Investigations of octylglyceryl dextran-*graft*-poly(lactic) acid nanoparticles for peptide delivery to the brain

Asme Boussahel, Daniel M. Ibegbu, Rhita Lamtahri, Julie Maucotel, Julien Chuquet, Benjamin Lefranc, Jérôme Leprince, Marta Roldo, Jean-Claude Le Mével, Darek Gorecki, Eugen Barbu

Abstract

Aim: Develop modified dextran nanoparticles showing potential to assist with drug permeation across BBB for the delivery of neuropeptides.

Methods: Nanoparticles loaded by emulsification with model macromolecular actives were characterized in terms of stability, cytotoxicity and drug release behavior. Peptide-loaded nanoformulations were tested in an in-vivo trout model and in food-deprived mice.

Results: Nanoformulations loaded with model peptides show good stability and appear nontoxic in low concentration against human brain endothelial cells. They were found to preserve the bioactivity of loaded peptides (angiotensin II) as demonstrated in-vivo using a trout model, and to induce a reduction in food consumption in mice when loaded with an anorexigenic octaneuropeptide.

Conclusion: Octylglyceryl dextran-*graft*-poly(lactic) acid nanoparticles formulated by emulsification demonstrate potential for peptide delivery.

Keywords nanoparticles; dextran; neuropeptides; drug delivery; brain.

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Introduction

The blood-brain barrier (BBB) continues to represent a major challenge for drug delivery as it prevents successful therapy with many neuropotent molecules including antibiotics, neuropeptides and antineoplastics [1-3]. This is exacerbated by the fact that historically relative little research has been directed towards enhancing drug delivery to the brain in comparison to the effort invested in drug discovery and development [4]; such imbalance is a major hurdle to treating CNS disorders, as most drugs developed have little access to their active sites in the brain due to the BBB [5].

At the neurovascular unit level, the BBB infrastructure lies mainly in the endothelial cells of the brain capillaries in combination with pericytes and astrocytes, and creates a physical transport and enzymatic barrier between the blood and the circulatory system that is necessary for regulating the internal environment of the brain (the composition of the extracellular fluid in the brain is maintained under constant and precise control independent of the composition of the blood, which helps to provide a stable environment for optimal neuronal function [6-8]. Such a barrier is very effective in preventing the uptake of most pharmaceuticals except for small hydrophilic molecules (< 150 Da) and some highly hydrophobic drugs (< 600 Da) that are able to diffuse through the endothelial cell membrane [9].

Various delivery strategies have been investigated historically in concerted efforts to overcome the BBB and enhance drug concentration in the brain through non-invasive methods. Osmotic opening of the tight junctions using hypertonic solutions is effective but problematic due to the high risk of potentially dangerous substances and pathogens also gaining access to the brain [10]. The re-design of existing drugs *via* chemical modification into lipophilic pro-drugs that can diffuse through the endothelial cells barrier (and convert back into the active drug once inside the brain) is limited to the number of active molecules open for such chemical modification, and the efficiency is drastically affected by the efflux pumps that eliminate the active drug [11]. An alternative approach exploits the endogenous transport system by employing existing BBB transporters (whose role is to transport molecules essential for brain function such as glucose, amino acids, certain peptides and proteins) to carry drug payloads across the barrier; it has been shown that it is possible to target these endogenous carrier systems by tagging neuroactives with antibodies or specific ligands [12].

Many recent research efforts have focused on the use of colloidal drug carriers such as liposomes and nanoparticles, which offer the advantage of easy conjugation with various ligands [13]. Among the various polymers investigated for the formulation of nanoparticles aimed at drug delivery to the brain, of particular interest are natural polysaccharides such as dextran, chitosan, cyclodextrins, starch and

cellulose, due to their biocompatibility, biodegradability and ease of chemical functionalization [14-15]. The structure of many polysaccharides offers the possibility of easy conversion into amphiphilic materials that can self-assemble into micro and nano structures, which can then act as optimal carriers for a variety of drugs. Common methods for the preparation of nanoparticles based on polysaccharides include emulsification followed by solvent evaporation, solvent diffusion, self-assembly, and/or dialysis [16-17].

Following from recent results suggesting that the assemblage of a drug-carrier system based on dextran that had been modified with alkylglyceryl moieties and the easily biodegradable poly(lactic acid) has potential to promote increased drug permeability towards the brain [18], we report here on the formulation and characterisation of octylglyceryl dextran-*graft*-poly(lactic acid) based nanoparticles loaded with model peptides *via* emulsification methods. Results of *in vitro* and *in vivo* investigations that were carried out to determine the potential of loaded peptides (angiotensin II and octaneuropeptide, a down-sized analogue of the octadecaneuropeptide ODN) to be delivered across the BBB are also reported.

Methods

Materials and equipment

Fluorescein isothiocyanate-dextran (150 kDa), 4-dimethylaminopyridine (DMAP), human angiotensin II (ANG2), ferricytochrome C, superoxide dismutase, adenosine 3,5-cyclic monophosphate, hydrocortisone, fetal calf serum (FCS), bovine serum albumin (BSA), BCA assay, Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), dextran (100 kDa), trehalose, Rhodamine B, Hoechst 33342, Triton X-100, NaOH solution (1 M), sodium dodecyl sulphate (SDS), and polyethylene glycol (PEG) 6 kDa were all purchased from Sigma-Aldrich, Gillingham, UK. Poly(vinyl alcohol) 16 kDa, L-glutamine, 2-mercaptoethanol, sodium pyruvate, MEM non-essential amino acids solution, Lab- ek eight-chamber glass slides, Corning matrigel basement membrane matrix phenol red free/DEV free, Dulbecco's modified eagle's medium (DMEM), antibiotic-antimycotic, and trypLE Express enzyme were purchased from Fisher Scientific, Loughborough, UK. HUVEC pooled pre-screened EGM2, EGM bullet kit medium, and reagent pack subculture kits were obtained from Lonza, Tewkesbury, UK. Cell titer Aqueous One solution cell proliferation assay was purchased from Promega, Southampton, UK.RO-20-1724 and forskolin were sourced from Enzo Life Science, NY, USA. Poly(lactic acid) with free carboxyl group (PLA; MW 15 kDa) was obtained from Purac, Gorinchem, Netherlands. Octaneuropeptide (OP) was synthesised as previously described in the literature [19]. An Ultra Turrax IKA- 18 homogeniser was used for emulsification and a PolarStar Optima plate reader (BMG Labtech, Aylesbury UK) was employed for assay readings.

