Formulations for use in the prevention and/or treatment of peripheral neuropathy and its associated diseases.

[001]. Field

[002]. The invention relates to formulations and methods of using formulations in the prevention and/or treatment of peripheral neuropathy and its associated diseases, particularly in the eye.

[003]. Background

[004]. The following discussion of the background to the invention is intended to facilitate understanding of the present invention. However, it should be appreciated that the discussion is not an acknowledgment or admission that any of the material referred to was published, known or a part of the common general knowledge in any jurisdiction as at the priority date of the application.

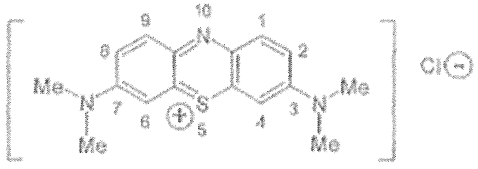
[005]. Our nervous system is divided in two components: the central nervous system (CNS), which includes the brain and the spinal cord, and the peripheral nervous system (PNS), which encompasses nerves fibres outside the brain and spinal cord.

[006]. Unlike the brain and the spinal cord of the CNS that are protected by myelin layers, the vertebrae and the skull, the nerves of the PNS are sometimes unmyelinated. Neurons are the cell bodies constituting the nervous system. Each neuron has a long process, known as the axon, which transmits the electrochemical signals through which neurons communicate. These axonal processes constitutes the PNS.The Dorsal Root Ganglion is part of the PNS and its nerve fibres conduct information from sensory inputs to go eventually to the brain for processing. Peripheral nerves supply important sensory units in the hands, feet and also the nerves of the cornea. Peripheral neuropathy is damage to the PNS. Peripheral neuropathy, often causes weakness, numbness and pain. Peripheral neuropathy can result from traumatic injuries, infections, metabolic problems, inherited causes and exposure to toxins. One of the most common causes is diabetes which results in hyperglycemia/ raised glucose level in the human body. Hyperosmolarity is also a sequelae of hyperglycemia potentially leading to hyperosmolarity induced peripheral nerve damage.

[007]. Diabetes Mellitus (DM), is a group of metabolic disorders characterised by high blood sugar levels over prolonged period of time. As of 2017, an estimated 425 million people had diabetes worldwide (Diabetes atlas 2017). In Diabetes Mellitus (DM), there is associated complications of peripheral neuropathy or damage to the PNS. Diabetic neuropathy is the most common complication in DM and affects all peripheral nerves including sensory neurons and motor neurons and less often affects the autonomic nervous system. Currently, there are no definitive management for hyperglycemia (high glucose) induced peripheral nerve injury besides good control of blood sugar level. Managing blood sugar levels can be very difficult for some patients especially those who have to be on insulin injections, due to

cost, and pain of injections. With the exception of managing blood sugar levels the only treatment for diabetic neuropathy is reducing pain and other symptoms. Degradation of nerve fibres, are common in type II diabetes, particularly in diabetes patients with proliferative diabetic retinopathy (Gao et al. Int J Ophthalmol, Vol 8, No.2, 2015, 358-364). Patients with DM demonstrate progressive decrease in corneal nerve density and reduction in corneal sensitivity (Han et al. Clinical interventions in Aging 2019: 14 p 53-63). Corneal nerve fibre length measured with confocal microscopy has been demonstrated to be capable of predicting the development of diabetic peripheral neuropathy (Han et al 2019). Due to hyperglycemia, the ocular surface environment can also become hyperosmolar, resulting in hyperosmolarity induced corneal nerve damage. There is a need for better management and treatment of diabetic neuropathy such preventing and/or treating peripheral nerve damage in the eye.

[008]. Methylthioninium Chloride (MTC) (also known as Methylene blue (MB); methylthionine chloride; tetramethylthionine chloride; 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; C.I. Basic Blue 9; tetramethylthionine chloride; 3,7-bis(dimethylamino) phenazathionium chloride; Swiss blue; C.I. 52015; C.I. Solvent Blue 8; aniline violet; and Urolene Blue®) is a low molecular weight (319.86), water soluble, tricyclic organic compound of the following formula:



MTC

[009]. Various synthetic methods for MTC are known and have been summarized in WO 2006/032879. WO 2006/032879 also discloses a number of applications of methylene blue, which include use as a medical dye, as a redox indicator, as an antiseptic, for the treatment and prevention of kidney stones, and for the treatment of melanoma, malaria, and viral infections. MTC has also been used as an oxidizing agent and as an antidote in the case of carbon monoxide, nitrite and aniline poisoning. MTC has also been proposed for treatment of Alzheimer's disease.

[0010]. MTC is soluble in water and alcohol. MTC is not recommended to be dissolved in saline because precipitation has been reported in cases where methylene blue has been diluted with sodium chloride 0.9%, saline (due to presence of chloride ions which have been shown to reduce the solubility of methylene blue). MTC is reported to be incompatible with caustic alkalis, iodides and dichromates, and oxidising and reducing substances.

[0011]. MTC when injected into the spinal cord compartment of cerebral spinal fluid has been reported to cause massive peripheral nerves damage and resultant total paraplegia (Sharr et al. J. Neurol. Neurosur, and Psychiatry 1978, 41, 384-386).

[0012]. Accidental use of MTC on the eye in higher concentrations, instead of Trypan Blue, during cataract surgery has led to irreversible blindness, corneal edema (Timucin et al. Arq. Bras. Oftalmol. vol.79 no.2, 2016), bullous keratopathy resulting in keratoplasty, and endophthalmitis with progression to corneal failure. Methylene blue is therefore generally considered a toxic dye not for use in the eyes. Further, as MTC is a dye its use on skin or mucosa generally results in unsightly blue discoloration and stains.

[0013]. Thus, there exists a need to develop formulations methods and uses which ameliorates at least one of the disadvantages outlined above.

[0014]. Summary

[0015]. An object of the invention is to ameliorate some of the above mentioned difficulties preferably formulations and methods of using formulations in the prevention and/or treatment of peripheral neuropathy, particularly in the eye.

[0016]. One aspect of the invention relates to a topical preparation for protecting against and/or treating peripheral nerve damage and its associated conditions comprising, less than or equal to 5 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer.

[0017]. Another aspect of the invention relates to a topical preparation as described herein, for use in preventing and/or treating peripheral nerve damage and its associated conditions.

[0018]. Another aspect of the invention relates to a method of preventing and/or treating peripheral nerve damage comprising administering to a subject in need of preventing and/or treating peripheral nerve damage and its associated conditions (a) a topical preparation comprising, less than or equal to 5 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer or (b) a topical preparation as described herein.

[0019]. Another aspect of the invention relates to use of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride in the manufacture of a topical preparation comprising, less than or equal to 5 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer or a carrier for prevention and/ or treatment of peripheral nerve damage and its associated conditions.

