Chitosan-based nanoparticles in Alzheimer's disease: messenger or message?

Eniko Manek, Georg A. Petroianu*

Cellulose is the most common natural (plant) polymer while its animal-kingdom close relative chitin comes in second. It is estimated that there are some 10 billion tons of chitin in the world (d'Ayala et al., 2008). Chitin may be described as cellulose with one hydroxyl group on each monomer replaced with an acetyl amine group. Chitin-producing organisms like protozoa, fungi, arthropods, and nematodes are often pathogens in other species. Despite the absence of endogenous chitin, mammals express chitinases (E.C 3.2.2.14) with enzymatic activity.

Chitosan, the only alkali polysaccharide believed to exist in nature, is a linear amino polysaccharide composed of glucosamine and n-acetyl glucosamine units and linked with β (1–4) glycosidic bonds, formed by n-deacetylation of chitin (**Figure 1A**).

The history of the water-soluble chitosan (*chitine modifiée*) dates to 1859 when French physiologist Charles Rouget (1824–1904), described the deacetylation of chitin through its boiling in the presence of concentrated potassium hydroxide. It was however not until 35 years later (1894) that such modified chitin received the name "chitosan", as given by the German physiologist and chemist Felix Hoppe-Seyler (1825–1895). It is conceivable that the name was derived from the merger of chitin with glucosamine, since chitosan is partially deacetylated chitin (to glucosamine). The term Chitosan represents a large group of structurally diverse chemical entities that may show different biodistribution, biodegradation and toxicological profiles. Mammalian chitosanases (EC 3.2.1.132) [glycosyl hydrolases that catalyse the endohydrolysis of β-1,4-glycosidic bonds of partially acetylated chitosan to release chitosan oligosaccharides (COS)] have -to our knowledge not yet been identified, but bacterial support is widely available. Chitosan also occurs naturally in different degrees of acetylation, often in close association with chitin. A mainly deacetylated form is a major component of the zygomycete fungal cell wall.

Chitosan's advantageous properties are manifold, including biocompatibility, low toxicity, low immunogenicity, flexibility in surface modification and antibacterial activity. Among polymeric carriers, chitosan-based nanoparticles shine as biodegradable yet stable vehicles for the delivery of central nervous system (CNS) medications. Unlike most polymers, chitosan also shows cationic and muco-adhesive character, which is particularly suitable for enhancing cellular uptake by ionic interactions as well as for promoting penetration of drugs via mucous membranes. Due to its D-glucosamine groups — which make chitosan structurally similar to sugars that are often used as cryoprotectants — chitosan can also serve as a cryoprotectant agent during the lyophilization of anti-Alzheimer therapeutics. Owing to these beneficial characteristics, chitosan is a widely reported nanocarrier of a vast array of small molecule drugs, genes as well as proteins. In view of CNS delivery, one of the most important attributes of chitosan is its biodegradability by different human as well as bacterial enzymes present in the gut flora (chitosanase, chitinase, chitin deacetylase, β-N-acetyl-hexose-amidinase and collagenase), as well as ubiquitously present lysozymes, lipases, and proteases. The metabolism of chitosan was addressed among others by the Swiss scientists Paul Karrer (1889–1971) and Albert Hofmann (1906–2008) (Karrer and Hofmann, 1929). Recently, Sonin et al. (2020) examined effect of chitosan nanoparticles with a size of ~100 nm and a weakly positive charge on blood coagulation, metabolic activity of cultured cardiomyocytes, general toxicity, biodistribution, and reactive changes in rat organs in response to their (nanoparticles; NPs) single intravenous administration at various doses. The authors point out that "potentially toxic attributes of nanomaterials (chitosan) are associated with the size, shape, and electrokinetic potential of the particles and less dependent on the chemical composition of the matrix and its biodegradation products".

Chitosan itself is widely regarded as being a non-toxic, biologically compatible polymer. It is approved for dietary applications in many countries showing a notably high lethal dose 50 value approximately equal to that of salt or sugar. It has also been approved by the Food and Drug Administration for use in wound dressings. The major product of the enzymatic degradation of chitosan involving deacetylation and depolymerization processes are COS, which can be further transferred to D-glucosamine units. These degradation products proved to be nontoxic, non-immunogenic and non-carcinogenic. In addition, COS exhibits a plethora of only partially understood beneficial effects such as suppression of nuclear factor-κB and molecular target of rapamycin by AMP-Activated Protein Kinase activation. Recent research revealed the potential of COS as an immune-stimulatory agent.

