Poly(ethylene glycol)-g-Chitosan Nanoparticles Functionalized with the Monoclonal Antibody OX26 for Brain Drug Targeting

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Aim: Drug targeting to the Central Nervous System (CNS) is challenging due to the presence of blood–brain barrier (BBB). We investigated Chitosan (Cs) nanoparticles (NPs) as drug transporter system across the BBB, based on mAb OX26 modified Cs. **Materials & methods:** Cs NPs functionalized with polyethylene glycol (PEG), modified and un-modified with OX26 (Cs-PEG-OX26) were prepared and chemico-physically characterized. These NPs were administered (i.p.) in mice to define their ability to reach the brain. **Results:** Brain uptake of OX26-conjugated NPs is much higher than of un-modified NPs, because: long-circulating abilities (conferred by PEG), interaction between cationic Cs and brain endothelium negative charges and OX26 TfR receptor affinity. **Conclusion:** Cs-PEG-OX26 NPs are promising drug delivery system to the CNS.

Keywords: Chitosan, nanoparticles, drug targeting, monoclonal antibody OX26, Blood-Brain Barrier (BBB).

Introduction

The blood–brain barrier (BBB) is considered as the most insurmountable barrier to protect the brain, constituting a homeostatic defense of the brain against pathogens and toxins. The BBB is formed by capillary endothelial cells, which are connected by tight junctions that govern the diffusion of molecules across the vessels. Some small molecules with appropriate solubility

features, molecular weight and charge are able to diffuse from blood into the Central Nervous System (CNS) [1], by passive or active transport mechanisms.

Passive transport includes para-cellular diffusion of hydrophilic compounds with a molecular weight lower than 150 Da, and trans-cellular transport which is used by small lipophilic molecules with a molecular weight lower than 400–600 Da [2]. Active transports include mainly carrier-mediated, adsorptive-mediated, receptor-mediated and cell-mediated transcytosis. Adsorptive-mediated transcytosis, also known as the pinocytosis route, is based on the electrostatic interaction between a positively charged substance and the negatively charged plasma membrane surface (i.e. heparin sulphate proteoglycans) [3], triggering vesicles formation and transport across the barrier. One of the most interesting processes (receptor-mediated transcytosis) provides a selective uptake of macromolecules taking advantage of the receptors for many different types of molecules such as transferrin (TfR), insulin, lipoprotein, diphtheria toxin, glutathione and leptin transporter on the BBB endothelial cells [4][5]. Finally, cell-mediated transcytosis, as transport mechanism, relies on immune cells such as monocytes or macrophages to cross the BBB [6].

Despite the wide variety of mechanisms, not all the therapeutically active substances are able to cross the BBB. In order to increase the drug concentrations in the CNS, different approaches were attempted for the treatment of neurodegenerative diseases exploiting both invasive [7][8] or non-invasive methods. Non-invasive strategies for brain targeting include chemical modifications of the drugs (i.e. prodrugs), biochemical approaches (i.e. conjugation with specific antibodies) [9] or colloidal approach, based on nano-medicines, in particular, biodegradable polymeric nanoparticles (NPs) [10][11][12]. Advantages of this last strategy rely on the high chemical and biological stability of NPs, on the feasibility of incorporating both hydrophilic and hydrophobic pharmaceuticals by adsorption or encapsulation and on the possibility to administer the NPs by different routes (including nose-to-brain, oral and parenteral) [1][13][14]. However, BBB crossing of drugs loaded in NPs could be improved, in terms of efficiency, by the surface modification with targeting ligands [15]. In particular, OX26 monoclonal antibody is one of the most widely used to direct NPs to the brain, due to its high selectivity towards the Transferrin receptor (TfR) that is present in the brain capillary endothelial cells [16][17][18].

A large number of polymers can be used in the preparation of NPs for brain targeting. Our choice fell on Chitosan (Cs), a linear, biocompatible [19], and biodegradable [20] polysaccharide, obtained from chitin by a deacetylation process involving alkaline hydrolysis [21]. Cs is composed by two repeating units randomly distributed, D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), linked by β-(1→4)-glycosidic bonds. One of the major advantages of Cs is its polycationic nature, since its high positive charge density leads to a high capacity of bioadhesion to cellular barriers. From the chemical point of view, Cs has the great advantage of owning reactive functional groups (amine and hydroxyl) providing a biopolymeric specific platform for side group attachment under mild reaction conditions, allowing the manipulation of the Cs properties through chemical modifications [22][23].

In view of its versatility, Cs is an excellent candidate for pharmaceutical applications. As a consequence, Cs NPs, obtained by different methodologies [24] (covalent cross-linking, precipitation, self-assembly, spray-drying and ionic cross-linking with tripolyphosphate), were employed in targeted therapy for colon or mucosal delivery [25], cancer treatment [26], or in delivering of vaccines, genes and peptides [27]. Moreover, as Cs NPs are unstable in the bloodstream owing to the activation of the reticuloendothelial system (RES), PEGylation of Cs NPs surface is strongly required [28]. The presence of PEG in the formulation does not produce damages on the brain cells as it was demonstrated by means of neuronal safety profiles of pegylated nanocarriers both *in vitro* and *in vivo* [29][30][31].

