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Insight on the fate of CNS-targeted nanoparticles. Part II: Intercellular neuronal cell-to-cell transport

Giovanni Tosi [a], Antonietta Vilella [b], Resham Chhabra [c], Michael J. Schmeisser [d], Tobias M. Boeckers [d], Barbara Ruozi [a], Maria Angela Vandelli [a], Flavio Forni [a], Michele Zoli [b], Andreas M. Grabrucker [c,d,*]

[a] Pharmaceutical Technology, Te. Far. T. I. group, Department of Pharmaceutical Sciences, University of Modena and Reggio Emilia, Italy
[b] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy
[c] WG Molecular Analysis of Synaptopathies, Neurology Dept., Neurocenter of Ulm University, Ulm, Germany
[d] Institute for Anatomy and Cell Biology, Ulm University, Ulm, Germany

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ABSTRACT

The application of polymeric nanoparticles (NPs) has a promising future for targeting and delivering drugs into the central nervous system (CNS). However, the fate of NPs once entered in the brain after crossing the blood–brain barrier (BBB) and taken up into neuronal cells is a neglected area of study. Thus, here, we investigate the possible mechanisms of a cell-to-cell transport of poly-lactide-co-glycolide (PLGA) NPs modified with a glycopeptide (*g7*-NPs), already demonstrated to be able to cross the BBB after *in vivo* administration in rodents. We also tested antibody (Ab) -modified *g7*-NPs both *in vitro* and *in vivo* to investigate the possibility of specific targeting. Our results show that *g7*-NPs can be transported intra- and inter-cellularly within vesicles after vesicular internalization. Moreover, 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*. The transport is dependent on F-actin and can be increased by induction of TNT-like structures overexpressing M-Sec, a central factor and inducer of TNT formation. Moreover, cell-to-cell transport occurs independently from NP surface modification with antibodies. These *in vitro* findings were in part confirmed by *in vivo* evidence after i.p. administration of NPs in mice.

1. Introduction

To circumvent the blood–brain barrier (BBB) for the delivery of drugs to the central nervous system (CNS), various strategies have been developed such as the disruption of the BBB [1,2], chemical modification of the drugs to facilitate membrane permeability [3,4] and carrier- or receptor-mediated drug delivery. Other alternative routes bypass the blood circulation altogether. For example, drugs can be delivered directly from nose to brain [5] or delivered by intraventricular infusion [6]. However, all these methods have limitations such as poor efficacy or invasivity. Thus, one of the major challenges of pharmaceutical research is to develop strategies for effective delivery of drugs to the CNS. To achieve this, an emerging approach is the use of nanosized carriers such as liposomes and polymeric nanoparticles (NPs) [7–17]. Specifically, engineered NPs could take advantage of various pathways for crossing the BBB, such as endocytosis or transcytosis. As shown in previous studies [18–25], we applied this approach by modifying polylactide-co-glycolide (PLGA) NPs with a glycopeptide (*g7*) to produce nanosystems (*g7*-NPs) that are able to enter the brain. The histological, biodistribution and pharmacological studies in rodents have shown that g7-NPs cross the BBB at a high rate following several modes of administration [18–25]. Additionally, *g7*-NPs were found to exploit endocytotic pathways, both to cross the BBB endothelium *in vivo* and to enter neurons or glial cells *in vitro* [26].

In the accompanying paper (Part I) [27], we focused on some still poorly described aspects, such as insights on dose/time-dependent accumulation of *g7*-NPs, tropism to specific brain regions and cell subpopulations after BBB crossing, and establishment of a correlation between *in vivo* and *in vitro* findings on the uptake mechanisms in neurons.

Despite the evidence obtained on these topics, some issues are still to be studied in depth to display a more clear picture of NP uptake, dynamics, transport and trafficking inside the CNS. In particular, we have investigated neuronal tropism, the possibility of cell targeting using antibody (Ab) modified NPs, the possible transport of NPs from cell to cell and the dynamics of intercellular NP trafficking.

In vivo, most cells can be found organized as specialized tissues. Within these functional assemblies, cells are able to perform cell-to-cell communication including the transport of intracellular components to organize physiological processes. Within the brain, neurons are closely surrounded by glial cells, leaving only a minimal amount of extracellular space. Thus, this part II study aims to evaluate the mechanisms of a possible intercellular transport of *g7*-NPs from cell to cell besides their transport within the extracellular fluid after crossing the BBB endothelium.