[0020]. Another aspect of the invention relates to a topical preparation for protecting against and/or treating peripheral nerve damage and its associated conditions comprising, less than or equal to 5 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a carrier.

[0021]. Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

[0022]. Brief description of the drawings

[0023]. In the figures, which illustrate, by way of example only, embodiments of the present invention include,

[0024]. **Figure 1.** Schematic of the test timeline.

[0025]. **Figure 2.** Cell viability by hoechst 33342 nucleic acid stain in cells treated with methylene blue under glucose toxicity condition. DRGs were treated with methylene blue 1h prior to 150 mM glucose and 48h later HTS assay was performed.

[0026]. **Figure 3.** Extremity number per neuron by beta III tubulin staining in cells treated with methylene blue under glucose toxicity condition. DRGs were treated with methylene blue 1h prior to 150 mM glucose and 48h later HTS assay was performed.

[0027]. **Figure 4.** Total length per neuron by beta III tubulin staining in cells treated with methylene blue under glucose toxicity condition. DRGs were treated with methylene blue 1h prior to 150 mM glucose and 48h later HTS assay was performed.

[0028]. **Figure 5.** Cell viability by WST8 staining in cells treated with methylene blue under glucose toxicity condition. DRGs were treated with methylene blue 1h prior to 150 mM glucose and 48h later HTS assay was performed.

[0029]. Detailed description

[0030]. Particular embodiments of the present invention will now be described with reference to the accompanying drawings. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention. Additionally, unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which the present invention belongs. Where possible, the same reference numerals are used throughout the figures for clarity and consistency.

[0031]. One aspect of the invention relates to a topical preparation for protecting against and/or treating peripheral nerve damage and its associated conditions comprising, less than or equal to 150 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer.

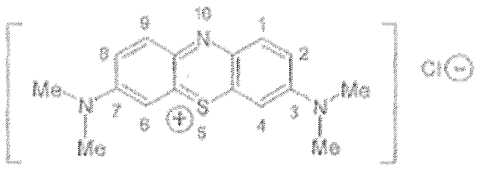
[0032]. While, methylene blue in the management of any eye related conditions has been strictly discouraged or prohibited, it has been devised that a solution having a lower concentration of methylene blue that has been buffered to a physiological pH range can be tolerated. Similarly, the solution can be tolerated by peripheral nerves. Advantageously, a lower concentration of methylene blue can be used for management of peripheral nerve diseases, particularly in various embodiments as a topical preparation in an eye related peripheral nerve disease and its associated conditions that may be caused by traumatic injuries, infections, metabolic problems, inherited causes, exposure to toxins such as chemotherapy, hyperglycemia and/or hyperosmolarity induced peripheral nerve damage, or

any other cause known in the art that results in peripheral nerve damage and its associated conditions.

[0033]. As used herein the term 'topical preparation' refers to any preparation for application to a particular place in or on a body. It may refer to solutions, suspensions, lotions, ointments, gels, powders, pastes, matrixes, transdermal patches or any other formulation that allows application to a particular place in or on a body. In various embodiments the particular place in or on a body refers to body surfaces such as skin or mucous membranes. In various embodiments the particular place in or on a body refers to directly at a nerve site for example a nerve site on the surface of the cornea. In various embodiments the particular place in or on a body refers to the eye.

[0034]. As used herein the term 'protecting', 'protect', or 'protection' or 'treating', 'treat' or 'treatment' of peripheral nerve damage refers to stopping or reducing damage or death of nerve cells, fibers or extensions from the PNS and/or providing an environment suitable for formation and growth of new nerve cells, fibers or extensions in the PNS. In various embodiments this may be achieved by reducing the negative sequelae resulting from the influx of glucose into the mitochondria of nerve cells resulting in cellular injury. Nerve cells are known to have greater mitochondrial volume than other cells. In various embodiments this may be achieved by reducing non-enzymatic bonding i.e. glycation of glucose to proteins or lipoproteins that may lead to damage of peripheral nerves. In various embodiments this may be achieved by reducing the toxic effects or toxins such as reducing the undesirable effects of chemotherapeutic drugs on nerves. In various embodiments this may be achieved by increasing the anti-oxidant defense capacity of the cell.

[0035]. As used herein the term '3,7-bis(dimethylamino) phenothiazin-5-ium chloride' may refer to Methythioninium Chloride (MTC) (also known as Methylene blue (MB); methylthionine chloride; tetramethylthionine chloride; C.I. Basic Blue 9; tetramethylthionine chloride; 3,7-bis(dimethylamino) phenazathionium chloride; Swiss blue; C.I. 52015; C.I. Solvent Blue 8; aniline violet; and Urolene Blue®) comprising a low molecular weight (319.86), water soluble, tricyclic organic compound of the following formula:



MTC

[0036]. In various embodiments the 3,7-bis(dimethylamino) phenothiazin-5-ium chloride may be formulated by any of the methods known in the art.

[0037]. In various embodiments the topical preparation comprises between 0.0001 μM and 150 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. In various embodiments the topical preparation comprises 149.5 μM or less of MTC. In various embodiments the topical preparation comprises 59.5μM or less of MTC. In various embodiments the topical preparation comprises 4.5 μM or less of MTC. In various embodiments the topical preparation comprises between 0.0001 μM and 50 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. In various embodiments the topical preparation comprises between 0.001 μM and 90 μM, or between 0.005 μM and 0.5 μM or between 0.0059 μM and 0.49 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. In various embodiments the topical preparation is selected from the group comprising 0.0005 μM, 0.005 μM, 0.05 μM 0.5 μM, 5μM, 50μM, 100μM and 150μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. In various embodiments the topical preparation comprises 0.05 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride.

[0038]. In various embodiments the physiological buffer comprises a hydrogen ion aqueous solution comprising a mixture of an acid and its conjugate base capable of maintaining a preparation at a physiological pH of about pH 4 to about pH8 when it comes into contact with additives or components of a biological environment. In various embodiments the physiological buffer is selected from the group comprising bicarbonate, bicine. Cacodylate, HEPES, MES, MOPS, PIPES, phosphate, TEPS, TAPSO, TES, Tricine, and Tris. In various embodiments the physiological buffer comprises sodium bicarbonate and sodium chloride. Surprisingly, despite technical prohibition to use MTC with sodium chloride when the sodium chloride is present together with sodium bicarbonate in a physiological buffer the MTC remains in solution, demonstrates protection of peripheral nerves and has a pH suitable for both direct use on peripheral nerves and ophthalmic use. In various embodiments the pH of the solution is from 4 to 8. In various embodiments the pH of the solution is from 4 to 7.5. In various embodiments the pH of the solution is 4. In various embodiments the pH of the solution is 5. In various embodiments the pH of the solution is 6. In various embodiments the pH of the solution is 7. In various embodiments the pH of the solution is 7.4. In various embodiments the pH of the solution is 7.5. In various embodiments the pH of the solution is 8.