Chitosan-based NPs are generally viewed as the most attractive carriers for the delivery of CNS medications in general and Alzheimer's disease in particular, as reviewed by Sarvaiya and Agrawal (2015) and more recently by Ouyang et al. (2017), Manek and Petroianu (2020), and Manek et al. (2020). The enhancements of drug blood-brain barrier penetration upon encapsulation in polymer NPs are usually attributed to the masking effect of the polymeric matrix, i.e., that the physicochemical properties of the drug will be predominantly determined by the characteristics of the nanoparticle delivery system itself. In addition to improved CNS accumulation, polymeric NPs can further improve drug pharmacokinetics by physically protecting the active pharmaceutical ingredients from degradation by enzymes of the blood and the blood-brain barrier, and by providing sustained drug release. As these above-mentioned benefits strongly depend on the morphology (size, shape, porous structure, etc.) and surface properties (hydrophilic-hydrophobic balance, surfactant coating, etc.) of the nano-carriers, preserving their integrity is key. While some of the chitosan polymers will be degraded into COS in the periphery and in the brain, quantification of this "first pass metabolism" was –to our knowledge- not performed. The enzymatic degradation process of chitosan usually starts with the random splitting of the β-1,4-glycosidic bonds (depolymerization), which is followed by splitting of the N-acetyl linkage (deacetylation).

However, it was shown that drug loading of chitosan NPs increases achievable drug C_{max} in the brain. It can be therefore concluded that chitosan metabolism is not so pronounced as to negate the reason of using it. Despite the high number of published studies, chitosan is not approved by the Food and Drug Administration for any product in drug delivery.

One interesting report was published by Hanafy et al. (2016) assessing galantamine-chitosan complex nanoparticles (drug loading ≈ 10 w/w%) in rats. They write: "*galantamine/chitosan complexation did not negatively alter the pharmacological efficiency of galantamine. Intriguingly, nasal galantamine-chitosan complex exhibited a significant decrease of acetylcholine esterase protein level and activity in rat brains compared to the oral and nasal galantamine solutions. No toxicity signs or histopathological manifestations were noticed. The nanoparticles were found intracellularly in the brain neurons*." Intriguing indeed as one would have expected an up-regulation of the inhibited enzyme. The authors assume that the duration of exposure was too short for such adaptive mechanisms to become relevant. As to the significantly more pronounced inhibition of cholinesterase in galantamine-chitosan complex nasally exposed rats (≈ 40% of untreated controls) versus same route galantamine only (60% of controls), they explain it by pharmacokinetic considerations.

While pharmacokinetics aspects certainly play a role, there is also a pharmacodynamics dimension to the issue. Drug loading (%) is defined as the (weight of drug in nanoparticles)/(weight of nanoparticles) × 100. In the described work the majority (by weight) of the administered galantamine-chitosan complex was chitosan, with a drug loading of ≈ 10%. Drug loading of less than 40% is noticed for most nanocarrier

drug combinations, the carrier being more abundant than the carried drug.

Lazaridou et al. (2020) show that for chitosan and the iron chelator deferoxamine with increasing loading, the size of the nanoparticles also increases. This is highly likely true for most if not all carrier drug combinations. When one administers vastly more carrier than drug it is questionable to assign any noticed effect exclusively to the carrier. The problem is of course with the cited Hanafy et al. (2016) contribution the lack of appropriate controls as no chitosan-only (carrier only) was examined. This is a major weakness of an-otherwise interesting-report.

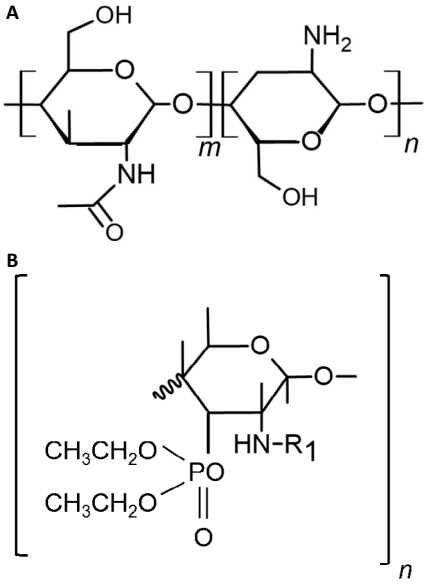
Shooting the messenger is a metaphor used to describe the act of blaming the bearer of bad news, sometimes extrapolated to the carrier of anything useless, unwelcomed or disliked. Here we have the flip side of the coin where one is so infatuated with the good news that the carrier is ignored.

