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(54) Title: HYDROGEN SULPHIDE FOR TOPICAL TREATMENT OF NAIL INFECTION

(57) Abstract: Disclosed is a topical source of hydrogen sulphide for use in the treatment of a nail infection, for example onychomycosis. Also disclosed is a system comprising the topical source of hydrogen sulphide.

WO 2025/017299 A1

[0001] This invention relates to a topical source of hydrogen sulphide for use in the treatment of a nail infections, for example fungal and bacterial nail infections, particularly onychomycosis. Also disclosed is a system comprising a topical source of hydrogen sulphide and a substantially airtight cover which adapted for topical application of hydrogen sulphide to an infected nail.

BACKGROUND

[0002] Medical conditions affecting the nail tissues are very difficult to treat because it is problematic to deliver therapeutic agents into this keratin-rich barrier. As a consequence, pathogenic infections of human nails, particularly fungal nail infections (known as onychomycosis) are a growing unmet medical need. Onychomycosis affects 10% of the global population and 50% of those aged over 70 (Ricardo JW et al., Safety of current therapies for onychomycosis, Expert Opinion on Drug Safety. 2020; 19(11):1395-14081). It is considered a global burden because it can cause mortality in immunocompromised patients via dissemination, that is, entry into the systemic circulation, which has a mortality of about 80% (Mays SR et al., Cutaneous fungal infections in the oncology patient: recognition and management, Am. J. Clin. Dermatol. 2006;7(1):31-432) and result in significant morbidity, including limb amputation in diabetic patients (Nather A et al., Epidemiology of diabetic foot problems and predictive factors for limb loss, J. Diabetes Complications, 2008; 22(2): 77-823). Furthermore, inadequacies in the current approaches are leading to an "alarming" increase in treatment-resistant infections (Gupta AK et al., The Growing Problem of Antifungal Resistance in Onychomycosis and Other Superficial Mycoses, Am. J. Clin. Dermatol., 2021 Mar;22(2):149-1574). These factors with the inadequacies of current treatment approaches make onychomycosis a significant unmet clinical need.

[0003] Current treatments of onychomycosis consist of commercially available oral or topical agents. Oral terbinafine and itraconazole are the clinician's treatments of choice, having mycological cure rates (commonly defined as negative culture and negative direct microscopy) of 70%, and 54%, and complete cure rates (commonly defined as 100% reversal of the disease) of 38% and 14% respectively (Baran R et al., Topical antifungal drugs for the treatment of onychomycosis: An overview of current strategies for monotherapy and combination therapy, J. Eur. Acad. Dermatology Venereol, 2005;19(1):21-95). However, oral treatments can require 4-6 months in fingernails and 12-18 months in toenails to achieve a complete cure. In addition, oral therapy has renal and hepatic toxicity, thus it is often not used in the elderly, who show the greatest prevalence of

the disease. As a result, topical antifungals, which are associated with minimal side effects, are an important second-line therapy choice.

[0004] There are four main topical preparations used to treat onychomycosis: ciclopirox (Penlac®), efinaconazole (Jubila®), tavaborole (Kerydin®), and amorolfine (Loceryl®).

5 Clinical data suggests that amorolfine mycological cure rates vary between 15% to 81% and complete cure rates range from 1 to 13%.

[0005] Ciclopirox clinical studies report mycological and a complete cure rate of 29-89.1% and 5.5-8.5% respectively (Iorizzo M et al., Ciclopirox 8% HPCH nail lacquer in the treatment of mild-to-moderate onychomycosis: A randomized, double-blind amorolfine
10 controlled study using a blinded evaluator, *Ski Appendage Disord.* 20157; and Gupta AK et al., Ciclopirox nail lacquer topical solution 8% in the treatment of toenail onychomycosis, *J. Am. Acad. Dermatol.* 2000/10/29. 2000;43(4 Suppl):S70-80).

[0006] Tavaborole studies report a mycological cure rate of 31.1-35.9% and a complete cure rate of 6.5-9.1% and efinaconazole studies have reported 83.3–87.2% and 15.8–
15 25.6% mycological and complete cure rates respectively (Elewski BE et al., Efficacy and safety of tavaborole topical solution, 5%, a novel boron-based antifungal agent, for the treatment of toenail onychomycosis: Results from 2 randomized phase-III studies, *J. Am. Acad. Dermatol.* 2015;73(1):62–98,9; and Tschen EH et al., Efinaconazole solution in the treatment of toenail onychomycosis: a phase 2, multicenter, randomized, double-blind
20 study, *J. Drugs Dermatol.* 2013;12(2):186–92).

[0007] Hydrogen sulphide (H₂S) is an endogenously produced gasotransmitter (Wang et al. Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J.* 2002;16(13):1792–8; and Abe et al., The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* 1996, 16, 1066–1071). Hydrogen
25 sulphide plays a key role in multiple physiological processes including vasodilation, angiogenesis, and inflammation (Stein et al., Redox biology of hydrogen sulfide: Implications for physiology, pathophysiology, and pharmacology, *Redox Biology*, Volume 1, Issue 1, 2013, Pages 32-39).

[0008] H₂S exhibits antifungal properties and has been shown to inhibit spore
30 germination, germ tube elongation, mycelial growth and increase intracellular reactive oxygen species (Fu et al., An antifungal role of hydrogen sulfide on the postharvest pathogens *Aspergillus niger* and *Penicillium italicum*. *PLoS One.* 2014 Aug 7;9(8)). Although hydrogen sulphide is used as an antifungal agent for crop protection, there are currently no treatments available for nail infections based on hydrogen sulphide as an
35 active agent.

[0009] The poor clinical efficacy of current topical therapies for nail infections is a consequence of the inability of the active agents to penetrate the nail plate (McAuley WJ et al., An investigation of how fungal infection influences drug penetration through onychomycosis patient's nail plates, Eur. J. Pharm. Biopharm. 2016;102:178–84).

- 5 Furthermore, the inability to use oral therapies in many patients, means that despite the availability of active agents against the pathogens responsible for nail infections, there remains a need for improved treatments of nail infections such as onychomycosis.

[0010] There are two methods that hydrogen sulphide could be administered to the nail: 1) using a chemical donor applied topically to the nail plate; and 2) using a cold plasma device. Cold atmospheric plasma (CAP) operates at ambient pressure and temperatures below 40°C, making it suitable for application on living cells or tissues. One of the most suitable applications for CAP is sterilisation, but other biomedical treatments, including wound healing have been suggested. Importantly, these applications can be performed without causing excessive heating, making cold plasma a versatile tool in both industrial and medical fields (Kong, M. G., Plasma medicine: An introductory review. New Journal of Physics, 2019, 11(11), 115012).

[0011] Cold plasma is typically generated by electrical discharges in gases at or near atmospheric pressure. Common techniques include dielectric barrier discharges and atmospheric pressure plasma jets (Attri *et al.*, Atmospheric pressure plasma jet using a DC-powered microplasma torch: The operating characteristics. Plasma Sources Science and Technology, 2013, 22(2), 025003). Plasma jets are one of the most common cold plasma generation devices for medical applications. The needle electrode within the plasma jet is attached to the voltage source whereas the ground external electrode surrounds the needle. As the carrier gas passes between the two electrodes at a high speed, it becomes ionised, excited, and dissociated to produce various reactive species before being accumulated and exiting through a nozzle as a beam. The beam stability is highly dependent on the type of carrier gas (Umair *et al.* Recent advances in plasma technology: Influence of atmospheric cold plasma on spore inactivation. Food Rev. Int., 2021, 1(1):1–23).

30 [0012] The reactive oxygen and nitrogen species generated during the ionisation process have shown the ability to kill microorganisms and fungi (Brun *et al.* Antibacterial efficacy and mechanisms of action of low power atmospheric pressure cold plasma: membrane permeability, biofilm penetration and antimicrobial sensitization. Journal of Applied Microbiology, 2018, 125, 398–408). For example, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by cold atmospheric pressure plasma (CAPP) has been shown to effectively inactivate bacteria and kill fungi (Brany, D. *et al.*

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Cold Atmospheric Plasma: A Powerful Tool for Modern Medicine. *Int. J. Mol. Sci.*, 2020, 21, 2932). The generation of ROS and RNS involves the ionisation of high-energy electrons from the plasma, which collide with nitrogen and oxygen molecules in the air, creating ions (Laroussi, M. & Lu, X., Atmospheric pressure plasma jet for biomedical applications. *Plasma Science, IEEE Transactions on*, 2005, 33(2), 745-753). During this process, the air molecules are also excited, temporarily becoming more energetic. As these molecules return to their normal state, they emit light, contributing to the visible glow of the plasma (Laroussi & Lu, 2005). During this process, the cold plasma also generates ozone (O₃), atomic oxygen (O), and hydroxyl radicals (OH), as well as RNS such as nitric oxide (NO) and nitrogen dioxide (NO₂) (Attri *et al.*, 2013). The plasma jet produces electric fields that can further ionise the surrounding air and influence the movement of charged particles. The distribution of positive and negative charges within the plasma affects the ion distribution in the air, impacting local electrical properties (Laroussi & Lu, 2005).

[0013] Microorganisms are thought to be killed through oxidative stress. This can be induced by Lipid Peroxidation where ROS, especially hydroxyl radicals (OH•), attack the lipid molecules in microbial cell membranes, triggering lipid peroxidation (Fridman *et al.*, Applied plasma medicine. *Plasma Processes and Polymers*, 2008, 5(6), 503-533). Organisms can also be killed using plasma via protein oxidation wherein reactive species oxidise amino acid residues in proteins, resulting in structural changes and loss of function, particularly in enzymes and structural proteins (Pawlat *et al.* Possibility of Humid Municipal Wastes Hygienisation Using Gliding Arc Plasma Reactor. *Water*, 2021, 13(2):194). Cold plasma can also cause DNA damage whereby ROS induce single and double-strand breaks in DNA, as well as oxidative modifications to nucleotides., which leads to mutations, replication errors, and impaired transcription (Fridman *et al.*, 2008). Through the formation of cold plasma, cell death can be induced by nitrosative stress. For example, the generated nitric oxide (NO) and peroxynitrite (ONOO-) cause nitrosative stress, resulting in nitration and nitrosylation of proteins and nucleic acids which will cause inactivation of enzymes and structural proteins and disruption of cells.