Preparation of octylglyceryl modified dextran

The synthesis of modified dextran was carried out as described in the literature [20], with some modifications. Briefly, dextran (2 g, 12.35 mmol glucose equivalent) was dissolved in anhydrous DMSO (150 mL), to which a solution of potassium tert-butoxide (1.38 g; 12.35 mmol) dissolved in anhydrous DMSO (50 mL) was added dropwise. The mixture was allowed to stir for 2 h before the dropwise addition of a solution of *n*-octyloxymethyloxirane (OX8; 12.6 mL; 63 mmol) in anhydrous DMSO (20 mL), then stirred for an additional 24 h at 40°C and followed by dialysis (MWCO 12-14 kDa; 3 day) against deionised water (10 L; exchanged 3 times per day). The content of the dialysing membrane was washed with diethyl ether (3 × 150 mL) in a separating funnel; the traces of organic solvent were removed from the aqueous layer in a rotary evaporator under reduced pressure and the product was lyophilised to afford 2-hydroxy-3-(octyloxy)propoxy dextran (DEX-OX8; named here octylglyceryl modified dextran) as off-white powder.

Synthesis of octylglyceryl-modified dextran-graft-poly(lactic) acid (DEX-OX8-PLA)

Dicyclohexylcarbodiimide (0.225 g; 1.09 mmol) dissolved in anhydrous DMSO (20 mL) was added to a solution of PLA (8.75 g) in anhydrous DMSO (100 mL). The mixture was stirred for 30 min at room temperature and was gradually added to a solution of DEX-OX8 (3.5 g) and DMAP (0.11 g; 0.90 mmol), both dissolved in anhydrous DMSO (150 mL) under stirring for 1 h. The reaction mixture was allowed to react for 24 h at room temperature with continuous stirring, and it was then filtered, dialysed for four days (against deionised water) and lyophilised. The obtained powder was washed with acetone and dried in a desiccator, then it was redispersed in water and finally lyophilised again to afford pure DEX-OX8-PLA as a fine, white powder.

Formulation of peptide-loaded DEX-OX8-PLA nanoparticles via the double emulsification method

DEX-OX8-PLA (40 mg) was dissolved in dichloromethane (DCM; 5 mL), BSA (9 mg) and peptide (1 mg; either ANG2 or OP) were dissolved separately in PBS (1 mL). The peptide solution was first emulsified with the polymer solution (DEX-OX8-PLA; 0.8% w/v) using an Ultra Turrax homogeniser (23,000 rpm; 3 min). A poly(vinylalcohol) solution (10 mL; 50 mg/mL; PVA, 16 kDa) was then added and the mixture was homogenised (23,000 rpm; 3 min) to form a water-in-oil-in-water (WOW) emulsion. Control nanoparticles were prepared using the same method except that the primary emulsion was formed with PBS alone (no peptide). The emulsions were stirred for 3 h (magnetic stirrer) to allow for solvent evaporation, and the nanoparticles were then collected by ultracentrifugation (50,000 rpm; 302,000 g; 30 min).

Formulation of peptide-loaded DEX-OX8-PLA nanoparticles using the solid-in-oil-in-water dispersion method

BSA (9 mg) and peptide (1 mg; either ANG2 or OP) were mixed with polyethylene glycol (PEG; 50 mg) and dissolved in deionised water (1 mL) by vortexing; the solution was frozen in liquid nitrogen and freeze-dried overnight to afford solid protein nanospheres that were further entrapped into DEX-OX8-PLA particles as follows. DEX-OX8-PLA (40 mg) was added to DCM (4 mL), followed by vortexing and allowing the mixture to dissolve completely on a shaker for 1 h. The protein nanospheres (the above lyophilisate of protein and PEG) were added to the polymer solution and the mixture was homogenised (3 min; 23,000 rpm). PVA solution (10 mL; 50 mg/mL) was then added and the mixture was further homogenised (3 min; 23,000 rpm) and then magnetically stirred for 3 h to evaporate the organic solvent. The suspension was then ultracentrifuged (50,000 rpm; 302,000 g; 30 min) and the supernatant removed; the collected nanoparticles were dried *in vacuo*. Blank control nanoparticles (with PEG but without peptide/protein) were prepared using the same procedure.

Determination of nanoparticle entrapment efficiency (EE%)

The amount of BSA and peptide (either ANG2 or OP) entrapped was determined by dissolving nanoparticles (4 mg) in a DMSO/SDS/NaOH solution and by using a BCA assay. By determining the BSA or peptide concentration in the solution of the dissolved nanoparticles it was possible to calculate the amount of protein and then calculate the entrapment efficiency (EE%) and the loading capacity of the nanoparticles (as degree of loading, DL%) using equations 1 and 2:

$$DL\% = \frac{A}{B} \times 100 \qquad \text{(eq. 1)}$$

$$EE\% = \frac{A}{C} \times 100 \qquad \text{(eq. 2)}$$

where:

A - mass of protein / peptide entrapped (mg)

B - mass of the loaded nanoparticles (mg)

C - initial mass of protein / peptide used (mg)

Characterisation of DEX-OX8-PLA nanoparticles

Nanoparticles were re-dispersed in deionised water by sonication (20 min) and analysed by Dynamic Light Scattering using a Malvern Zetasizer Nano ZS instrument and by Nanoparticle Tracking Analysis using a Nanosight LM10 instrument equipped with a 532 nm green laser module. The stability of nanoparticle suspensions was tested by repeating the measurements after 24 h. The zeta

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potential of nanoparticles was determined via electrophoretic mobility measurements (EPM) using the same Malvern Zetasizer Nano ZS instrument.

