UNIVERSITY OF GOTHENBURG

Nano- neurotherapeutics for neonatal cerebral damage

Preparation and evaluation of Chitosan-based nanoparticles as delivery systems of therapeutic proteins. An experimental study.

Degree project in Medicine

Moshtak Karma

Supervisor: Carina Mallard PhD

Co supervisor: Syam Nair PhD

Department of Neuroscience and Physiology, Gothenburg University, Sweden

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Abstract

Degree project in medicine, Programme in Medicine at University of Gothenburg
Title: Preparation and evaluation of Chitosan-based nanoparticles as delivery systems of therapeutic proteins. An experimental study.
Author: Moshtak Karma, supervised by Syam Nair and Carina Mallard
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Institution: Institution of neuroscience and physiology at Sahlgrenska Academy
City: Gothenburg
Country: Sweden
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Nanoparticles (NPs) are an example of controlled delivery systems, where an active therapeutic drug can be incorporated in a polymeric structure to later be released in a predefined manner. NPs have colossal applications that could be important for the treatment of brain injury. NPs can readily transmigrate across the blood-brain-barrier (BBB) without compromising its integrity. Studies show Death-Associated-Protein-kinase-1-inhibitors (DAPK1-inhibitor) to be neuroprotective in both in vitro and in vivo ischemic models. However, the potential therapeutic application of DAPK1 has been severely restricted by anatomical features that prevent drugs from being delivered to the central nervous system across the BBB. Thus, it is highly relevant to study nanoparticle-based systems to improve drug administration. The aim of this project was to fabricate and characterize chitosan nanoparticles loaded with a DAPk1-inhibitor. NPs were fabricated by a well-established method and different surface modifications were added. Encapsulation efficiency (EE), loading capacity (LC) and in vitro release was evaluated. High EE (96.3% ±3.7) indicates that a high amount of the drug is entrapped in the NPs. The low LC (7.3% ±1.8) implies that the NPs are much larger than the volume of drug. Release test in vitro shows an initial small burst release followed by a sustained release. In conclusion, the study showed that NPs could succesfully be fabricated, loaded and afterwards release the drug. Thus, chitosan NPs show interesting and important perspectives as drug carriers.

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Introduction

Nanoparticles

Nanotechnology refers to the science of materials within the dimensional range of 2 to 300 nm.[1] In the field of medicine, nanotechnology gave rise to the concept *"nanomedicine"* which in definition is '*the process of diagnosing, treating and preventing disease and traumatic injury, relieving pain, preserving and improving human health using molecular tools and molecular knowledge of the human body'*.[2]

Nanoparticles (NPs) are an example of controlled delivery systems, where an active therapeutic drug is incorporated in a molecular structure to later be released in a controlled manner. Drug release is the outcome of NP-degradation. Controlled release depends on NPs' stability.[3,4] NPs have colossal applications that could change the treatment of brain injury. NPs can readily transmigrate across the blood-brain-barrier (BBB) without compromising its integrity. Nanocarriers have shown to protect drugs from enzymatic degradation.[5] Nanoparticle drug carriers consist of solid biodegradable particles in size ranging from (2 –300 nm). NPs diffuse through the BBB to deliver their content into the brain parenchyma, without compromising the integrity of the drug.[6] NPs are also used in imaging systems such as magnetic resonance, positron emission tomography.[7,8] They allow therapeutic agents to selectively target organ, cell and tissue specific levels and minimize exposure of drugs to healthy tissue.

There are studies on several synthetic and natural polymers as drug carriers, one can vary the method of preparation, use different biodegradable materials or add different ligands or functional groups to make target specific NPs.[9]

NPs can be modified to change characteristics such as drug uptake, transport across BBB and release time which can differ from a few hours to a month or several years.[4,10] As seen in figure 1 below, NPs can be divided into polymers and non-polymers, the main difference being that polymers are large molecules composed of many repeated subunits whereas non-polymers are not composed of similar subunits.[11] Polymeric nanoparticles (PNPs) are stable and have a controlled-release profile, they can be surface modified to avoid activation of the inflammatory system. A study using PNPs showed protection against oxidative damage induced by cerebral ischemia.[12] PNPs can migrate through the BBB and carry a high amount of drug molecules without degrading the drug load. However, they are also prone to systemic enzymatic attack in the peripheral circulation and are constantly removed from the central nervous system (CNS) through efflux pump systems lining the capillary wall of the BBB.[13]

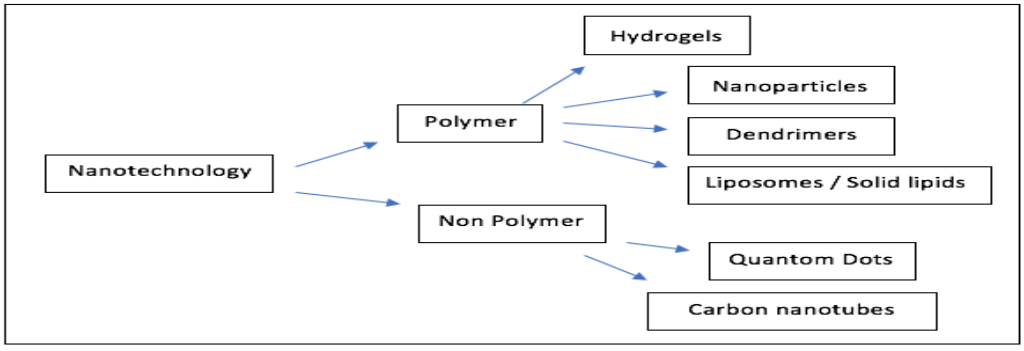

Fig 1. Example of categorization of different nanomaterials, here roughly divided in polymers and non-polymers.

Hydrogels

Also known as nanogels, are hydrophilic, three-dimensional, crosslinked, entangled polymer networks that can entrap larger quantities of fluids without dissolving. They often have ionic and non-ionic chains, enabling higher loading capacity.[14] There are several reviews that recommend the usage of hydrogels as a drug delivery system (DDS) [9,15] However, some of these are not very biodegradable and tend to cause local inflammation.[16]

Liposomes

Liposomes are one of the earliest forms of DDS, they are vesicles of phospholipid membrane and can vary in size (30nm to several μm).[17]

Dendrimers

Branched macromolecules, which consist of a central core, interior dendritic part (branches) and an exterior surface with room for functional groups. They have diverse applications and several properties suited for the medical field.[18] However, a decrease in drug encapsulation is seen in solutions with high concentration of these nanomaterials due to steric crowding of the branching arms.[19]

Quantum dots

Tiny nanocrystals of semiconducting material with diameters between 2-10 nanometres. They can be used for in vivo imaging due to their bright fluorescence and narrow emission. Quantum dots are highly toxic, which limits their applicability in medicine, they can however be used in cancer therapy.[20,21]

Carbon nanotubes

Very strong crystals with unique electrical properties, they can conduct and isolate. The walls can be single or multi layered. Nanotubes are mostly used in industry. [22]

Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are formulated from solid lipids such as triglycerides, complex glyceride mixtures and waxes and have been used as controlled DDS since 1990.[23] They have good biocompatibility, biodegradability, are small sized and solid in room temperature, making them perfect for drug release.[24] A study demonstrated the use of SLNs for administrating mineral iron.[25] However, the production technique is not suited for proteins due to the high temperature, pressure and shear stress.[26]

Chitosan nanoparticles

Chitosan (CS) is a natural polysaccharide, a polymer that can be used in a number of formulations such as films, hydrogels and particles based on its excellent pharmacological properties such as biodegradable, biocompatible [27], bio adhesive [28], permeation-enhancement, antibiotic [29], antitumor etc. properties.[30]. CS was also found to have wound-healing properties.[31]

CS is obtained from the deacetylation of chitin, a naturally occurring polysaccharide. The biocompatibility and nontoxic properties of CS make this polymer a good candidate for pharmaceutical applications such as vaccine delivery, mucosal delivery or targeted drug delivery across the BBB by means of receptor-mediated

endocytosis.[32] Its properties allow for delivery of hydrophilic and hydrophobic drugs. It is approved by the US Food and Drug Administration for usage as wound dressing [33] and studies have shown that even high doses have no adverse (toxic) effect on humans.[34,35] CS-NPs can circumvent the immune system and deliver drugs with higher efficiency in comparison to micro particles.[36] Due to its excellent pharmacokinetic properties, CS makes an excellent choice for NP formulations.