[0014] Cold plasma has several advantages in the treatment of topical infections in the skin and nails. For example, it is non-invasive unlike surgical treatments, cold plasma is painless, providing a more comfortable patient experience. It is effective against a wide range of pathogens, including bacteria, fungi, and viruses, reducing the likelihood of secondary infections. Cold plasma also does not rely on precise mechanism of action, making it an effective alternative for treating drug-resistant fungal strains. Furthermore, treatment sessions are typically short, and the effects can be seen relatively quickly compared to traditional chemical treatments.

[0015] As a result of these advantages, cold plasma has been used to treat topical infections of the skin and nail. For example, when targeted against bacterial inactivation to improve wound healing, Brun *et al.*, (2018) reported that the exposure for 2 min to the afterglow originated from the plasma source successfully inactivated *P. aeruginosa* and *S. aureus* independently from the mode of growth, planktonic or biofilm. Another study describes activity also in the human nail, e.g., the alleged eradication of *Candida albicans* using non-thermal atmospheric plasma (NTAP) (Bulson *et al.*, Non-thermal atmospheric plasma treatment of onychomycosis in an in vitro human nail model. *Mycoses*, 2020, 63, 225–232). The plasma was applied to both suspended cultures and toenails using single-use patch electrodes made of flexible conductive copper mesh surrounded by medical-grade rubber insulation. This study revealed that NTAP effectively eliminated *C. albicans* on agar within one minute of direct exposure, highlighting its strong antifungal properties compared to untreated controls. However, this was only shown to treat superficial infections and the major disadvantage of this approach is that it has not been shown to be effective beyond the most superficial layers of skin and nail tissue. This was exemplified in Lux *et al.* (Inactivation of Dermatophytes Causing Onychomycosis and Its Therapy Using Non-Thermal Plasma. *J. Fungi*, 2020, 6, 214) who demonstrated in a study involving 40 patients with onychomycosis that plasma was most effective when applied in the early stages of fungal growth. Conversely, exposure to NTAP in later stages, when the fungi are more developed and sporulated, proved to be less effective. Thus, there also remains a need for improved plasma treatments of nail infections such as onychomycosis.

BRIEF SUMMARY OF THE DISCLOSURE

[0016] The inventors have found that topical application of a topical source of hydrogen sulphide to the surface of a nail and maintaining the topical source of hydrogen sulphide under a substantially airtight cover results in rapid penetration of high concentrations of hydrogen sulphide into and through the nail plate to provide an anti-infective effect against pathogens (e.g. fungi) in the nail plate and/or underlying nail bed and surrounding tissues. The Examples herein illustrate that the transungual mass-transfer rate of hydrogen sulphide using sodium hydrosulphide (also known as sodium hydrogen sulphide or NaHS) as a topical source of hydrogen sulphide is more than 175,000, times faster than topically applied efinaconazole (compared to published permeation rate) and approximately 640 times faster than topically applied ciclopirox and rapidly provides concentrations of hydrogen sulphide in excess of the minimum inhibitory concentration (MIC) of pathogens which cause nail infections, such as onychomycosis. The transungual delivery method described herein has the potential to provide much faster treatment of nail infections than currently known topical treatments for nail infections. Maintaining the topically source of

hydrogen sulphide under a substantially air-tight cover during treatment reduces the loss of hydrogen sulphide gas into the atmosphere and enhances hydrogen sulphide penetration into and through the nail plate thereby maximising exposure to pathogens to the anti-infective (e.g. anti-fungal) effects of hydrogen sulphide.

5 **[0017]** In accordance with the present invention there is provided a topical source of hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment,

10 wherein the topical source of hydrogen sulphide comprises a composition comprising a hydrogen sulphide donor,

 wherein the hydrogen sulphide donor is selected from sodium hydrosulphide (NaHS), ammonium tetrathiomolybdate (ATTM), diallyl trisulphide (DATS) and 4-hydroxybenzothioamide (HBTA).

15 **[0018]** Also disclosed, is a topical source of hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.

[0019] In certain embodiments the topical source of hydrogen sulphide provides a
20 solution comprising hydrogen sulphide which is topically applied to the surface of the infected nail. In some embodiments the topical source of hydrogen sulphide comprises a solution comprising hydrogen sulphide. Suitably the topical source of hydrogen sulphide comprises a composition comprising a hydrogen sulphide donor. Alternatively, the topical source of hydrogen sulphide may be a plasma comprising ionised hydrogen sulphide,
25 wherein the plasma is contacted with the surface of the infected nail. Accordingly, the topical source of hydrogen sulphide may be a plasma comprising ionised hydrogen sulphide or a composition comprising a hydrogen sulphide donor.

[0020] In embodiments where the topical source of hydrogen sulphide comprises a composition comprising a hydrogen sulphide donor, the hydrogen sulphide donor may be
30 any agent which is capable of generating or releasing hydrogen sulphide into the composition. Suitably at least a proportion of the hydrogen sulphide generated or released by the hydrogen sulphide donor into the composition is dissolved in the composition to provide a hydrogen sulphide solution. Hydrogen sulphide donors are well-known to the skilled person, for example as described in Magli et al., H₂S Donors and Their Use in
35 Medicinal Chemistry. Biomolecules 2021, 11, 1899; Chadwick et al., A Review of

Hydrogen Sulfide (H₂S) Donors: Chemistry and Potential Therapeutic Applications

Biochem. Pharmacol. 2018 March ; 149: 110–123; and Zaorska et al., Hydrogen Sulfide in Pharmacotherapy, Beyond the Hydrogen Sulfide-Donors, Biomolecules 2020, 10, 323, the disclosures of which are incorporated herein by reference.

- 5 **[0021]** In certain embodiments the hydrogen sulphide donor is selected from an inorganic sulphide salt (e.g. CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS, SiS₂, preferably NaHS), ammonium tetrathiomolybdate, an allyl-substituted polysulphide (e.g. S-(prop-2-en-1-yl) prop-2-ene-1-sulfinothioate (allicin), diallyl sulphide, diallyl disulphide or diallyl trisulphide), an isothiocyanate derivative (e.g. allyl isothiocyanate (AITC), erucin, or an
- 10 aryl isothiocyanate (e.g. benzyl isothiocyanate or 4-hydroxybenzyl isothiocyanate)), Lawessonn's Reagent and analogues thereof, a phosphonamidodithioate derivative (e.g. GYY4137), a phosphonodithioate derivative, a phosphonamidothioate derivative (e.g. a JK donor), a dithiolthione derivative (e.g. a 1,2-dithiole-3-thione derivative), an N-mercapto derivative (e.g. an N-benzoylthiobenzamide derivative), an S-arylothiooxime derivative, an
- 15 acyl perthiol derivative, a dithioperoxy-anhydride, a tetrasulphide derivative, a thioamide derivative (e.g. an aryl thioamide derivative such as 4-hydroxybenzothioamide), a gem dithiol derivative, a N-thiocarboxyanhydride derivative, a thiocarbamate derivative, a phosphoramidodithioate derivative, thioamino acid and a thioester prodrug.

- [0022]** In certain embodiments the hydrogen sulphide donor is selected from an
- 20 inorganic sulphide salt (e.g. CaS, KHS, NaHS, MgS, SrS, BaS, SiS₂, preferably NaHS), ammonium tetrathiomolybdate, an allyl-substituted polysulphide (e.g. diallyl sulphide, diallyl disulphide or diallyl trisulphide), an isothiocyanate derivative (e.g. an aryl isothiocyanate (e.g. benzyl isothiocyanate or 4-hydroxybenzyl isothiocyanate)), Lawessonn's Reagent and analogues thereof, a phosphonamidodithioate derivative (e.g.
- 25 GYY4137), a phosphonodithioate derivative, a phosphonamidothioate derivative (e.g. a JK donor), a dithiolthione derivative (e.g. a 1,2-dithiole-3-thione derivative), an N-mercapto derivative (e.g. an N-benzoylthiobenzamide derivative), an S-arylothiooxime derivative, an acyl perthiol derivative, a dithioperoxy-anhydride, a tetrasulphide derivative, a thioamide derivative (e.g. an aryl thioamide derivative such as 4-hydroxybenzothioamide), a gem
- 30 dithiol derivative, a N-thiocarboxyanhydride derivative, a thiocarbamate derivative, a phosphoramidodithioate derivative, thioamino acid and a thioester prodrug.

- [0023]** In certain embodiments the hydrogen sulphide donor is selected from an inorganic sulphide salt (e.g. CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS, SiS₂, preferably NaHS), ammonium tetrathiomolybdate, Lawessonn's Reagent and analogues thereof, a
- 35 phosphonamidodithioate derivative (e.g. GYY4137), a phosphonodithioate derivative, a

phosphonamidothioate derivative (e.g. a JK donor), a phosphoramidodithioate derivative, a gem dithiol derivative and a dithiolthione derivative (e.g. a 1,2-dithiole-3-thione derivative).

[0024] In certain embodiments the hydrogen sulphide donor is selected from an inorganic sulphide salt (e.g. CaS, KHS, NaHS, MgS, SrS, BaS, SiS₂, preferably NaHS), ammonium tetrathiomolybdate, Lawesson's Reagent and analogues thereof, a
5 phosphonamidodithioate derivative (e.g. GYY4137), a phosphonodithioate derivative, a phosphonamidothioate derivative (e.g. a JK donor), a phosphoramidodithioate derivative, a gem dithiol derivative and a dithiolthione derivative (e.g. a 1,2-dithiole-3-thione derivative).

[0025] In certain embodiments the hydrogen sulphide donor is selected from ammonium
10 tetrathiomolybdate, an N-mercapto derivative (e.g. an N-benzoylthiobenzamide derivative), an S-arylothiooxime derivative, an acyl perthiol derivative, a dithioperoxy-anhydride, a tetrasulphide derivative, a thioamide derivative (e.g. an aryl thioamide derivative such as 4-hydroxybenzothioamide) an isothiocyanate derivative, and a gem dithiol derivative.

[0026] In certain embodiments the hydrogen sulphide donor is selected from an N-
15 thiocarboxyanhydride derivative and a thioester prodrug.

[0027] In certain embodiments the hydrogen sulphide donor is selected from a thioamino acid.

[0028] In certain embodiments the hydrogen sulphide donor is selected from a thiocarbamate derivative.

[0029] In certain embodiments the hydrogen sulphide donor is selected from sodium
20 hydrosulphide (NaHS), disodium sulphide (Na₂S), ammonium tetrathiomolybdate (ATTM), diallyl trisulphide (DATS) and 4-hydroxybenzothioamide (HBTA).