From the safety point of view, Cs NPs were deeply investigated in order to assess if cationic polymer-based NPs were toxic or not. In particular, regarding chitosan NPs aimed to brain delivery, it was reported [32] that the maximum tolerated dose (after oral administration) of chitosan NPs loaded with an antidepressant-like drug (minocycline) was in the range of 350 mg/kg for loaded chitosan NPs versus 319 mg/Kg recorded for the drug alone, clearly indicating a modest or rather absent toxicity of the vehicle. More generally, as reported recently [33], CS is biodegraded by several enzymes present in human mucosas and in other physiological fluids [20][34] and, even if very old studies performed in rabbits and dogs declared the begin of signs of cytoxicity after subcutaneous injection in the range of 5–50 mg/kg/day [35], the presence of at least 15 clinical trials [36] listing chitosan-based systems as an interventions could be considered as a good proof of absence of significant toxicity of chitosan-based drug delivery system. The degree of cell toxicity with chitosan nanoparticles depends on the concentration and the surface charge density of the particles, the last one can be modulated with the degree of deacetylation and the molecular weight of the polymer [37][20]. To reduce the cytotoxicity, hydrophilic molecules like polyethylene glycol (PEG) have been employed. PEG not only reduces toxicity but also prevents any unintended interaction of the encapsulated therapeutic molecule with cellular or serum proteins, thus enhancing stability of the nanoparticle and avoiding macrophage uptake [38].

On the basis of these considerations, this study aims to further investigate a Cs NPs drug delivery system able to reach and cross the BBB [39]. To increase the BBB crossing efficiency, the positively charged Cs NPs were thus conjugated with both polyethylene glycol (PEG) to enhance the plasma residence time and with the monoclonal antibody OX26 (using the hydroxysuccinimide/carbodiimide approach) to optimize their passage through the BBB.

Materials & methods

Animals

BALB/c mice weighing 25 ± 5 g (Charles River, Lecco, Italy) were used for the *in vivo* experiments. The animals were maintained at 25°C for an average period of 15 days before the experiments, on standard diet and water *ad libitum*. The experiments were carried out in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) for experimental care.

Chemicals

Chitosan (Cs) (molecular weight 80 kDa; degree of deacetylation 82%), fluorescein isothiocyanate (FITC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), succinic anhydride, *N*-hydroxysuccinimide (NHS), poly(ethylene glycol)methyl ether (mPEG; average Mw 5 kDa) and 2-(N-morpholino)ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (St.Louis, Missouri; USA). Pentasodium tripolyphosphate (TPP) was obtained from Protokimica Chemicals (Medellín; Colombia). Purified monoclonal mouse anti–rat transferrin receptor IgG2a (clone OX26) was purchased from BD Biosciences (Milan; Italy), Nuclear antigen (NeuN) from Millipore (Billerica, MA, USA) and Mouse IgG2a ELISA quantification kit (Cat. No. E99-107) from Bethyl Laboratories (Montgomery, AL; USA). A Milli-Q water system (Millipore) supplied with distilled water provided high-purity-water. All the other chemicals and solvents were obtained from commercial sources and used without purification.

Synthesis and characterization of Succinic Anhydride-Conjugated Chitosan (SCs)

Cs (500 mg) was firstly dissolved in 125 mL 1% (v/v) acetic acid and the resulting solution was freeze-dried without any cryoprotector (FreeZone®Freeze Dryers, LABCONCO, Kansas City, USA) to obtain an acetate salt form of Cs. Chitosan acetate (200 mg) was dissolved in high-purity-water (30 mL) and succinic anhydride (39 mg) was added keeping the solution under magnetic stirring (300 rpm) for 1 h. The pH of the mixture was adjusted to 8.0 with 0.2 M $NaHCO_3$ and the reaction proceeded under magnetic stirring (300 rpm) at r.t. for 15 h. The obtained succinic anhydride-conjugated chitosan (SCs) was precipitated in an excess of acetone, centrifuged to remove the solvent and then washed three times with ethanol, and subsequently dried at 60 ^{0}C for 24 h.

To analyze and detect the functional groups on Cs and SCs, Fourier Transform Infrared (FTIR) KBr pellets spectra were recorded between 4000-450 cm^{-1} at r.t. using a Spectrum One equipment (Perkin-Elmer, Waltham, MA; USA).

The degree of succinylation and molecular weight of the modified Cs were determined by ^{1}H-NMR (D_2O/CH_3COOH) with an Avance 400 BrukerBiospin spectrometer (Billerica, MA, USA).

Solubility of polymers, which is indicative of modification in polarity of conjugated compounds, was evaluated at different pH values through transmittance measurements of Cs and SCs solutions with a UV/Vis spectrophotometer (Lambda35, Perkin-Elmer) at 600 nm. Briefly, 2 mL of Cs or SCs (0.01 mM in 1% (v/v) acetic acid) were taken and the pH was adjusted to different values with the addition of 5 M NaOH. The polymers were considered as insoluble when the transmittance of the solution was lower than 50%, compared to that of a control solution of 1% (v/v) acetic acid.