Investigations of the effect of process parameters on the ANG2-loaded nanoparticle size and ANG2 entrapment efficiency using the WOW method

The effect of several variables such as polymer, protein and PVA concentration, and the duration and speed of homogenization on the characteristics of the ANG2 loaded nanoparticles was investigated. Polymer concentrations in the range 0.5 to 1.5% (w/v), protein concentrations in the range 10 to 30 mg/mL, and PVA concentrations in the range 30 to 50 mg/mL were investigated. Homogenization speed was varied between 15,000 and 23,000 rpm for either 1 or 3 min.

Studies of ANG2 release from DEX-OX8-PLA nanoparticles

Nanoparticles (4 mg) were suspended in PBS (1 mL) and incubated at 37 °C with continuous orbital shaking. At different time points, the nanoparticles were centrifuged down (13,000 rpm; 14,800 g; 30 min) and the supernatant was collected for HPLC analysis (peptide content) and then replaced with fresh PBS.

Biological activity of the ANG2 released from nanoparticles

HUVEC cells were cultured in 24 well plates in EGM-2 medium, which was then removed and replaced with ANG2 aqueous solution (200 µl) of different concentrations (either 0.05, 0.01, or 0.1 µg/mL) and ferricytochrome C (200 µl; 140 µM). The amount of superoxide anion produced was monitored after incubation at 37 °C at different time points (30, 60, 90 and 120 min) using a ferricytochrome C reduction assay(measuring the absorbance at 550 nm). . To determine the bioactivity of the loaded and then released ANG2, nanoparticles were added to the HUVEC cells at a predetermined concentration that would release 0.01 µg/mL of ANG2 in the medium. The amount of superoxide anion released was monitored and compared to both positive and negative controls.

Cytotoxicity investigations of ANG2 loaded DEX-OX8-PLA nanoparticles using bEnd3 and hCMEC/D3 cells

Mouse brain endothelial cells (bEnd3) were seeded in 96 well plates at a density of 25,000 cells/well in modified DMEM (10% FCS, 2mM L-glutamine, 5µM sodium pyruvate, 1% non-essential amino acids). Human cerebral microvascular endothelial cells (hCMEC/D3) immortalised with hTERT/SV40 Lagre T antigen (Institute Cochin, INSERM, Paris, France) were cultured in EGM-2 media and treated following the same protocol. The cells were left to attach to the plates overnight and the media was replaced by fresh one containing ANG2 loaded DEX-OX8-PLA DEX-OX8 nanoparticles suspended in deionised water (1 mg/mL). At different time points, an MTT assay was

carried out to measure the cell viability; following the addition of the MTT assay reagent (20 µL), the plates were incubated for 2 h at 37 ˚C and the absorbance at 490 nm was measured.

bEnd3 cell uptake studies using Rhodamine B and ANG2 loaded nanoparticles
bEnd3 cells were seeded onto Lab- ek eight-chamber glass slides at a density of 5×10^4 cells per well and left to attach overnight in 400 µL of modified DMEM, as above. ANG2 loaded DEX-OX8-PLA nanoparticles were labelled with Rhodamine B by mixing a nanoparticle suspension with Rhodamine B solution at a weight ratio nanoparticle:Rhodamine of 1:1; the nanoparticles were then recovered through centrifugation (13,000 rpm; 14,800 g; 1 h), re-suspended in growth medium, and used to incubate the bEnd3 cells cultured on the Lab- ek glass slides. Control slides were incubated with media. After 3 h, the media was recovered and the cells were washed with PBS, fixed with paraformaldehyde (4.0%, w/v) at 4 °C for 10 min, permeabilised with Triton X-100 (0.1%, v/v) for 20 min and finally stained with Hoechst 33342 (5.0 µg/mL) for 40 min at room temperature. The cells were then visualised using a confocal microscope (LSM 710, ZEISS; 405 nm for Hoechst, 543 nm for Rhodamine B).

Investigations of the effect of intra-arterial injection of ANG2 loaded nanoparticles on the blood pressure and heart rate in an in vivo trout model
Animal handling and experimental protocols were approved by the local ethical committee (CEFEA 02142.01). Non anaesthetised rainbow trout (*Oncorhynchus mykiss)* were prepared with two electrocardiographic (ECG) AgCl electrodes and a dorsal aorta cannula. Either 50 µL of vehicle (Ringer's solution) as negative control, free ANG2 (50 pmol dose) as positive control, or a suspension of ANG2-loaded DEX-OX8-PLA nanoparticles (at an equivalent ANG2 loaded dose of 50 pmol) were injected intra-arterially through the dorsal aorta. The effects of the released ANG2 from the nanoparticles on the heart rate and blood pressure of the trout were recorded using standardized electronic devices and analysed using a PowerLab 4/30 data acquisition system (ADInstruments, Oxford, England) and LabChart Pro software (v.7.0; ADInstruments, Oxford, England) [21] and compared to that of the controls.

Investigations of the effect of intra-venous injection of OP loaded nanoparticles on the food intake of mice
Animal manipulations were performed according to the European Community Council Directive (86:609:EEC), were approved by the local Ethical Committee (authorization numbers: N/10-04-04-12 and N/13-04-04-15) and were conducted by authorized investigators. Male Swiss albinos CD1 mice (Iffa-Credo/Charles River, Saint-Germain-sur-l'Arbresle, France), weighing 22–25g, were housed 20 in Makrolon cages, with free access to standard semisynthetic laboratory diet (U.A.R., Villemoisson-sur-Orge, France) and tap water. The animals were kept in a ventilated room at a temperature of 22±1

°C under a 12h light/dark cycle. All the experiments were carried out between 9am and 6pm in testing rooms adjacent to the animal rooms. Two days before the experiments, mice were anesthetized with isoflurane (5% for the induction and 1.75% during the surgery) and the tail vein was canulated with a 0.25mm external diameter nylon catheter filled with saline and closed by a top. Mice were then isolated in individual cages with free access to water and the pellets of food laid down the floor of the cages, in order to make the animals accustomed to the test conditions. Fifteen hours before (6:00 p.m. to 9:00 a.m.), animals were totally deprived of food but had *ad libitum* access to water. Then, mice received a ≈5g weighed food pellet deposited on the floor of the cage 10 min after intravenous (100 µL) administration of saline (0.9% NaCl) or OP-loaded NPs (equivalent to 10 µg of OP) through the tail catheter during 4 days. Thereafter, the pellet was briefly (<20s) removed with forceps and weighed every 3h, for 9h and a 6-day period.