[0030] In certain embodiments the hydrogen sulphide donor is selected from sodium
25 hydrosulphide (NaHS), ammonium tetrathiomolybdate (ATTM), diallyl trisulphide (DATS) and 4-hydroxybenzothioamide (HBTA). It may be that the hydrogen sulphide donor is selected from sodium hydrosulphide (NaHS), ammonium tetrathiomolybdate (ATTM), and diallyl trisulphide (DATS).

[0031] In certain embodiments the hydrogen sulphide donor is sodium hydrosulphide (NaHS) or disodium sulphide. Preferably the hydrogen sulphide donor is sodium
30 hydrosulphide (NaHS).

[0032] In certain embodiments, the hydrogen sulphide donor is not disodium sulphide (Na₂S).

[0033] Suitably at least a proportion of the hydrogen sulphide released from the hydrogen sulphide donor is dissolved in a liquid present in the composition thereby

providing a solution comprising hydrogen sulphide which is applied to the surface of the infected nail. Accordingly, the composition topically applied to the infected nail suitably comprises a hydrogen sulphide donor and a hydrogen sulphide solution (e.g. an aqueous solution). Upon topical application to the nail plate the hydrogen sulphide solution
5 penetrates into and through the nail plate and provides an anti-infective effect on the pathogens present in the nail plate, the nail bed, and the surrounding tissues. Once the hydrogen sulphide solution has penetrated into and through the nail plate hydrogen sulphide gas may be released from the hydrogen sulphide solution (see, for example, Example 3). The anti-infective effect may be provided by the hydrogen sulphide solution
10 and/or hydrogen sulphide gas released in the nail plate and nail bed. It may be that the anti-infective effect of the composition topical applied to the nail is two-fold. Firstly, an anti-effective effect may be provided by the hydrogen sulphide solution (e.g. NaHS solution) as it permeates through the nail plate. Once the hydrogen sulphide solution (e.g. NaHS solution) has permeated the nail plate, hydrogen sulphide gas is released (as shown in
15 Example 3). Thus, secondly, an anti-effective effect may also be provided by the hydrogen sulphide gas as it permeates back through and out of the nail plate.

[0034] Topical application of a composition comprising a hydrogen sulphide donor to the nail plate (e.g. as a solution of the hydrogen sulphide donor) may also enable the hydrogen sulphide donor to penetrate into and through the nail plate and to release
20 hydrogen sulphide directly at the site of infection in the nail plate and/or nail bed and surrounding tissues.

[0035] In certain embodiments the composition comprising the hydrogen sulphide donor comprises a liquid. Suitably the liquid is one in which hydrogen sulphide is soluble. For example the liquid may comprise water, a hydrocarbon solvent, a halogenated
25 hydrocarbon solvent, an alcohol, an ether, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile or a sulfoxide. In certain embodiments the composition comprising the hydrogen sulphide donor comprises a liquid selected from water and a polar organic solvent, or a mixture thereof. In some embodiments liquid comprises water and/or a polar organic solvent
30 selected from, for example, an alcohol, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile or a sulfoxide. For example, the liquid may be selected from one or more of water, methanol, ethanol, glycerol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone.

35 **[0036]** In certain embodiments the composition comprising the hydrogen sulphide donor is an aqueous composition. In certain embodiments the composition comprises a

hydrogen sulphide donor, water and optionally one or more polar organic co-solvent(s). In certain embodiments the composition comprises water and one or more co-solvents selected from methanol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone. In certain embodiments the composition
5 comprises a hydrogen sulphide donor and water and methanol (e.g. a 1:1 v/v mixture of water and methanol). In certain embodiments the composition comprises a hydrogen sulphide donor and water.

[0037] The hydrogen sulphide donor provides a source of hydrogen sulphide in the composition and where the composition comprises a liquid (e.g. an aqueous liquid), at
10 least a portion of the hydrogen sulphide is present in the composition as a hydrogen sulphide solution.

[0038] In certain embodiments the hydrogen sulphide donor is dispersed or dissolved in the composition. In another embodiment the hydrogen sulphide donor is dissolved in the composition. For example, it may be that the composition is an aqueous composition
15 comprising a solution of the hydrogen sulphide donor.

[0039] In certain embodiments the composition comprising the hydrogen sulphide donor further comprises an agent to activate or enhance release of hydrogen sulphide from the hydrogen sulphide donor. For example, the composition further comprises a thiol (e.g. glutathione, cysteine, homocysteine or N-acetylcysteine), a catalyst, an enzyme, a pH
20 adjusting agent, a base (e.g. a bicarbonate), or an oxidising agent (e.g. hydrogen peroxide).

[0040] In certain embodiments the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) is in the form of a solution, a dispersion, an emulsion, a foam, a lotion, a cream or a gel. In
25 some embodiments the composition comprising the hydrogen sulphide donor is in the form of a gel composition. For example, the composition may be in the form of a hydrogel composition. In some embodiments the hydrogen sulphide donor may be present in the composition as a dispersion wherein at least a proportion of the available hydrogen sulphide is dissolved in the composition. The dispersion may comprise any suitable
30 particle size of the hydrogen sulphide donor, including nano-dispersions. Also contemplated are compositions wherein a hydrogen sulphide donor in the form of a solid (e.g. a powder) is placed on the surface of the nail and in use the powder is brought into contact with a suitable medium (e.g. aqueous liquid or gel) resulting in the formation of a hydrogen sulphide solution in-situ. In some embodiments the topical source of hydrogen
35 sulphide may comprise a hygroscopic hydrogen sulphide donor. When applied to the surface of the nail (e.g. as a powder or a layer) the hygroscopic hydrogen sulphide donor

absorbs water to provide a solution of hydrogen sulphide which penetrates into and/or through the nail plate. Similarly the topical source of hydrogen sulphide may be a composition comprising a hydrogen sulphide donor and hygroscopic material. Suitably the composition is applied to the surface of the nail (e.g. as a powder or a coating) where it

5 absorbs water to provide a solution of hydrogen sulphide in contact with the nail to be treated. The composition may comprise, for example, a simple mixture or granule comprising the hydrogen sulphide donor and hygroscopic material. Alternatively the hydrogen sulphide donor may be dispersed within a matrix formed by the hygroscopic material. Suitable hygroscopic materials are well known. For example the hygroscopic

10 material may be a deliquescent salt (e.g. calcium chloride, magnesium chloride, zinc chloride or carnallite) or a desiccant (e.g. silica gel or sucrose). In some embodiments the topical source of hydrogen sulphide may be a composition comprising a hydrogen sulphide donor or hydrogen sulphide solution that is encapsulated in a lipid or polymer (e.g. as micro- or nano-particles). During treatment hydrogen sulphide is released from the

15 encapsulated hydrogen sulphide donor or solution for absorption into and through the nail plate.

[0041] H_2S is a weak acid that has two dissociation constants in aqueous solution of $\text{pK}_{\text{a}1} = 7.04$ and $\text{pK}_{\text{a}2} = 11.96$. Hydrogen sulphide solutions are therefore in an equilibrium between H_2S , HS^- and S^{2-} , the predominant species being determined by, for

20 example, pH and the temperature of the solution. The hydrogen sulphide in an aqueous hydrogen sulphide solution is volatile and is released as hydrogen sulphide gas from the solution. Accordingly, the concentration of hydrogen sulphide in a solution will rapidly reduce if the solution is open to the atmosphere as a result of volatilisation of H_2S gas. The inventors have found that by maintaining the composition comprising a hydrogen

25 sulphide donor under a substantially airtight cover after application to the nail minimises loss of hydrogen sulphide gas from the hydrogen sulphide solution present in the composition applied to the nail and also delays release of hydrogen sulphide from the solution until the hydrogen sulphide solution has penetrated into and/or through the nail plate. The Examples show that simply exposing a nail to hydrogen sulphide as a gas does

30 not result any significant permeation of the hydrogen sulphide into and through the nail plate. In contrast topical application of a composition comprising a solution of hydrogen sulphide results in rapid permeation of hydrogen sulphide into and through the nail plate when the composition is maintained under a substantially airtight cover. The Examples also show that applying a composition comprising solution of hydrogen sulphide to a nail

35 without maintaining the composition under an airtight cover significantly reduces or prevents permeation of hydrogen sulphide into the nail. Without wishing to be bound by theory, it is thought that ionised hydrogen sulphide (i.e. HS^-) is able to penetrate into and

through the nail plate much more effectively than gaseous hydrogen sulphide.

Accordingly, in preferred embodiments the topical source of hydrogen sulphide applied to the infected nail comprises a solution of hydrogen sulphide. More particularly in preferred embodiments, the topical source of hydrogen sulphide in contact with the surface nail plate
5 comprises a hydrogen sulphide donor and a solution (e.g. an aqueous solution) of hydrogen sulphide. In each case the composition applied to the infected nail is maintained under a substantially airtight cover during treatment.

[0042] In certain embodiments the cover is adapted to provide a substantially airtight chamber over the topical source of hydrogen sulphide on the infected nail. The cover may
10 be adapted to provide a substantially airtight chamber enclosing at least a proportion of the topical source of hydrogen sulphide on the nail surface when the cover is placed over the topical source of hydrogen sulphide. Suitably the cover provides an airtight chamber over at least 50% of the topical source of hydrogen sulphide present on the surface of the nail. Preferably the cover provides a substantially airtight chamber over at least 60%, at
15 least 70%, at least 80% at least 90% or still more preferably substantially all of the topical source of hydrogen sulphide on the surface of the nail.

[0043] In certain embodiments the cover is, or comprises, a substantially air-impermeable polymer. In some embodiments the cover is or comprises a substantially air-impermeable polymer selected from a polyester, a polyurethane, a polytetrafluoroethylene,
20 a polyethylene, a polysiloxane, a polyisocyanate, a polycarbonate poly(ethylene terephthalate), an acrylic polymer (e.g. a poly(methyl methacrylate), a poly(ethyl methacrylate) or a polyacrylonitrile)) or a polyvinylchloride, or a co-polymers of two or more thereof. Polytetrafluoroethylene (PTFE) is particularly stable to hydrogen sulphide. Accordingly, in a preferred embodiment the cover is or comprises PTFE.