The pH-dependent charge profile was monitored by evaluating the ζ potential (ZetasizerNano ZS, Malvern Instrument, 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 ¼ 25 °C) of Cs or SCs solutions (0.01 mM in 1% (v/v) acetic acid) at various pH values ranging from

3 to 11. The isoelectric point was considered as the value where the ζ potential was equal to zero [40][41].

Preparation and characterization of poly(ethylene glycol)-g-chitosan (mPEG-g-Cs)

In a first step mPEG was modified with succinic anhydride to obtain carboxylic acid terminal groups (mPEG-COOH) for the grafting reaction with Cs. Briefly, mPEG (20 mg), dissolved in DMF (50 mL) at r.t., was added to succinic anhydride (mPEG:succinic anhydride, 1:1 molar ratio). The reaction was carried out at 60 ^{0}C over 15 h in the presence of pyridine as catalyst, and the product was precipitated in ethyl ether, filtered under vacuum, and washed several times with ethyl ether. The solid mPEG-COOH was vacuum dried at 50 ^{0}C.

In a second step mPEG-COOH was grafted onto Cs, using the carbodiimide coupling between the -COOH groups of mPEG and -NH_2 groups of Cs. Briefly, Cs (500 mg) was dissolved in 125 mL 0.1 M HCl and the resulting solution was freeze-dried to obtain the Cs·chlorohydrate salt (Cs HCl), which was dissolved in high-purity-water. Then, mPEG-COOH (15 mg), and NHS (1.7 mg) were added to 15 mL of Cs·HCl water solution (6.6 mg/mL) and the mixture was magnetically stirred for 5 min. Finally EDC (22 mg) was slowly added and the reaction was carried out at r.t. for 22 h. The mixture was dialyzed against Milli-Q water over 3 days with a cellulose regenerate membrane (MWCO 6-8 kDa, Spectrum Labs, Rancho Dominguez, CA; USA), and finally lyophilized without any cryoprotector (FreeZone®Freeze Dryers, LABCONCO) to give mPEG-g-Cs (weight yield about 90%).

Preparation and characterization of FITC-labeled Chitosan (FCs)

Fluorescein isothiocyanate (FITC) was covalently linked to the –NH_2 groups of Cs by well-known procedures [42]. Briefly, a solution of Cs [0.25% (w/v) in 1% (v/v) acetic acid] was added to an ethanol solution of FITC (0.34 mg/mL, molar ratio FITC:NH_2 groups 0.06:1) and the reaction was kept at r.t. over 16 h under darkness and magnetic stirring (300 rpm). The product was precipitated with 5 M NaOH, centrifuged and separated from unreacted FITC by washing with a mixture ethanol:water (70/30 v/v) until no evidence of FITC was found (checked by absorbance measurements at 490 nm). Finally, the FITC-labeled Cs was re-dissolved in 1% (v/v) acetic acid and dialyzed under darkness through regenerated cellulose membrane (MWCO 6-8 kDa, Spectrum Labs) against Milli-Q water for 48 h. The final product was obtained by freeze-drying without any cryoprotector.

To determine the FITC labeling efficiency, the visible absorbance intensity (λ = 490 nm) of an exact amount of FITC Cs (25 mg), dissolved in 1% (v/v) acetic acid and diluted 2.5 times with a pH 8.0 phosphate buffer solution, was measured. The UV method was previously standardized with solutions of 0.1 to 1.0 µg/mL of FITC prepared by diluting a methanol stock solution of FITC with pH 8.0 phosphate buffer (the calibration curve is shown in the Figure. S1, supplementary information).

The FITC labeling efficiency was assessed in the order of 13.5% molar percentage of FITC per molar unit of Cs.

Preparation of the nanoparticles (NPs) from the modified polymers

NPs were prepared using the ionotropic gelation method [43] through the electrostatic interaction between positively charged Cs and negatively charged TPP.

SCs, mPEG-g-Cs (PCs) and Cs were dissolved separately (1 mg/mL in 1% (v/v) acetic acid solution) under stirring (400 rpm). Then SCs, PCs and Cs solutions (50/40/10, w/w) were mixed and the solution pH value was adjusted to 5.0 with 5 M NaOH. TPP was dissolved in high-purity-water (1 mg/mL) and the pH value was adjusted to 9.0. NPs were formed adding dropwise the TPP solution to SCs/PCs/Cs solution (SCs/PCs/Cs:TPP ratio 3:1 w/w) under magnetic stirring (500 rpm) for 1 h at r.t. NPs were purified through ultra-filtration using a regenerated cellulose membrane with MWCO of 1 kDa (Millipore Corporation), washed several times with Milli Q water and finally recovered in pH 6.0 MES buffer to obtain SCs/PCs/Cs NPs.

To prepare the FITC labeled NPs (SCs/PCs/FCs NPs), the same procedure was followed but FCs was added to the polymeric solution [SCs/PCs/FCs (50/40/10 w/w)] before the addition of TPP. Polyvynil-alchol (PVA), as surfactant, could be alternatively be used to stabilize the suspension of NPs (0.1 % m/m) [44][45][46].