[0039]. In various embodiments the physiological buffer solution comprises HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), preferably in the presence of sodium bicarbonate and sodium chloride. In various embodiments the physiological buffer comprises a neurobasal medium.

[0040]. In various embodiments the topical preparation comprises a carrier. In various embodiments the carrier may comprise polymers such as hydrophilic polymers of high molecular weight, polyvinyl alcohol, poloxamers, hyaluronic acid, carbomers, cellulose

derivatives (gellan gum, xanthan gum) dexamethasone, pilocarpine, 2-hydroxypropyl-beta-cyclodextrin, poly(2-hydroxyethlmethacrylate), ethylene glycol dimethylacrylate, acrylamide, N-vinylpryrrolidone and ethylacrylate or combinations thereof. In various embodiments the carrier may comprise excipients that may increase penetration into the eye such as chelating agents, surfactants or cyclodextrins. In various embodiments the carrier may comprise inclusion complexes such as nanoparticles, microparticles, niosomes, discosomes, dendrimers, in situ gels, wafers or embedded in contact lenses. In various embodiments the carrier may comprise sugar molecules such as sucrose, glucose, lactose D-glucose (dextrose). In various embodiments the endogenous glucose naturally occurring in the peripheral nerve environment may form part of the carrier in solution. This is particularly relevant in subjects with DM who have hyperglycemia involving a high concentration of glucose. In various embodiments any endogenous glucose that interacts with the solution may be removed from the body together with the solution washing away with tears.

[0041]. In various embodiments the carrier modulates the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride over a pre-determined time period. Any known carrier that is able to modulate the rate of MTC release into the peripheral nerve environment would be suitable. In various embodiments the carrier modulates the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride to a predetermined rate below the rate of release of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride per se . In various embodiments a suitable carrier may comprise polymers such as hydrophilic polymers of high molecular weight, polyvinyl alcohol, poloxamers, hyaluronic acid, carbomers, cellulose derivatives (gellan gum, xanthan gum) dexamethasone, pilocarpine, 2-hydroxypropyl-beta-cyclodextrin, poly(2-hydroxyethlmethacrylate), ethylene glycol dimethylacrylate, acrylamide, N-vinylpryrrolidone and ethylacrylate or combinations thereof. In various embodiments a suitable carrier may comprise inclusion complexes such as nanoparticles, microparticles, niosomes, discosomes, dendrimers, in situ gels, wafers or may be embedded in contact lenses. In various embodiments the carrier slows down or reduces the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride over a pre-determined time period.

[0042]. In various embodiments the carrier increases the viscosity to a range of about 15 to 150 milli-Pascals per second. In various embodiments the carrier increases the viscosity to a range of about 15 to 100 milli-Pascals per second. In various embodiments the carrier increases the viscosity to a range of about 15 to 50 milli-Pascals per second. In various embodiments the viscosities are those at room temperature. Increased viscosity may have the advantage of enabling longer contact time between MTC and the corneal surface, thus increasing its bioavailability. In various embodiments the polymers, sugar molecules, nanoparticles, microparticles, niosomes, discosomes, dendrimers carriers may increases the viscosity of the solution.

[0043]. In various embodiments the carrier modulates the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. In various embodiments the carrier is a micro-particle with a sustained release profile. This has the advantage of increasing the viscosity of the preparation and it also reduces the need for frequent applications of eye drops by increasing the pre-corneal retention time of the topical preparation. The sustained release formulation also has the advantage of reducing unsightly blue stains on the skin as the increased viscosity reduces dripping and/or release with tears causing less stains and discoloration. In various embodiments the carrier micro-particle with a sustained release profile comprises a poly (lactide-co-glycolide) (PLGA) microspheres however any microparticle known in the art to be able to provide a slow/ sustained release profile would be suitable. In various embodiments the PLGA microspheres comprise a polymer ratio of PLGA (50:50), PLGA (75:25), or PLGA (85:15). Such ratios may be able to modulate the release profile of MTC. In various embodiments the sustained release profile of MTC may be modulated to 1, 3, or 6 months with the use of different polymer ratios in the microspheres.

[0044]. Another aspect of the invention relates to a topical preparation as described herein, for use in preventing and/or treating peripheral nerve damage.

[0045]. In various embodiments the peripheral nerve damage results in or comprises neurotrophic keratopathy. In various embodiments the peripheral nerve damage comprises peripheral neuropathy caused by traumatic injuries, infections, metabolic problems, inherited causes, exposure to toxins such as chemotherapy, hyperglycemia induced peripheral nerve damage, or any other cause known in the art that results in peripheral nerve damage.

[0046]. In various embodiments the topical preparation is for ophthalmic use. In various embodiments the topical preparation is in the form of eye drops or ointment.

[0047]. Another aspect of the invention relates to a method of preventing and/or treating peripheral nerve damage comprising administering to a subject in need of preventing and/or treating peripheral nerve damage (a) a topical preparation comprising, less than or equal to 150 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer or (b) a topical preparation as described herein above, or (c) a topical preparation comprising, less than or equal to 150 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a carrier.

[0048]. In various embodiments the topical preparation is an ophthalmic topical preparation.

[0049]. As used herein a subject refers to a patient or an individual diagnosed with, at high risk or having or suspected of having peripheral neuropathy. In various embodiments the patient or individual may be diagnosed with DM or pre-diabetes or suspected of having or developing DM or an individual with dry eye disease. In various embodiments the patient or individual may be diagnosed with neurotrophic keratopathy. In various embodiments the patient or individual may have peripheral neuropathy caused by traumatic injuries, infections,

metabolic problems, inherited causes, exposure to toxins such as chemotherapy, hyperglycemia and/or hyperosmolarity induced peripheral nerve damage, or any other cause known in the art that results in peripheral nerve damage.

[0050]. In various embodiments the topical preparation is administered directly to the nerve site. In various embodiments the topical preparation is administered into the eye.

[0051]. In various embodiments the method further comprises the step of measuring parameters of corneal nerves to determine whether the subject is in need of preventing and/ treating peripheral nerve damage prior to administration of the topical preparation. In various embodiments the parameters measured comprise nerve fiber length, nerve fiber density. In various embodiments the parameters are measured with *in vivo* or *in situ* confocal microscopy of the cornea. The condition of corneal nerve fibers has been demonstrated to correlate to DM and peripheral neuropathy in other parts of a subjects body (Han et al. 2019).

[0052]. In various embodiments the method further comprises the step of measuring parameters of corneal nerves after administration of the topical preparation. In various embodiments the parameters measured comprise nerve fiber length, nerve fiber density. In various embodiments the parameters are measured with *in vivo* or *in situ* confocal microscopy of the cornea. This may allow monitoring of the effectiveness of the prevention and/or treatment.

