Drug Deliv. and Transl. Res. (2014) 4:395–399
DOI 10.1007/s13346-014-0208-9

SHORT COMMUNICATION

Gelatin nanoparticles enhance the neuroprotective effects of intranasally administered osteopontin in rat ischemic stroke model

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Published online: 8 November 2014

Abstract As a leading cause of death and adult disability, ischemic stroke requires the development of non-invasive, long-acting treatments. Osteopontin (OPN) is an endogenous protein shown to have neuroprotective effects in the post-ischemic brain of rats when administered through the non-invasive, intranasal pathway. Previously, gelatin microspheres (GMSs) have been shown to enhance the neuroprotective effects of OPN when used as a carrier during instrastriatal administration, but GMSs are generally too large to enter the brain parenchyma following intranasal administration. Here, gelatin nanoparticles (GNPs) were investigated as a carrier for intranasal delivery of an OPN peptide for the treatment of ischemic stroke. We not only successfully fabricated GNPs with a uniform shape, but also demonstrated the ability of these GNPs to pass into the brain parenchyma following intranasal administration. Critically, the use of GNPs as a carrier allowed for a 71.57 % reduction in mean infarct volume and extended the therapeutic window of intranasally administered OPN peptide to at least 6 h post-middle cerebral artery occlusion (MCAO). Our findings support the development of GNPs as a promising drug delivery platform for the intranasal treatment of ischemic stroke and, potentially, other neurologic disorders.

Keywords Osteopontin · Neuroprotection · Ischemic stroke · Gelatin nanoparticles · Intranasal delivery

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Ischemic stroke is a leading cause of death and adult disability worldwide. In the USA alone, stroke accounted for approximately 1 in every 19 deaths in 2010 and costs approximately $36.5 billion annually [1]. Current stroke treatment focuses on (1) preventing clot formation by controlling risk factors, such as hypertension, hypercholesterolemia, diabetes mellitus, physical inactivity, and smoking [2], and (2) reperfusion of tissue by removing or reducing clots through invasive endovascular therapies [3], thrombolytic drugs [4], such as tissue plasminogen activator (tPA), and anti-platelet agents [5]. However, no currently available therapy can be used to repair damaged tissue and reduce infarct volume once blood flow has been restored. Additionally, current stroke treatments have a narrow window of therapeutic efficacy of about 3 h, but less than 30 % of stroke victims reach treatment centers within this time frame [1]. Improved treatment options focused on neuroprotection, increased therapeutic window, convenience, and decreased invasiveness are clearly needed for ischemic stroke patients.

Osteopontin (OPN) is a widely distributed, glycosylated, secreted phosphoprotein found in numerous tissues including

bone, cartilage, kidney, activated macrophages, and brain [6]. OPN has been shown to have cell migratory, pro-inflammatory, anti-inflammatory, and anti-apoptotic actions [6]. Though its main role is thought to be in bone formation and remodeling [6], OPN has also been shown to have neuroprotective effects in the middle cerebral artery occlusion (MCAO) model of ischemic stroke [7, 8]. These neuroprotective effects depend on a short, RGD-containing sequence on the N-terminal side of the thrombin cleavage site [7]. In this work, an 11 amino acid peptide (GRGDSLAYGLR) incorporating the crucial RGD motif was employed.

The intranasal pathway is a rapid, non-invasive method for drug delivery to the brain [9]. Materials in the nasal cavity may pass through the olfactory mucosa and enter the central nervous system (CNS) by following the olfactory nerves without having to cross the blood-brain barrier (BBB) directly [10]. Due to its non-invasive nature, intranasal administration has been investigated for the delivery of therapeutics to treat neurological disorders such as Alzheimer's disease [11–13]. Typically, intranasal delivery to the brain is highly inefficient with less than 1 % of the administered drug reaching the brain [9]. However, the use of nanocarriers can increase the efficacy of CNS delivery via the intranasal pathway [14, 15]. In this work, we employed gelatin, a natural polymer of hydrolyzed collagen, as a carrier for intranasal delivery of an OPN peptide. Not only is gelatin biocompatible, biodegradable, and classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), but it can also passively target damaged brain tissue due to upregulation of gelatinases in these regions [16]. In addition, gelatins with different charges at physiological pH are readily available allowing for flexibility in drug choice.