Functionalization of nanoparticles with OX26 antibody (SCs/PCs/Cs-OX26 NPs, and SCs/PCs/FCs-OX26 NPs)

To conjugate antibody onto NPs surface, NHS-EDC technology was employed [47]. Briefly, NPs (un-labelled SCs/PCs/Cs NPs and labeled SCs/PCs/FCs) (15 mg) dispersed in pH 6.0 MES buffer (6 mL) reacted with NHS and EDC (NPs/NHS, 1/1 w/w ratio and NPs/EDC, 1/3 w/w ratio). The NPs dispersion was stirred at r.t. for 6 h. Then, the activated NPs were separated by ultra-filtration using a regenerated cellulose membrane with MWCO of 1 kDa (Millipore Corporation). Subsequently, 200 µl of OX26 in MES buffer (0.5 mg/mL) was added to the dispersion of activated NPs (2 mg/mL) and the reaction was carried out under magnetic stirring (300 rpm) at r.t. for 15 h. The mixture was centrifuged at 20,000 rpm for 60 min at 4 ^{o}C (UC1 NPs) and this pellet, after water washing, was re-centrifuged (UC2 NPs) in order to remove the non-bonded OX26 antibody.

Quantification of OX26 antibody in the conjugated nanoparticles

The quantification of OX26 was performed on un-labeled NPs in order to avoid any possible interference of the FITC labeling compound during the analysis. The supernatants obtained by the purification of NPs after the OX26 coupling procedure were submitted to reaction with the Mouse IgG2a ELISA quantification kit, according to the standard procedure (see supplementary information). The amount of OX26 bound to NPs was calculated indirectly, subtracting the measured amount of unbound OX26 present in the supernatants from the initial amount of OX26 used for the OX26-NPs coupling.

Particle size/ζ potential/TEM analysis of the NPs

Particle size and ζ potential of all the NPs samples (see Table 1) were measured at 25^{o}C, before and after each process involved in the functionalization with the OX26 antibody, using a Zetasizer Nano ZS (Malvern Instrument, Photon-Correlation Spectroscopy, PCS). The results were normalized with respect to a polystyrene standard solution.

The morphological and microstructural characterization was carried out through FEG-SEM microscopy (field emission gun scanning electron microscopy, Nova NanoSEM 450, FEI Co., Hillsboro, OR, USA) by using a scanning transmission electron microscopy detector (STEM II detector; TEM mode). Briefly, 10 uL of suspension of antibody (SCs/PCs/FCs-OX26 NPs) and antbody free (SCs/PCs/FCs NPs) samples were placed on a 200-mesh formvar copper grid (TABB Laboratories Equipment, Berks, UK). The liquid was evaporated at room temperature and the samples observed operating under an acceleration voltage of 30 KV, spot size 3 um, field free lens mode and working distance of about 8 mm.

Measurement of the storage stability of NPs

In order to analyze the colloidal stability of the nanoparticles during storage time, the variation of the mean diameter of nanoparticles was used. The nanoparticles in MES buffer (pH 6.0) were stored in polypropylene vials at 4 °C for 4 weeks, and the particle size was measured at various time intervals.

***In vivo* experiments: NPs brain uptake**

In vivo experiments were performed to determine the ability to cross the BBB of both SCs/PCs/FCs-OX26 NPs and non OX26 conjugated NPs (SCs/PCs/FCs). As a control standardization, a preliminary analysis on the emission/excitation profile of both labeled polymer and labeled NPs was performed with confocal microscopy (Leica DM IRE2, Bannockburn, IL, USA; Leica Confocal System: scan head multiband 3 channels Leica TCS SP2 with AOBS, laser diode blue (405 nm / 25 mW), Laser Ar (458 nm / 5 mW) (476 nm / 5 mW) (488 nm / 20 mW) (496 nm / 5 mW) (514 nm / 20 mW), Laser HeNe (543 nm / 1.2 mW), Laser HeNe (594 nm) (Orange), Laser HeNe (633 nm / 102 mW) (see Figure S3, supplementary information).

In vivo experiments were carried out by injecting via i.p. in BALB/c mice targeted SCs/PCs/FCs-OX26 NPs and control SCs/PCs/FCs NPs suspended in saline solution (100 µL, 2 mg/mL). The mice were sacrificed 2 h after injection through intracardiac perfusion with 4% (w/v) paraformaldehyde solution, in order to fix the tissues for immunohistochemistry studies [48][49]. The brain was then removed and sections (thickness approximately 50 µm) of different brain regions (hippocampus, cortex, striatus, corpus callosum and thalamus) were subjected to an immunohistochemical treatment. Briefly, after five washes with 1x PBS pH 7.4 for 10 min, blocking was performed for 1 h at r.t. in a 1x PBS solution containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA). Incubation with primary antibodies diluted in 0.3% Triton X-100, 1% normal serum (NS) and 1x PBS was performed overnight at 4 °C. After three washes in PBS / 0.1% Triton X-100, incubation with goat anti mouse Alexa 594 (1:200) or goat anti rabbit Alexa 594 (1:200) secondary antibody (Life Technologies, Carlsbad, CA, USA) in 0.2% Triton X-

100, 1% NS and PBS was carried out for 90 min at r.t. After washing three times with PBS for 10 min, brain sections were placed on gelatinised glass slides, dried and, after incubation with DAPI (which forms fluorescent complex with natural double-stranded DNA) [48][49], mounted for confocal microscopy analysis.