[0044] In certain embodiments the cover is adapted to be sealably attached to the subject over the topical source of hydrogen sulphide during treatment. Sealing the cover to the subject provides an airtight seal between the cover and the subject such that the topical source of hydrogen sulphide on the nail is maintained in an airtight environment during treatment. In some embodiments the cover placed over the topical source of
30 hydrogen sulphide on the nail and is sealed onto the dorsal surface of the nail. Also contemplated is placing the cover over the topical source of hydrogen sulphide on the nail and sealing the cover to the skin of the finger or toe surrounding the infected nail.

[0045] In some embodiments the cover is sealed to the subject using an adhesive. Adhesive may be applied to the surface of the nail so as to form a perimeter of adhesive
35 which surrounds the topical source of hydrogen sulphide on the nail, the cover is then placed onto the adhesive thereby sealing the topical source of hydrogen sulphide in a

substantially airtight environment. In a further embodiment the cover is provided with an adhesive and is attached directly to the surface of the nail (and/or surrounding skin) so as to cover the topical source of hydrogen sulphide. In some embodiments the cover comprises a sealing surface, for example an elastomer, and provides a pressure seal
5 between the sealing surface and the subject (e.g. the dorsal surface of the nail plate). In this configuration the cover may be secured in place using, for example, a suitable adhesive tape or strapping so as to maintain a substantially airtight seal between the cover and the nail.

[0046] In some embodiments the cover is conveniently sealed to the surface of the nail
10 over the topical source of hydrogen sulphide. Suitably, the cover (and topical source of hydrogen sulphide) extends over substantially the whole dorsal surface of the nail being treated to maximise the surface area of the nail in contact with the hydrogen sulphide solution. However, because the rate of permeation of hydrogen sulphide into and through the nail plate and underlying nail bed is high it is also possible to place the cover on a
15 smaller area of the nail. This is particularly advantageous in some subjects where the nail infection has damaged the integrity of the nail plate which may make it difficult to form a seal to the damaged nail plate, especially if the damage is at the edge of the nail plate. Sealing the cover to an undamaged surface of the nail plate provides the desired airtight chamber over the topical source of hydrogen sulphide. In some embodiments the cover
20 extends over at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 95% of the dorsal surface of the nail plate. For example from 20% to 95%, 40% to 95%, 40% to 80% , 20% to 50% or 30 to 50% of the dorsal surface of the nail plate.

[0047] In certain embodiments the cover is an occlusive dressing or occlusive patch. The dressing or patch may be made from any material provided it provides a substantially
25 air-impermeable cover over the topical source of hydrogen sulphide applied to the nail surface. Thus the cover may comprise a substantially air-impermeable occlusive wound dressing or patch. For example, the wound dressing or patch may comprise one or more air-impermeable polymer layers such as one of the air-impermeable polymers described herein.

30 **[0048]** In certain embodiments the cover comprises the topical source of hydrogen sulphide and the topical source of hydrogen sulphide is applied to the infected nail by contacting the cover with at least a portion of the surface of the infected nail.

[0049] In certain embodiments the cover is in the form of an artificial nail. The use of an artificial nail as a cover enables the cover to resemble the natural shape and colour of the
35 subject's nail during treatment, thereby improving the visual appearance of the infected nail during treatment and potentially enhancing patient compliance with the treatment. In

some embodiments the cover, e.g. artificial nail may be coloured to disguise or mask the topical source of hydrogen sulphide under the cover.

[0050] In certain embodiments the cover comprises one or more layer or reservoir containing the topical source of hydrogen sulphide, wherein the one or more layer or reservoir is in fluid communication with the dorsal surface of the infected nail when the cover is placed on the infected nail.

[0051] In certain embodiments the cover is in the form of a patch comprising a dorsal surface and a ventral surface;

wherein the dorsal surface comprises a substantially air impermeable layer;

the ventral surface comprises an adhesive layer;

the patch comprises one or more layer or reservoir comprising the topical source of hydrogen sulphide in fluid communication with the ventral surface; and

wherein the patch is sealably attached to the subject by means of the adhesive layer to provide a substantially air-tight chamber over at least a portion of the infected nail.

[0052] In certain embodiments the patch further comprises a removable substantially air impermeable backing layer over the adhesive layer which maintains the topical source of hydrogen sulphide in a substantially airtight environment within the patch prior to use. The backing layer is removed prior to sealably attaching the patch to the subject.

[0053] Also provided herein is a plasma comprising ionised hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the plasma to an infected nail. Suitably, the plasma is formed from a gaseous mixture comprising hydrogen sulphide and one or more inert gases (e.g. helium, argon, krypton or neon).

[0054] In some embodiments, the plasma is not maintained under a substantially airtight cover during treatment. In other embodiments, the plasma is maintained under a substantially airtight cover during treatment.

[0055] In some embodiments the topical source of hydrogen sulphide is a plasma comprising ionised hydrogen sulphide. In some embodiments the plasma is formed from a gaseous mixture comprising hydrogen sulphide. In some embodiments the plasma may be formed from a mixture of hydrogen sulphide gas and one or more inert gases (e.g. helium, argon, krypton or neon). It may be that the plasma is formed from a mixture of hydrogen sulphide gas and argon.

- [0056] Suitably the plasma is a non-thermal or “cold plasma” formed by providing an electrical discharge through gaseous hydrogen sulphide. The plasma is then directed to the surface of the infected nail where the ionised hydrogen sulphide penetrates into and through the nail plate. Systems for the generation of non-thermal plasmas for the treatment of nail infections are known, for example as described in WO 2016/020407 and WO 2018/175327. When the topical source of hydrogen sulphide is a plasma, the cover may be a chamber placed over the infected nail. The cover may also be formed by a conduit in gaseous communication with source of the plasma and the surface of the nail. For example, the conduit may be provided with a means for providing an air-tight seal between the surface of the nail and the conduit, for example an elastomeric seal (e.g. in the form of an O-ring) between the distal end of the conduit and the dorsal surface of the nail plate. The cover may also be in the form of a chamber wherein the subjects hand or foot is placed in the chamber containing the plasma and the chamber is sealed around the wrist or lower leg of the subject, thereby exposing the infected nail to the plasma.
- [0057] Also provided is a method for treating a nail infection in a subject, the method comprising applying an effective amount of a topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment. In some embodiments the method for treating the nail infection is a therapeutic treatment. In some embodiments the method for treating the nail infection is a non-therapeutic or cosmetic method for the treatment of a nail infection.
- [0058] In certain embodiments the nail infection is a fungal, yeast and/or bacterial infection. It may be that the nail infection is in the form of a biofilm, for example a fungal, yeast and/or bacterial biofilm.
- [0059] In certain embodiments the nail infection is a fungal nail infection. In certain embodiments the nail infection is onychomycosis.
- [0060] In certain embodiments the nail infection is a bacterial nail infection. In certain embodiments the nail infection is paronychia.
- [0061] Also provided is a system comprising:
- a cover; and
 - a topical source of hydrogen sulphide;
- the cover being adapted to provide a substantially airtight chamber over the topical source of hydrogen when the topical source of hydrogen sulphide is applied to an infected nail in a subject.

[0062] In some embodiments of the system the cover comprises the topical source of hydrogen sulphide. In some embodiments of the system the cover comprises one or more layer or reservoir containing the topical source of hydrogen sulphide. Suitably the cover further comprises a removable, substantially air impermeable backing layer which is adapted to maintain the topical source of hydrogen sulphide in the cover in a substantially airtight environment prior to use.

[0063] In some embodiments the system further comprising a means for affixing the cover to a surface tissue of a subject (for example a means for affixing the cover to the dorsal surface of a nail). Suitably the means for affixing the cover is an adhesive, for example an acrylic adhesive.

[0064] In some embodiments of the system the cover is in the form of a patch comprising a dorsal surface and a ventral surface;

wherein the dorsal surface comprises a substantially air impermeable layer;
the ventral surface comprises an adhesive layer; and

the patch comprises one or more layer or reservoir comprising the composition in fluid communication with the ventral surface.

[0065] In some embodiments of the system the cover is an occlusive dressing or occlusive patch. In a particular embodiment the cover is in the form of an artificial nail. In some embodiments of the system the cover is, or comprises, an air-impermeable polymer, for example PTFE.

[0066] In certain embodiments the cover further comprises a removable, substantially air impermeable backing layer which is adapted to maintain the topical source of hydrogen sulphide in the cover in a substantially airtight environment prior to use.

BRIEF DESCRIPTION OF THE DRAWINGS

[0067] Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

Figure 1 is a schematic illustrating a cover in the form of an artificial nail containing a topical source of hydrogen sulphide for use the treatment of a nail infection. Stage 1 illustrates an infected nail and exemplary length and width dimensions of the nail. Stage 2 shows a cover in the form of an artificial nail dimensioned to overlay the dorsal surface of the infected nail. Stage 3 shows the topical source of hydrogen sulphide in the form of a gel containing a hydrogen sulphide donor located in a chamber formed by the convex shape of the inner surface of the artificial nail. The artificial nail has a peripheral edge which has a coating of a suitable adhesive (e.g. acrylic adhesive, dotted line in stage

3) such that a substantially airtight seal is formed between the artificial nail and the dorsal surface of the infected nail when the artificial nail is placed on the infected nail, thereby providing a substantially airtight chamber containing the topical source of hydrogen sulphide. Stage 4 shows the artificial nail attached to the infected nail during treatment.

- 5 The schematic on the right hand-side of Stage 4 illustrates the penetration of dissolved hydrogen sulphide (HS^-) into the and through the nail plate and the equilibrium between dissolved and gaseous H_2S within the nail plate and nail bed. Stage 5 shows the removal of the artificial nail following the completion of the treatment.

Figure 2A shows the release of H_2S from H_2S donors over 24 hours under a
10 constant airflow of 650 mL/min. The H_2S donors were matched via their molar concentration. Points represent mean H_2S concentration $\pm$ SD, $n=3$. Figure 2B shows the amounts of H_2S gas and dissolved H_2S following addition of solid H_2S donor to an aqueous liquid medium after 2, 6 and 24 hours of holding in an airtight container with no air flow. The black bar represents the amount of H_2S in the liquid and the grey bar the amount of
15 H_2S gas. In Figures 2A and 2B NaHS= sodium hydrosulphide, HBTA= hydroxybenzothioamide, DATS= diallyl trisulphide, and ATTM= ammonium tetrathiomolybdate.

Figure 3A shows the effect of pH on the kinetic release of H_2S in the liquid phase from a solution of NaHS at pH 8.5 (large data points) and pH 4 (small data points).