* Corresponding author at: Ulm University, WG Molecular Analysis of Synaptopathies, Anatomy and Cell Biology, Albert-Einstein Allee 11, 89081 Ulm, Germany. Tel.: +49 731 500 23214 (office); fax: +49 731 500 1223214.
E-mail address: andreas.grabrucker@uni-ulm.de (A.M. Grabrucker).

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Furthermore, in this paper, for the first time, we investigate the pathway of NP transport, taking into consideration the recent discovery of tunneling nanotubes (TNTs). This type of cell-to-cell communication was found to be facilitated by the formation of membranous F-actin-rich structures between cells. TNTs mediate the intercellular transport of various cellular components by generating membrane continuity between cells [28]. That way, the direct transfer of cargos between cells in cell-to-cell communication is possible. Intriguingly, previous findings on Quantum Dot NPs showed that these systems are actively transported via membrane nanotubes between cardiac myocytes in a bidirectional mode (with a mean velocity of 1.23 μm/s). Specific labeling of F-actin clearly demonstrated the pivotal role of F-actin in transport between myocytes [29]. Moreover, the presence of TNTs in neurons and their pivotal role in transporting prions from cell to cell was shown [30], as well as the possibility of decreasing or increasing TNT number by specific inhibitors (i.e., Latrunculin B) [31] and TNT formation enhancers (i.e., M-Sec) [32].

2. Material and methods

2.1. Chemicals and reagents

Latrunculin B was purchased from Sigma-Aldrich (Milan, Italy) and prepared as 5 μM solution. Exo1 was purchased from Tocris (Tocris Bioscience, Bristol, UK). Abs were purchased from the following companies: M-Sec from Santa Cruz Biotechnology (Dallas, TX, USA), microtubule-associated protein 2 (Map2) from Chemicon (Temecula, CA, USA), and vesicular GABA transporter (vGAT) from Synaptic Systems (Goettingen, Germany). Neural cell adhesion molecule 1 (NCAM1) and CD44 antibodies were purchased from BD Biosciences (Buccinasco, Italy) and used as described previously [17]. FM1-43FX and FM4-64 were purchased from Invitrogen (Monza, Italy) and used according to the manufacturer's manual. Poly(D, L-lactide-co-glycolide) (PLGA RG503H, MW near to 11,000) was used as received from the manufacturer (Boehringer-Ingelheim, Ingelheim am Rhein, Germany). Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-Glucose)-$CONH_2$ (*g7*) was prepared and conjugated with PLGA to obtain *g7*-PLGA [9–16]. PLGA conjugated with Rhodamine B piperazine amide (Sigma-Aldrich) (R-PLGA) was prepared as previously described [33]. A MilliQ water system (Millipore, Bedford, MA, USA) provided high-purity water (18 MΩ). All other chemicals were of analytical grade and purchased from Sigma-Aldrich.

2.2. Preparation and characterization of nanoparticles

2.2.1. Preparation of rhodamine labeled g7-NPs (R-g7-NPs)

NPs were prepared as described in literature [34] with some modifications in the preparation procedure already described before [18–25].

2.2.2. Preparation of antibody-modified R-g7-NPs (NCAM-R-g7-NPs and CD44-R-g7-NPs)

Ab modified *g7*-NPs were prepared starting from *g7*-NPs (obtained as described before [26]), and well known methodologies were used for Ab-surface engineering of NPs [26,35,36]. Briefly, g7-NPs were first activated in the presence of EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, 170 mg) and NHS (N–hydroxy-succinimide, 30 mg) (g7-NPs-NHS), and then the free primary amine groups on the g7-NPs surface were conjugated with the carboxylic groups of the Ab molecules. Desired amounts of g7-NPs-NHS (50 mg) were suspended and stirred at RT for 1.5 h in MES (2-(N-morpholino)ethanesulfonic acid, Sigma Aldrich) buffer with designated volume of Anti-NCAM1 Ab (50 μl of a 1 mg/ml stock solution) or Anti-CD44 Ab (50 μl of a 0.5 mg/ml stock solution), in order to obtain NCAM-*g7*-NPs and CD44-*g7*-NPs respectively.