Statistical treatment of data

All results were presented as mean ± standard error of the mean, with a minimum number of replicates n = 3. Anova with Tukey post-hoc tests were used for normally distributed data, alternatively non-parametric K independent samples test with Mann-Whitney tests were carried out; multiple T-tests, with the level of significance set at 0.05, were also carried out using SPSS 21 software.

Results and discussion

The chemical functionalisation of dextran with 2-[(octyloxy)methyl]oxirane followed by grafting with PLA were carried out as previously reported in the literature [18, 20], with some adjustments. ^{1}H-NMR data confirming the chemical modification of dextran (Figure 1) revealed the resulting material had a degree of substitution with OX8 (defined as the number of octylglyceryl chains attached to 100 glucose residues) of 28.4 %, and a degree of grafting with PLA (defined as the number of lactic acid units grafted, as PLA polymer chains, per 100 glucose residues) of 163.9 %.

Figure 1

Employed as model protein and peptides, BSA, ANG2 and OP were loaded into DEX-OX8-PLA polymer nanoparticles using two alternative methods, which combine emulsification with solvent evaporation. In the double emulsion method (WOW), reported as suitable for peptide loading into nanoparticles [22, 23], an initial W/O emulsion of the model active aqueous solution in an organic solution of the polymer was further emulsified in aqueous PVA. In the solid/oil/water dispersion method (SOW, previously employed to load proteins into microparticles [24]), the model drug was

first loaded by phase separation into small nanospheres, which were subsequently dispersed into an organic solution of the polymer, and the resulting primary solid-in-oil dispersion was further emulsified in aqueous PVA. The size of the BSA-loaded DEX-OX8-PLA nanoparticles was in the range 150 to 300 nm, as measured by both DLS and NTA; a comparison of the main characteristics of the nanoparticles prepared using these two procedures is presented in Figure 2. Unloaded nanoparticles (prepared using the same protocols but without protein/peptide) were employed as controls (labelled in Figure 2 as 'cNP'),

Figure 2

 The results indicate that the entrapment of BSA did not significantly affect the size of the particles formulated using either of these two techniques. Though the control nanoparticles (cNP) prepared using the WOW method appear to present a smaller particle size (as measured by DLS) compared to those prepared *via* the SOW technique, this was not supported by the results of the NTA measurements (Figure 2a). To investigate the stability of BSA-loaded nanoparticle in suspension, samples were measured again after 24 h (results presented in Fig 2a as DLS24), when only negligible agglomeration and no statistically significant difference in particle size were observed. All nanoparticles presented negative zeta potential values (around -30 mV, Figure 2b), indicative of the good overall stability observed (for comparison, the zeta potential of DEX-OX8-PLA nanoparticles before formulation/loading *via* SOW/WOW was about -20 mV). The BSA entrapment efficiency values are presented in Figure 2c, with the difference between the SOW and WOW methods not being statistically significant. The ANG2 entrapment efficiency appeared slightly higher for the SOW method compared to WOW (Figure 2d), but there was no significant difference in size between the ANG2-loaded nanoparticles produced using the two methods. The ANG2 entrapment was found to be slightly lower *via* SOW compared to WOW (116 µg/mg). The ANG2-loaded nanoparticles presented zeta potential values in the range -20 to -25 mV, with those prepared by WOW being more negatively charged compared to those obtained via SOW (Figure 2e). SEM investigations confirmed the spherical morphology of DEX-OX8-PLA nanoparticles (Figure 3).

Figure 3

In an attempt to optimise the loading of ANG2 into DEX-OX8-PLA nanoparticles using the WOW technique, the effect of processing parameters on the particle size and entrapment efficiency was investigated. It was found that an increase in the DEX-OX8-PLA polymer concentration from 0.5% to 1.5% (w/v) led to a significant improvement in the peptide entrapment efficiency (one way Anova with Tukey post hoc test; $p < 0.05$), with the size of the nanoparticles plateauing around 350-400 nm

(Fig. 4a); a similar effect was previously reported for PLGA [25] when it was attributed to an increase in the solution viscosity with increasing polymer concentration.

Figure 4

Increasing the peptide concentration in the range 10-30 mg/mL significantly enlarged the particle size (Fig. 4b); interestingly, the entrapment efficiency reached a maximum (51%) at 20 mg/mL, followed by a significant drop (to 10%) when the concentration was further increased to 30 mg/mL. At the same time, the nanoparticle ANG2 load also decreased from 128 to 80 µg/mg. A rise in the concentration of the PVA surfactant gradually increased not only the entrapment efficiency but also the nanoparticle size (that showed a maximum for 40 mg/mL PVA; Figure 4c). In an attempt to better control the characteristics of the loaded nanoparticles, we also varied the speed (15,000 *vs.* 23,000 rpm) and duration of the homogenisation steps (1 *vs.* 3 min), and found that a combination of high speed and longer homogenisation time resulted in high entrapment efficiency while still yielding a reasonably small nanoparticle size (Figure 4d). The adjusted parameters (10 mg/mL peptide; 1.5% w/v DEX-OX8-PLA ; 50 mg/mL PVA; stirring 23,000 rpm for 3 min) were further used to prepare nanoparticles loaded with either ANG2 or OP (characteristics presented in Table 1).

The release of model peptide ANG2 from DEX-OX8-PLA nanoparticles was investigated over a period of four days (Figure 5a). About 50% of the model peptide was discharged within 24 h, followed by a gradual release (with about 90% ANG2 released over four days).

Figure 5

The adverse effects of the loading process on protein biological activity has been previously studied by Zilberman, who reported up to 18% loss of activity for complex proteins loaded *via* double emulsification [26], though small size peptides are expected to demonstrate better stability. To assess the potential loss of ANG2 biological activity during the nanoparticle formulation process, a biological assay based on the fact that ANG2 stimulates HUVEC cells to release superoxide anions [27] was employed. Figure 5b shows the effect of ANG2 released from DEX-OX8-PLA nanoparticles (loaded using the WOW method) at 0.01 µg/mL compared to that of free ANG2 at the same concentration, after two hours incubation. No significant difference was found in the superoxide anion production, indicating that the entrapment and processes do not exert a key detrimental effect on the bioactivity of the released ANG2.