Functionalization and surface modification of Nanoparticles

Functionalization means adding functional groups that modify the surface and structure of NPs which in turn can strengthen the NPs, allowing better penetration and drug release.[37-39] This is a crucial part of nanoscale DDS. Drug carrying capability is as important as transport, tissue targeting and release capabilities. NPs used in tomographic imaging have been modified with fluorescents.[7,8] Figure 2 shows an example of how NPs can be modified with different components.

Biotin (BIO) is a water-soluble B-vitamin. Biotinylation is a method where a protein is conjugated with biotin. Biotin binding proteins with extremely high affinity and specificity, such as avidin, neutravidin and streptavidin (SA), can isolate biotinylated proteins from a sample. Beads are formed and structures binding to the biotinylated molecule will stay, while other unbound molecules can be washed away. BIO-SA-linkage is one of the strongest non-covalent interactions. [40,41]

Polyethylene glycol (PEG) copolymers are popular vehicles for drug delivery. PEG acts as a bio protectant, preventing particle aggregation and reducing mechanical stress during freezing and drying processes.[42] PEG also gives a protective outer layer that shields the core and stabilizes the particles.[43]

TfRMAb is an anti-mouse transferrin receptor monoclonal antibody. Like a Trojan horse, it is used for targeting transferrin receptors on the BBB, this is used mostly in vivo.[44,45] It can bind to NPs through established BIO-SA-linkage.

Ionic gelation

There are in general three methods of fabricating nanoparticles; dispersion of preformed polymers, ionic gelation of polymers and polymerization of monomers.[46] Ionic gelation refers to when CS is cross-linked by either glutaraldehyde, polyaspartic acid or sodium tripolyphosphate (TPP) as counter ions to form NPs (we will mainly be focusing on TPP). Negative groups of TPP cross-link with positively charged amino groups of CS. When TPP is used as anionic cross-linking agent, one can manipulate the particle size by changing pH values.[47,48]

Using ionic gelation to form nanoparticles has several advantages since the process is simple, mild and avoids usage of organic solvents and high temperature, which otherwise can compromise the activity, thus allowing better encapsulation of fragile molecules, such as proteins. [49-52] This was seen by Bodmeier et al, 1989 [53] when dropping chitosan in TPP-solution. However, NPs prepared by ionic gelation are known to aggregate after preparation or have limited stability when stored for an extended time. It is important that both carrier and material must be biocompatible

and biodegradable.[54] According to Calvo et al. 1997, the CS-solution must be in an appropriate concentration range to the TPP, to receive a opalescent suspension in which NPs can be further proved. TPP is used to overcome the otherwise large burst release effect of CS-NPs. [55] Burst release refers to when a large amount of drug is released within the first hours from NPs.

According to Rampino et al 2013 [42] the concentration ratio of CS:TPP affects the formation and structure of NPs. At low concentration TPP (0.25 mg/ml) the CS coils are either randomly distributed or the anionic TPP interacts with cationic groups on CS-chains which through intramolecular links fold over themselves.
Higher concentration of TPP leads to constant size and a more compact particle structure. Sharma et al 2008 observed that forming NPs at lower pH (<6) gave more positively charged CS-NPs with smaller particle size. They also noticed that PEG-NPs had longer stability.[56]

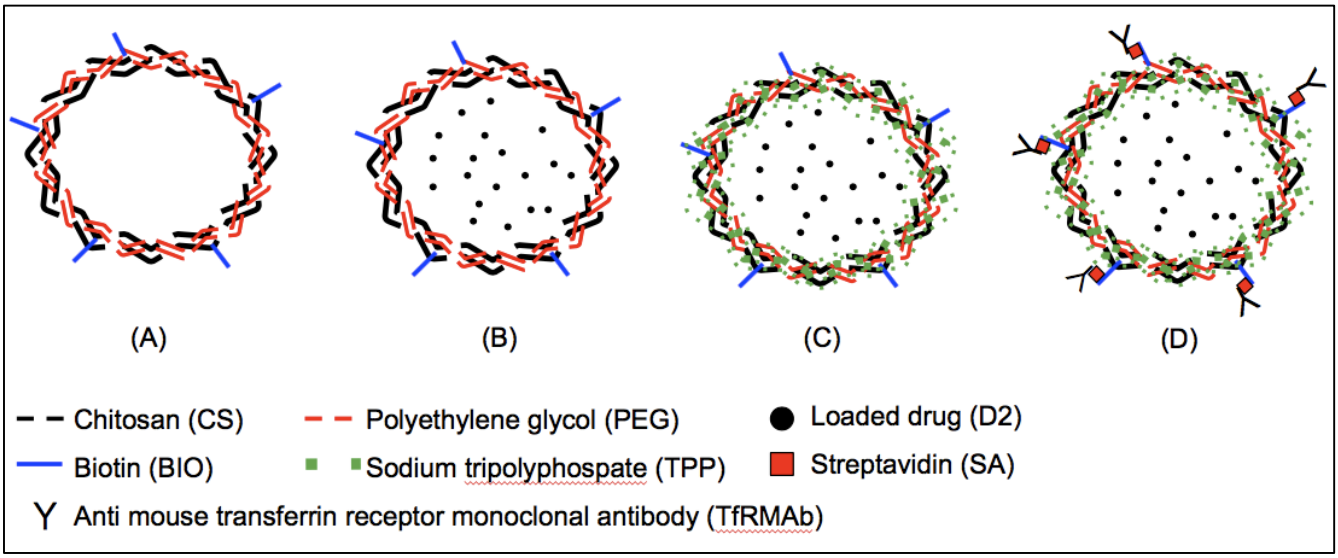

Fig 2. (A): CS-PEG-BIO. However, note that CS-PEG-BIO does not form cavities as the figure shows until it reacts with TPP. (B): Drug loaded. (C): Ionic gelation with TPP results with D2-CS-PEG-BIO-NPs. (D): TfRMAb is conjugated through the established BIO-SA-linkage.

