Chapter 24

Intrathecal Delivery of Therapeutic Oligonucleotides for Potent Modulation of Gene Expression in the Central Nervous System

Zachary Kennedy, James W. Gilbert, and Bruno M. D. C. Godinho

Abstract

Therapeutic oligonucleotides hold tremendous potential for treating central nervous system (CNS) disorders. The route of administration of oligonucleotides significantly impacts both distribution and silencing efficiency. Here, we describe a technically simple, clinically relevant method to administer oligonucleotide compounds into the CNS via direct intrathecal injections. This method achieves distribution throughout the CNS rapidly and permits high-throughput testing of oligonucleotide efficacy and potency in mammals.

Keywords siRNA, Antisense oligonucleotides, CSF infusion, IT dosing, Intrathecal injections, Modified oligonucleotides, Gene silencing, Therapeutic oligonucleotides, CNS administration

1 Introduction

Therapeutic oligonucleotides hold tremendous potential for treating disorders of the central nervous system (CNS), but numerous factors can hinder the delivery of these compounds. Among the major determinants affecting delivery of drugs to the CNS is the route of administration.

Drug delivery to the CNS can be performed through two general approaches: (a) systemic, where oligonucleotides are initially delivered outside of the CNS, or (b) direct, where oligonucleotides are delivered directly within the CNS. Systemic delivery strategies (e.g., intravenous or subcutaneous) are technically simple and easy to perform, but require significantly higher doses and result in very low efficacy in the CNS, mostly due to the inability of oligonucleotides to pass the blood–brain barrier [1–3]. Direct delivery methods, on the other hand, are comparatively more technical, but require much lower doses and enable significantly better

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distribution, efficacy, and potency throughout the CNS than systemic methods [4, 5]. The most commonly used direct delivery methods, especially in rodent models, are intracerebroventricular (ICV) injections or lumbar intrathecal (IT) injections.

From a technical perspective, IT injections are easier to perform than ICV injections—they require less setup/procedural time and do not require stereotactic equipment [6, 7]. IT administration is also favored from a clinical perspective because it is less invasive than ICV injections; IT injections do not require brain surgery and can be performed as an outpatient procedure. IT injections can be performed with or without a catheter. Currently, IT injections are the only clinically approved route of administration for therapeutic oligonucleotide treatment of CNS disorders [8].

Given its ability to deliver to the CNS, the comparatively quick operation time, and the clinical relevance of the route of administration, IT injections are an attractive delivery method for which to screen oligonucleotide compounds for CNS applications. In this chapter, we describe how to perform direct IT injections in mice without using a catheter. Special attention is given to the preparation of the oligonucleotide compounds, prepping the mouse for the injections, and ensuring successful delivery.

2 Materials

2.1 Preparation of Test Oligonucleotides

1. SpeedVac™ concentrator.
2. NanoDrop™.
3. Bench top centrifuge.
4. 3 kDa cutoff Amicon spin column (0.5 mL or larger).
5. 5 mM sterile calcium chloride (CaCl₂) solution.
6. Sterile phosphate-buffered saline (PBS).

2.2 Direct Intrathecal Injections

1. Gas tight Hamilton syringe, 25 µL 1702RN with a 30 G needle, 0.5 in. long, and point style 4 (12° angle bevel).
2. Electric shaver.
3. Dry bead sterilizer.
4. Weigh scale.
5. Cotton-tipped applicators (Q-tips).
6. Eye lubricant.
7. Anesthetics (Isoflurane or Ketamine/Xylazine).
8. Analgesic (Ketoprofen or Meloxicam SR).
9. Antiseptic Povidone-Iodine Solution (5–10%).
10. Ethanol solution (70%).

11. Oligonucleotide preparation (antisense oligonucleotides, ASOs; small interfering RNAs, siRNAs) at desired concentration.
12. Sterile gloves.

3 Methods

Oligonucleotides must be prepared aseptically in a laminar flow cabinet to avoid contamination and maintain sterility. All procedures performed in live animals must be approved by the Institutional Animal Care and Use Committee (IACUC) prior to executing the experiment.

3.1 Preparation of Test Oligonucleotides

It is recommended that test oligonucleotides be treated with a calcium solution to minimize acute in vivo toxicities (*see Note 1*). ASOs can be treated immediately after synthesis, whereas siRNAs should be treated after duplexing of the guide and passenger strands (*see Note 2*).