Figure 6

The cytotoxicity of ANG2-loaded DEX-OX8-PLA nanoparticles was investigated following incubation at different concentrations and for different periods of time with mouse brain endothelial cells (bEnd3), and using a typical MTT assay (living cells convert the yellow tetrazole (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan, which can be quantified photometrically). At a concentration of 1 mg/mL, no significant differences were observed - for the same incubation time - between the cytotoxicity of ANG2-loaded nanoparticles and that of empty nanoparticles used as control (cNP), or that of the main polymeric components used in the formulation of the nanoparticles (PLA, DEX, and DEX-OX8); at this concentration, an apparent increase in the observed viability at 48 h might be explained by minor cell multiplication. The viability of bEnd3 cells incubated with ANG2-loaded nanoparticles was observed to significantly decrease with concentration (towards4 mg/mL), in particular at longer incubation times (Figure 6a). The cytotoxicity of DEX-OX8-PLA nanoparticles was also investigated using human brain endothelial cells (hCMEC/D3), which were incubated for 24 h with either empty or ANG2-loaded nanoparticles at 1 mg/mL concentration; no significant differences in cell viability were found between ANG2-loaded and unloaded nanoparticles, and the media control (Figure 6b).

The uptake of ANG2-loaded nanoparticles by bEnd 3 cells was confirmed by confocal microscopy combined with Z-stacking experiments; nanoparticles were labelled with Rhodamine B as fluorescent marker and are visible (in red) as agglomerates (Figure 7).

Figure 7

To preliminary investigate *in vivo* the delivery from DEX-OX8-PLA nanoformulations, and to confirm the ability of the optimized nanoparticle formulation process to preserve the biological activity of the loaded peptides, ANG2-loaded nanoparticles prepared using the WOW method were investigated using a trout model; as indicated by previous studies, an injection of ANG2 (50 pmol) in trout causes an increase in the dorsal aortic blood pressure [21]. In our experiments (Figure 8a), an apex of 85% in the blood pressure was reached 2-3 min after the injection, with the effect clearing almost completely after 30 min (5%). Following the injection of ANG2-loaded nanoparticles containing an equivalent amount of peptide, a similar effect was observed, although the increase in blood pressure was smaller than that induced by free ANG2 (reaching only 35% within 2-3 min). However, the effect observed was more sustained and was extended beyond the duration of the *in vivo* experiment (30 min). A two way ANOVA analysis revealed that between 5 and 25 min after the injection, both free and released ANG2 caused a significant increase in blood pressure, which was significantly higher for free ANG2. At the end of the *in vivo* experiment (25 min after the injection), the effect of the ANG2 being gradually released from nanoparticles still caused a significant increase in blood pressure (16%) which was not matched by that of the free ANG2 (5%). A two way ANOVA

test also revealed that ANG2, whether free or released from nanoparticles, did not significantly affect the heart rate of the tested trout (Figure 8b), confirming previous results reported in the literature [21].

Figure 8

The endozepine ODN (octadecaneuropeptide) is a glial neuropeptide involved in various neurobiological processes such as neuroprotection [28-30] and central glucose sensing [31], and it is now well established that its C-terminal octaneuropeptide ($ODN_{(11-18)}$; OP) mimics the effects of ODN [19]. The intracerebroventricular (icv) injection of OP produces an anorexigenic effect in food-deprived mice [32] similar to that of ODN [33], however it was found that intravenous administration of high doses of ODN (200 fold the icv effective dose) does not affect food intake in mice, suggesting that these peptides do not cross the BBB [33]. Here we investigated the *in vivo* delivery of OP *via* DEX-OX8-PLA nanoparticulate carriers in a mice model, by monitoring the weight and food consumption following intravenous administration; the results were compared to those obtained using intravenously injected saline control.

Figure 9

The effect of semi-chronic (4 days) administration of OP-loaded NPs was tested by using a daily intravenous injection (equivalent of 10 µg of OP) in food-deprived mice. While we found the weight monitoring results inconclusive, and in control mice (saline) no modification in food intake was observed during the 4-day treatment period, intravenous administration of OP-loaded NPs provoked a significant reduction (P = 0.016) in food consumption during the fourth 9 h diurnal period, suggesting the possibility that the OP loaded into the DEX-OX8-PLA nanoparticles had been successfully delivered into the brain. Following cessation of treatment (days 5 and 6), animals recovered to a quantitative normal diet without rebound in food consumption (Figure 9).

Conclusions

Angiotensin II was successfully loaded as a model short peptide into octylglyceryl dextran-*graft*-poly(lactic) acid nanoparticles (DEX-OX8-PLA) using two emulsion-based formulation techniques; both methods afforded ANG2-loaded nanoparticles of about 250 nm size and negative zeta potential (approx. -30 mV, indicative of the observed good stability of the nanoformulations), with an entrapment efficiency of about 40%.

DEX-OX8-PLA nanoparticles were able to sustain the release of ANG2 *in vitro* over more than four days, with almost 50% of the dose being released over the first 24 h. The results of the *in vitro* bioactivity assay using HUVEC cells indicated that the biological activity of the loaded ANG2 peptide did not appear affected by the formulation process. This was also confirmed by the ANG2 released from the nanoformulation following intra-arterial injection in a trout model, which produced a lower but more prolonged effect on the blood pressure compared to the free ANG2 control, suggesting a sustained release *in vivo*. The ANG2-loaded DEX-OX8-PLA nanoparticles were shown to be non-toxic against human brain endothelial cells (hCMEC/D3; 1 mg/mL), but the viability of mouse brain endothelial cells (bEnd3) was found to be significantly affected in particular at higher concentrations (up to 4 mg/mL).

The results of *in vivo* studies investigating the delivery of octaneuropeptide OP from loaded DEX-OX8-PLA nanoparticles in a mice model revealed a significant reduction in food consumption compared to the saline control, though without noticeable weight reduction over the duration of the experiment.