Drug delivery

In pharmacokinetics and pharmacology, drugs and their delivery system in general is divided in four different phases: **Absorption** (how the drug is given and moved in the body e.g. orally, intravenously, subcutaneously), **Distribution** (how the drug is transferred through the body to its desired location), **Metabolism** (how the drug is broken down and activated) and **Elimination** (how the wastes are eliminated from the organism).[57]

Clinical outcome for drugs depends, among other factors, on their bioavailability, meaning how the drug reaches and affects the tissue in mind.[15] Systemically, administered drugs have low bioavailability and can decrease fast in blood plasma concentration, requiring higher re-administration which in turn can lead to bad compliance or the possible overdose. When it comes to developing medicine, non-invasive routes such as mucosal (nasal, oral, vaginal) is attracting great interest due to the fact that it enables local delivery to target tissue.[58] Micro and nanoparticles show great potential in these areas, they were first described for pharmaceutical applications by Birrenbach and Speiser.[59]

Overview of blood-brain-barrier

The BBB protects the brain and maintains the CNS homeostasis. It consists of specialized endothelial cells with high resistance tight junctions, the capillary endothelia lack fenestrations which inhibit significant para-cellular and trans-cellular transport.[60,61] Astrocytes, microglia cells and membrane transporters, all protect the CNS against changing environments. [62] Membrane transporters do not distinguish between harmful molecules and active therapeutics.[63]

Small amount of pharmaceutical compounds can have side effects on the central nervous system [64] since only a low number of molecules with high lipid solubility and molecular mass < 400 Daltons, can actually cross the BBB.[65] This leads to limited delivery of many therapeutic "neuroprotectants" and diagnostic compounds to the brain.[60,66] Transient osmotic opening of the BBB[67] and chemotherapy[68] are techniques of delivering drugs across the BBB, however these methods have undesirable side effects and make the brain vulnerable to damage or infection.[69]

Receptor mediated endocytosis

There are different methods of transport across the BBB (see fig 3) such as trans cellular, transport through tight junctions, adsorptive transcytosis, receptor mediated endocytosis etc. Transport through tight junctions is extremely rare and requires decreased trans endothelial electric resistance or relaxation of junctions. This is usually seen in presence of inflammation and recreational drugs such as methamphetamine, cocaine etc.[70]

Receptor mediated endocytosis (also called clathrin-dependent endocytosis) is an energy mediated transport where ligands bind to receptors on the plasma membrane which then invaginates, forming a clathrin-coated vesicle which surrounds the macromolecule as specific cargo. The BBB has receptors for several endogenous peptides such as insulin, transferrin, albumin and opioid peptides.[71] Thus, NPs conjugated with ligands of surface receptors on brain endothelial cells, act as Trojan horses where they can cross the BBB and release their loaded therapeutics to neuronal cells and tissues.

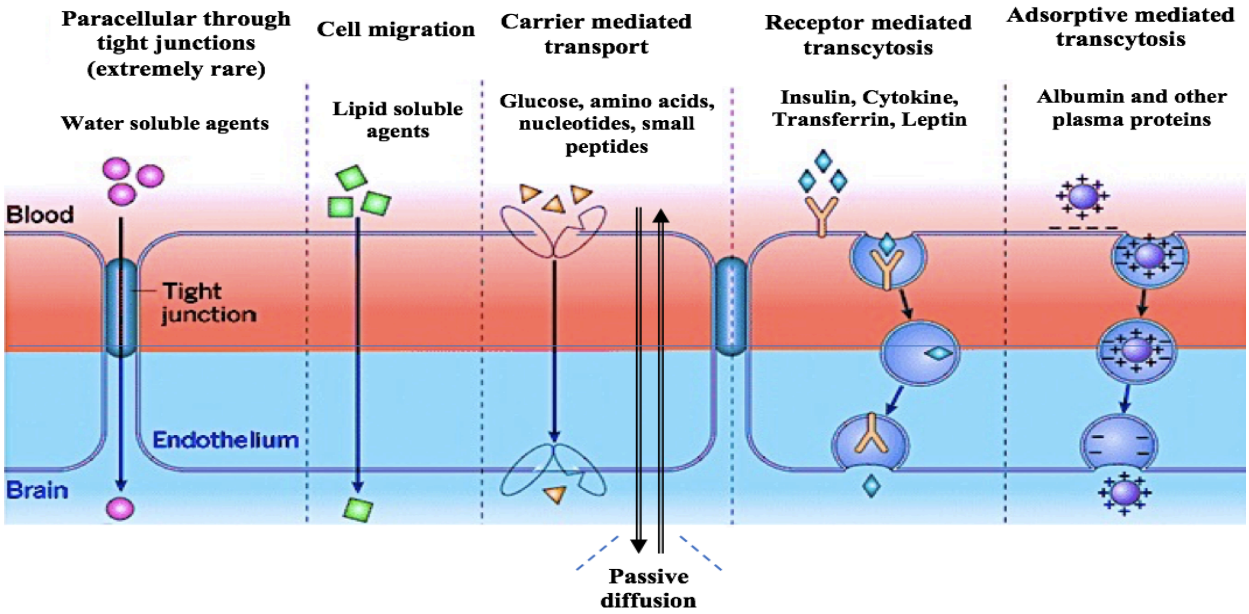

Fig 3. Shows potential transport mechanisms across the blood-brain-barrier (BBB). Nanoparticles modified with transferrin-ligands can cross the BBB through receptor mediated transcytosis. (Modified from [72])

Death associated protein kinase-1

Cerebral ischemia leads to a cascade of neurotoxic events [73], resulting in glutamate release which in turn leads to activation of N-methyl-D-aspartate receptors (NMDAR) resulting in intracellular Ca^{2+} influx. This leads to damage of several cell components, ultimately leading to cell death.[74,75] NMDAR-antagonists are not recommended due to their unwanted side effects and narrow therapeutic windows.

Death associated protein kinase-1 (DAPK1) is a protein in a family of calcium/calmodulin dependent kinases that are involved in cell death pathways. Studies show that DAPK1 interacts with a subunit of NMDARs, known as NR2B, this interaction acts as a central mediator for ischemic neuronal damage.[76] This molecular component is localized at extra synaptic sites that have been linked to cell death but not with NMDARs' physiological functions.

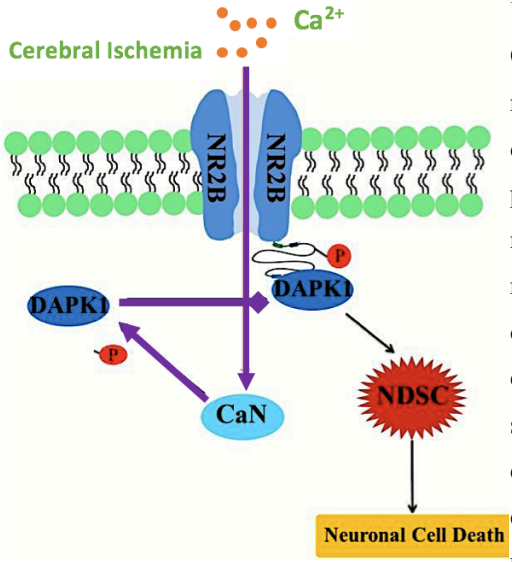

Fig 4. Proposed DAPk1-mediated cell death pathway:

Cerebral ischemia leads to Ca2+ influx through the NMDA receptor channel. Calcineurin (CaN) is activated and dephosphorylates DAPk1 at pS308. Activated DAPK1 phosphorylates the NR2B subunit of the NMDA receptor, resulting in higher Ca2+-influx and in turn more recruitment of DAPk1. This leads to the activation of death-signalling proteins in a neuronal death signalling complex (NDC). The NDC include all neuronal death-signalling proteins that closely associate with the NMDAR channel pore like the NR2B–PSD95–nNOS signalling complex, ultimately leading to cell death. Modified and used with permission from Nair et al 2013. [77]

Studies reveal that cell death pathways differ between adult and immature brains, apoptotic pathways are more prominent in the immature brain.[78,79] NMDAR and DAPKs are more highly expressed in the immature brain which implies that DAPK1-mediated mechanisms may play a particular important role in the injured developing brain.[80-82] Also shear stress has been reported to activate and up-regulate expression of DAPK1 in endothelial cells [83], which suggests DAPK1 may have a role in other types of brain injuries such as traumatic brain injury.