In order to give a semi-quantitative measurement of the number of NPs per brain areas, the confocal images of brain sections were submitted to an image analysis software (ImageJ) [50] using well established protocols [48][49][51]. For detection and numbering of NPs signals, we set up parameters in order to consider as single positive signal a number of pixel corresponding to 250 nm, indicating therefore one single NPs. Regarding confocal images analzyed, NPs profiles were selected from background using the threshold function and optimizing pre-selected parameters (see supplementary file, Figure S3). In particular, confocal analyses were conducted on FCs polymer and FCs NPs, after excitation at 488 nm and recording emission profile every 5 nm. Maximum peak of emission was recorded at 515-525 nm for both samples. Moreover, for image analysis quantification we did not manipulate or over-expose any image, by maintaining constant the potency of the laser, the excitation and emission ranges, the gain of signals, threshold limits and all other confocal parameters (pinhole opening, between-line modalities, etc.). Thereafter, for each treatment, 10 cells/section in 3 animals were analyzed and the number of NPs/brain areas was calculated.

Statistical analysis

All data were expressed as averages with standard deviations. Mean comparisons were made by the Student t-test. The difference was considered significant when the p-value was less than 0.05. ANOVA was used to determine the statistical difference among the groups, and then pair-wise comparison was made using the paired t-test. A p-value < 0.05 on a 2-tailed test was considered statistically significant.

Results & discussion

Characterization of SCs

The -NH_2 groups into the Cs backbone reacted with succinic anhydride to form amide bonds, leaving free pendant carboxylic acid groups, which were then used for anchoring antibody onto the NPs surface.

In Figure 1, Cs spectrum shows the characteristic absorption bands. The broad band at 3380 cm^{-1} is assigned to the stretching vibrations of inter-molecular hydrogen bonded of -OH and -NH or –NH_2 groups. The bands at 1597 cm^{-1} and 1647 cm^{-1} correspond to the asymmetric bending of –NH_2 band and the C=O stretching, respectively. The adsorption at 1382 cm^{-1} corresponds to C–N stretching vibration. In addition, the absorption bands at 1153 cm^{-1} (asymmetric stretching of C-O-C bridge), 1078 cm^{-1} and 1030 cm^{-1} (skeletal vibrations due to C–O stretching) are all characteristic vibration frequencies of the Cs polysaccharide structure. Compared to the IR spectrum of Cs, SCs showed absorption bands centered at 1663 cm^{-1} and 1567 cm^{-1} assigned to C=O stretching and to N-H flexion of the amide formed with

succinic anhydride. These results confirm the succinyl group substitution at the N-position of Cs chains.

The ^{1}H-NMR spectrum of Cs is shown in Figure 2A. According to previous reports [52], the assignment of signals is as follows: δ= 3.15 (H2 of glucosamine); δ = 3.45–4.20 (H2` of N-acetyl glucosamine, H3, H4, H5, and H6); δ=4.86 (H1). The ^{1}H-NMR spectrum of SCs (Figure 2B) shows a new signal at δ= 2.4-2.7 (NH-CO-C**H$_2$**C**H$_2$-**COOH, a-a* protons) [52]. The average population of succinyl groups substituted at the N-positions of the Cs chains (i.e., the degree of substitution, DS = 28%) was determined from the ratio of the integral peak of -C**H$_2$**C**H$_2$-** at 2.4-2.7 ppm and the integral peak of H2 at 3.15 ppm in the Cs structure, according to equation (1). This corresponds to a molecular weight about 91 kDa for the SCs.

$$DS(\%) = \frac{I_{2.4-2.7\ ppm}}{4 * I_{3.15\ ppm} + I_{2.4-2.7\ ppm}} * 100 \qquad (1)$$

The solubility of Cs and SCs, determined by transmittance measurements at 600 nm, (Figure 3) showed that unmodified Cs started to precipitate from a solution at pH 6.5, when the value of the transmittance drops down to 50%. On the contrary, SCs was practically soluble throughout the analyzed pH range, since the value of transmittance remains about 100%. The significant increase in solubility of Cs-derivative confirm the conjugation of -COOH groups to the Cs backbone.

Moreover, to further confirm the modification of Cs with succinic anhydride, the charge of the modified polymer, expressed as ζ potential, was measured at different pH values. The finding (Figure 4) correlates with the pH-dependent solubility profile. The unmodified chitosan presented an isoelectric point (IEP) of 7.9, as shown in the insert of Figure 4, which corresponds to the pH value at which the turbidity started to reach a plateau (Figure 3). SCs presented an isoelectric point of 7.2. Below this value (acid pH) its surface charge is positive due to the protonation of NH_2 groups and turned negative at basic pH due to deprotonation of -COOH groups. These findings are consistent with those reported in the literature [53].