Summary points:

- Despite recent efforts in drug discovery leading to the development of new promising macromolecular neuro-therapeutic agents, clinical success in delivering actives to the central nervous system has been hindered by the inability of many macromolecules to cross the blood-brain-barrier (BBB).

- Following from previous research results revealing the potential of modified dextran nanoparticles to assist with drug permeation across the BBB, this work focuses on the development of these materials for the delivery of peptides.

- Biocompatible dextran that has been modified with *n*-octyloxymethyloxirane and poly(lactic acid), to increase its amphiphilic character, was used to prepare nanoparticles loaded with bovine serum albumin, angiotensin II (ANG2) and octaneuropeptide (OP) as model macromolecular actives.

- Both emulsification methods used for nanoparticle preparation (i.e. double emulsion and solid/oil/water dispersion) led to the formation of nanoparticles 2-300 nm in size and having negative zeta potential (-30 mV to -40 mV), indicative of the observed good stability of these nanoformulations.

- The effect of the process parameters on the nanoparticle characteristics was also investigated, and release studies revealed a significant burst release (up to 50%), followed by approximately 30% of the loaded model peptide (ANG2) being gradually released over one week.

- Nanoparticles were shown to be nontoxic when tested in low concentration against human brain endothelial cells (MTT assay), and were found to preserve the bioactivity of loaded ANG2 as demonstrated in-vivo by experiments using a trout model.

- Nanoparticles loaded with octaneuropeptide OP were injected intravenously into food-deprived mice, when a significant reduction in food consumption compared to the saline control was observed.

- The results of the investigations of octylglyceryl dextran-*graft*-poly(lactic) acid nanoparticles formulated by emulsification indicate their potential as non-viral vehicles for the delivery of peptides.

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Table 1: Characteristics of DEX-OX8-PLA nanoparticles loaded with either angiotensin (ANG2) or octaneuropeptide (OP) using the optimised WOW method (EE - entrapment efficiency; DL - degree of loading).

	ANG2-loaded NPs	OP-loaded NPs
EE (%)	40.0 ± 1.4	32.2 ± 6.4
DL (%)	48.5 ± 1.5	28.35 ± 3.1
Size (nm)	230.0 ± 20.5	232.1 ± 1.1
Zeta potential (mV)	-35.8 ± 2.1	-26.4 ± 1.7

List of figures

Figure 1: Structure and ^{1}H-NMR spectrum of the 2-[(octyloxy)methyl]oxyrane modified-dextran-*graft*-poly(lactic acid), DEX-OX8-PLA.

Figure 2: Characteristics of DEX-OX8-PLA nanoparticles formulated using different techniques (WOW and SOW): (a) BSA-loaded particle size, as determined by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA); measurements were repeated after 24 h (DLS24) to assess stability; (b) zeta potential of BSA-loaded nanoparticles; (c) BSA entrapment efficiency (%); (d) ANG2 entrapment efficiency and ANG2-loaded nanoparticle size; (e) zeta potential of ANG2-loaded nanoparticles (n = 3; error bars represent SEM; WOW – water/oil/water; SOW – solid/oil/water).

Figure 3: SEM micrographs of ANG2-loaded DEX-OX8-PLA nanoparticles prepared using WOW (a) and SOW (b) methods.

Figure 4: The effect of processing parameters on the peptide entrapment efficiency and on the particle size for the preparation of ANG2-loaded DEX-OX8-PLA nanoparticles *via* the WOW method: (a) polymer concentration; (b) ANG2 concentration ; (c) surfactant concentration; (d) stirring speed and duration (n = 3; error bars represent SEM).

Figure 5: (a) *In vitro* cumulative release profile of ANG2 from DEX-OX8-PLA nanoparticles; (b) bioactivity of ANG2 released from nanoparticles (NP ANG2) compared to free ANG2 (equivalent concentration, 0.01 µg/mL). n = 3 (error bars represent SEM).

Figure 6: Viability of brain endothelial cells (as % relative to media control): (a) mouse bEnd3 cells incubated with either cNP or the main polymeric components (1 mg/mL), or with ANG2-loaded DEX-OX8-PLA nanoparticles at different concentrations, measured at different incubation time points; (b) human hCMEC/D3 cells incubated with ANG2-loaded DEX-OX8-PLA nanoparticles vs. empty DEX-OX8-PLA nanoparticles at 1 mg/ml, measured at 24 h (n = 3; error bars represent SEM).

Figure 7: Confocal images of mouse bEnd3 cells (in green) incubated with: (A) ANG2-loaded DEX-OX8-PLA nanoparticles that have been labelled with Rhodamine B (red); (B) media, as control (no nanoparticles); (C) and (D) higher magnification.

Figure 8: The effect of intra-arterial injection of 50 pmol ANG2, equivalent ANG2 dose loaded into DEX-OX8-PLA nanoparticles, and a control vehicle (Ringer salt) on the dorsal aortic blood pressure P_{DA} (a) and heart rate HR (b) in trout. The arrow indicates the time point when the injection was administered (n = 8-11; error bars represent SEM; * $P < 0.05$ compared to vehicle at all corresponding time points except for free ANG2 at 30 min).