[0053]. Another aspect of the invention relates to the use of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride in the manufacture of a topical preparation comprising, less than or equal to 150 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer or a carrier for prevention and/or treatment of peripheral nerve damage.

[0054]. In various embodiments the topical preparation for prevention and/or treatment of peripheral nerve damage is as described herein.

[0055]. In various embodiments the use is for ophthalmic use. In various embodiments the use is for treatment of dry eye. In various embodiments the use is for prevention of corneal peripheral neuropathy.

[0056]. Another aspect of the invention relates to a topical preparation for protecting against and/or treating peripheral nerve damage comprising, less than or equal to 150 µM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride and a carrier that increases the viscosity of the preparation to a range of about 15 to 150 milli-Pascals per second.

[0057]. In various embodiments the topical preparation comprises a carrier. In various embodiments the carrier may comprise polymers such as hydrophilic polymers of high molecular weight, polyvinyl alcohol, poloxamers, hyaluronic acid, carbomers, cellulose derivatives (gellan gum, xanthan gum) dexamethasone, pilocarpine, 2-hydroxypropyl-beta-cyclodextrin, poly(2-hydroxyethlmethacrylate), ethylene glycol dimethylacrylate, acrylamide,

N-vinylpryrrolidone and ethylacrylate or combinations thereof. In various embodiments the carrier may comprise excipients that may increase penetration into the eye such as chelating agents, surfactants or cyclodextrins. In various embodiments the carrier may comprise inclusion complexes such as nanoparticles, microparticles, niosomes, discosomes, dendrimers, in situ gels, wafers or embedded in contact lenses. In various embodiments the carrier may comprise sugar molecules such as sucrose, glucose, lactose D-glucose (dextrose). In various embodiments the endogenous glucose naturally occurring in the peripheral nerve environment may form part of the carrier in solution. This is particularly relevant in subjects with DM who have hyperglycemia involving a high concentration of glucose. In various embodiments any endogenous glucose that interacts with the solution may be removed from the body together with the solution washing away with tears.

[0058]. In various embodiments the carrier reduces the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. Any known carrier that is able to slow release profile such as any carrier able to reduce the rate of MTC release into the peripheral nerve environment would be suitable. In various embodiments the carrier reduces the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride to a predetermined rate below the rate of release of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride alone. In various embodiments a suitable carrier may comprise polymers such as hydrophilic polymers of high molecular weight, polyvinyl alcohol, poloxamers, hyaluronic acid, carbomers, cellulose derivatives (gellan gum, xanthan gum) dexamethasone, pilocarpine, 2-hydroxypropyl-beta-cyclodextrin, poly(2-hydroxyethlmethacrylate), ethylene glycol dimethylacrylate, acrylamide, N-vinylpryrrolidone and ethylacrylate or combinations thereof. In various embodiments a suitable carrier may comprise inclusion complexes such as nanoparticles, microparticles, niosomes, discosomes, dendrimers, in situ gels, wafers or may be embedded in contact lenses.

[0059]. In various embodiments the carrier increases the viscosity to a range of about 15 to 150 milli-Pascals per second. In various embodiments the carrier increases the viscosity to a range of about 15 to 100 milli-Pascals per second. In various embodiments the carrier increases the viscosity to a range of about 15 to 50 milli-Pascals per second. In various embodiments the viscosities are those at room temperature. Increased viscosity may have the advantage of enabling longer contact time between MTC and the corneal surface, thus increasing its bioavailability. In various embodiments the polymers, sugar molecules, nanoparticles, microparticles, niosomes, discosomes, dendrimers carriers may increases the viscosity of the solution.

[0060]. In various embodiments the carrier reduces the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride. In various embodiments the carrier is a micro-particle with a slow release profile. This has the advantage of increasing the viscosity

of the preparation and it also reduces the need for frequent applications of eye drops. The slow release formulation also has the advantage of reducing unsightly blue stains on the skin as the increased viscosity reduces dripping and/or release with tears causing less stains and discoloration. In various embodiments the carrier micro-particle with a sustained/slow release profile comprises a poly (lactide-co-glycolide) (PLGA) microspheres however any microparticle known in the art to be able to provide a sustained/slow release profile would be suitable. In various embodiments the PLGA microspheres comprise a polymer ratio of PLGA (50:50), PLGA (75:25), or PLGA (85:15). Such ratios may be able to reduce the release profile of MTC. In various embodiments the release profile of MTC may be modulated to 1, 3, or 6 months with the use of different polymer ratios in the microspheres.

[0061]. In various embodiments the carrier comprises a microparticle as described herein.

[0062]. In various embodiments the carrier comprises a gel as described herein.

[0063]. As used herein the tem 'gel' may refer to a cross linked network comprising a 3 dimensional semi-solid matrix containing or able to contain a high percentage of liquid content.

[0064]. Methylene blue has never been shown to be efficacious in any PNS disorders. The devised topical preparation has an indication to manage peripheral neuropathy that may be caused by traumatic injuries, infections, metabolic problems, inherited causes, exposure to toxins such as chemotherapy, hyperglycemia induced peripheral nerve damage, or any other cause known in the art that results in peripheral nerve damage. This can be preventive and/or therapeutic.

[0065]. Throughout the specification, unless otherwise indicated to the contrary, the terms "comprising", "consisting of", and the like, are to be construed as non-exhaustive, or in other words, as meaning "including, but not limited to".

[0066]. Throughout the specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0067]. Throughout the specification, unless the context requires otherwise, the word "include" or variations such as "includes" or "including", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0068]. As used herein, the term "about" typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0069]. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by a skilled person to which the subject matter herein belongs. As used herein, the following definitions are supplied in order to facilitate the understanding of the present invention.

[0070]. Throughout this document, unless otherwise indicated to the contrary, the terms "comprising", "consisting of", "having" and the like, are to be construed as non-exhaustive, or in other words, as meaning "including, but not limited to".

[0071]. Furthermore, throughout the specification, unless the contest requires otherwise, the word "include" or variations such as "includes" or "including" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0072]. As used in the specification and the appended claims, the singular form "a", and "the" include plural references unless the context clearly dictates otherwise.

[0073]. Examples.

[0074]. Example 1: Prevention of damage of peripheral nerves by glucose.

[0075]. In-vitro cell assay based experiment demonstrated the ability for methylene blue to protect the dorsal root ganglion neurones and its nerve extensions from the insult of high glucose concentration with just 1 hour of pre-insult incubation.