Gelatin nanoparticles (GNPs) were fabricated using a modified double-desolvation method [17]. Briefly, 50 mL of 5 % type A gelatin (175 bloom) solution was precipitated with 50 mL acetone at 40 °C for 60 s. The precipitate was dried and used in the second desolvation. For this step, 55 mL of acetone was added to 20 mL of 7 mg/mL gelatin solution (pH 2.5) at 40 °C with continuous stirring. After acetone addition, particles were crosslinked with 0.0667 % glutaraldehyde (GA) overnight before washing with ethanol three times at 4 °C and lyophilizing. Based on scanning electron microscope (SEM) observation (Hitachi S-4800, Hitachi, Japan), GNPs were 183±57.3 nm in diameter (Fig. 1).

To verify their delivery to the brain via nasal route, GNPs were suspended in saline solution and administered intranasally to adult male Sprague-Dawley rats. Rats were anesthetized with a 3:1 ketamine/xylazine injection: 20 μL of GNP-saline solution was administered into the nasal cavity with the head at a 90° angle (10 μL per nostril in two allotments of 5 μL each). Six hours after intranasal administration, rats were sacrificed with isoflurane gas and perfused with 4 % paraformaldehyde (PFA) solution. Whole brains were extracted and

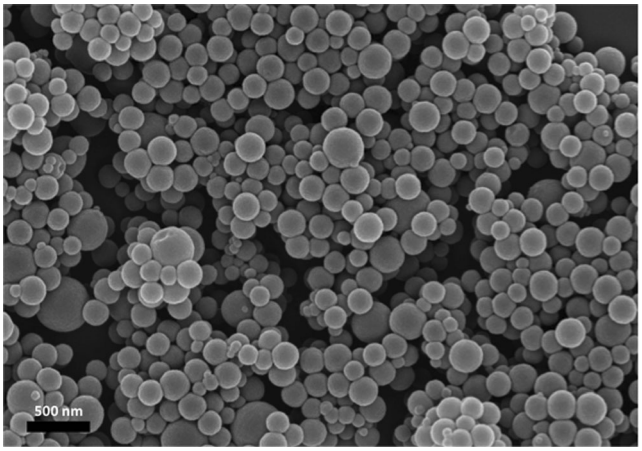

Fig. 1 Scanning electron micrograph of GNPs showing spherical shape

preserved in a 4 % PFA, 1 % GA solution overnight. Regions of interest, including the olfactory bulb (OB), olfactory tract (OT), cortex (CTX), striatum (STR), amygdala (AMY), and hypothalamus (HYP), were dissected and prepared for SEM observation (Fig. 2). Briefly, samples were washed with 0.1 M phosphate buffer (PB), fixed in a 1 % OsO_4 solution, washed again with PB, and dehydrated with an ethanol series (50, 70, 80, 90, 95, and 100 % ethanol). As seen in Fig. 2, all brain regions investigated were found to contain GNPs. The GNPs were easiest to find in the OT, CTX, and STR. Few GNPs were observed in the OB, indicating that, at the 6-h mark, the particles had already traveled to deeper brain regions. These images clearly show that the GNPs are able to enter the brain parenchyma following intranasal administration and confirm results from other studies using radiolabeled [18] and fluorescently tagged [19] NPs. Interestingly, GNPs were often observed in clusters, as well as individually, in the brain parenchyma; it is not clear if this aggregation occurs before or after passage through the nasal mucosa.