Characterization of mPEG-g-Cs

In Figure 5A and 5B, the FTIR spectra of mPEG and mPEG-COOH are shown. Characteristic absorptions of mPEG at 1113 cm^{-1} (C-O-C stretching) and at 2887 cm^{-1} (aliphatic CH_2 stretching) and of COOH groups at 1733 cm^{-1} (C=O stretching), are present. In particular, the defined signal for COOH groups confirms the functionalization of mPEG with COOH groups.

Regarding the conjugation of mPEG-COOH with Cs, the grafting of mPEG to Cs was confirmed by the increase in intensity of the bands at 1649 cm^{-1} and 1400 cm^{-1} corresponding to the secondary amide group (C=O and C-N stretchings) formed between the carboxylic acid of

mPEG and the amine group of Cs (Figure 5D). This result was also highlighted by the intensity increase of the signal at 2887 cm^{-1} (CH_2 stretching) in the mPEG-g-Cs spectrum, occasioned by the CH_2 groups of the mPEG grafted into the Cs.

The copolymer mPEG-g-Cs was also characterized by ^{1}H-NMR analysis (Figure 2C), which clearly indicates the coupling of mPEG by the presence of an intense signal at δ = 3.70 ppm corresponding to the mPEG methylene units (c protons in the spectrum), and signals at δ = 3.36 ppm (C**H**$_3$-O, d protons in the spectrum) and δ = 2.66 ppm (NH-CO-C**H$_2$**C**H$_2$**-CO, b protons). The other signals, at δ = 3.15 ppm (H2 of glucosamine) and δ = 3.45–4.20 ppm (H2`of N-acetyl glucosamine, H3, H4, H5, and H6), correspond to Cs.

A degree of pegylation of 1.72% was determined from the ^{1}H-NMR spectrum by using the equation (2) with the integration values of the signals at 2.66 and at 3.15 ppm, respectively, corresponding to the b and H2 protons in the mPEG-g-Cs structure.

$$DS(\%) = \frac{I_{2.66\ ppm}}{4 * I_{3.15\ ppm} + I_{2.66\ ppm}} * 100 \qquad (2)$$

Nanoparticle characterization

The formation of the NPs occurred spontaneously upon incorporation of the counteranion TPP into the corresponding polymer solutions. In fact, NPs resulted from the ionic interactions between the negative TPP and the positively charged Cs amino groups. Cs NPs show a mean particle size close to 150 nm with a homogeneous distribution confirmed by low polidispersity index (PDI) values (Table 1).

Interestingly, the particle size was not significantly modified during the activation process and the modification with OX26 antibody, suggesting that the functionalization of NPs with the antibody did not influence the NPs stability.

As further comments **(see Figure S2, supplementary file)** on the impact of surface modification on the size of NPs, NPs conjugation with a mean number of antibodies over than 100 units could result in an increase in the size of NPs, but in the case of OX26, other reports in literature clearly indicated that the conjugation of OX26 to NPs (of different material, as poly-lactide-co-glycolide) did not have impact on the NPs size (variation of less than 10 nm) [18]. Similarly, our Cs-NPs with OX26 surface engineering did not suffer a very relevant increase or even any aggregation. This is also confirmed by PDI values which remain almost constant amongst the samples indicating good homogeneity and maintenance of reproducibility. Moreover, surface charges did not decrease under “critical” values (may leading to aggregation) which are normally considered close to neutrality.

This concept is nicely valuable when the samples are not submitted to strong purification processes. Notwithstanding this fact, these processes are required and therefore we tested the changes in size, surface charge and homogeneity after two different purification processes,

namely ultra-filtration and ultra-centrifugation. These processes (ultra-filtration or ultra-centrifugation) significantly ($p<0.05$) lead to an increase in particle sizes (for: SCs/PCs/Cs post UF NPs, SCs/PCs/FCs post UF NPs, SCs/PCs/Cs-OX26 post UC NPs, and SCs/PCs/FCs-OX26 post UC NPs) compared to those of un-purified NPs. The same trend (slight increase in terms of values) was recorder considering PDI values when purification processes are applied. Both of these evidences are not symptomatic of aggregation processes since, as reported in literature in many papers, values of PDI under 0.3 normally mean still good homogeneous population of NPs, while values over this threshold describe populations of NPs not homogeneous. A possible explanation of the mild particle size increase can be find into a swollen effect during the purification which could happen when using Chitosan as starting polymer and the pH of the medium is changed [54]

Regarding the surface charge of NPs, all particles showed positive charges, with ζ potential values around +20mV, due to the protonated NH_2 groups. The modification with the antibody (SCs/PCs/Cs-OX26 NPs) did not produce any significant change in the surface charge of the NPs since the reaction with the antibody involves the COOH groups of the NPs rather than their protonated NH_2 groups.