1. Add the entire oligonucleotide solution into a 3 kDa cutoff Amicon spin column. For a 0.5-mL Amicon column, add ~300 μ L of 5 mM CaCl_2 solution, sterilized using an autoclave.
2. Elute by centrifugation at $14,000 \times g$ for 10 min (min) at room temperature (RT) and discard the flow-through. Repeat **steps 1 and 2**.
3. Remove excess CaCl_2 , add ~400 μ L of nuclease-free water, centrifuge at $14,000 \times g$ for 10 min at RT, and discard flow-through. Repeat this step.
4. Wash compounds by adding ~400 μ L of $1 \times$ sterile PBS, centrifuge at $14,000 \times g$ for 10 min at RT, and discard flow-through.
5. Transfer compounds to sterile tubes by adding $1 \times$ PBS to the Amicon column and mixing the solution up and down (*see Note 3*). To recover as much compound as possible, invert the Amicon filters into the collection tubes and centrifuge at $2000 \times g$ for 5 min at RT.
6. Determine the concentration of the test oligonucleotide preparation using a NanoDropTM. Adjust to the desired concentration using sterile $1 \times$ PBS.

While the aforementioned CaCl_2 wash steps minimize toxicity, recovery of oligonucleotides from the columns is often incomplete, resulting in reduced yields. If the initial amount of oligonucleotides is low, an alternative strategy would be to evaporate oligonucleotides in SpeedVac (using the no temperature option for drying rate) and resuspend at the desired concentration with the chosen buffer.

3.2 Direct Intrathecal Injection

1. Gather all materials and set up a clean area for the procedure. If possible, the procedure should be done in a laminar flow cabinet (Fig. 1a).
2. Record the weight of each animal. This is important to perform prior to injections, in order to monitor the well-being of the animal after the procedure concludes.
3. Anesthetize the mouse according to the approved IACUC protocol (e.g., isoflurane, ketamine/xylazine, avertin). If isoflurane is used, induction is carried out with 4–5%, and maintenance with 1.5% by continuous administration through a nose cone.
4. Ensure the mouse is sufficiently sedated by gently pinching hind paws. If properly sedated, the mouse should not respond to the pinch.
5. Cover both the eyes with eye lubricant using a sterile cotton-tipped applicator.
6. Using an electric shaver, shave hair around the lower spine, starting from the base of the tail and spanning an area approximately 3×3 cm.
7. Clean the shaved area with an antiseptic 5–10% povidone-iodine solution, then wipe with 70% ethanol. Repeat this step three times.
8. Place a 15-mL conical tube under the abdomen of the mouse to expose a bigger area of the interspinous ligament, which is punctured by the needle to access the intradural space (see Fig. 1b). Positioning the mouse on a 15-mL conical tube also makes it easier to find and grasp the iliac crest.
9. Identify the iliac crest by finding the two pits formed at the interface of the muscle and the hip bone (see Fig. 1b, black dotted lines).
10. The injection site (L5–L6) will be directly above the iliac crest (marked with an asterisk in Fig. 1b). Determine the position of the L6 vertebra by identifying the most protruding spinal process (see Fig. 1b). Injection at this position reduces the possibility of spinal damage, since this site is where the spinal cord ends and the cauda equina begins.
11. Prepare and load the Hamilton Syringe with a test compound (5–10 μ L) (see Note 4). The same syringe can be used for multiple animals; however, use only one syringe per compound to avoid cross contamination. Syringes should be cleaned and the tip sterilized in the bead sterilizer between animals (see Note 5).

12. Grasp the animal by pinching just above the hips, taking care to ensure that the tail is visible (see Fig. 1c).
13. Insert the needle between the groove of L5 and L6 vertebrae at a $\sim 30^\circ$ angle, and to a depth of approximately 0.5 cm. Once the needle has successfully entered the intradural space, the tail of the mouse will move or “flick” (see Note 6, and Fig. 1c, d).
14. After observation of the tail flick, slowly and continuously inject oligonucleotides. After the entire volume of test oligonucleotide has been injected, keep the needle in the intradural space for 5–10 s to prevent or reduce backflow.
15. Carefully remove the needle and inspect the site for backflow.
16. Administer analgesia as per IACUC protocol.
17. Return the animal to its cage and monitor for pain and discomfort for at least 30 min. Until animals regain sternal recumbency, supplemental heat should be provided. This can be achieved by keeping the cage on top of a heating pad. Monitor animals daily for 3 days after the procedure, and weekly thereafter. IT injections may be repeated as necessary (see Notes 7 and 8).

When establishing this methodology in the lab, it is recommended to first practice using Evans blue dye. A successful injection will result in immediate distribution of the dye throughout the spinal cord and periphery of the brain (see Fig. 1e). It is also important to consider that distribution of therapeutic oligonucleotides is highly dependent on its conjugated ligand modality. Thus, in studies investigating biodistribution, oligonucleotides are often labeled with fluorescent tags, such as Cy3. Figure 2 shows the distribution of a Cy3-labeled Di-siRNA scaffold (20 nmol in 10 μ L) 48 h after a single IT injection.