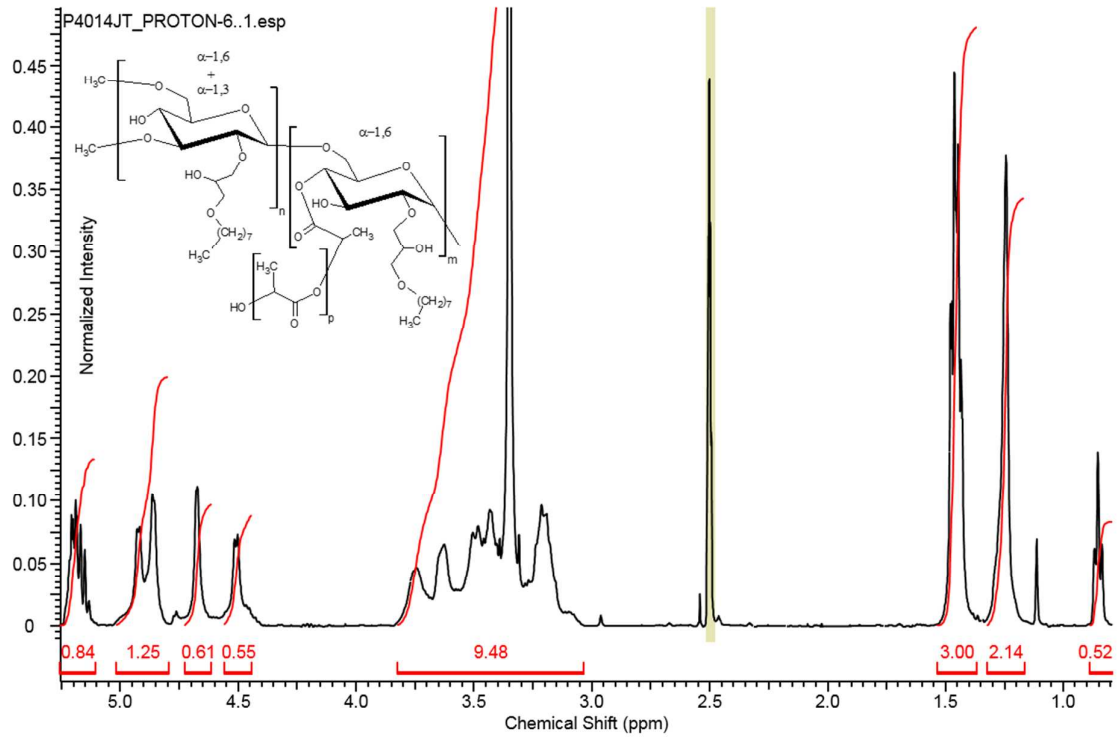**Figure 9**: Food consumption of mice intravenously injected with OP-loaded DEX-OX8-PLA nanoparticles compared to saline (n = 3; error bars represent SEM).
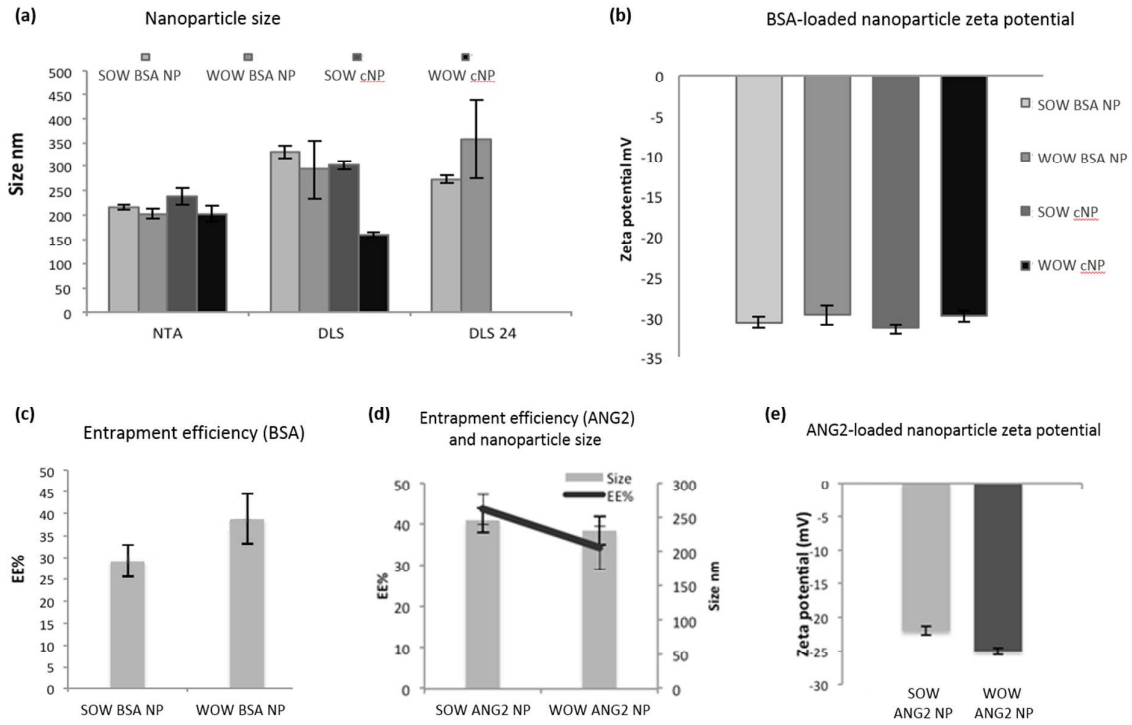