NPs were also investigated in regards of their structures and architectures. As examples, SCs/PCs/FCs NPs and SCs/PCs/FCs-OX26 NPs were investigated by SEM-FEG in TEM modality (without any fixing agent) as shown in Figure 6 and Figure 7, respectively.
In particular, SCs/PCs/FCs NPs seem to be not perfectly spherical, slightly twisted, probably in consequence of the vacuum conditions used in the analysis (Figure 6A). These NPs show compact and matricial structures, along with a certain level of aggregation probably due to the purification process. Interestingly, the presence of PEG in the formulation is clearly recognizable around the surface of NPs (Figure 6B). The size of NPs is close to 400-500 nm, higher than that observed in PCS modality; this evidence is normally attributed to the difference in procedures of analysis and stands for a slight distortion of the NPs due to their deposition on the support for analysis.
NPs with OX26 surface engineering (SCs/PCs/FCs-OX26 NPs) display a compact internal architecture but more heterogeneous structure (Figure 7A). The high discontinuity in the contrast recorded in these samples, clearly evident in Figures 7B and 7C (obtained at 60.000x and 80.000x, respectively), suggest a higher complexity of the structure, due to the presence of antibody conjugated onto their surface. Also in these samples, twisting of the NPs (with size around 400-500 nm) were recorded and are due to the process of analysis.

The colloidal stability of the SCs/PCs/Cs (post UF) and SCs/PCs/Cs-OX26 (post UC2) NPs, with and without fluorescent marker, was studied by particle size measurements of the systems stored for 30 days at 4^0C (Figure 8). No significant statistical differences in particle size were found in both samples after the insertion of the fluorescent marker and purification processes

((SCs/PCs/FCs (post UF) and SCs/PCs/FCs-OX26 (post UC2)). Moreover for all NPs, in MES buffer during 30 days the particle size remains approximately constant with low PDI indexes. This means that no aggregation or formation of larger particles occurred in the period analyzed.

Quantification of OX26 antibody

The amount of OX26 bonded to the NPs, determined by means of the Mouse IgG2a ELISA quantitative Kit, was 30 ± 1 µg OX26/mg NPs. This value agrees with the data reported in the literature for the OX26 conjugated PEG-PLA micelles [55]. Based on this result, it was possible to calculate the theoretical average number of molecules (N_{OX26}) of OX26 per nanoparticle, using the equation (3) [55][56].

$$N_{OX26} = \frac{C * M_{nparticle} * 10^{-3}}{M_{nOX26}} \qquad (3)$$

Where C: µg OX26/mg NPs determined by ELISA method; M_{nOX26}: 95 kDa/mol; $M_{nparticle}$: $4\pi R^3 NA\rho/3$ (R is the radius of the particle determined from light scattering; NA is the Avogadro constant; ρ=1.05 g/mL).

The results showed that every NP should display onto the surface a mean number of 345 OX26 antibody molecules. This value is higher than that found for PEG-PLA micelles [55]. This may be due to the larger particle size of the Cs NPs and therefore the presence of more functional groups (about 100 COOH groups per chain) available for reaction with the antibody than in the case of the PEG-PLA micelles (one reactive group per chain). Moreover, we use an antibody with smaller molecular weight than that used for the PEG-PLA micelles.

***In vivo* experiments: NPs brain uptake**

Mice were treated with NPs modified with OX26 monoclonal antibody (SCs/PCs/FCs-OX26) and with un-modified NPs as control group (SCs/PCs/FCs). The semi-quantitative analysis (Figure 9) were conducted in order to make a comparison between brain areas and therefore not connected, as percentage to injected dose of NPs which remains constant over the experiments. The results clearly indicated that the majority of NPs (independently from the kind of NPs) is located in the hippocampus (dentate gyrus and CA3) compared with other brain regions investigated (cortex, striatus, corpus callosum and thalamus) and that the difference is more dramatic in the case of SCs/PCs/FCs-OX26 than for SCs/PCs/FCs. In fact, the accumulation of SCs/PCs/FCs-OX26 in CA3 or dentate gyrus is over 2-3 folds higher than the accumulation of the same NPs in striatum, corpus callosum or thalamus. The higher accumulation of NPs in the hippocampus is closely agreeing with previous results on other kind of NPs (i.e. PLGA NPs modified with glycopeptides), accumulating in the same areas due to Rab5-linked neuronal uptake [48][49]. Thus, we could hypothesize that the same accumulation

process took place also in the case of SCs/PCs/FCs-OX26. The accumulation process is currently under investigation for a complete understanding of the dynamics and trafficking of this kind of NPs.
The same difference in accumulation was not detected with the un-modified NPs (SCs/PCs/FCs) since there was not significantly increase in hippocampus (CA3 or dentate gyrus) with respect to the other analyzed brain areas.

Furthermore, comparing the effect of surface modification on accumulation degree, it is also evident how the modification with OX26 significantly increases the tropism of NPs for selected areas. In hippocampus (CA3 and dentate gyrus), cortex and striatum, the accumulation of NPs modified with OX26 monoclonal antibody (SCs/PCs/FCs-OX26) is remarkably different respect to that of un-modified NPs (SCs/PCs/FCs) ($p<0.01$). On the contrary, this difference, in corpus callosum and thalamus areas, decreased significantly ($p<0.05$).
In general, above a regional tropism of both kinds of NPs for specific areas, considering the impact of the surface modification on the accumulation, the semi-quantitative analysis clearly evidenced the supremacy of OX26 modified NPs in BBB crossing with respect to un-modified Cs NPs. This evidence is the consequence of the BBB crossing of NPs modified with OX26 triggered by receptor-mediated transcytosis mechanism produced by the recognition between OX26 antibody present onto NPs surface and TfRs present at BBB level [16].