Importantly, and different from traditional NMDA receptor antagonists, evidence suggests that inhibition of death-associated protein kinase 1 (DAPk1) prevents excessive NMDA receptor activation without interfering with physiological functions, and in turn provides neuroprotection in models of stroke.[84] Studies show that a selective small-molecule DAPK1-inhibitor was neuroprotective in both in vitro and in vivo ischemic models. [76,84]

Preliminary, unpublished results (Nair. et al) show that intracerebral injections of a novel DAPK1-inhibitor were neuroprotective in neonatal mice. When binding to DAPK1, it stays in a phosphorylated state, which prevents DAPK1 from binding and activating NMDAR and thereby prevents neuronal death. We will refer to this inhibitor as D2. However, a better way of delivering the drug must be found. The long-term aim of this study is to evaluate the efficacy of biodegradable and functionalized chitosan nanoparticles as an effective targeted delivery tool of novel DAPK1 inhibitors to the brain.

Neonatal cerebral damage

Perinatal brain injury is a major cause of neurological deficits in new-borns leading to lifelong consequences such as cerebral palsy and delayed cognitive and behavioural deficits.[73] Worldwide, more than 1 million babies die annually from complications of birth asphyxia. The condition causes more than 800,000 deaths in the neonatal period per year worldwide and a substantial fraction of the infants that survive suffer neurological sequelae. [73,85] Today, hypothermia is the only established method of treating the new-born after asphyxia. Numbers needed to treat (NNT) is 6, meaning that 1 out of 6 avoids cerebral palsy or death. Therefore, one could argue that the neuroprotective effects are limited and it is only moderately injured children who benefit from this treatment. [86-88] The increasing prevalence of neonatal brain injury and related health risks, combined with the lack of effective therapies, highlight the urgent need for continued research for exploring the safe and effective drugs, leading to neuroprotection and extend the benefit to a larger number of infants diagnosed with perinatal asphyxia. Numerous preclinical studies have reported very promising results using “neuroprotectants”, all of which have failed at clinical trials because of either

safety issues or lack of efficacy. The delivery of many potentially therapeutic neuroprotectants and diagnostic compounds to specific areas of the brain is restricted by the BBB.[6]

In Sweden, about 500 children per year develop permanent neurological problems as a consequence of events that occur around birth, and about 200 of these develop cerebral palsy. Emerging evidence indicates that there is great potential to improve treatment of acute brain injuries in these children and also opportunities to develop more effective regenerative treatment.[89]

Therefore, it is imperative that we continue our efforts to identify mechanisms of injury and repair in the developing brain and to develop new therapeutic strategies.

Objectives

Fabrication of Drug loaded chitosan-based nanoparticles (CS-NPs) will be fabricated. Characteristics of different properties of CS-NPs, such as loading capacity, encapsulation efficiency and in vitro release of DAPk1 inhibitors will be determined.

Material and methods

Solutions

CS-PEG-BIO was prepared by mixing 10 mg CS-PEG-BIO (gift from professor Eduardo Fernandez-Megia, Departamento de Quimica Organica, Spain) with 10 ml water to obtain a concentration of 1 mg/ml, the solution was put on a magnetic stirrer for 60 min (500 rpm/g).

5 mg D2 (DAPk-1-inhibitor (2-(1,3-O_2-1,3-H_2-2H-isoindol-2-yl)-N-(4-sulfamoylphenyl) acetamide)) (Synthetized at Chembridge) was mixed with 557 µl Dimethyl sulfoxide (DMSO, a great solvent used for chemicals and medicine [90]) (D2650-5x5 ml SIGMA-ALDRICH). This gave a stock solution of 25 mM. (MW 359 Dalton). The solution was put on a magnetic stirrer for 60 min (500 rpm/g) and afterwards stored in fridge until usage.

21 mg TPP (Sodium tripolyphosphate) (72061-500G SIGMA-ALDRICH) was mixed with 25 ml Milli-Q water (also known as ultra pure water) to obtain a concentration of 0.84 mg/ml, the solution was put on a magnetic stirrer for 60 min (500 rpm/g) and afterwards stored in room temperature until usage.

40 mg 2-iminothiolane (26101, Thermo Fischer, Scientific Inc., Pierce, USA) was mixed with 10 ml water to obtain a concentration of 4 mg/ml, it was stirred for 5 min (Vortex Genie 2) and afterwards stored in fridge until usage.

TfRMAb, also known as CD71 was bought with the desired concentration of 1 mg/ml (14-0711 Affymetrix).

Borate Buffer with 0,1 M EDTA was prepared by mixing 3.4 mg NaOH (S5886-1 kg SIGMA ALDRICH) , 31 mg boric acid (B6768-1 kg SIGMA ALDRICH), 37.3 mg potassium chloride (P-4504 SIGMA ALDRICH) and 3.8 mg tetrasodium ethylenediaminetetracetic acid (EDTA) (03701-250G Fluka) with 10 ml Milli-Q water. The solution was put on a magnetic stirrer for 60 min (500 rpm/g) and afterwards stored in room temperature until usage.

500 µg MBS (m-maleimidobenzoyl- N -hydroxysuccinimide solution) (22311 Thermo Fischer, Scientific Inc, Pierce, USA) was mixed with 100 µl dimethylformamide to obtain a concentration of 5 mg/ml. The solution was put on a magnetic stirrer for 30 min (500 rpm/g) and afterwards stored in fridge until usage.

1 tablet PBS (Phosphate buffered Saline) (18912–014 Gibco, Thermo Fischer, Scientific Inc, Pierce, USA) was mixed with 500 ml Milli-Q water. The solution was put on a magnetic stirrer for 60 min (500 rpm/g) and afterwards stored in room temperature until usage.

Preparation of the nanoparticles

NPs were prepared by a protocol modified from Calvo et al. in 1997.[55] Concentration and ratio of CS-PEG-BIO and TPP was based on results from preliminary screenings that showed a concentration of 0.84 mg/ml TPP and a ratio of 5:2 CS:TPP gave best characteristics.

Protocol – preparation of NPs

6 Eppendorf tubes (1.5 ml) were marked and weighed (A1-A6, 1-3 would later contain D2 and 4-6 were blanks). Every sample was prepared in triplicates. 3.5 ml of CS-PEG-BIO was measured in two larger vials marked “D2” and “D0”. Dissolve 19.6 µl of D2 was dissolved in the vial marked D2 which gave a concentration of 100 µM D2. The ionic gelation was made by adding 1.4 ml TPP dropwise to polymer solution in “D2” and “D0” while stirring the vials on a Vortex Genie 2. Suspensions were afterwards stirred for an additional 5 min (Vortex Genie 2). Both vials were sonicated for 10 min (Bramson model B1200E-1) and then divided in vials marked A1-A6 described earlier. Eppendorf tubes were centrifugated at 20 000 rpm (9,277 g), 4 °C for 1 hour (Heraeus Fresco 21 by thermo scientific). Supernatant was removed and filtered through a 13 mm diameter 0.22 µm cellulose acetate syringe filter to other vials marked B1-B6, which were stored in fridge until usage. Vials A1-A6 were weighed and the weight of the pellets was determined. Pellets were put in new vials marked C1-6, with estimation of weight afterwards. Using a lyo-dryer, pellets were freeze dried (Lyophilisation) overnight (approximately 18 hours). This is a dehydration process used to preserve materials. [91] All vials were weighed, only batches with no difference in weight between the triplicates were included. Pellets from vials were put in new eppendorf tubes marked ”D2” respectively ”D0” (in total

2 tubes instead of 6). These 2 vials were diluted with Milli-Q water to obtain a concentration of 1 mg/100 µl, they were sonicated (Vortex Genie 2) for 5 minutes and stored in fridge until usage. Time taken to complete one preparation was roughly 3 hours.