[0076]. The objective of the present study is to explore the dose-dependent neuroprotective effects of methylene blue against the neurotoxicity induced by glucose using a multi-parametric cell-based protocol that reflects the mechanisms of action. The assay was carried out using freshly isolated rat dorsal root ganglion neurons (DRG) cultured in 96-well-plates pre-treated with different concentrations of MTC compound (0.0005 μM, 0.005 μM, 0.05 μM, 0.5 μM, 5 μM and 50 μM) prior to exposure to 150 mM glucose for 48 hours.

[0077]. In vitro injury was induced in DRGs culture by glucose treatment (150 mM) for 48 hours. DRGs were incubated with six increasing concentrations of the test compound (0.0005 μM, 0.005 μM, 0.05 μM, 0.5 μM, 5 μM and 50 μM) for 1 hour prior to the glucose treatment. After treatments, cells were fixed and stained with anti-beta III tubulin, and were then analyzed with BD Pathway 855 (Becton Dickinson). By using HCS technology, cell parameters associated with neurite outgrowth - an indication of pre-lethal cytotoxic effects - were measured at single cell level, which allows high-throughput screening.

[0078]. In order to compare the degree of neuroprotection of the compound at different concentrations, the level of neuroprotection of each concentration of the compound was studied and four different scores of neuroprotection were established according to the level of variation when compared with control cells: No neuroprotection, low neuroprotection, moderate neuroprotection, and high neuroprotection.

[0079]. Primary cultures of dorsal rat ganglion neurons were prepared from the dorsal ganglions of neonatal Sprague-Dawley rats at day 8. Ganglions were removed from spine under a binocular microscope and neurons were enzimatically dispersed twice with collagenase I (Worthington) 1.25 mg/ml during 1hour at 37°C and plated on poly-L-lysine (Sigma Aldrich # Cat.P1524) wells with neurobasal medium (Invitrogen # Cat.21103-049) supplemented with B27(Invitrogen # Cat.17504-044) with a number of 30,000 cells per well. Cells were maintained in neurobasal medium supplemented with B-27 component for 8 days at 37°C in a humidified 5% CO_2 atmosphere.

[0080]. Methylene blue compound (Tocris) was solubilized in ethanol to a final concentration of 10mM. The concentration of the test compound was then adjusted in neurobasal medium (Invitrogen # Cat.21103-049) supplemented with 20% B27 (Invitrogen # Cat.17504-044).

[0081]. At day 8, cells were pre-treated for 1 hour with 6 increasing concentrations of methylene blue (0.0005 μM, 0.005 μM, 0.05 μM, 0.5 μM, 5 μM, and 50 μM) in complete medium. Then, medium was replaced with neurobasal medium without the B27 component (80%) plus neurobasal medium supplemented with B27 component (20%) for 48h in the presence of glucose 150 mM. Some cells were also incubated with ethanol 0.5% as a vehicle control which is the equivalent maximum concentration found in the treatments (50 μM). See figure 1 for the testing timeline.

[0082]. The neurobasal medium are basal media formulated to meet the neuronal cells special requirements. It allows for long-term maintenance of the normal phenotype and growth of neuronal cells, and maintain pure populations of neuronal cells without the need of an astrocyte feeder layer serum free. The use of the medium results in a methylene blue solution with a higher pH in the range of 4 to 8 which is suitable for use with neuronal cells. The solution increases the viscosity.

Components of neurobasal medium	**Molecular Weight**	**Concentration (mg/L)**	**mM**
Amino Acids			
Glycine	75.0	30.0	0.4
L-Alanine	89.0	2.0	0.02247191

Components of neurobasal medium	Molecular Weight	Concentration (mg/L)	mM
L-Arginine hydrochloride	211.0	84.0	0.39810428
L-Asparagine-H_2O	150.0	0.83	0.0055333334
L-Cysteine	121.0	31.5	0.2603306
L-Histidine hydrochloride-H2O	210.0	42.0	0.2
L-Isoleucine	131.0	105.0	0.8015267
L-Leucine	131.0	105.0	0.8015267
L-Lysine hydrochloride	183.0	146.0	0.7978142
L-Methionine	149.0	30.0	0.20134228
L-Phenylalanine	165.0	66.0	0.4
L-Proline	115.0	7.76	0.06747826
L-Serine	105.0	42.0	0.4
L-Threonine	119.0	95.0	0.79831934

Components of neurobasal medium	Molecular Weight	Concentration (mg/L)	mM
L-Tryptophan	204.0	16.0	0.078431375
L-Tyrosine	181.0	72.0	0.39779004
L-Valine	117.0	94.0	0.8034188
Vitamins			
Choline chloride	140.0	4.0	0.028571429
D-Calcium pantothenate	477.0	4.0	0.008385744
Folic Acid	441.0	4.0	0.009070295
Niacinamide	122.0	4.0	0.032786883
Pyridoxal hydrochloride	204.0	4.0	0.019607844
Riboflavin	376.0	0.4	0.0010638298
Thiamine hydrochloride	337.0	4.0	0.011869436
Vitamin B12	1355.0	0.0068	5.0184503E-6

Components of neurobasal medium	Molecular Weight	Concentration (mg/L)	mM
i-Inositol	180.0	7.2	0.04
Inorganic Salts			
Calcium Chloride ($CaCl_2$) (anhyd.)	111.0	200.0	1.8018018
Ferric Nitrate ($Fe(NO_3)_3 \cdot 9H_2O$)	404.0	0.1	2.4752476E-4
Magnesium Chloride (anhydrous)	95.0	77.3	0.8136842
Potassium Chloride (KCl)	75.0	400.0	5.3333335
Sodium Bicarbonate ($NaHCO_3$)	84.0	2200.0	26.190475
Sodium Chloride (NaCl)	58.0	3000.0	51.724136
Sodium Phosphate monobasic (NaH_2PO_4-H_2O)	138.0	125.0	0.9057971
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	288.0	0.194	6.736111E-4

Components of neurobasal medium	Molecular Weight	Concentration (mg/L)	mM
Other Components			
D-Glucose (Dextrose)	180.0	4500.0	25.0
HEPES	238.0	2600.0	10.92437
Phenol Red	376.4	8.1	0.021519661
Sodium Pyruvate	110.0	25.0	0.22727273

[0083]. **High Content Screening assay:** Methylene blue neuroprotection was determined by high content screening (HCS) analysis, which includes the following endpoints: neurite outgrowth, cell number, and cell viability.

[0084]. 1. Cell number: For determining cell number, hoechst 33342 nucleic acid stain was used. Cells were stained with 5 μg/ml hoechst 33342 (Invitrogen # Cat.H1399), washed 3 times and with fluorescence measured at an excitation wavelength of 380 nm and emission wavelength of460 nm. This dye allows a sensitive cell number determination by fluorescence microscopy.