Owing to the preferential accumulation of NPs in the hippocampus, we decided to focus the investigation on this region (Figure 10), although Figure S4 and S5 (in Supplementary information) show the confocal microscopy images of all the analyzed brain regions.

As evidenced from Figure 10, only few green signals due to the FITC labeling of the un-modified NPs (SCs/PCs/FCs) were detected in the analyzed area (Figure 10C). The presence of un-modified NPs in the CNS could be related to the positive charge that is intrinsic to these nanoparticles, by which they can exhibit an adsorptive-mediated transcytosis [3]
On the contrary, a stronger presence of green spots was detected after administration of NPs modified with OX26 (SCs/PCs/FCs-OX26). In this case, SCs/PCs/FCs-OX26 were detected closely in contact with nuclear structures as evidenced by DAPI staining and inside neuronal cytoplasm, thus demonstrating their ability in being up-taken by cells.
To further investigate on the BBB crossing dynamics, we deeply analyzed the brain microvessels (Figure 11).

OX26-modified NPs (SCs/PCs/FCs-OX26) were found close to brain microvessels, both in neurons and endothelial cells with some differences (Figure 11A), even 2 h after administration, evidencing a rather strong interaction with TfR. These findings are in line with previous hypothesis [2] describing the hitch of mAb (OX26)–modified nanocarriers to be released

(exocytosis) from brain endothelial cells after endo-transcytotic pathway mediated by TfR interaction. On the contrary, un-modified NPs (Figure 11B) were only detected far from microvessels and not in close association with endothelial cells. In this case, un-modified NPs are supposed to be unable to interact with receptors and able to cross the BBB with a pathway (i.e. adsorptive mediated transcytosis), which is less efficient (in terms of amount of NPs reaching the CNS).

Conclusions

The preparation of Cs NPs functionalized with polyethylene glycol and OX26 antibody, with sizes in the range of 140-200 nm and positively charged has been successfully achieved. Furthermore, these nanoparticles showed a high colloidal stability in a pH 6.0 buffer medium for 30 days at 4^0C. *In vivo* experiments (i.p administration in mice) demonstrated the ability of these nanoparticles to cross the BBB, discovering that they can be a promising delivery system for targeting drugs to the central nervous system. Moreover, in this paper we also confirmed that the rate of BBB crossing of Cs-NPs could be greatly implemented after functionalization with OX26 antibody.

Future perspective

The findings found in this research can be useful in the field of neuro-nanomedicine to assist in the design of systems that are selectively planned to the delivery of drugs to CNS, and more specifically systems with the ability to cross the BBB. In this view, coupling different strategies for BBB crossing (i.e. by taking advantage of different BBB crossing pathways as adsorptive and receptor-mediate endocytosis) would favor the increase of percentage of carriers reaching the CNS, maybe avoiding possible drawbacks which limit single pathways. As future developments, the use of fragments (epitope) of antibody against receptors over-expressed at BBB level, as TfR, will probably help in circumventing both the endogenous saturation of the receptors and the too strong binding of antibody with receptor, which may not lead to a complete process of transcytosis.

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Executive summary

- By exploiting the amino groups availability, the Chitosan (Cs) was chemically modified with carboxylic acid groups, polyethylene glycol (PEG) and fluorescein isothiocyanate. [subheading: production of modified chitosan polymers]

- Different Cs derivatives were used for preparing nanoparticles (NPs) by the ionotropic gelation method. [subheading: production of chitosan NPs]
- The hydroxysuccinimide/carbodiimide approach was used to obtain surface modification NPs with OX26 monoclonal antibody (SCs/PCs/Cs-OX26 NPs). [subheading: production of antibody-modified chitosan NPs]
- Both modified and un-modified nanoparticles showed high colloidal stability at pH 6.0 buffer medium over 30 days at 4^0C. [subheading: stability of chitosan NPs over 30 days]
- Fluorescently labeled SCs/PCs/Cs-OX26 NPs and SCs/PCs/Cs NPs were administered via i.p in mice aiming to define their ability to cross the Blood Brain Barrier (BBB) and to reach the Central Nervous System. [subheading: in vivo testing for CNS targeting]
- Confocal microscopy analysis of brain tissues showed the capability of SCs/PCs/Cs-OX26 and SCs/PCs/Cs NPs to cross BBB. [subheading: proof of BBB crossing by OX26-modified chitosan NPs]
- A deep analysis of the tropism of SCs/PCs/Cs-OX26 and SCs/PCs/Cs NPs was performed highlighting the most predominat accumulation of NPs in the hippocampus, higher for NPs functionalized with the OX26 antibody. [subheading: semi-quantitative analysis indicates tropism for brain areas by OX26-modified NPs]
- The ability of NPs modified with OX26 to cross the BBB is probably exploited by receptor-mediated transcytosis mechanism based on the selective recognition between OX26 antibody present onto NPs surface and TfRs present at BBB level. Meanwhile, the presence of un-modified chitosan NPs in the CNS could be related to the positive charge that is an intrinsic feature of these kind of NPs leading to adsorptive-mediated transcytosis.

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