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Ionizing radiation-engineered nanogels as insulin nanocarriers for the development of a new strategy for the treatment of Alzheimer's disease

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ABSTRACT

A growing body of evidence shows the protective role of insulin in Alzheimer's disease (AD). A nanogel system (NG) to deliver insulin to the brain, as a tool for the development of a new therapy for Alzheimer's Disease (AD), is designed and synthetized. A carboxyl-functionalized poly(N-vinyl pyrrolidone) nanogel system produced by ionizing radiation is chosen as substrate for the covalent attachment of insulin or fluorescent molecules relevant for its characterization. Biocompatibility and hemocompatibility of the naked carrier is demonstrated. The insulin conjugated to the NG (NG-In) is protected by protease degradation and able to bind to insulin receptor (IR), as demonstrated by immunofluorescence measurements showing colocalization of NG-In^{FITC} with IR. Moreover, after binding to the receptor, NG-In is able to trigger insulin signaling via AKT activation. Neuroprotection of NG-In against dysfunction induced by amyloid β (Aβ), a peptide mainly involved in AD, is verified. Finally, the potential of NG-In to be efficiently transported across the Blood Brain Barrier (BBB) is demonstrated. All together these results indicate that the synthesized NG-In is a suitable vehicle system for insulin deliver in biomedicine and a very promising tool to develop new therapies for neurodegenerative diseases.

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1. Introduction

Alzheimer's disease (AD) is clinically characterized by progressive cognitive decline and, at tissue level, by the increasing presence of extracellular senile plaques composed primarily of amyloid-β peptide (Aβ) and intracellular neurofibrillary tangles formed by Tau protein. Aβ is a small peptide (39–43 amino acids) obtained by the sequential proteolytic cleavage of a larger precursor, the Amyloid Precursor Protein (APP), by the β- and γ-secretases [1]. The neurofibrillary tangles are mainly made up of hyperphosphorylated Tau protein, a biomolecule mostly present in the axons of neurons where it is strictly attached to the microtubules. It has been demonstrated that in its hyper-phosphorylated state Tau is no longer able to bind to microtubules, thus causing cell collapse [2].

In recent years, a growing body of evidence has linked insulin resistance and insulin action to AD, a condition also referred to as Type 3 Diabetes (T3D) [3,4]. Insulin and insulin receptors (IRs), distributed throughout the hippocampus and cerebral cortex, play a vital role for learning and memory, and their decreased presence is a feature of the insulin resistance. Studies on cultured neurons

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have demonstrated that IRs are redistributed or decreased in number under Aβ stimulation [5–7]. At molecular level, insulin resistance corresponds to impairment in insulin signaling and reduced activation of specific pathways involved in metabolism and growth. High levels of IRs are present in the healthy hippocampus, in contrast to the reduced levels reported in post mortem cerebral material from AD patients, supporting the hypothesis that impaired insulin signaling is linked to cognitive and functional brain deficits in the elderly [5,8]. Recently, it has been demonstrated that insulin is capable of reducing toxicity induced by Aβ oligomers by inhibition of the intrinsic apoptotic pathway [9]. Moreover, activation of insulin signaling provides a neuroprotective mechanism to counteract oxidative stress, mitochondrial damage and neurodegeneration triggered by Aβ oligomers in neuroblastoma cells [7]. Such a heterogeneous landscape of the disease can be only partly described by the brain's tendency to accumulate processed, misfolded and aggregated/oligomeric proteins. Thus, other factors, such as energy metabolism, oxidative stress, mitochondrial dysfunction, neuro-inflammation, insulin and IGF (insulin growth factor) resistance, and insulin/IGF deficiency in the brain should be considered altogether to formulate a valid and realistic therapeutic approach to AD. The cited evidences strongly encourage in pursuing the administration of insulin to the brain in a potential therapy for AD.

Insulin delivery to the brain is a very challenging task. The insulin present in the adult Central Nervous System (CNS) is primarily derived from pancreatic β-cells and transported by Cerebrospinal Fluid (CSF) into the brain [10–14]. This insulin crosses the Blood Brain Barrier (BBB) mostly via a carrier-mediated, saturable, adjustable, and temperature sensitive active process [11–14], that is limited by the barrier system formed by the tight junctions between endothelial cells [15]. Thus, it is not surprising that an acute increase in peripheral insulin levels leads to higher CSF insulin, whilst chronic peripheral hyperinsulinemia (as occurs in insulin resistance) down-regulates insulin receptors (IRs) levels at BBB, impairing insulin transport into the brain [16].

