrin-dependent and caveolin-dependent endocytosis, respectively.

2.4 Primary cell cultures, cell measurements and statistical analysis

Hippocampal neuronal cultures were prepared using a modified Banker culture protocol as reported in previous papers [4,5]. Immunofluorescence and immune-histochemistry were applied. Images were taken with a spinning disk confocal microscope from Zeiss and a fluorescence microscope. Quantification of fluorescence data was performed using ImageJ 1.47d for Macintosh and Axiovision.

3. RESULTS and DISCUSSION

3.1 Nanoparticle characterization

All the batches of NPs produced were analyzed for the surface properties (size and surface charge) and morphology. The results obtained by PCS analysis showed that both g7-NPs and C-NPs were sizing around 170 nm (±18 nm), with a polydispersity index (PDI) with values close to 0.02 (± 0.01) indicating a good homogeneity in dimensions. The surface charge, expressed as zeta potential (z-p), showed slightly negative charge values (-18 mV ± 5 mV). The SEM and AFM images demonstrated that g7-NPs are featured by spherical shapes with regular surfaces, also confirming the size distribution assessed by PCS analysis.

3.2 Nanoparticle accumulation in brain areas and cell type: in vivo evidence

In order to clarify the possible accumulation of g7-NPs in specific brain areas or cell populations, we injected g7-NPs and analyzed samples of different brain areas by confocal microscopy. Six hours after the injection, g7-NPs were widely distributed across brain regions, with higher concentrations in grey matter areas than in white matter areas (**Fig.1a,b** cerebral cortex vs. corpus callosum). Next, we performed detailed analysis within the hippocampal region, using different cell-type specific markers (NeuN, NPY for neuronal populations, GFAP for astroglia and Iba-1 for microglia). By far the largest population of cells with clear g7-NP accumulation were NeuN-ir neurons (**Fig.1i**). A clear accumulation of g7-NPs was also observed in some Iba-1-ir microglia (**Fig.1k**), whereas g7-NPs could only rarely be attributed to GFAP-ir astroglia (**Fig.1l**). g7-NP neuronal accumulation was heterogeneous, with preferential accumulation in interneurons of the stratum oriens and lacunosum-moleculare of Cornu Ammonis (CA) subfields as well as of the hilus of the dentate gyrus (DG), especially NPY interneurons (**Fig.1j**). Regarding the other major classes of hippocampal neurons, the g7-NP labeling was clearly present in CA3 pyramidal neurons, though at a level lower than in NPY interneurons, but labeling was almost undetectable in granule cells of the DG or CA1 pyramidal neurons.

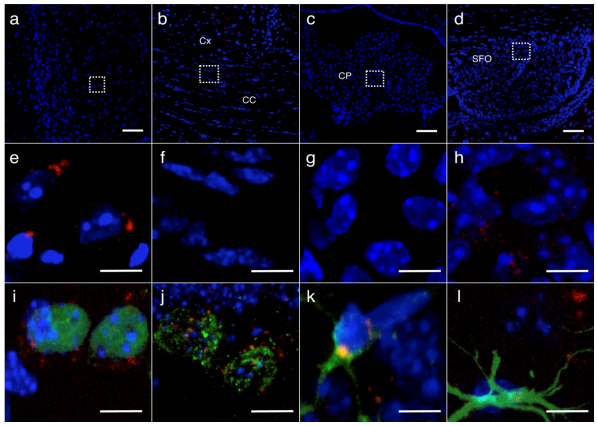

Figure 1

3.3 Nanoparticle uptake mechanisms

Recent studies reported that layered double hydroxide NPs internalization in neurons is mediated by clathrin-dependent endocytosis [6] and that NPs are targeted mainly to the clathrin endocytotic machinery [6,7]. Accordingly, g7-NP-containing vesicles showed characteristics of early endosomes derived from clathrin coated vesicles in primary hippocampal cultures and were negative for the caveola-related early endosome marker EEA1 (**Fig.2a-e**), but strongly positive for the clathrin-related early endosome marker Rab5 (**Fig.2f-j**).