20 Figure 3B shows the effect of pH on the liquid-air equilibrium of H_2S over 2 hours, 6 hours and 24 hours at pH 8.5, 6 and 4 after holding in an airtight container with no air flow. The black bars show the H_2S in the liquid phase and the gray bars the H_2S gas released (μg). In Figures 3A and 3B the number of repeats was 3 for each value. The error bars represent the standard deviation between repeats.

25 Figure 4A shows the permeation of H_2S through a nail plate using an airtight microchamber on top of the nail surface with H_2S gas (square data points) compared to HS^- liquid (NaHS solution applied to the nail surface, circular data points). The y-axis in Figure 4A shows the cumulative H_2S mass/area ($\mu\text{g}/\text{cm}^2$). The donor concentration was NaHS concentration of 5.6 mg/200uL.

30 Figure 4B shows the cumulative permeation of H_2S through a nail plate using a NaHS solution in an airtight microchamber on top of the nail surface (circular data points) compared to NaHS solution applied to the nail surface in a chamber that was open to the atmosphere (square data points). The y-axis in Figure 4B shows the cumulative H_2S mass/area ($\mu\text{g}/\text{cm}^2$). The donor concentration was NaHS concentration of 11.2 mg/200uL.

Figure 4C shows the cumulative permeation of H₂S through a nail plate using comparison of two hydrogen sulphide donors, sodium hydrosulphide (NaHS, circular data points) and ammonium tetra thiomolybdate (ATTM, square data points). In both cases the topical source of hydrogen sulphide was applied to the nail using an airtight chamber over the topical source of hydrogen sulphide. Figure 4C also shows the permeation of ciclopirox through the nail plate (triangular data points). In Figures 4A, 4B and 4C n=8 and the human nail plates were standardised to 30 µm thickness.

Figure 5 shows the permeation of H₂S through a human nail using a solution of NaHS at pH 8.5 (circular data points) and pH 4 (square data points). Data points represent the mean ± SD, n=8.

Figure 6 shows H₂S gas released from human nail clippings after treatment with different H₂S donor solutions as H₂S mass/mg of nail. The molar H₂S donor concentration was matched in the treatment solutions. Bars represent mean H₂S concentration ± SD, n=3. The minimum inhibitory concentration for *T. rubrum* (MIC) is the horizontal line and was calculated using the exemplar MIC from the NaHS donor. NaHS= sodium hydrosulphide, HBTA= hydroxybenzothioamide, DATS= diallyl trisulphide, and ATTM= ammonium tetrathiomolybdate.

Figure 7 shows the effect of H₂S on *T. rubrum conidia* from NaHS at different treatment times of 1, 3, 6 and 24 hours (n=3, biological replicates but only one shown).

Figure 8 shows shows the effect of H₂S on *T. rubrum conidia* from ATTM at different treatment times of 1, 3, 6 and 24 hours (n=3, biological replicates but only one shown).

Figure 9 shows the effects of Effect of H₂S on *T. rubrum conidia* that were incubated for different times of 0, 1, 3, 6 and 24 hours prior to a 24 h treatment with H₂S in order to understand the effects on different stages of fungi growth compared to a control sample before treatment, (n=3, biological replicates but only one shown). The upper panel shows visual observation and the lower panel with microscopy at 60x magnification.

Figure 10, panel A shows the effects of NaHS applied in the liquid form on inhibiting the growth of *T. rubrum* isolates TR 188, TR719, TR420 and TR 936. Figure 10, panel B shows the effects of NaHS applied in liquid form on inhibiting the growth of *Candida albicans* (CA), *Microsporum canis* (MC), *Aspergillus niger* (AN), and *Fusarium oxysporum* (FO) in liquid administration. (n=3).

Figure 11 panels A and B show the viable conidia (%) of *T. rubrum conidia* grown for 7 days and then treated for 24 h with A) H₂S in liquid or B) ciclopirox olamine. (n=3,

biological replicates each). *= denotes a statistical difference in the viable conidia (%). NS= denotes no statistical difference in the viable conidia (%). "LOD" is the limit of detection.

Figure 12 shows viable cells (%) of *T. rubrum conidia* after treatment with H₂S with and without an airtight chamber on the infected nail model described in the Examples.

5 "LOD" is the limit of detection.

Figure 13 shows the results of a human skin and human nail toxicity study. A, B, C, and D illustrate the confocal images of A. untreated human skin, B. Neg control (skin placed in cell culture media), C positive control (glycolic acid (10% w/v)), and D hydrogen sulphide treated skin (H₂S, 3400 µg/mL), respectively. The application of all solutions was repeated in 2 hour intervals over 6 hours. E, F, G and H, illustrate the skin integrity of E. untreated human skin, F. negative control, G positive control, and H treated skin, respectively. SC= stratum corneum, EP= epidermis, and D= dermis. I and J show the second derivative of the Amide I region and the Raman spectra of the disulphide and

10 sulphhydryl regions of human nail keratin after I phosphate buffered saline (PBS) and J H₂S treatment, respectively. The nails were incubated in PBS and H₂S (680 µg/mL) solutions over 24 hours before testing in Raman spectroscopy and ATR-FTIR machines.

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Figure 14 shows the effect of H₂S treatment on the production of ROS in *T. rubrum conidia*: A) untreated, B) treated with H₂S (gas) and no microchamber, C) treated with H₂S (liquid) (no) microchamber, D) treated with H₂S (gas) with microchamber, and E) treated with H₂S (liquid) with microchamber (Calcofluor white stained the fungal cell blue while DCFH-DA stained the ROS green. Images were taken at magnification x20).

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Figure 15 shows the effect of H₂S treatment on the production of ROS in *T. rubrum* hyphae: A) untreated, and B) H₂S (liquid) with microchamber (Calcofluor white stained the fungal cell blue while DCFH-DA stained the ROS green. Images were taken at magnification x20).

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Figure 16 illustrates artificial nails prepared using a 3D printer after measuring the dimensions and curvature of the nail of a subject. Each artificial nail included a cavity in the dorsal side of the artificial nail (the side to be applied to the subject's nail when in use), wherein in use, the cavity accommodates the topical source of hydrogen sulphide. Figure 16 panel A shows the dimensions of "large", "medium" and "small" artificial nails. Panel B shows the artificial nails produced by 3D printing using a Formlab printer and clear resin. Panel C shows an artificial nail attached to the thumbnail of a subject (shown without the topical source of hydrogen sulphide in the cavity of the artificial nail).

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Figure 17 shows the gel stability of four different polymers with sodium hydrogen sulphide. The first row in Figure 17 shows the appearance of the gels formed using

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methylcellulose E4M (MC E4M), methylcellulose E15 (MC E15), hydroxyethylcellulose (Natrosol M ((NM)) and Carbopol 934 (CP 934) immediately after preparing the gel. The lower row shows the appearance after 7 days at 45°C. The hydroxyethylcellulose gel was the most stable gel and maintained pH, appearance, and consistency over seven days.

- 5 Methylcellulose E4M and E14 resulted in precipitation, while Carbopol 934 showed a yellow coloration indicating chemical reaction and gel instability.

Figure 18 panels A and B illustrate two variants of artificial nail models, leaking and non-leaking designs, respectively. Each model was produced in three sizes: large, medium, and small, with nearly identical length, width, and curvature across each design. The distinguishing factor lies in the adhesive margin between the gel pocket (chamber) and the nail edge, highlighted on the right of each design. The non-leaking designs (B) featured a larger margin for adhesive application of 4.1 mm, 3.6 mm or 3.3 mm, thereby enhancing sealability of the artificial nail to the nail to be treated compared to the “leaking design” (A), which had adhesive margins of 1 mm. The black arrow in the illustration signifies the total width or length of the nail, while the red dotted line indicates one side margin, excluding the top and bottom.

Figure 19 shows a comparative analysis of hydrogen sulphide leakage from different designs of artificial nails. Panel A shows a comparison of H₂S gas release from a NaHS liquid and gel loaded onto unsealed artificial nail (i.e. covers that are open and acting as controls 100% leakage) vs a sealed nail cover (no leaking) and a nail cover that is not sealed (leaking gas). Panel B shows the Area Under Curve (AUC) for release of hydrogen sulphide gas into a sealed test vessel from a simple NaHS solution, a NaHS gel, a leaking artificial nail containing a NaHS gel that was glued to a model fingernail, and a non-leaking artificial nail containing a NaHS gel glued to a model fingernail. This panel shows the relative leakage of H₂S gas from each configuration over the test period. Panel C shows methylene blue dye containing gel in the leaking artificial nail, with noticeable dye leakage visualized, indicating poor sealing of the artificial nail to the model fingernail used in the experiment. Panel D demonstrates the successful sealing performance of an optimized artificial nail design with an NaHS gel and methylene blue dye in the chamber of the artificial nail glued to the model fingernail. No dye or H₂S leakage was detected during the test, confirming the efficiency of this design and successful sealing of the artificial nail to a model fingernail. N= 3 for each gas release profile.

Figure 20 shows the effect of H₂S against *C. albicans* from NaHS at different treatment concentrations (5.6 µg/mL, 14 µg/mL, 28 µg/mL, 56 µg/mL, 112 µg/mL, 280 µg/mL and 560 µg/mL) against organisms at different cell numbers (OD) on Sabouraud Dextrose (SD) agar plates (N=3, biological replicates but only one shown).

Figure 21 shows the effect of H₂S against *S.aureus* from NaHS at different treatment concentrations (5.6 µg/mL, 14 µg/mL, 28 µg/mL, 56 µg/mL, 112 µg/mL, 280 µg/mL and 560 µg/mL) against organisms at different cell numbers (OD) on tryptone soy agar (TSA) agar plates (N=3, biological replicates but only one shown).

5 Figure 22 shows the ability of NaHS, ciclopirox and amorolfine to kill *T.rubrum* infected in human nails (N=3 biological replicates). Figure 22A shows the raw ATP bioluminescence data (au). Figure 22B shows the kill percentage.

 Figure 23 shows the killing effect of NaHS, Ciclopirox and Amorolfine against *C.albicans* in human infected nails (N=3, biological replicates). NaHS, Ciclopirox and
10 Amorolfine were tested at the following treatment concentrations: 0, 2, 4, 8, 16, 32, and 64 µg/mL. Figure 23A shows the kill effect (raw ATP bioluminescence data (au)) of Hydrogen sulphide (NaHS), Ciclopirox and Amorolfine against *C.albicans* after 24 h. Figure 23B shows the kill percentage of Hydrogen sulphide (NaHS), Ciclopirox and Amorolfine against *C.albicans*.