Antibody conjugation

1 mg SA was dissolved in 100 µl borate buffer containing 0.1 M EDTA and 5 µl Traut's reagent, this was put in a vial marked "SA" which was put on a magnetic stirrer for 90 min. 100 µl TfRMAb solution was mixed with 5 µl MBS solution, this was put in a vial marked "CD71" which was put on a magnetic stirrer for 30 min. Solutions in the 2 vials were mixed and later put on a magnetic stirrer for 30 min. All stirring was done at 500 rpm/g, in room temperature. For every mg of lyophilised CS-PEG-BIO, 10 µl SA/TfRMAb-conjugate was added and the solution was incubated for 30 min.

Characterization of Nanoparticles

Standard Curve

The standard curve, also called calibration curve, is a means to generate a curve based on known concentrations and is used to back calculate how much drug there is in a solution. With the help of a standard curve of known drug concentrations, one can calculate the amount of drug in our unknown solutions of NPs. Spectrophotometer is used to quantify a material by its reflection of wavelengths, one can also find the exact absorbance (wavelength where the material of interest is found) by analysing at different wavelengths.

Following solutions were used:

- Supernatant from blank NPs
- Supernatant from blank NPs with added D2 (100 µM)
- Supernatant from drug loaded NPs (unknown concentration of D2)

Serial dilution (2X) was made of supernatant from blank NPs, using a 96-well plate. 50 µl of supernatant from blank NPs was pipetted in all wells in column 1-3 except for row A. 325 µl of blank supernatant was mixed with 1.3 µl D2 (100 µM). 100 µl of blank supernatant mixed with D2 (100 µM), was pipetted down in row A. Serial dilution was made from row A down to row G with 50 µl, last 50 µl in each row was discarded, posing a serial dilution from 100 µM (row A) to 1.5 µM (row G).

50 µl of supernatant from D2 loaded nanoparticles was pipetted in all wells in column 4-6. These wells were considered as unknown as the amount of drug was calculated. Supernatant from blank NPs was used as blanks (wells in row H, column 1-3). The

plate was later analysed by spectrum spectrophotometry 200-300nm (Spectramax PLUS).

Determination of Entrapment and Loading efficiency

Encapsulation efficiency (EE) describes how much of the drug is successfully absorbed into the NPs. For example, %EE 40 means that 40% of the initial loaded drug is incorporated into the NPs.

$$\text{Drug Encapsulation Efficiency (\%EE)} = \frac{\text{Da} - \text{Ds}}{\text{Da}} \text{ x } 100$$

Loading capacity (LC) reveals the drug content per NP. For example, %LC 20 means that 20% of the NPs' weight is composed of the drug (1 mg NP is made of 0.2 mg drug).

$$\text{Drug Loading capacity (\%LC)} = \frac{\text{Da} - \text{Ds}}{\text{Na}} \text{ x } 100$$

Da: Total amount of drug = Concentration (mM) x Volume (ml) x Molecular Weight (MW)

Da = 0.1 mM (concentration of D2 in final solution) x 4,9 ml (volume of solution CS-TPP-D2) x 359 (MW) = 175.91 µg/ml

Ds: Amount of drug in supernatant after centrifugation = mean value concentration (given after spectrophotometer) x MW of D2 x 10^{-6} x 10^{3} (since g/l is converted to µg/ml

Ds = [[mean value concentration]] x 359 x 10^{-6} x 10^{3}

Na: Total weight of NPs containing D2 = Weight of pellet (D2) after freeze drying (lyophilisation).

Concentration of D2 in the supernatant was determined using UV-visible spectrophotometer at 260 nm. By comparing a standard curve (supernatant from blanks with added D2) with the supernatant from drug loaded D2-CS-NPs, Ds could be calculated. Na was received when pellets were weighed after lyophilisation overnight. EE and LC were determined by calculations presented above.

Release study (In Vitro)

Also known as dissolution testing, is an important method of monitoring consistency of drug release from different drug products. It can be used for predicting the physiological availability in vivo.

$$Drug\ Release\ = \frac{D(t)}{D(0)}\ x\ 100$$

D (t) = amount of drug released to supernatant at specific time point. Same calculation as Ds.

D (0) = amount of drug loaded to NPs. Same calculation as Da.

Phosphate buffered saline (PBS) (pH 7.4) was used as release medium due to its low ionic strength and therefore low risk of affecting the release. Literature also states that PBS simulates human body fluid more than Milli-Q water.[91] A 96-well plate was prepared as seen below (table 1). D2 loaded NPs, conjugated with antibody were diluted in PBS to a concentration of 1 mg/100 µl. A total volume of 1 ml was made and put in an Eppendorf tube that in turn was put in a horizontal water bed shaker at 60 rpm, 37 °C. At each time point, 60 µl PBS (release medium) was taken from the Eppendorf and added to 3 wells (20 µl in each). Using the standard curve prepared

earlier (table 1), drug concentration was measured at different time points with the help of Spectra MAX PLUS (260 nm). Percentage of drug release was calculated and plotted against the time points. No other materials in the solutions interfered with the absorbance of the drug. Equal volume of PBS was added after each sampling to replace taken solution from the Eppendorf tube.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Serial dilution of D2 in PBS (10 µM down to 0.078125 µM)			D2h0025 (15 min)			D2h12 (12 hours)			D2h60 (60 hours)		
B				D2h0050 (30 min)			D2h18 (18 hours)			D2h66 (66 hours)		
C				D2h01 (1 hour)			D2h24 (24 hours)			D2h72 (72 hours)		
D				D2h02 (2 hours)			D2h30 (30 hours)			EMPTY		
E				D2h03 (3 hours)			D2h36 (36 hours)					
F				D2h04 (4 hours)			D2h42 (42 hours)					
G				D2h06 (6 hours)			D2h48 (48 hours)			Blanks (only PBS)		
H				D2h08 (8 hours)			D2h54 (54 hours)					

Table 1. shows how the wells were distributed in a 96-well plate for the release study. Serial dilution of Death-associated-protein-kinase-1-inhibitor in phosphate buffer (10 µM down to 0.078125 µM) in column 1-3. Blue marked wells contained samples from different time points. Each well contained 20 µl solution.

Statistical method

All samples were made in triplicates. All data is presented as mean value +/- standard deviation (SD) unless otherwise is stated. 2 tailed Student's TTEST was performed in order to evaluate if the results were statistically significant which was considered when $p < 0.05$. This was done with Word Excel 2016.

Ethics

Project has received ethical clearance from the animal committee, Gothenburg Ethical Clearance No: 18-2015

Results

Preparation of NPs

CS-PEG-BIO-D2-NPs spontaneously formed via the electrostatic attraction between positively charged primary amino groups on chitosan chains and negative groups of the TPP. This reaction is known as ionic gelation. The resulting opalescent suspensions were determined as CS-NPs. 7 batches were made (T1-T7), all using the same ratio CS:TPP, concentration D2, instruments and chemicals. The weight of pellet containing drug loaded NPs was significantly higher than the controls, the difference in weight being 0.7 mg +/- 0.3 mg ($p<0.03$). The estimation of weight, described in the methods for preparation of NPs (A1-A6) was only measured to see if the lyophilisation had an effect. These numbers are of no interest for the study and therefore not presented.