To evaluate the efficacy of GNPs as a drug delivery vehicle, OPN peptide-loaded GNPs (OPN-GNPs), unencapsulated OPN peptide, and empty GNPs were administered intranasally to male Sprague-Dawley rats (1 μg OPN per rat, n=3–6) 1, 3, or 6 h following 1 h of MCAO using a nylon filament plug, as described previously [20]. To prepare OPN-GNPs, lyophilized particles were rehydrated in an aqueous solution of OPN peptide (1 μg OPN/25 μg GNPs) at room temperature for 2 h. Just prior to administration, volume was adjusted to 20 μL per rat with phosphate buffered saline solution (PBS, pH 7.4). OPN enters the GNPs as the water is absorbed into the hydrogel matrix and becomes trapped due to electrostatic interactions [21]. Previously, we have shown that a dose of 100 ng/rat administered via intrastriatal injection confers neuroprotection following MCAO. For this study, a larger dose was chosen to account for anticipated loss during passage through the nasal mucosa [9]. Preliminary dose and loading ratio experiments (data not shown) indicated that a dose of

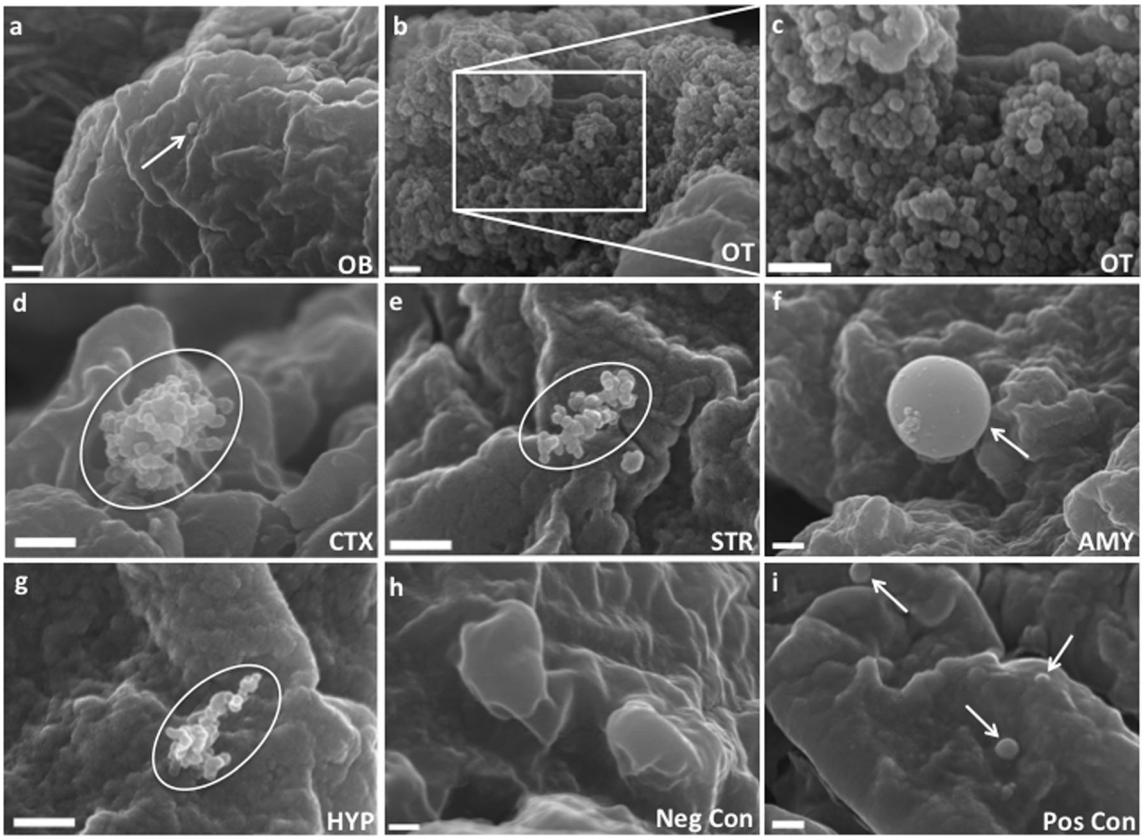

Fig. 2 Scanning electron micrographs of GNPs in the olfactory bulb (**a**), olfactory tract (**b**), detail of same olfactory tract region (**c**), cortex (**d**), striatum (**e**), amygdala (**f**), and hypothalamus (**g**) of a normal rat following intranasal administration with a sample from an empty brain region (**h**) and the striatum following intrastriatal injection (**i**) for comparison. *Arrows* and *ovals* indicate GNPs. *Scale bar* 200 nm