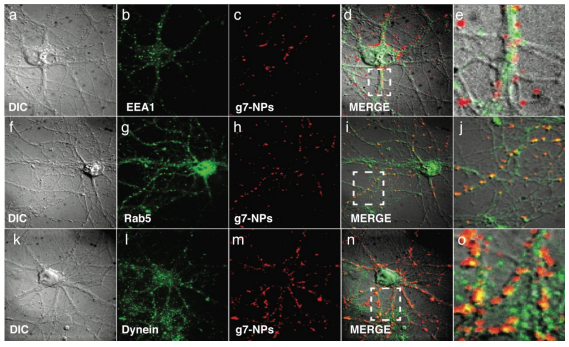

Figure 2

Analysis of the staining for clathrin revealed that a subset of g7-NPs in closer proximity to the plasma membrane colocalizes with clathrin positive signals (**Fig.3a-d**). In line with previous studies showing that inhibition of clathrin-coated pit endocytosis decreases intracellular uptake, whereas inhibition of caveola-coated pit endocytosis does not affect NP uptake [4,5], chlorpromazine hydrochloride significantly reduced the amount of endocytosed g7-NPs, while nystatin had no significant effect (**Fig.3e-m**). However, some g7-NPs were still found intracellular, suggesting that additional mechanisms, such as membrane permeabilization or fluid-phase endocytosis [7] may provide additional mechanisms for g7-N cell entry.

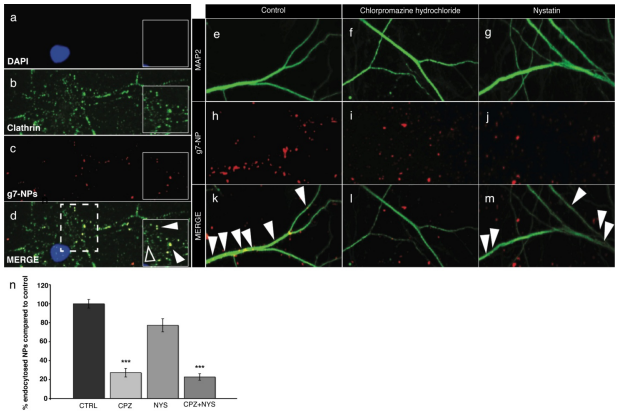

Figure 3

3.4 In vivo evidence

Based on these *in vitro* data, we sought to verify whether these uptake mechanisms of g7-NPs are active *in vivo* in the mouse brain upon peripheral g7-NP administration. First, we studied the possible co-localization of g7-NPs with the early endosome markers Rab5 and EAA1 (**Fig.4a-d**). In accordance with the *in vitro* data, there was an impressive co-localization of g7-NP and Rab5 signals in hippocampal interneurons (**Fig.4a,b**), but only minimal co-localization with EAA1 signals in the same cells (**Fig.4c,d**). Next, we tested whether neuronal uptake of g7-NPs *in vivo* is clathrin-dependent and/or caveolin-dependent. Thus, we infused chlorpromazine hydrochloride or staurosporine (an inhibitor of caveolin-dependent endocytosis) unilaterally within mouse hippocampus and 15 min later, injected g7-NPs (300 ag/300 µL /mouse) peripherally. Infusion of the respective vehicles was performed into the contra-lateral hippocampus. In accordance with the *in vitro* data, we observed a marked decrease of g7-NP signal

accumulation within hippocampal neurons in chlorpromazine-treated hippocampus (**Fig.4e,f**) but not in staurosporine-treated hippocampus (**Fig.4g,h**).

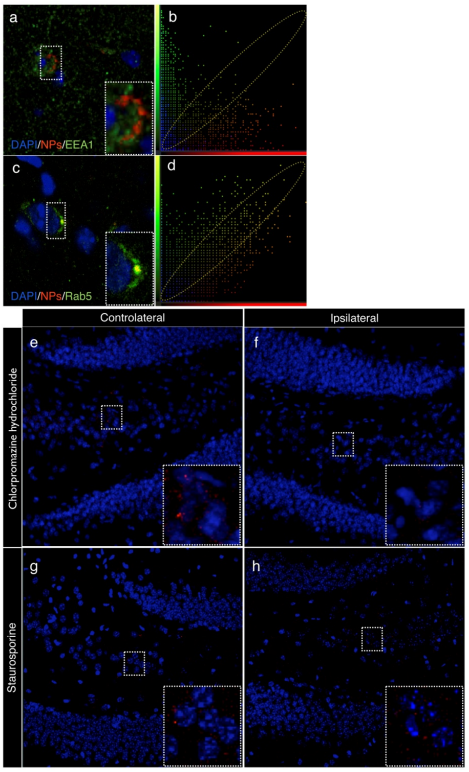

Figure 4

4. Conclusion

Drug delivery and targeting to the CNS represent a major challenge for pharmaceutical nanotechnologists. The fate of nanocarriers, once they have crossed the BBB, remains an open question, due to the lack of *in vivo* evidence and attempts to correlate *in vitro* and *in vivo* findings. Several insights were given in this research, regarding the dose/time-dependent accumulation of NPs in the brain, their regional distribution and cell tropisms, uptake mechanisms, inter- and intra-cellular transport, to finally show existing in vivo/in vitro correlations.

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