Standard Curve

A 96-well plate was filled with different solutions as described in methods. Figure 5 shows the interpolation of the different wells as their absorbance was measured with a spectrophotometer, one sees a possible peak at 260 nm where it is assumed that D2 can be detected.

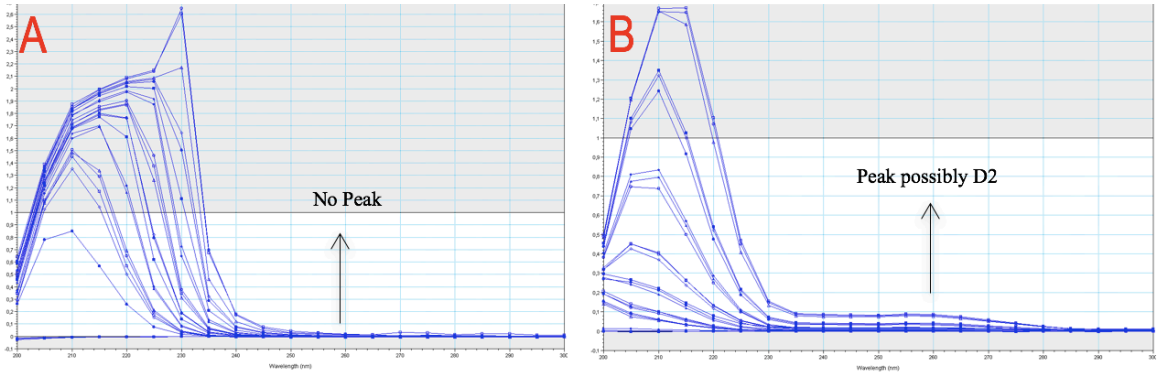

Fig 5. Standard Curve, absorbance measured with spectrophotometer. (A): all wells. (B): supernatant from D2 with added D2. The interpolation on the graph shows a difference between D0 and D2 which is the peak at 260 nm (where D2 can be detected). Y-axis: absorbance (0-1.6). X-axis: wavelength (200-300 nm).

Determination of Entrapment and Loading efficiency

Figures 6-7 show the EE and LC of NPs. T1-T7 stands for 7 different batches, made with the same concentrations and procedure. Mean %EE = 96.3 ±3.7 indicating a high drug encapsulation. Mean %LC = 7.3 ±1.8 meaning the loaded drug stands for roughly 7 % of the total NP-weight.

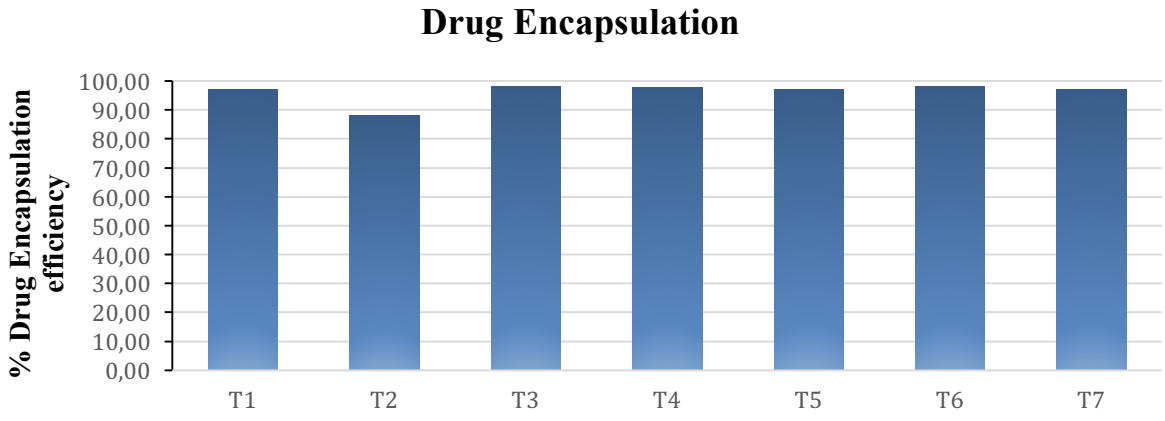

Fig 6 Entrapment efficiency (EE) shows how much of the drug is successfully absorbed into the nanoparticles. T1-7 represent different batches of nanoparticles. Mean %EE = 96.3 ±3.7.

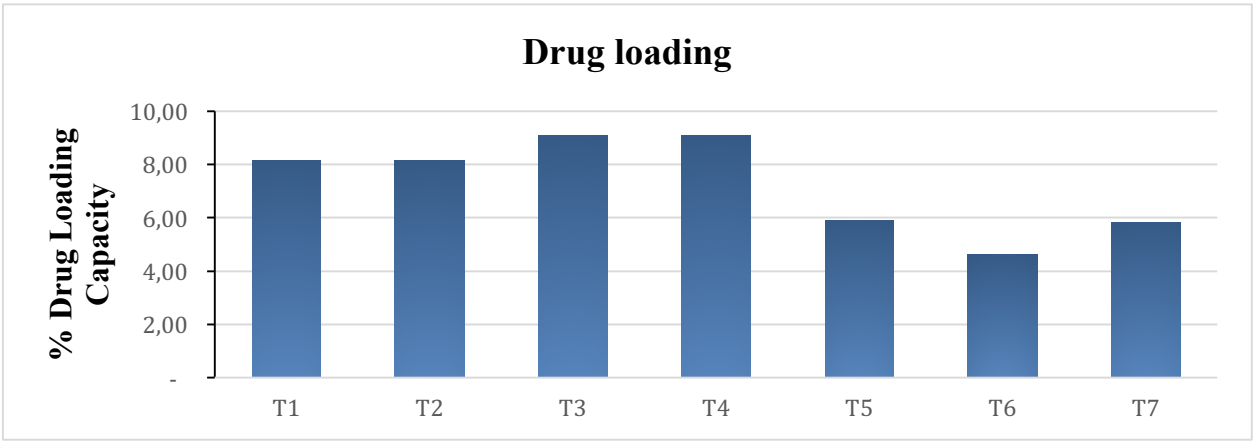

Fig 7 Loading capacity (LC) reveals the weight of drug content in comparison to the weight of the nanoparticles. T1-7 represent different batches of nanoparticles. Mean %LC = 7.3 ±1.8.

Release study (In Vitro)

Figure 8 below shows in vitro drug release profile for drug loaded chitosan nanoparticles from batch T4, samples taken at different time points. Analysed with spectrophotometer, absorbance **260** nm. One sees a moderate initial burst release first 12 hours followed by a slow release.