15 Figure 24 shows the effect of cold plasma treatment with argon and a combination of H₂S with argon on the growth of *T.rubrum* under a cover. Figure 24A shows the effect of the cold plasma gases against *T.rubrum* agar plates (N=3, biological replicates). Figure 24B shows the kill percentage of the cold plasma gases against *T.rubrum* agar plates (N=3, biological replicates).

20 Figure 25 shows the effects of cold H₂S plasma vs traditional chemical anti-infectives on *C.albicans* biofilms without a cover. In particular, the following treatments were tested: amorolfine, ciclopirox, amphotericin-b (amp(b)), sodium hydrogen sulphide (NaHS), the carrier gas Argon (Arg(g)), argon as a plasma (Arg(p)), gaseous H₂S (H₂S(g)) and gaseous H₂S mixed with argon as a plasma (Arg+H₂S) (N=3, biological replicates).
25 Mean data is shown plus and minus the experiment standard deviation.

 Figure 26 shows the miniaturised H₂S application system designed for the rat *in vivo* studies. The left panel shows the device design in CAD software 3DS max. The right panel shows the use of the 3D printed device on the rat's paw.

 Figure 27 shows the H₂S deposited into rat nails after application of three different
30 H₂S gel concentrations: 8.5, 17, and 34 mg/mL (N=4 to 6 paws, 12 to 18 nail digit). Results are expressed as mean ±SD.

 Figure 28 shows the animal plasma levels pre- and post-H₂S topical exposure with 34 mg/mL NaHS gel. Results are expressed as mean ±SD, N = 3.

DETAILED DESCRIPTION

Definitions

[0068] Unless otherwise stated, the following terms used in the specification and claims have the following meanings set out below.

- 5 **[0069]** The terms “sulphide” and “sulfide” are used interchangeably, thus “hydrogen sulphide” and “hydrogen sulfide” both refer to H_2S .

[0070] Reference to “substantially airtight” or “substantially air-impermeable” refer to the cover minimising the escape or leakage of hydrogen sulphide gas through, or out of, the cover during treatment. However, it is not essential that the cover completely eliminates
10 hydrogen sulphide leakage and generally a cover is considered to be “substantially airtight” or “substantially air-impermeable” if, when the cover is sealed to the subject (e.g. the surface of the nail to be treated), no leakage of the topical source of hydrogen sulphide (e.g. solution or gel) from the cover is observed over a 12 hour period. Visualisation of leakage of the topical source of hydrogen sulphide (e.g. solution or gel) can be enhanced
15 by including a suitable dye (e.g. methylene blue) to indicate the presence of hydrogen sulphide as illustrated in the examples. Conveniently leakage of the topical source of hydrogen sulphide (e.g. solution or gel) from a cover to assess if the cover is “substantially airtight” may be simulated by attaching the cover to a suitable artificial substrate and monitoring if any leakage of the topical source of hydrogen sulphide (e.g. solution or gel) is
20 observed over a 12-hour period. For example where the cover is in the form of an artificial nail, leakage of the topical source of hydrogen sulphide from the cover may be monitored following gluing the artificial nail to a substrate (e.g. a 3D printed model finger prepared using FormLabs “Dental Model Resin” (a methyacrylate-based resin)), optionally using a dye such as methylene blue to highlight the hydrogen sulphide containing liquid or gel
25 present in the system.

[0071] In certain embodiments a cover may be considered to be to “substantially airtight” or “substantially air-impermeable” when the cover is sealed onto the subject (e.g. glued to the dorsal surface of a nail) if the cover retains at least about 70% of the available H_2S gas present in the topical source of hydrogen sulphide under the cover during treatment.
30 Accordingly, the cover is “substantially airtight” or a layer in the cover is “substantially air-impermeable” when less than about 30% of the available H_2S gas present in the topical source of hydrogen sulphide leaks from, or permeates through, the cover/layer into the atmosphere when the cover is secured to the subject during treatment. In preferred embodiments the cover or impermeable layer prevents less than about 25%, 20%, 15%,
35 10%, 5%, 2%, 1%, 0.5 % or 0.1% of the total available H_2S gas present in the topical source of hydrogen sulphide leaking from the cover into the atmosphere during treatment.

Accordingly, in some embodiments the cover is considered to be “substantially airtight” if it retains about 70% to about 100% (e.g. about 80% to about 100%, about 85% to about 100%, about 90% to about 100%, or about 95% to about 100%) of the available H₂S gas present in the topical source of hydrogen sulphide under the cover when the cover is sealed to the subject.

[0072] The amount of available H₂S gas present in a topical source of hydrogen sulphide may be determined as described in Example 12. Accordingly, the total available H₂S gas present in a topical source of hydrogen sulphide may be determined by placing a sample of the topical source of hydrogen sulphide in an airtight sealed chamber (the open sample) and measuring the concentration of H₂S gas released from the topical source of hydrogen sulphide into the sealed chamber every minute for a period of 24 hours (or until evolution of H₂S stops) using a suitable H₂S gas analyser (e.g., using a Teledyne T101 gas analyser using a 10 V analogue output range, and a data output once every 1 min as in the examples). The total H₂S gas evolved by the topical source of hydrogen sulphide from the open sample is determined by calculating the AUC (µg/ml*h) of the H₂S gas released. The amount of H₂S gas that leaks from, or permeates through, the cover (e.g. in the form of an artificial nail) may be determined by sealing the cover comprising the topical source of hydrogen sulphide to a model of a human nail and placing the model nail with the attached cover in an sealed airtight chamber (the sealed sample). The concentration of H₂S gas that escapes from the cover into the sealed container is determined by sampling the H₂S concentration in the sealed chamber every minute for 2.8 days or until no further H₂S gas evolution is detected and then calculating the AUC (µg/ml*h) of the H₂S gas that leaks from the cover sealed to the model of the nail. The % of available H₂S gas that leaks from, or permeates through, the cover is calculated as:

% H₂S gas leakage = $\frac{\text{H}_2\text{S gas AUC from cover sealed to model of fingernail}}{\text{H}_2\text{S gas AUC from open topical source of H}_2\text{S}} \times 100\%$

[0073] As will be recognised by the skilled person, the topical source of hydrogen sulphide used to determine the % H₂S gas leakage should be identical in the open and the sealed samples, that is the concentration of the hydrogen sulphide donor and its formulation are the same in both the open sample and sealed sample. The measurement of H₂S concentrations released from the open and sealed samples is suitably performed at room temperature (20°C) and atmospheric pressure (1 atmosphere, 101.325 kPa). Suitably the model of the human fingernail to which the cover is sealed in the closed sample may be any plastic material that can mimic the sealing of the nail cover to the human nail. For example a model finger prepared using a biomimetic dental model resin

(e.g. FormLabs “Dental Model Resin” (a methyacrylate-based resin) as illustrated in the Examples.

[0074] The terms “treating”, or “treatment” refer to any beneficial effect in the treatment or amelioration of an injury, disease, pathology or condition associated with a nail infection, for example a fungal or bacterial nail infection, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the injury, pathology or condition more tolerable to the patient; slowing in the rate of degeneration or decline; modifying the progression of a disease or condition, making the final point of degeneration less debilitating; improving a patient’s physical or mental well-being. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination. The term “treating” and conjugations thereof, includes prevention of an injury, pathology, condition, or disease (i.e., prophylaxis or prevention). For example, the term “treating” and conjugations thereof, include prevention of a pathology, condition, or disease associated with a nail infection (e.g., reducing or preventing symptoms or effects of the disease or condition or preventing or inhibiting progression of the disease or condition). The treatment of a nail infection herein (e.g., onychomycosis and/or tinea pedis) may reduce or eliminate at least one clinical symptom associated with the infection (e.g. disfiguration, discoloration or breakage of the nail, or dry or scaly skin in the vicinity of the infected nail). The treatment may be result in the killing or reduction in cell viability of a fungus or bacteria. For example treatment of a fungal infection may kill the fungal colony, for example by preventing or inhibiting conidia or hyphae, killing fungal spores or preventing sporulation. The treatment of a fungal infection may also disrupt and/or eradicate a fungal biofilm. Treatment of bacterial infection may, for example kill bacteria or reduce their viability and or replication.

[0075] An “effective amount” is an amount sufficient to accomplish a stated purpose. For example an amount sufficient to achieve the effect for which it is administered, treat a nail infection, or reduce one or more symptoms of a nail infection. An example of an “effective amount” is an amount sufficient to contribute to the treatment, prevention, or reduction of a symptom or symptoms of a nail infection or, or modify the progression of a nail infection, which could also be referred to as a “therapeutically effective amount.” A “reduction” of a symptom or symptoms means decreasing of the severity or frequency of the symptom(s), or elimination of the symptom(s) associated with a nail infection.

[0076] The therapeutically effective amount of hydrogen sulphide and/or the topical source of hydrogen sulphide can be initially estimated from *in-vitro* determination of the MIC of active against the pathogen that has caused the nail infection using methods known in the art. Target concentrations will be those concentrations of active compound that are capable

of achieving the therapeutic effect described herein, as measured using the methods described herein or known in the art. Generally the topical source of hydrogen sulphide is administered in an amount sufficient to provide a hydrogen sulphide concentration at the site of infection in the nail and/or nail bed which exceeds the MIC of the pathogen, for example at a concentration of at least about 1x, 2x, 3x, 5x or 10x the MIC.

[0077] Therapeutically effective amounts for use in humans can also be determined from animal models using known methods. For example, a dose for humans can be formulated to achieve a concentration that has been found to be effective in animals. The dosage in humans can be adjusted by monitoring compound effectiveness and adjusting the dosage upwards or downwards, as described above. Adjusting the dose to achieve maximal efficacy in humans based on the methods described above and other methods is well within the capabilities of the ordinarily skilled artisan.

[0078] Dosages may be varied depending upon the requirements of the subject. The dose administered to a subject in the context of the present invention should be sufficient to effect a beneficial therapeutic and/or cosmetic response in the subject over time. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects. Determination of the proper dosage for a particular situation is within the skill of the practitioner. Dosage amounts and intervals can be adjusted individually to provide levels of the administered active compound effective for the particular indication being treated.

[0079] Reference to "about" in the context of a numerical is intended to encompass the value +/- 10%. For example, about 20% includes the range of from 18% to 22%.