3.3 Suggestions for Tissue Collection and Processing

For microscopy, the whole spine may be fixed in decalcifier/formalin after perfusion of the animal with cold $1\times$ PBS. For RNA and protein analyses, the spinal cord can be either dissected or flushed out of the spine. After identifying the different segments of the spinal cord, these can be divided longitudinally to generate two samples per region for: (1) RNA assessment and (2) protein evaluation. RNA analyses can be carried out by RT-qPCR, bDNA, or other methods. Protein analyses can be performed by Western blot, ELISA, or other methods.

Fig. 1 (continued) maximum exposure of the cartilage between the L4 and L5 spinal segments. Successful needle insertion is often accompanied with a subtle tail-flick, which can be observed at the base of the tail or the very tip (see arrows), (d) Placement of needle during procedure. Dotted lines indicate the superficial depression formed by the spinal cord and iliac crest, where the hip meets the spinal cord. (e) Ventral view of brain and spinal cord immediately after IT injection of Evans blue dye

Fig. 2 Distribution pattern of Cy3-labeled oligonucleotide within the spinal cord. Horizontal cross sections of spinal cord segments following intrathecal injection of Cy3 labeled Di-siRNA oligonucleotides (20 nmol). Mice were euthanized 48 h post injection and tissues processed for microscopy. Tiled fluorescent images were acquired using 10 $\times$ objective and displayed as overlay images: Cyan (DAPI, Nuclei), red (Cy3-labeled oligo). Scale bar 1 mm

4 Notes

1. Upon injection into the CSF, the negatively charged phosphate backbone of therapeutic oligonucleotides can rapidly sequester divalent ions (Ca^{2+} , Mg^{2+}), causing acute adverse events (e.g., seizures). To minimize toxicity in vivo, a calcium treatment can be performed to saturate every phosphate position.
2. ASOs and siRNAs can be either synthesized in-house or acquired through a vendor or a contract research organization. If test oligonucleotides are delivered in dried form, they should be resuspended in nuclease-free water or other appropriate buffers. In the case of siRNA, guide and passenger strands can be annealed in equimolar amounts after the determination of initial concentration.

3. To ensure compounds are not overdiluted, it is important to start with small volumes (e.g., 50–100 μL) and wash the filter several times.
4. Based on previous literature, we found that 10 μL injections in adult mice are well-tolerated and do not cause neuronal loss, astrogliosis, or microgliosis [9]. We do not recommend injecting in less than 5 μL due to higher chance of pipetting error while preparing the compounds.
5. Syringes should be cleaned right after use to avoid clogging. Rinse the syringe with: Milli-Q purified (or deionized) water (5 $\times$), diluted Hamilton cleaning solution (P/N 18311) (5 $\times$), then Milli-Q purified (or deionized) water (5 $\times$) again. Sterilize the tip by dipping it on a dry bead sterilizer for 15–20 s and allow it to cool. Rinse the syringe with 1 $\times$ PBS before drawing the compound for the next injection. Disinfectants, such as Microcide SQ[®] (P/N 3995–01), can be used to eliminate a variety of common bacteria, fungi, and viruses. Before storage, and after using a cleansing agent or disinfectant, rinse with Milli-Q purified (or deionized) water and with acetone. Air dry and store in case.
6. The flick of the tail can be very subtle; while advancing the needle into the skin, it is important to focus your attention on the tail. If the tail flick event is missed, and the needle is pulled back and re-inserted, the likelihood of observing a tail flick on a second attempt is significantly lower. Tail flicks are somewhat inversely correlated with the deepness of the anesthesia—very deep anesthesia will make it harder to see the tail flick event occur.
7. Immediately after the procedure, mice should be warmed with thermal support (i.e., a heating pad or a heat lamp) and observed until they are ambulatory. After mice are ambulatory, return mice to their cages and make sure food and water are easily accessible to mice. This can be achieved by adding food pellets to the floor of the cage and supplanting the cage with gel water. After the procedure, mice should be monitored at least once every 24 h for 3 days. Monitor for signs of stress that could indicate pain, including weight loss, lethargy, reduction in rearing behavior, excessive barbering, urine stain, and changes in behavior. If at any point, the mice show signs of pain, they should be given analgesics or euthanized in accordance with the IACUC-approved protocol.
8. Depending on the dosing strategy and the individual IACUC protocol, IT injections can be performed on a single mouse up to three times per 24 h.

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