[0085]. Glucose treatment resulted in 54% of mortality in neurons after exposure at 150 mM concentration and methylene blue could prevent glucose-induced mortality in all concentrations with the exception of the highest concentration (50 μM). Moreover, it was also observed that this concentration of the compound in addition to glucose induced an increase in mortality (figure 2 and table 1).

Table 1: data of HOECHST analysis. Values represent the mean at each condition (triplicates average).

DAPI	Cell viability by Hoechst			mean	Standard deviation		Mean % of control	Standard deviation
Control	223	224	218	221.666667	3.21455025	Control	100	1.4545476
Glucose	125	104	81	103.333333	22.0075745	Glucose	46.7571644	9.9581784

0.0005 uM	187	236	242	221.666667	30.1717307	0.0005 uM	100.301659	13.652366
0.005 uM	255	290	218	254.333333	36.0046293	0.005 uM	115.082956	16.291687
0.05 uM	270	253	241	254.666667	14.571662	0.05 uM	115.233786	6.5935122
0.5 uM	234	217	273	241.333333	28.7112057	0.5 uM	109.200603	12.991495
5 uM	168	103	157	142.666667	34.7898453	5 uM	64.5550528	15.742011
50 uM	74	69	62	68.3333333	6.02771377	50 uM	30.920060 3	2.7274723
etOH	88	139	75	100.666667	33.8279963	etOH	45.550527 9	15.306785

[0086]. Different grades of neuroprotection were observed with the rest of the concentrations tested therefore, methylene blue supplementation significantly reduced cell death and improved survival of neurons and nerve projections.

[0087]. 2. WST-8 assay: For detection of viable cells, 10 μl of CCK -8 reagent (WST-8) were added to each well and the plate was incubated at 37°C. After 1 hour, absorbance was measured at 450 nm using the Synergy II microplate reader.

[0088]. Glucose treatment resulted in 22% of viability reduction in DRGs when measured by WST8 after exposure but the compound could not prevent glucose induced viability decrease (figure 5). It was also observed that the highest concentrations of the compound (50 μM) in addition to glucose induced a decrease in viability.

[0089]. 3. Beta-III tubulin staining: Beta-III tubulin staining was determined by Immunohistochemistry (IHQ). Cells were washed with PBS (Sigma Aldrich # Cat.D8537) and fixed with methanol for 10 minutes. After the fixation step the samples were washed three times with PBS and permeabilized with PBS + 0.3% triton for 10 minutes. The samples were then blocked with PBS + Bovine Serum Albumine (BSA) for 30 minutes and finally anti-beta tubulin III antibody (Abcam # Cat.ab7751) were added at 1/1000 in PBS + 0.5% BSA for 60 minutes at room temperature. After three washing steps, the secondary antibodies, anti-goat-antimouse Alexa 633 (Abcam # Cat.A21050) were added at 1/100 for 60 minutes to react against the primary antibody. The samples were then washed three times and measured under automated fluorescent microscope. To investigate the role of neurite extension, two geometric patterns were mainly employed in this study: total neurite length (total length of the path from a neuron body), and number of extremities (number of terminating neurite segments per neuron).

[0090]. At the end of the assay, for measuring viable cells, cells were firstly stained with WST-8 for 1 hour and then fixed with methanol for beta-III tubulin staining and imaged again using BD Pathway 855. In order to acquire enough cells for the analysis, nine fields per well were imaged. The 20x objective was used to collect images for the distinct fluorescence

channels. Dyes were excited and their fluorescence was monitored at the excitation and emission wavelengths with appropriate filter settings. In the setting up of the procedure, exposure times were adjusted in order to avoid overlapping emission between different probes. The collected images were analyzed using a module that allows the simultaneous quantification of subcellular structures, which are stained by different molecular probes to measure the fluorescence intensity associated with predefined nuclear and citoplasmic compartments.

[0091]. To investigate the role of neurite extension two geometric patterns was mainly employed in this study; neurite total length per neuron and extremity count/neuron. Glucose treatment result in a decrease of 38% and 31% in neurite total length and neurite extremity count respectively compared to untreated cells, and on the other hand, methylene blue could only re-establish the affected neurite outgrowth induced by glucose with 0.05 μM concentration (figure 3, 4, Tables 2 and 3). Therefore, methylene blue supplementation significantly increased neurite outgrowth and improved survival of neurons.

Table 2: data of extremity count/neuron. Values represent the mean at each condition (triplicates average).

	Extremity count			mean	Standard deviation		Mean % of control	Standard deviation
Control	1.14349776	1.28125	1.31192661	1.24555812	0.08970787	Control	100	7.2345056
Glucose	0.82	0.9862069	0.7818118	0.86267503	0.10867178	Glucose	69.5705666	8.76385297
0.0005 uM	1.05839416	0.8612903	0.9783625	0.96601569	0.09913029	0.0005 uM	77.9044908	7.99437846
0.005 uM	0.8645161	1.1896551	1.0050314	1.01973425	0.16306741	0.005 uM	82.236633	13.150597 2
0.05 uM	1.34468085	1.0594059	1.1407624	1.18161642	0.14695995	0.05 uM	95.2916466	11.851609 1
0.5 uM	0.6567164	0.8552050	0.7783882	0.76343658	0.10008546	0.5 uM	61.5674662	8.07140792
5 uM	0.8558823	0.5369458	0.5777070	0.65684506	0.17357204	5 uM	52.9713756	13.997745
50 uM	0.2833333	0.2344827	0.2625	0.26010536	0.02451317	50 uM	20.976239	1.97686833
etOH	1.0681818	0.9496402	0.76	0.9259407	0.1554518	etOH	74.6726373	12.536435 2

Table 3: data of total length analysis. Values represent the mean at each condition (triplicates average).

	Total length			mean	Standard deviation		Mean % of control	Standard deviation
Control	65.9581897	71.3820715	74.911945	70.7507354	4.51014107	Control	100	6.3747577
Glucosa	42.9495352	52.9692737	37.5623844	44.4937311	7.81866133	Glucosa	62.8886659	11.051111 4

0.0005 uM	50.9571179	40.1309287	59.35768	50.1485755	9.63884317	0.0005 uM	70.8813789	13.6238066
0.005 uM	45.834792	61.1928946	50.5044719	52.5107195	7.87315701	0.005 uM	74.2200982	11.1281371
0.05 uM	62.9112639	50.3896069	69.8008052	61.033892	9.84083578	0.05 uM	86.2669852	13.9093085
0.5 uM	37.3358324	47.7689148	34.0662692	39.7236721	7.15660239	0.5 uM	56.146533	10.1153391
5 uM	53.6118527	42.2031616	32.2964971	42.7038371	10.6664944	5 uM	60.3587804	15.0763172
50 uM	18.1810885	12.6422943	17.4525766	16.0919865	3.00964523	50 uM	22.7448572	4.25391551
etOH	51.697476	53.6595957	52.7696797	52.7089171	0.98247007	etOH	74.5002362	1.38865027

[0092]. It was also observed that the highest concentrations of the compound (5 and 50 μM) in addition to glucose induced neurite outgrowth reduction.