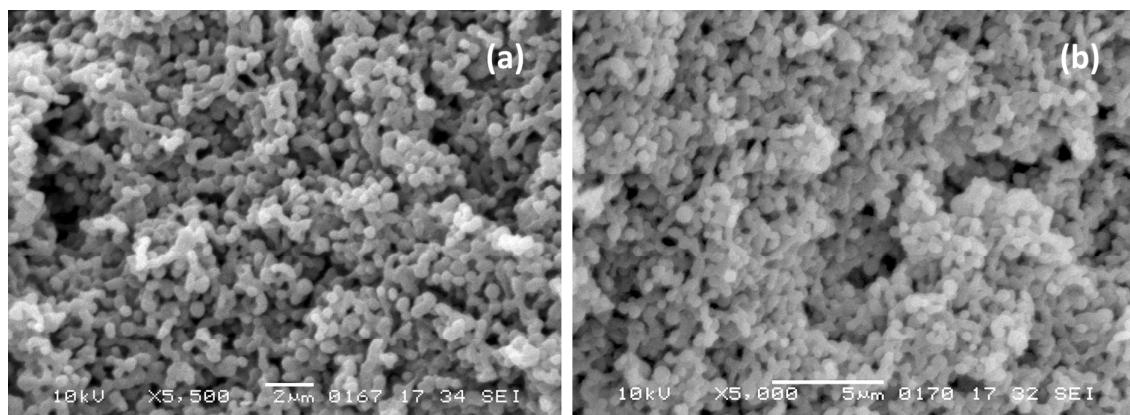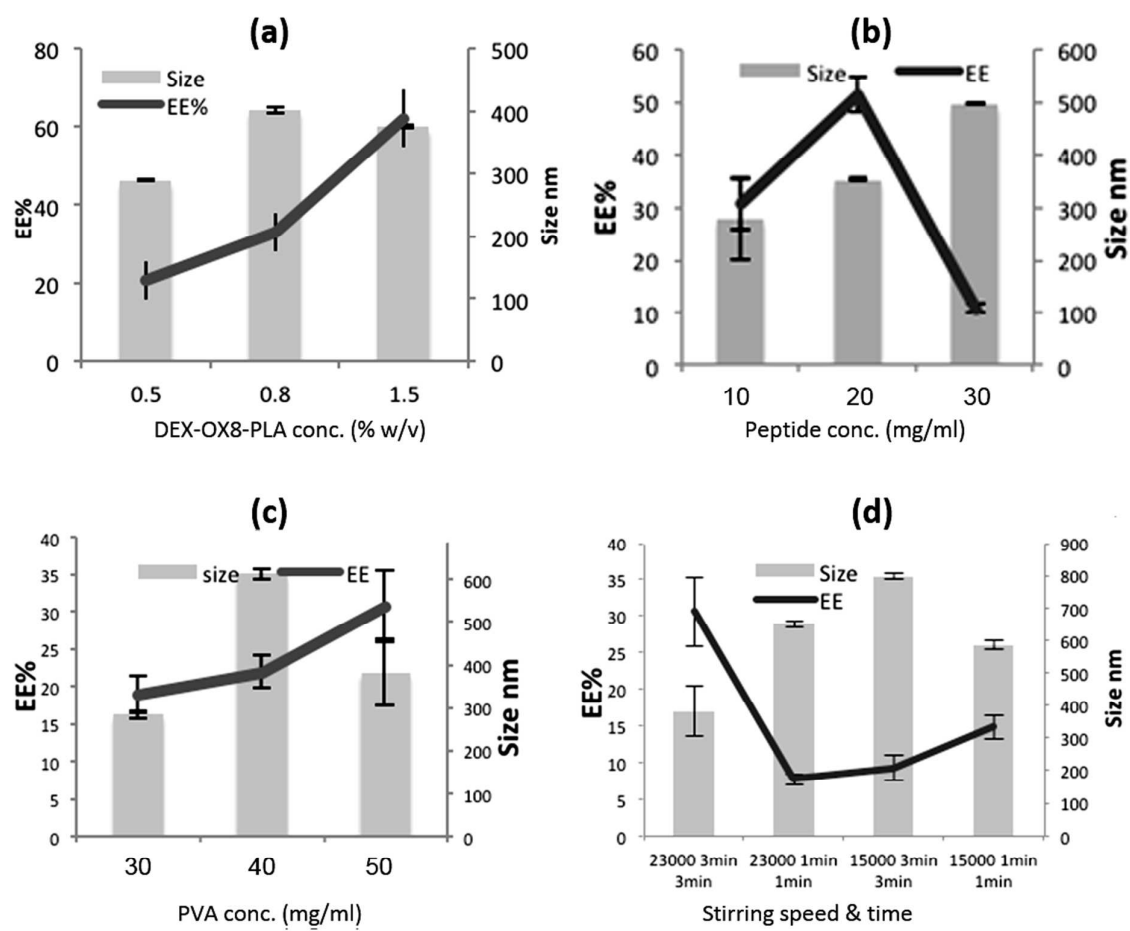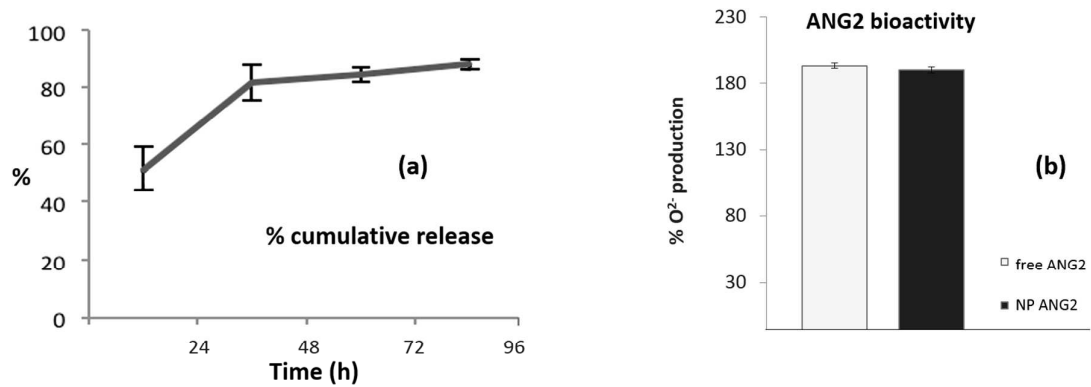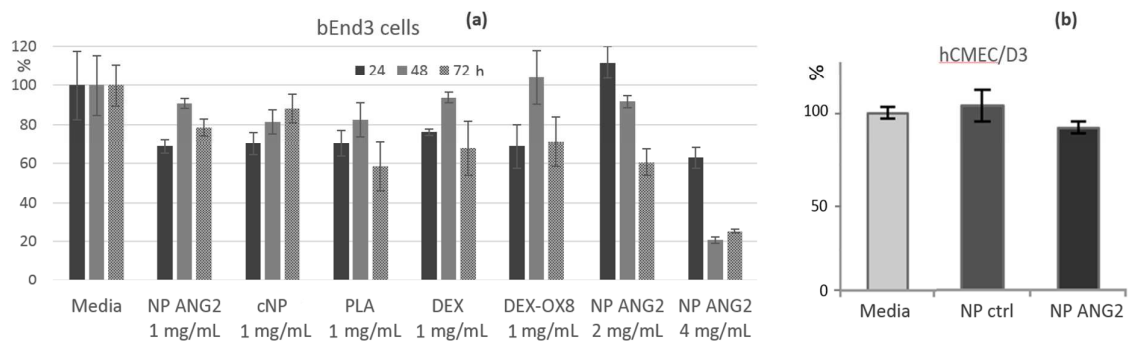
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

(a)

(b)

Fig. 8

Fig. 9