[0080] The term "gel" is used herein refers to a semi-solid, apparently homogeneous substance that may be elastic and jelly-like (as in, for example, gelatin). The gel comprises a three-dimensional polymeric or inorganic matrix within which is dispersed a liquid phase. The liquid phase may comprise an aqueous (e.g. water to provide a hydrogel) or non-aqueous liquid (to provide an anhydrous gel). The matrix of the gel comprises a network of physically or chemical cross-linked polymers or copolymers that swell but do not dissolve in the presence of a solvent (for example water). The cross-linking within the gel matrix may be physical cross linking (for example by hydrogen bonding or ionic cross-linking) or may be covalently cross-linked. The gel may be prepared by forming a solution or dispersion of the hydrogen sulphide donor and/or hydrogen sulphide in the liquid and then mixing the solution or dispersion with a suitable gel-forming polymer. Alternatively, a hydrogen sulphide donor or hydrogen sulphide may be incorporated into a gel by simply dissolving or dispersing the hydrogen sulphide and/or hydrogen sulphide donor into a gel. The gels are preferably clear in appearance; however, turbid gels are also contemplated. Generally, the gel-forming agent, for example gel-forming polymer, is present in the gel in an amount of from about 0.5-

15% weight/volume (w/v), typically 0.5-5% w/v. For example, when the gel-forming agent is hydroxyethyl cellulose, it may be present in the gel composition in an amount of about 3% w/v.

5 [0081] Reference to a “non-aqueous” composition (e.g. a non-aqueous topical composition), or “in the absence of water” includes compositions that are substantially water free. For example, the non-aqueous compositions disclosed herein contain less than 5%, less than 1% or suitably less than 0.01%, preferably less than 0.001% by weight water. Preferred non-aqueous compositions are those which are anhydrous and contain no detectable water.

10 [0082] The term “halo” or “halogen” refers to one of the halogens, group 17 of the periodic table. In particular the term refers to fluorine, chlorine, bromine and iodine. Preferably, the term refers to fluorine or chlorine.

[0083] The term C_{m-n} refers to a group with m to n carbon atoms.

15 [0084] The term “ C_{1-6} alkyl” refers to a linear or branched hydrocarbon chain containing 1, 2, 3, 4, 5 or 6 carbon atoms, for example methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *iso*-butyl, *sec*-butyl, *tert*-butyl, *n*-pentyl and *n*-hexyl. “ C_{1-4} alkyl” similarly refers to such groups containing up to 4 carbon atoms. Alkylene groups are divalent alkyl groups and may likewise be linear or branched and have two points of attachment to the remainder of the molecule. Furthermore, an alkylene group may, for example, correspond to one of those alkyl groups
20 listed in this paragraph. For example, C_{1-6} alkylene may be $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH(CH_3)-$, $-CH_2CH_2CH_2-$ or $-CH_2CH(CH_3)CH_2-$.

[0085] The term “ C_{1-6} haloalkyl”, e.g., “ C_{1-4} haloalkyl”, refers to a hydrocarbon chain substituted with at least one halogen atom independently chosen at each occurrence, for example fluorine, chlorine, bromine, and iodine. The halogen atom may be present at any
25 position on the hydrocarbon chain. For example, C_{1-6} haloalkyl may refer to chloromethyl, fluoromethyl, trifluoromethyl, chloroethyl e.g., 1-chloromethyl and 2-chloroethyl, trichloroethyl e.g., 1,2,2-trichloroethyl, 2,2,2-trichloroethyl, fluoroethyl e.g., 1-fluoromethyl and 2-fluoroethyl, trifluoroethyl e.g., 1,2,2-trifluoroethyl and 2,2,2-trifluoroethyl, chloropropyl, trichloropropyl, fluoropropyl, trifluoropropyl. A haloalkyl group may be, for
30 example, $-CX_3$, $-CHX_2$, $-CH_2CX_3$, $-CH_2CHX_2$ or $-CX(CH_3)CH_3$ wherein X is a halo (e.g., F, Cl, Br, or I). A fluoroalkyl group, i.e., a hydrocarbon chain substituted with at least one fluorine atom (e.g., $-CF_3$, $-CHF_2$, $-CH_2CF_3$ or $-CH_2CHF_2$).

[0086] The term “heteroalkyl,” refers to a stable linear or branched chain alkyl, including at least one carbon atom and at least one heteroatom (e.g., O, N, P, Si, and S), and wherein
35 the nitrogen and sulfur atoms may optionally be oxidized, and the nitrogen heteroatom may

optionally be quaternized. The heteroatom(s) (e.g., N, S, Si, or P) may be placed at any interior position of the heteroalkyl group. The heteroalkyl is a non-cyclic group. "2 to 8 membered heteroalkyl" refers to a heteroalkyl in which there are a total of 1, 2, 3, 4, 5, 6, 7 or 8 carbon atoms and heteroatoms (e.g., O, N, P, Si, and S) in the heteroalkyl group.

5 Examples include, but are not limited to: -CH₂-O-CH₃, -CH₂-CH₂-O-CH₃, -CH₂-NH-CH₃, -CH₂-CH₂-NH-CH₃, -CH₂-N(CH₃)-CH₃, -CH₂-CH₂-N(CH₃)-CH₃, -CH₂-S-CH₂-CH₃, -CH₂-S(O)-CH₃, -CH₂-S(O)₂-CH₃, -CH₂-CH₂-S-CH₃, -CH₂-CH₂-S(O)-CH₃, -CH₂-CH₂-S(O)₂-CH₃, -CH₂-CH=N-OCH₃. Up to two or three heteroatoms may be consecutive, such as, for example, -CH₂-NH-OCH₃ and -CH₂-O-Si(CH₃)₃. A heteroalkyl moiety may include one heteroatom (e.g., O, N, S, Si, or P). A heteroalkyl moiety may include two optionally different heteroatoms (e.g., O, N, S, Si, or P). A heteroalkyl moiety may include three optionally different heteroatoms (e.g., O, N, S, Si, or P). A heteroalkyl moiety may include four optionally different heteroatoms (e.g., O, N, S, Si, or P).

15 **[0087]** The term "C₂₋₆ alkenyl" includes a branched or linear hydrocarbon chain containing at least one double bond and having 2, 3, 4, 5 or 6 carbon atoms. The double bond(s) may be present as the *E* or *Z* isomer. The double bond may be at any possible position of the hydrocarbon chain. For example, the "C₂₋₆ alkenyl" may be ethenyl, propenyl, butenyl, butadienyl, pentenyl, pentadienyl, hexenyl and hexadienyl.

20 **[0088]** The term "C₂₋₆ alkynyl" includes a branched or linear hydrocarbon chain containing at least one triple bond and having 2, 3, 4, 5 or 6 carbon atoms. The triple bond may be at any possible position of the hydrocarbon chain. For example, the "C₂₋₆ alkynyl" may be ethynyl, propynyl, butynyl, pentynyl and hexynyl.

25 **[0089]** The term "C₃₋₆ cycloalkyl" includes a saturated hydrocarbon ring system containing 3, 4, 5 or 6 carbon atoms. For example, the "C₃₋₆ cycloalkyl" may be cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, bicyclo[2.1.1]hexane or bicyclo[1.1.1]pentane. Suitably the "C₃₋₆ cycloalkyl" may be cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl.

30 **[0090]** The term "heterocyclyl", "heterocyclic" or "heterocycle" includes a non-aromatic saturated or partially saturated monocyclic or fused, bridged, or spiro bicyclic heterocyclic ring system. Monocyclic heterocyclic rings may contain from about 3 to 12 (suitably from 3 to 7) ring atoms, with from 1 to 5 (suitably 1, 2 or 3) heteroatoms selected from nitrogen, oxygen or sulfur in the ring. Bicyclic heterocycles may contain from 7 to 12-member atoms in the ring. Bicyclic heterocyclic(s) rings may be fused, spiro, or bridged ring systems. The heterocyclyl group may be a 3-12, for example, a 3- to 9- (e.g. a 3- to 7-) membered non-aromatic monocyclic or bicyclic saturated or partially saturated group comprising 1, 2 or 3
35 heteroatoms independently selected from O, S and N in the ring system (in other words 1, 2 or 3 of the atoms forming the ring system are selected from O, S and N). By partially

saturated it is meant that the ring may comprise one or two double bonds. This applies particularly to monocyclic rings with from 5 to 7 members. The double bond will typically be between two carbon atoms but may be between a carbon atom and a nitrogen atom. Bicyclic systems may be spiro-fused, i.e. where the rings are linked to each other through a single carbon atom; vicinally fused, i.e. where the rings are linked to each other through two adjacent carbon and/or nitrogen atoms; or they may share a bridgehead, i.e. the rings are linked to each other through two non-adjacent carbon or nitrogen atoms (a bridged ring system). Examples of heterocyclic groups include cyclic ethers such as oxiranyl, oxetanyl, tetrahydrofuranyl, dioxanyl, and substituted cyclic ethers. Heterocycles comprising at least one nitrogen in a ring position include, for example, azetidiny, pyrrolidiny, piperidiny, piperaziny, morpholiny, thiomorpholiny, tetrahydrotriaziny, tetrahydropyrazoly, tetrahydropyridiny, homopiperidiny, homopiperaziny, 2,5-diaza-bicyclo[2.2.1]heptanyl and the like. Typical sulfur containing heterocycles include tetrahydrothienyl, dihydro-1,3-dithiol, tetrahydro-2H-thiopyran, and hexahydrothiepine. Other heterocycles include dihydro oxathioly, tetrahydro oxazoly, tetrahydro-oxadiazoly, tetrahydrodioxazoly, tetrahydrooxathiazoly, hexahydrotriaziny, tetrahydro oxaziny, tetrahydropyrimidiny, dioxoliny, octahydrobenzofuranyl, octahydrobenzimidazoly, and octahydrobenzothiazoly. For heterocycles containing sulfur, the oxidized sulfur heterocycles containing SO or SO₂ groups are also included. Examples include the sulfoxide and sulfone forms of tetrahydrothienyl and thiomorpholiny such as tetrahydrothiene 1,1-dioxide and thiomorpholiny 1,1-dioxide. A suitable value for a heterocyclyl group which bears 1 or 2 oxo (=O), for example, 2 oxopyrrolidiny, 2-oxoimidazolidiny, 2-oxopiperidiny, 2,5-dioxopyrrolidiny, 2,5-dioxoimidazolidiny or 2,6-dioxopiperidiny. Particular heterocyclyl groups are saturated monocyclic 3 to 7 membered heterocyclyls containing 1, 2 or 3 heteroatoms selected from nitrogen, oxygen or sulfur, for example azetidiny, tetrahydrofuranyl, tetrahydropyranyl, pyrrolidiny, morpholiny, tetrahydrothienyl, tetrahydrothienyl 1,1-dioxide, thiomorpholiny, thiomorpholiny 1,1-dioxide, piperidiny, homopiperidiny, piperaziny or homopiperaziny. As the skilled person will appreciate, any heterocycle may be linked to another group via any suitable atom, such as via a carbon or nitrogen atom. For example, the term "piperidino" or "morpholino" refers to a piperidin-1-yl or morpholin-4-yl ring that is linked via the ring nitrogen.