[0093]. For all the studied parameters, a variation of at least 20% in fluorescence intensity or in the corresponding morphological parameter in relation to untreated cultures was considered. In order to compare the degree of neuroprotection of methylene blue, four different scores of neuroprotection were established according to the level of variation when compared with control cells: 0 (no neuroprotection or variation lower than 20%), 1 (variation 20-40%), 2 (variation 40-60%), 3 (variation 60-100%), 4 (variation 100-200%). Moreover, some concentrations presented certain degree of injury, maybe because they are toxic or maybe because they are toxic in combination with glucose, and similar scores of injury were established according to the level of variation when compared to glucose-treated cells: -1 (variation 20-40%), -2 (variation 40-60%) and -3 (variation 40- 60%). The sum of each individual score resulted in the total level of neuroprotection for each compound and was defined as its degree of neuroprotection. From this calculation, a neuroprotective scale was established: high (>6), moderate (4-5), low (1-3), and no neuroprotection (0).

[0094]. In order to compare the degree of neuroprotection of the tested compound at different concentrations, different scores were created according to the level of the alteration (the group formation criterion is described in material and methods section) and then a value was assigned to each parameter. The sum of all the values for each parameter and concentration tested was calculated, and four distinct groups were created according to their level of neuroprotection. From this analysis, the degree of neuroprotection of each compound studied was defined (table 4).

Table 4. Degree of neuroprotection of methylene blue after analyzing data for each concentration in glucose-treated DRGs. The score of neuroprotection was establish as described in material and methods. The boxes highlighted in red represent toxicity.

	0.0005 µM	0.005 µM	0.05 µM	0.5 µM	5 µM	50 µM
HOECHST	3	3	3	3	1	-1
Extremity count	0	0	1	0	-1	-3
Total length	0	0	1	0	0	-1
WST8	0	0	0	0	0	-1
Total	3	3	5	3	0	-6
Neuroprotection degree	low	low	moderate	low	no	toxic

[0095]. Briefly, methylene blue scored moderate neuroprotection at 0.05 μM, low neuroprotection at 0.0005 μM, 0.005 μM, and 0.5 μM, no neuroprotection at 5 μM and toxicity at 50 μM.

[0096]. Negative control with vehicle (ethanol) showed similar toxicity with glucose-treated cells in cell number and neurite outgrowth measures. Despite this, the toxicity produced by 0.5% ethanol was lower than that observed by 50 μM methylene blue, which has the same amount of ethanol.

[0097].

[0098]. The neuroprotection effect of methylene blue is shown in the table 5 below:

Table 5: Classification of the degree of neuroprotection provided by different concentrations of methylene blue prior to assault with 150 mM glucose.

	0.0005 µM	0.005 µM	0.05 µM	0.5 µM	5µM	50 µM
Neuroprotection degree	low	low	moderate	low	no	no Toxic

[0099]. According to all parameters studied, methylene blue showed some neuroprotection against glucose-induced DRGs toxicity at concentrations from 0.0005 μM to 0.5 μM and moderate neuroprotection effect against glucose-induced DRGs toxicity at 0.05 μM.

[00100]. In the present study, glucose toxicity resulted in a decrease in neurite outgrowth and cell number and viability. The preventive effects of methylene blue against glucose toxicity is associated with increase of cell viability and restoration of neurite

outgrowth. Attending to all parameters studied, methylene blue supplementation scores moderate neuroprotection levels at 0.05 μM.

[00101]. Example 2: Testing of topical Methylene blue preparation tolerance in the human eye *(in vivo)*

[00102]. To establish the concentrations of Methylene blue topical preparations that can be used in the human eye, including and up to 0.005 % (160μM), a series of topical ophthalmic preparations concentrations were prepared and tested. Methylene blue compound (Bleu de Methylene 1%, STEROP) was solubilized in sterile lubricant eye gel (Systane Gel Drop) to a volume of 10 mls to a final maximum concentration of 160microM. The concentration of the topical preparations was adjusted to 0.05μM, 0.5μM, 5μM, 50μM, 60μM, 150μM and 160μM. The viscosity of the eye drops were increased with the use of the lubricant eye gel (Systane Gel Drop). One drop of each of the topical preparations was applied to each human eye once per day. The eyes were examined immediately after application, at 6 hours and 24 hrs after application using standard optometry scan and displayed no adverse effects at all the time points.

[00103]. In all the topical preparations including at 150μM and below the methylene blue topical preparations were observed to be fully tolerable to the eye without causing any damage nor discomfort. Above 150μM, it gives a slight stingy sensation to the eye causing temporary mild discomfort which is self-limiting in nature. The viscosity of this gel-like topical preparation allows the topical preparations to be retained within the eye and not streak down the eyelid onto the face. This "non drip" effect is very desirable.

[00104]. Example 3: prevention of peripheral nerve damage of the eye.

[00105]. Methylene blue compound (Tocris) will be solubilized in sterile water to a final concentration of 10mM. The concentration of the test compound will be adjusted to 0.05μM, 0.5μM, 5μM, 50μM, or 100μM in 10mM HEPES buffer containing sodium bicarbonate and sodium chloride (preparation A). An untreated group only contained 10mM HEPES buffer containing sodium bicarbonate and sodium chloride. A second group of the same test solutions will be made with the viscosity adjusted to be increased with 5g/l gellan gum (preparation B). A third group of the same test solutions will be made with the viscosity adjusted to be increased using a slow release micro-particle (preparation C).

[00106]. Preparation A

[00107]. Methylene blue compound (Tocris) will be solubilized in sterile water to a final concentration of 10mM. The concentration of the test compound will be adjusted to 0.05μM, 0.5μM, 5μM, 50μM, or 100μM in 10mM HEPES buffer containing sodium bicarbonate and sodium chloride.

[00108]. Preparation B

[00109]. Fluid gels will be produced by first dissolving low acyl gellan gum (Kelco gel CG LA, Azelis, UK) in deionized water. Gellan powder will be added to deionized water at ambient temperature in the correct ratio to result in a 1% (w/v) solution. The sol will be heated to 70 °C under agitation, on a hotplate equipped with a magnetic stirrer, until all the polymer has dissolved. Once dissolved, gellan sol will be added to the cup of a rotational rheometer equipped with cup and vane geometry (cup: 35mm diameter, vane: 28mm diameter). The system will then be cooled to 40 °C. Methylene blue compound (Tocris) will be solubilized in sterile water to a final concentration of 10mM. The concentration of the test compound will be adjusted to 0.05μM, 0.5μM, 5µM, 50µM, or 100µM in phosphate-buffered saline (PBS) (4.76 mg/ml) and aqueous sodium chloride (0.2 M). Following this, the mixture will be cooled at a rate of 1 °C/min under shear (450/s) to a final temperature of 20 °C. The sample will then be removed and stored at 4 °C until further use. In the case of fluid gels without Methylene blue compound, ratios will be adjusted so that the final eye drop has a composition of 0.9% (w/v) gellan, 10mM NaCl.