After the reaction, the Ab-modified NPs (NCAM-*g7*-NPs or CD44-*g7*-NPs) were collected by centrifugation, and were further purified by washing twice with distilled water. Some amounts of Ab-modified NPs were re-suspended in ultrapure water for further characterization, and rest of the NPs were lyophilized to prepare stocks (trealose, in ratio 1:1 w/w with respect to NPs was added as cryoprotector).

As previously established in literature [26,36], the efficiency of surface engineering of *g7*-NPs with Ab moieties was demonstrated by Electron Spectroscopy for Chemical Analysis (ESCA) study, performed on a 04–153 X-ray source analysis system (PHI, Uvalca-PHI, Tokyo, Japan) and an EA11 hemispherical electron analyzer (Leybold Optics, Alzenau, Germany) using MgKα1,2 radiations. The spectra were recorded in fixed retardation ratio (FAT) mode with 190 eV pass energy. The pressure in the sample analysis chamber was approximately 10–9 mbar. The data were acquired and processed using the RBD AugerScan 2. ^{1}H-HRMAS NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance 400 instrument and D2O was added to the sample, which was then spun at 4000 Hz. All observations were recorded at RT.

2.2.3. Chemico-physical characterization of NPs

All batches of NPs (*g7*-NPs, NCAM-*g7*-NPs and CD44-*g7*-NPs) were characterized regarding their surface properties, size and shape. A scanning electron microscope (SEM) (XL-40 Philips, Eindhoven, The Netherlands) (×10,000) was used to evaluate the morphology of NPs. Before the SEM analysis, the samples were coated under argon enriched atmosphere with a 10 nm layer of palladium gold (Emitech K550 Supper Coated, Emitech, U.K.). Moreover, the morphology of the NPs was evaluated using an atomic force microscope (AFM) (Park Instruments, Sunnyvale, CA, USA) at room temperature (about 20–25 °C) operating in air and in non-contact mode. Briefly, triangular silicon tips were used for this analysis. The resonant frequencies of this cantilever were found to be about 160 kHz. A drop of each sample suspension was diluted with water (0.01 mg/ml) before being applied on a small mica disk (1 cm × 1 cm); after 2 min, the excess of water was removed using filter paper. Two types of images were obtained: a topographical image and an "error signal". The error signal is obtained by comparing two signals: the first one representing the amplitude of vibrations of the cantilever, and the other one representing the amplitude of a reference point. The images obtained by this method show small superficial variations of the samples. Images were flattened using second-order fitting to remove sample tilt.

All NPs in distilled water were analyzed for particle size and zeta potential (z-p) by photon correlation spectroscopy and laser Doppler anemometry using a Zetasizer Nano ZS (Malvern, UK; Laser 4 mW He–Ne, 633 nm, Laser attenuator Automatic, transmission 100% to 0.0003%, Detector Avalanche photodiode, Q.E. > 50% at 633 nm, T = 25 °C). The results were normalized with respect to a polystyrene standard solution.

The stability of all NP formulations was studied by incubating a fixed amount of NPs (1 mg) in 1 ml of saline solution, ringer solution or serum with stirring at 37 °C for 24 h. The NPs were then washed and purified by multiple centrifugations and re-suspended in distilled water for analysis aiming to establish surface properties (size, charge, surface composition) and functionality.

In order to further assess the presence of Ab on the surface of NPs, we incubated the Ab-modified NPs (NCAM-g7-NPs or CD44-g7-NPs) with nanogold (mono-sulfo-N-Hydroxy-succinimido Nanogold®, Nanoprobes, Inc., NY 11980–2301 USA) of 1.4 nm in size and able to react with Ab, to provide a qualitative identification of Ab. As control, we performed the same coupling reaction using *g7*-NPs.

Following the procedures given by the manufacturer, a weighted amount (1 mg) of NPs (g7-NPs, NCAM-g7-NPs, CD44-g7-NPs) was suspended in phosphate buffer (pH 7.8) and added to 300 μl of a resuspension of nanogold, made to a final volume of 3 ml of solution. After purification, the NPs coupled with nanogold were processed for Transmission Electron Microscopy (TEM) analysis. In particular, negative staining TEM was used for the evaluation of the inner morphology of all batches of NPs. A drop of a water-diluted suspension of the purified nanoparticles (about 0.05 mg/ml) was placed on a 200-mesh formvar copper grid (TABB Laboratories Equipment, Berks, UK), and allowed to adsorb. The excess suspension was removed by filter paper. A drop of