Lee et al. (2009) published a report titled *Chito-oligo-saccharides suppress the level of protein expression and acetylcholinesterase activity induced by Aб (25–35) in PC12 cells*. Aβ (25–35) is a fragment of the amyloid-β peptide generated by proteolytic cleavage of Aβ (1–40) peptide. Aβ (25–35) is the shortest fragment capable of forming β-sheet fibrils and represents the fragment functionally required for neurotoxicity. The (clonal line of rat adrenal pheochromocytoma cells) PC12 the authors used was established in the seventies by Lloyd Greene and Arthur Tischler at Harvard and is widely used in neurosciences research. PC12 cells are very sensitive to the toxic amyloid beta protein.

COS with different degree of deacetylation (90% and 50%) and molecular weight (range 1000–10,000 Da) were obtained by hydrolysis of chitosan in order to carry out an evaluation of their respective acetylcholine esterase (AChE) inhibitory effects. The authors concluded that high degree of deacetylation and high MW COS are the more potent AChE inhibitors (≈ 65% inhibition) and depressors of enzyme protein expression.

Same year Yoon et al. (2009) synthetized novel COS [with different substitution groups, including aminoethyl, dimethylaminoethyl and diethylaminoethyl] and reported their ability to inhibit acetyl-cholin-esterase. These derivatives displayed AChE inhibitory activities with IC_{50} values of 56, 24 and 9 µg/mL, respectively. To put things in perspective, physostigmine has an IC_{50} value for the same enzyme three orders of magnitude lower, while galantamine is approximately one order of magnitude lower (Yoon et al., 2009; Balkrishna, 2019).

Surprisingly, these compounds exhibited no activity against butyrylcholinesterase. The authors conclude that these COS derivatives exert selective inhibitory effects against AChE and that among the COS derivatives, diethylaminoethyl-COS, which is the most hydrophobic, showed strongest AChE inhibitory activity and this indicate that there is a hydrophobic interaction between



R_1=H, $COCH_3$

Figure 1 | **Chemical structures of chitosan and chitosan diethyl phosphate.**
(A) Chemical structure of chitosan; (B) chemical structure of chitosan diethyl phosphate.

diethylaminoethyl group and AChE.

Cárdenas et al. (2002) reported work on the synthesis and insecticidal properties of chitosan diethyl phosphate (**Figure 1B**). They found that while phosphorylated chitosan was three orders of magnitude less toxic than conventional pesticide (chlorpyrifos) it reduced (butyryl-cholinesterase) enzyme activity to about 15% of baseline.

Overall, chitosan appears to be a gratifying substrate to work with, combining low intrinsic toxicity with a willingness to allow organic chemistry alterations and some desirable properties on his own, cholinesterase inhibition being just one among many. Considering the loading factor of chitosan nano-carriers, a significant dose of "carrier" is co-administered whatever the drug contained in it. It is mandatory to have a carrier-only control group when performing studies attempting to demonstrate any carried drug effect.

With respect to cholinesterase inhibition, it appears likely that some proportion of effect is due to the chitosan carrier molecules and COS, a case where the messenger became the message.

We are still far away from finding the ideal messenger, but structural modifications of chitosan, weight optimization and deacetylation, might deliver the ideal symbiosis between message and messenger or even the situation where the messenger is the message.

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Date of submission: November 8, 2020
Date of decision: December 28, 2020
Date of acceptance: January 28, 2021
Date of web publication: March 25, 2021

https://doi.org/10.4103/1673-5374.310685
How to cite this article: *Manek E, Petroianu GA (2021) Chitosan-based nanoparticles in Alzheimer's disease: messenger or message? Neural Regen Res 16(11):2204-2205.*

Open peer reviewers: *Wen Hu, New York State Institute for Basic Research in Developmental Disabilities, USA; Leonardo Hernandez-Hernandez, CUCS-Universidad de Guadalajara, Mexico.*
Additional file: *Open peer review report 1.*

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P-Reviewers: Hu W, Hernandez-Hernandez L; C-Editors: Zhao M, Qiu Y; T-Editor: Jia Y