[00110]. Preparation C

[00111]. Oil in Water emulsions will be generated using a glass capillary microfluidic setup. The axisymmetric coaxial glass capillary flow-focusing device will be assembled using a square and round capillary. The surface of round capillary will be hydrophilized with the treatment of oxygen plasma (100 Watts) for 120 seconds. The aqueous continuous phase (W) will be prepared by mixing PVA (1 % w/v) in water. The dispersed phase (O) will be one of the following three: i) Methylene blue compound (Tocris) 0.5µM, ii) Methylene blue compound (Tocris) 50µM, iii) Methylene blue compound (Tocris) 150µM respectively). PLGA concentration in dichloromethane (DCM) (50 mg per mL) will be kept constant for all the three cases. W and O phases will be infused from the two ends of the square capillary through the outer coaxial region using syringe pumps (Harvard PHD 22/2000 series) at flow rates of 150 and 50 μL per min respectively. The fluids will be hydro-dynamically flow focused through the nozzle of the round capillary resulting in the formation of emulsion droplets. 6 cm ID glass wells will be used for sample collection. Approximately 300 μL of O/W emulsions will be dispensed directly into a glass well containing a predispensed film (3 mL) of continuous phase to prevent droplet coalescence. Solvent evaporation will be performed at room temperature (24 °C).

[00112]. The solutions will be dropped in a diabetic mouse eyes once daily for 2 weeks. In-vivo Confocal Microscopy will be performed using a modified Heidelberg Retina Tomograph Rostock Corneal Module. Before scanning, mice were anesthetized with a single injection of 50 mg/kg ketamine and 5 mg/kg xylazine and one drop of topical proparacaine 0.5% ophthalmic solution (Bausch & Lomb, Tampa, FL). Lubrication was maintained on the contralateral eye using Refresh Plus (Allergan, Irvine, CA). To enhance

epithelial viewing, a silicon washer was placed on the outer edge of the TomoCap (Heidelberg, Germany) to generate a thin layer of space between the TomoCap tip and the surface epithelium.17 The dimensions of the washer were as follows: 1.2-cm outer diameter, 3-mm inner diameter, and 600-mm thick (Specialty Silicone Products, Inc., Ballston Spa, NY). Image acquisition was performed as previously described.17 Corneas were scanned at a lens speed of 30 mm per second with 1-mm increment steps. A minimum of three scans was performed on each cornea.

[00113]. The repeated treatment is envisioned to increase the effectiveness of the topical methylene blue ophthalmic preparations at 0.05 μM, and the increase viscosity of the solution at 5μM, 50μM, and 100μM is envisioned to increase the effectiveness of the methylene blue ophthalmic solution.

Claims:

1. A topical preparation for protecting against and/or treating peripheral nerve damage and its associated conditions comprising, less than or equal to 150 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer.
2. The topical preparation according to claim 1, wherein the topical preparation comprises between 0.0001 μM and 60 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride.
3. The topical preparation according to claim 1, wherein the topical preparation comprises 0.0005 μM, 0.005 μM, 0.05 μM, 0.5 μM, 5 μM, 50μM, or 100μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride.
4. The topical preparation according to any one of claims 1 to 3, wherein the physiological buffer comprises sodium bicarbonate and sodium chloride.
5. The topical preparation according to any one of claims 1 to 4, wherein the physiological buffer comprises 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
6. The topical preparation according to any one of claims 1 to 5, wherein the physiological buffer comprises a neurobasal medium.
7. The topical preparation according to any one of claims 1 to 6, wherein the topical preparation comprises a carrier.
8. The topical preparation according to claim 7, wherein the carrier increases the viscosity to a range of about 15 to 150 milli-Pascals per second.
9. The topical preparation according to claim 7 or 8, wherein the carrier modulates the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride.
10. The topical preparation according to any one of claims 7 to 9, wherein the carrier comprises a microparticle.
11. The topical preparation according to any one of claims 1 to 10, for use in preventing and/or treating peripheral nerve damage and its associated conditions.
12. The topical preparation according to claim 11, wherein the peripheral nerve damage comprises neurotrophic keratopathy.
13. The topical preparation according to claim 11, wherein the use is for ophthalmic use.
14. A method of preventing and/or treating peripheral nerve damage and its associated conditions comprising administering to a subject in need of preventing and/or treating peripheral nerve damage and its associated conditions (a) a topical preparation comprising, less than or equal to 150 μM of 3,7-

bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer or (b) a topical preparation according to any one of claims 1 to 8, or (c) a topical preparation comprising, less than or equal to 150 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a carrier.

15. The method according to claim 14, wherein the topical preparation is an ophthalmic topical preparation.

16. The method according to claim 14, wherein the topical preparation is administered directly to the nerve site.

17. The method according to any one of claims 14 to 16, wherein parameters of corneal nerves are measured to determine whether the subject is in need of preventing and/or treating peripheral nerve damage and its associated conditions prior to administration of the topical preparation.

18. The method according to any one of claims 14 to 17, wherein parameters of corneal nerves are measured after administration of the topical preparation.

19. The method according to any one of claims 14 to 18, wherein the peripheral nerve damage and its associated conditions comprises neurotrophic keratopathy.

20. Use of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride in the manufacture of a topical preparation comprising, less than or equal to 150 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride; and a physiological buffer or a carrier for prevention and/ or treatment of peripheral nerve damage and its associated conditions.

21. Use of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride in the manufacture of a topical preparation according to any one of claims 1 to 9, for prevention and/or treatment of peripheral nerve damage and its associated conditions.

22. Use according to claim 20 or 21, wherein the peripheral nerve damage and its associated conditions comprises neurotrophic keratopathy.

23. Use according to claim 20 or 21, for ophthalmic use.

24. A topical preparation for protecting against and/or treating peripheral nerve damage and its associated conditions comprising, less than or equal to 150 μM of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride and a carrier that increases the viscosity of the preparation to a range of about 15 to 150 milli-Pascals per second.

25. The topical preparation according to claim 24, wherein the carrier modulates the release profile of 3,7-bis(dimethylamino) phenothiazin-5-ium chloride.

26. The topical preparation according to claim 24 or 25, wherein the carrier comprises a microparticle.

27. The topical preparation according to claim 24 or 25, wherein the carrier comprises a gel.

28. The topical preparation according to claim 24 or 25, wherein the carrier is a sustained release platform.