1 μg/rat and an OPN/GNP loading ratio of 1:25 should result in neuroprotection; an optimal dose and loading ratio have not yet been determined. However, based on our previous work with gelatin microspheres (GMSs), we anticipate the loading efficiency to be ≥96 % [20]. Two days after the MCAO procedure, rats were again sacrificed and their brains harvested. Each brain was sectioned into 2-mm-thick slices, stained with 2,3,5-triphenyl tetrazolium chloride (TTC) for 3–5 min, and fixed in PFA solution for 24 h before photographing for infarct volume calculation using ImageJ. Groups were compared with one-way ANOVA and Tukey post hoc test implemented with R statistical software (www.r-project.org). As anticipated from previous work using intrastriatal injections [20], when administered at 1 h post-MCAO, bare OPN peptide is able to significantly reduce mean infarct volume to 70.22 % that of untreated controls; but if not administered until 6 h after MCAO, the bare OPN peptide has no effect (Fig. 3). If administered at 3 h post-MCAO, the bare OPN peptide has a variable effect with some animals showing a strong response (79.43, 57.54, and 79.49 % reduction in mean infarct volume compared to MCAO) and other animals a comparatively weak response (18.71 and 21.61 % reduction). In contrast, when OPN-GNPs were administered 6 h after MCAO, the mean infarct volume was again reduced (71.57 % reduction in comparison to MCAO). Previously, using GMSs to deliver OPN to the post-ischemic brain via intrastriatal injection, we observed minimal difference between bare OPN and OPN encapsulated in GMS when administered at 1 h post-MCAO [20]; therefore, the benefits of GNP-mediated delivery at 1 and 3 h post-MCAO are anticipated to be small and were not investigated in the present study. Many promising neuroprotective agents have failed in clinical trials due to a short therapeutic window [22]; therefore, extension of the therapeutic window of OPN is a key benefit of GNP-mediated delivery.

The increased efficacy of OPN-GNPs over bare OPN peptide may be attributable to the following hypotheses: (1) GNPs provide sustained release of OPN over the course of hours to days, (2) GNPs protect OPN from degradation by proteases, and (3) higher OPN concentration in ischemic brain regions due to increased degradation of GNPs in these regions. Drug release from GNPs is controlled by a combination of diffusion and degradation [21]. The diffusive and degradative properties of GNPs can be tailored during fabrication to create particles that release encapsulated drugs over a period of hours to days by controlling the processing parameters, such as particle size and gelatin crosslink density [20, 21, 23]. By packaging OPN in GNPs, the release of OPN into the brain can be sustained