Peripheral administration of insulin is not viable owing to the risk of hypoglycemia or induction and/or exacerbation of peripheral insulin resistance. A new therapeutic strategy is the normalization of brain insulin levels through intranasal administration; the drug can be directly transported to the central nervous system via bulk flow along olfactory and trigeminal perivascular channels, thus bypassing the periphery. A follow up study examined daily intranasal insulin treatment for 4 months in patients with AD or amnestic mild cognitive impairment [17–20]. Compared with the placebo group, the insulin treated subjects showed improved memory performance without affecting plasma insulin or glucose levels. Unfortunately, together with positive effects this method presents side effects such as irritation and damage of the nasal mucosa [21] and, more importantly, increase of the systolic, diastolic and arterial blood pressures [22]. However, Kamei and Tekeda-Morishita demonstrated that insulin could not penetrate effectively into the brain after intranasal administration without any additional delivery strategies [23].

For decades now, hydrogels have been engineered and applied as drug delivery devices, because of their high biocompatibility, soft and rubbery consistency resembling that of natural tissues and stimuli-responsiveness. More recently, their nanoscalar analogs, nanogels (NGs), have been considered as drug nanocarriers. These water-swollen, crosslinked polymer nanoparticles, with hydrodynamic size in the 10 to ~100 nm range, may be dispersed in an aqueous medium whilst maintaining their fixed conformation. The main advantage of NGs is that their size, surface electric charge, network density and chemical functional groups can be opportunely controlled and tailored to obtain the desired structural and functional properties. NGs can be produced via high-energy irradiation of polymer aqueous solutions in the presence of small amounts of functionalizing monomers, so that polymer cross-linking, monomer grafting to the network and product sterilization can be obtained in a single step. A proper combination of system composition and irradiation conditions enables the production of colloidal stable nanogels with the desired particle size and reactive groups for subsequent conjugation reactions to bind fluorescent probes and biomolecules of interest [24–29].

A clinically relevant area of research is the design of nanogels that can be administered systemically to deliver drugs and biomolecules. Some systems have been specifically designed for the smart delivery of insulin to control the glucose levels in the blood. Injectable nanogels, made of modified dextran, coated with alginate or chitosan, and encapsulating insulin and specific enzymes, have been demonstrated able to release insulin in hyperglycemic condition [30]. The smart delivery of insulin has also been faced by Wu and coworkers, who developed nanogels able to sense glucose in the blood and self-regulate insulin delivery [31,32].

In the present work the potential therapeutic applications of an insulin-nanogel-loaded system able to cross a BBB cell model and tested on a cellular AD system to be use as nasal spray for achieving neuro-protection is demonstrated.

2. Materials and methods

2.1. Materials

Poly(N-vinyl pyrrolidone)-co-acrylic acid nanogels (NG) with $D_h = 65 \pm 20$ nm, determined by DLS analysis, Mw = 4.5 ± 0.2 MDa from Zimm plot analysis, ζ-potential = −32 ± 5.6 mV from Laser Doppler Velocimetry and concentration of 5 mg mL^{-1} were used as substrate [33]. An average number of carboxyl groups per nanoparticle equal to about 50 ± 5 was estimated via a colorimetric method [34] *N*-Boc-1,5-diaminopentane (N-Boc-Cad), 2-(N-morpholino)ethanesulfonic acid hydrate (MES), human insulin HEPES solution (In, 10 mg mL^{-1}), human insulin labeled with fluorescein isothiocyanate (FITC), (In^{FITC}), Tetramethylrhodamine isothiocyanate mixed isomers (TRITC), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS). All chemicals were purchased from Sigma Aldrich and used as received.

2.2. Preparation of NG-In conjugated variants

Grafting of In and In^{FITC} on NG was carried out following a EDC/Sulfo-NHS protocol (Fig. 1a). Firstly, a given volume of NG aqueous dispersion was mixed with EDC and Sulfo-NHS aqueous solution (isotonic phosphate buffered saline pH 7.4) for 30 min, then given volumes of In or In^{FITC} were added, corresponding to various In/NG carboxyl groups ratios (namely, 1:1, 1:2 and 1:10). The reaction was conducted for further six hours under gentle stirring at room temperature. The insulin conjugated NG systems were then purified through prolonged dialysis (7 days) against isotonic PBS at room temperature, using 100 kDa cut-off membrane to remove unreacted reagents. The conjugation degree, that is given by molecules of insulin per nanoparticle, was estimated by UV–VIS absorption spectroscopy on the NG-In^{FITC} variants at $\lambda_{(Max\ Abs)}$ = 480 nm (Jasco V 670 spectrophotometer at room temperature, scan-speed 100 nm min^{-1}, band width 1 nm). Synthesis and purification of NG-In conjugates were conducted under dark conditions. The final concentration of NG-In was 1.6 mg mL^{-1}. Characterization of solutions eluted from 100 kDa cut-off centrifuge-filtered NG-In^{FITC} suspensions was performed by both UV–VIS absorption and fluorescence spectroscopy (JASCO FP-6500

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