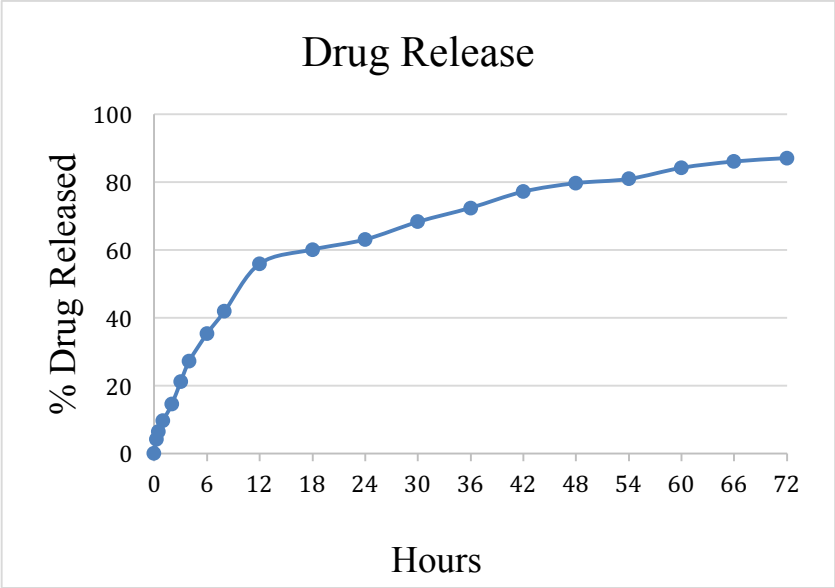

Fig 8. In vitro drug release profile for drug loaded chitosan nanoparticles. Analysed with spectrophotometer, absorbance **260** nm. Moderate initial burst release first 12 hours followed by a slow release.

Discussion

CS-PEG-BIO-NPs were successfully synthesized and loaded with DAPk1-inhibitor D2. They were also characterized to explore properties that affect drug delivery and efficacy. Difference in weight was 0.7 mg +/- 0.3 mg ($p<0.03$). The SD however shows a high spread in the samples. This could be optimized by increasing the time of lyophilisation to avoid any unwanted solution such as moisture to affect the weight difference.

The conjugation with a transferrin receptor antibody (TfRMAb) was done merely as an example of how to modify NPs to enable transport over the BBB, as seen by previous studies.[92] Transferrin receptors however are expressed in many parts of the human body such as kidneys, blood cells, BBB, microphages etc.[93] The quality of antibody conjugation needs to be analysed with an analytical technique such as western blot, immunocytochemistry, immunohistochemistry, enzyme-linked immunosorbent assay etc.

Vials were weighed before and after lyophilisation, the reason was to evaluate whether or not the overnight drying had any effect on the weight. One saw a clear difference in weight.

Standard Curve

Interpolation of the graph (fig 5) shows a difference between D0 and D2 which is the peak at 260 nm. This is concluded to be the absorbance of D2. Peaks between 200 and 240 nm may be the result of other particles such as borate buffer or DMSO. Further analyse can be done by comparing the other included solutions.

Determination of Entrapment and Loading efficiency

The high entrapment efficiency likely depends on the high electrostatic interactions between drug and CS-polymer.[94] Studies have shown this to also depend on the small size of CS-NPs since they have high surface-volume ratio (discussed below under release).

Batch T2 of the drugs shows low entrapment efficiency and high amount of drug in supernatant. When extracting supernatant, parts of pellet could have been taken as well. The weight of the pellet after lyophilisation is marginally lower than the other batches.

Lower loading capacity in batch T5-T7 indicates that these NPs are larger than the other batches since same amount of drug was loaded to all batches. Since the entrapment efficiency does not differ marginally, one could presume that the low loading capacity could be the outcome of non-efficient drying which lead to increased weight of pellets.

Release study (In Vitro)

One sees a burst release in the first 12 hours in pH 7.4 at 37 °C followed by a slow release in the following hours. Gan et al. 2007 and Zhang et al 2010 studied release from CS-NPs, both studies showed higher initial burst release within first 4 respectively 8 hours. The burst release implies that a larger amount of the loaded drug is actually localized on the surface of the NPs and in fact not embedded in the NPs' inner structure. [94,95] Others have also discussed release mechanisms such as diffusion of PBS into the NPs which dissolves the entrapped drug, as well as erosion of the

NPs. [96] However, the initial release is neither rapid nor large, this may be on account of CS being hydrophobic. CS chains and their high electrostatic interaction may hinder diffusion of D2 from the NPs.[95] Factors such as rpm of the horizontal shaker being too low, too small Eppendorf tube, surface modifications, ratio of CS:TPP etc. could have also affected the release study.

Further research has to be made in order to know how the NPs act in vivo. It is known that factors such as pH can cause leakage and degradation of NPs, ultimately affecting the drug release.[54] There are several ways to affect the drug release from NPs, such as pH-adjustment, stabilizing the NP (avoiding degradation) and usage of linkers between the drug and NP which can be "cleaved" by a chosen substance.[97]

The cerebral immune system could also affect the NPs and their characteristics or simply eliminate the NPs. Macrophages can be activated by factors such as size, however, pathogens like bacteria are in the size range 2-3 μm whereas loaded NPs have shown to be around 500 nm.[10,98]

NP-degradation is unavoidable and in fact important since biodegradability is an important key for future medical applications. Degradation of NPs ultimately leads to drug release, therefore it is important to postpone the degradation until the NPs have crossed the BBB. Otherwise, one cannot accurately calculate the amount of drug released at the target tissue since it will start degrading and releasing loaded drug before even entering in vivo.

Methodological considerations

Many factors can influence the formation and outcome of the experiments, therefore the same stirring rate, same beaker, magnetic stirrer, vortex, centrifuge, sonicator were used on all samples.

Practical yield (PY) compares how much product we should have gotten against the actual weight of produced NPs, it is calculated using following equation: $\text{Practical Yield } (\%) = \frac{\text{Weight of lyophilized NPs}}{\text{Theoretical mass (NPs+D2+TPP)}} \text{ x } 100$

It is a quantitative analysing method that measures effectiveness of the NP-fabrication. PY can appear to be higher than 100%, however, this is a sign of impurity as weight of undesired by-products such as water (due to inefficient lyophilisation) affect the calculation.[99] Thus, one should further investigate to see level of loss in the process or inefficiency of the chemical reaction.

A consideration would be to further analyse the influence of different D2 concentrations and CS:TPP ratio on characteristics such as encapsulation efficiency, loading capacity and drug release. Usage of 0.84 mg/ml TPP and 5:2 ratio of CS:TPP is based mainly on particle size distribution, earlier seen with scanning electron microscope (SEM).

TTEST is used to see if results are statistically significant, however it usually requires more data in order to accurately compare drug loaded NPs and controls. TTEST depends on the data to be normally distributed, in this case one cannot guarantee this since we did not test for normality. Parametric tests assume you have a normal distribution of data and usually require sample sizes > 30. Therefore, only one TTEST

has been added to the thesis as an example. The experiments need to be repeated in order to accurately analyse the statistical significance.[100]

Cumulative percentage release was not calculated, if release studies were to be repeated, one could determine the cumulative percentage for further analysation.[101]

Diffusion through dialyzer tubes should be considered instead of regular Eppendorf tubes for the release study. In that way, one would exclude the risk of sampling aggregated NPs since the membrane allows only smaller particles to cross. By having D2 loaded NPs in the dialyzer, which in turn is put in a larger vial filled with PBS, one could sample PBS from the larger vial to study the release of D2 from the NPs. However, there is potential risk that the release will seem lower/slower due to the membrane decreasing amount of D2 outflow to the PBS in the outer vial.

Kannan et al 2012 studied dendrimers loaded with N-acetyl-L-cysteine (NAC) which is a known anti-oxidant and anti-inflammatory substance, and has been studied for possible usage for perinatal brain injury, fetal inflammation, autism etc.[97,102] Most brain damage is first seen well after birth. Microglia are immune cells in the brain, they are important for remodelling and brain development in the fetal and postnatal period.[103] However, these cells can also give a powerful inflammatory response, where even astrocytes (cells that protect neurons) cannot uphold their neuroprotective role, ultimately leading to brain injury.[104,105]

Kannan et al 2012 tried to target activated microglia during ongoing neuroinflammation. It was seen that NAC reduces neuroinflammation in the postnatal

period, suggesting possible treatment of cerebral palsy after birth. They used cleavable linkers between drug and NPs where in the absence of the "cleaver" the NP-conjugates did not release drug for over 72 hours. They also noticed a burst release when the "cleaver" (in their case glutathione) was present.[97] If applicable, usage of linkers should be further investigated.