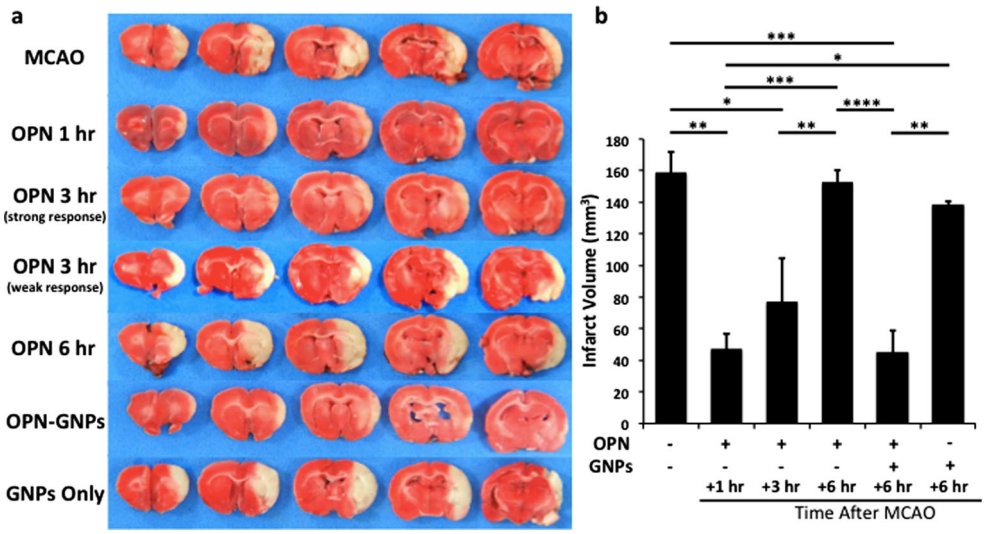

Fig. 3 Comparison of effect of intranasally administered osteopontin and osteopontin-loaded GNPs on infarct volume following MCAO. Representative images (**a**) and infarct volumes (**b**). *Error bars* represent the standard error of the mean. *$p<0.05$; **$p<0.005$; ***$p<0.001$; ****$p<0.0005$

during the healing process, which may contribute to the reduced infarct volumes observed [20]. Similarly, encapsulation may protect the OPN peptide from degradation by proteases in the post-ischemic brain and, thereby, explain the recovery of neuroprotection seen at 6 h post MCAO for the OPN-GNPs (Fig. 3). Finally, the use of GNPs as a delivery vehicle helps target the OPN peptide to damaged brain regions due to upregulation of matrix metalloproteinases (MMP) 2 and 9, also known as gelatinase A and B, in damaged areas of the brain following an ischemic event [16]. GNP degradation, and thus, drug release, will be significantly higher in brain regions with elevated levels of MMPs 2 and 9. Additionally, it was reported that nanoparticles administered systemically accumulate in the ischemic core and penumbra region during a stroke [24], providing further support for the concentration of OPN in damaged brain regions. Because gelatin and, therefore, GNPs are a substrate for MMP-9 and decreasing MMP-9 activity following ischemic stroke has been shown to reduce infarct volume [25], it may be inferred that one of the modes of action of OPN-GNPs in stroke is blocking MMP-9 activity: MMP-9 cleaves GNPs instead of its normal targets. However, in practice, this mechanism does not seem to contribute to the benefits of OPN-GNPs because unloaded GNPs have no effect on infarct volume.

We have demonstrated that GNPs are not only able to penetrate into the brain parenchyma following intranasal administration, but are also able to enhance the neuroprotective effects of an OPN peptide when used as a drug delivery vehicle. Though the results clearly demonstrate the beneficial effects of GNPs as an intranasal drug delivery vehicle as well as confirm the neuroprotective and therapeutic effects of OPN, additional studies are needed to fully elucidate the neuroprotective effects of OPN-GNPs in ischemic stroke. Crucially, functional assessments of the effects of intranasally delivered OPN-GNPs are needed as clinical studies use functional outcomes, not infarct volumes, to assess therapeutic outcomes [22]. Similarly, infarct volumes and functional assessments need to be conducted at longer time points (>48 h) to determine if the benefits of OPN-GNP administration remain in the long term. Finally, in order to fully assess clinical utility, a further exploration of the therapeutic window of OPN-GNPs, as well as a potential optimization of GNP release kinetics, is needed.

Acknowledgments This work was financially supported by Research Grants funded by the National Research Foundation of Korea Global Research Network (NRF-220-2011-E00027), Trionix Research Laboratory, Inc., and by a Whitaker International Summer Grant from the Institute of International Education. This work was carried out in part in the Frederick Seitz Materials Research Laboratory Central Facilities, University of Illinois.

All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of interest All authors declare that they have no conflict of interest.

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