By evaluating the characterization and improving the efficiency of specific DAPk1 inhibitor-chitosan nanoparticle conjugates, we will in the future be able to develop target-specific biocompatible chitosan nanoparticles for DAPK1 inhibitors and test the BBB permeability and neuroprotective potential against neonatal hypoxic-ischemic injury. Furthermore, it could in the future be possible to design a treatment specifically for cerebrovascular disorders and patent the technology for future clinical use.

There is a bright future for the field of nanomedicine. Nanotechnology, as an industry, has grown vastly to improve drug delivery and molecular imaging. Despite nanoparticles being extremely small, larger multidisciplinary collaborations between medicine, engineering and media are needed in order to gain precious knowledge. There are still many challenges in the field aside from entrapment, loading, toxicity, solubility and manufacturing, such as long-term storage, dosage, animal models, cost etc. [106,107]

Shoaib et al 2006 studied the possibility of using NPs containing Ibuprofen to develop a once-daily tablet in order to improve compliance and reduce fluctuation of drug level in the body.[108] Caspases have an important role as mediators of cell death,

inhibitors of caspases need intra-cerebro-ventricular administration however, Karatas et al 2009 showed promising results in brain protection when using NPs to transport caspase inhibitors.[44] These studies and many more are an example of the importance of evaluating NPs and their drug release kinetics. In conclusion, chitosan nanoparticles show interesting and important perspectives as drug carriers.

Conclusion and Implications

PEGylated, biotin coated, biodegradable chitosan nanoparticles (CS-PEG-BIO-NPs) were prepared and several characterizations have been made. High EE (96.3% ±3.7) indicates that a high amount of the drug is entrapped in the NPs. The low LC (7.3% ±1.8) implies that the NPs are much larger than the weight of drug. Release study in vitro shows an initial burst release followed by a sustained release. Further evaluation of different fabrication methods, surface modifications and conjugations should be done in order to gain a better perspective on the matter.

Future studies will establish the neuroprotective efficacy of DAPk1 inhibitor-chitosan nanoparticles in a model of neonatal global cerebral hypoxia-ischemia (HI) by a well-established method.[109] This procedure induces transient cerebral HI in the hemisphere ipsilateral to the carotid ligation and results in mainly cerebral subcortical and cortical damage. Newly synthesized loaded chitosan nanoparticles will be administered by intraperitoneal-injection to the pups before, immediately after and 3 h after HI, followed by neuropathological assessment. If the outcome is promising, the neuroprotective efficacy of post-insult alone treatment will also be investigated. Well-established immunohistological methods will be used to determine brain infarction and white matter damage at 7 days after HI. The total volume of tissue loss will be calculated according to the Cavalieri Principle.

Populärvetenskaplig sammanfattning på svenska

LÄA110 Examensarbete, Göteborgs Universitet

Titel: Tillverkning och utvärdering av chitosan-baserade nanopartiklar för transport av terapeutiska proteiner. En experimentell studie. 2018

Institutionen för neurovetenskap och fysiologi, Göteborgs Universitet

Student: Moshtak Karma

Handledare: Syam Nair, Carina Mallard

Syrebrist under förlossning eller nyföddhetsperioden kan leda till hjärnskador som i sin tur orsakar utvecklingsstörningar och neurologiska funktionshinder som exempelvis cerebral pares. En speciellt utsatt grupp är barn som föds för tidigt, där behandlingsalternativ saknas idag. Kylmössa är enda metoden tillgänglig för barns som föds med syrebrist vid fullgången tid, men det finns utrymme för bättre behandlingseffekt. Nya behandlingsstrategier utvecklas kontinuerligt, dock faller många potentiella läkemedel mellan stolarna då de inte kan administreras på ett säkert sätt. I detta arbete nämns en grupp av substanser med god behandlingseffekt på hjärnskada hos nyfödda möss. Problemet är dock självaste administreringen då man i studierna har injicerat substanserna direkt till hjärnan genom skallen.

Nanopartiklar är samlingsnamnet för mycket små partiklar. Biologiskt nedbrytbara nanopartiklar är målinriktade bärarsystem och leveransverktyg. Istället för att injicera eventuella substanser direkt i exempelvis hjärnan, kan nanopartiklar fyllas med den tänkta substansen, ges som en sedvanlig spruta var som helst eller oralt, och sedan navigera sin väg till tänkta målorganet eller målceller där de sakta frisätter substansen (likt en disksvamp som suger upp vatten och sedan kramas ut).

Vårt immunförsvar skyddar kroppen från virus, bakterier och andra främmande partiklar, därför måste egenskaper så som storlek och ytan av dessa nanopartiklar modifieras för att kunna fly undan från immunsystemet. Detta kan göras genom att inkorporera och omsluta nanopartiklarna med olika ämnen.

Denna studie förklarar hur nanopartiklar kan användas i behandlingssyfte med fokus på hjärnskador och nanopartiklar som kan ta sig över blodhjärnbarriären till hjärnan. Syftet var att tillverka och karakterisera chitosan nanopartiklar (en av många olika sorter nanopartiklar) med en hjärnskyddande substans. Jämförelse har gjorts emellan tomma nanopartiklar respektive de som innehöll substansen. Faktorer så som inkapslingsförmåga (hur mycket av tillsatta substansen som tas upp av nanopartikeln), laddningskapacitet (hur stor del av nanopartiklarna som utgörs av substansen) och frisättningsförmåga (hur substansen sedan frisätts från nanopartiklarna) beräknades med hjälp av olika nanotekniska mätinstrument.

Resultatvis ser man att en hög mängd av substansen togs upp. Utvärdering av frisättningen visar att substansen sedan töms sakta ut ur de skapade nanopartiklarna. Slutsatsen är att chitosan nanopartiklar besitter en stor potential som läkemedelsbärare som kan sakta frisätta mindre doser av läkemedlet. Vidare utvärdering av tillverkningen och mindre modifieringar behöver göras för att få vidare perspektiv av området.

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Abbreviations

Ds	Amount of drug in supernatant after centrifugation
TfRMAb	Anti mouse transferrin receptor monoclonal antibody
BIO	Biotin
BBB	Blood-brain-barrier
CaN	Calcineurin
CNS	Central nervous system
CS	Chitosan
D2	DAPk1-inhibitor
DAPK-1	Death-associated-protein-kinase-1
DMSO	Dimethyl Sulfoxide
DDS	Drug delivery system
EE	Encapsulation efficiency
EDTA	etrasodium ethylenediaminetetracetic acid
HI	Hypoxia-Ischemia
LC	Loading capacity
LMW	Low molecular weight
NAC	N-acetyl-L-cysteine
NMDAR	N-methyl-D-aspartate receptor
NPs	Nanoparticles
NDC	Neuronal death signalling complex
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PNP	Polymeric nanoparticle
SEM	Scanning electron microscope
TPP	Sodium tripolyphosphate
SLN	Solid lipid nanoparticles
SD	Standard deviation
SA	Streptavidin
Da	Total amount of drug
Na	Total weight of nanoparticles containing the drug

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