[0091] The term "bridged ring systems" includes ring systems in which two rings share more than two atoms, see for example Advanced Organic Chemistry, by Jerry March, 4th Edition, Wiley Interscience, pages 131-133, 1992. Suitably the bridge is formed between two non-adjacent carbon or nitrogen atoms in the ring system. The bridge connecting the bridgehead atoms may be a bond or comprise one or more atoms. Examples of bridged heterocyclyl

ring systems include, aza-bicyclo[2.2.1]heptane, 2-oxa-5-azabicyclo[2.2.1]heptane, aza-bicyclo[2.2.2]octane, aza-bicyclo[3.2.1]octane, and quinuclidine.

5 [0092] The term “spiro bi-cyclic ring systems” includes ring systems in which two ring systems share one common spiro carbon atom, i.e., the heterocyclic ring is linked to a further carbocyclic or heterocyclic ring through a single common spiro carbon atom. Examples of spiro ring systems include 3,8-diaza-bicyclo[3.2.1]octane, 2,5-diaza-bicyclo[2.2.1]heptane, 6-azaspiro[3.4]octane, 2-oxa-6-azaspiro[3.4]octane, 2-azaspiro[3.3]heptane, 2-oxa-6-azaspiro[3.3]heptane, 6-oxa-2-azaspiro[3.4]octane, 2,7-diaza-spiro[4.4]nonane, 2-azaspiro[3.5]nonane, 2-oxa-7-azaspiro[3.5]nonane and 2-oxa-6-azaspiro[3.5]nonane.

10 [0093] “Heterocyclyl- C_{m-n} alkyl” includes a heterocyclyl group covalently attached to a C_{m-n} alkylene group, both of which are defined herein; and wherein the Heterocyclyl- C_{m-n} alkyl group is linked to the remainder of the molecule via a carbon atom in the alkylene group. The groups “aryl- C_{m-n} alkyl”, “heteroaryl- C_{m-n} alkyl” and “cycloalkyl- C_{m-n} alkyl” are defined in the same way.

15 [0094] “- C_{m-n} alkyl substituted by -NRR” and “ C_{m-n} alkyl substituted by -OR” similarly refer to an -NRR” or -OR” group covalently attached to a C_{m-n} alkylene group and wherein the group is linked to the remainder of the molecule via a carbon atom in the alkylene group.

[0095] The term “aromatic” when applied to a substituent as a whole includes a single ring or polycyclic ring system with $4n + 2$ electrons in a conjugated π system within the ring or
20 ring system where all atoms contributing to the conjugated π system are in the same plane.

[0096] The term “aryl” includes an aromatic hydrocarbon ring system. The ring system has $4n + 2$ electrons in a conjugated π system within a ring where all atoms contributing to the conjugated π system are in the same plane. An aryl may be a single ring or multiple rings (preferably from 1 to 3 rings) that are fused together (i.e., a fused ring aryl) or linked
25 covalently. A fused ring aryl refers to multiple rings fused together wherein at least one of the fused rings is an aryl ring. For example, the “aryl” may be a C_{6-12} aryl, suitably phenyl or naphthyl. The aryl system itself may be substituted with other groups. The term “aryl” also covers partially aromatic bi- or polycyclic ring systems wherein at least one ring is an aromatic ring and one or more of the other ring(s) is a non-aromatic, saturated or partially
30 saturated ring.

[0097] The term “heteroaryl” includes an aromatic mono- or bicyclic ring incorporating one or more (for example 1-4, particularly 1, 2 or 3) heteroatoms selected from nitrogen, oxygen or sulfur. The ring or ring system has $4n + 2$ electrons in a conjugated π system where all atoms contributing to the conjugated π system are in the same plane.

[0098] Examples of heteroaryl groups are monocyclic and bicyclic groups containing from five to twelve ring members, and more usually from five to ten ring members. The heteroaryl group can be, for example, a 5- or 6-membered monocyclic ring or a 9- or 10-membered bicyclic ring, for example a bicyclic structure formed from fused five and six membered rings or two fused six membered rings, also referred to as a “fused bicyclic heteroaryl”. Bicyclic heteroaryl groups can be vicinally fused, i.e., where the rings are linked to each other through two adjacent carbon and/or nitrogen atoms. Each ring may contain up to about four heteroatoms typically selected from nitrogen, sulfur and oxygen. Typically, the heteroaryl ring will contain up to 4, for example up to 3 heteroatoms, more usually up to 2, for example a single heteroatom. In one embodiment, the heteroaryl ring contains at least one ring nitrogen atom. The nitrogen atoms in the heteroaryl rings can be basic, as in the case of an imidazole or pyridine, or essentially non-basic as in the case of an indole or pyrrole nitrogen. In general, the number of basic nitrogen atoms present in the heteroaryl group, including any amino group substituents of the ring, will be less than five.

[0099] Examples of heteroaryl include furyl, pyrrolyl, thienyl, oxazolyl, isoxazolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, triazolyl, tetrazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazenyl, benzofuranyl, indolyl, isoindolyl, benzothienyl, benzoxazolyl, benzimidazolyl, benzothiazolyl, benzothiazolyl, indazolyl, purinyl, benzofurazanyl, quinolyl, isoquinolyl, quinazolinyl, quinoxalinyl, cinnolinyl, pteridinyl, naphthyridinyl, carbazolyl, phenazinyl, benzisoquinolyl, pyridopyrazinyl, thieno[2,3-b]furanyl, 2H-furo[3,2-b]-pyranyl, 1H-pyrazolo[4,3-d]-oxazolyl, 4H-imidazo[4,5-d]thiazolyl, pyrazino[2,3-d]pyridazinyl, imidazo[2,1-b]thiazolyl, imidazo[1,2-b][1,2,4]triazinyl, imidazo[1,2-a]pyridine, imidazo[1,2-a]pyrazine, imidazo[1,2-a]pyrimidine, imidazo[1,2-b]pyridazine, triazolo[1,5-a]pyridine, [1,2,3]triazolo[1,5-a]pyridine,.

Examples of heteroaryl groups comprising at least one nitrogen in a ring position include pyrrolyl, oxazolyl, isoxazolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, triazolyl, tetrazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazenyl, indolyl, isoindolyl, benzoxazolyl, benzimidazolyl, benzothiazolyl, benzothiazolyl, indazolyl, purinyl, benzofurazanyl, quinolyl, isoquinolyl, quinazolinyl, quinoxalinyl, cinnolinyl and pteridinyl.

[00100] “Heteroaryl” also covers partially aromatic bi- or polycyclic ring systems wherein at least one ring is an aromatic ring and one or more of the other ring(s) is a non-aromatic, saturated or partially saturated ring, provided at least one ring contains one or more heteroatoms selected from nitrogen, oxygen or sulfur. Partially aromatic heteroaryl bicyclic ring systems can be vicinally fused, i.e., where the rings are linked to each other through two adjacent carbon and/or nitrogen atoms. Examples of partially aromatic heteroaryl

groups include for example, tetrahydroisoquinoliny, tetrahydroquinoliny, 2-oxo-1,2,3,4-tetrahydroquinoliny, dihydrobenzthienyl, dihydrobenzfuranyl, 1,3-dihydroisobenzofuran, 2,3-dihydro-benzo[1,4]dioxiny, benzo[1,3]dioxolyl, 2,2-dioxo-1,3-dihydro-2-benzothienyl, 4,5,6,7-tetrahydrobenzofuranyl, indoliny, 1,2,3,4-tetrahydro-1,8-naphthyridiny, 5 1,2,3,4-tetrahydropyrido[2,3-*b*]pyraziny and 3,4-dihydro-2*H*-pyrido[3,2-*b*][1,4]oxaziny.

[00101] Examples of five-membered heteroaryl groups include but are not limited to pyrrolyl, furanyl, thienyl, imidazolyl, furazanyl, oxazolyl, oxadiazolyl, oxatriazolyl, isoxazolyl, thiazolyl, isothiazolyl, pyrazolyl, triazolyl and tetrazolyl groups.

10 **[00102]** Examples of six-membered heteroaryl groups include but are not limited to pyridyl, pyraziny, pyridaziny, pyrimidiny and triaziny.

[00103] Particular examples of bicyclic heteroaryl groups containing a six-membered ring fused to a five-membered ring include but are not limited to benzofuranyl, benzothiophenyl, benzimidazolyl, benzoxazolyl, benzisoxazolyl, benzothiazolyl, benzisothiazolyl, isobenzofuranyl, indolyl, isoindolyl, indoliziny, indoliny, isoindoliny, 15 puriny (e.g., adeniny, guaniny), indazolyl, benzodioxolyl, pyrrolopyridine, and pyrazolopyridiny groups.

[00104] Particular examples of bicyclic heteroaryl groups containing two fused six membered rings include but are not limited to quinoliny, isoquinoliny, chromanyl, thiochromanyl, chromenyl, isochromenyl, chromanyl, isochromanyl, benzodioxanyl, 20 quinoliziny, benzoxaziny, benzodiaziny, pyridopyridiny, quinoxaliny, quinazoliny, cinnoliny, phthalaziny, naphthyridiny and pteridiny groups.

[00105] The term “oxo,” or “=O” as used herein, means an oxygen that is double bonded to a carbon atom.

25 **[00106]** The invention contemplates pharmaceutically acceptable salts of the compounds disclosed herein. These may include the acid addition and base salts of the compounds. These may be acid addition and base salts of the compounds.

[00107] Suitable acid addition salts are formed from acids which form non-toxic salts. Examples include the acetate, aspartate, benzoate, besylate, bicarbonate/carbonate, bisulfate/sulfate, borate, camsylate, citrate, edisylate, esylate, formate, fumarate, 30 gluceptate, gluconate, glucuronate, hexafluorophosphate, hibenzate, hydrochloride/chloride, hydrobromide/bromide, hydroiodide/iodide, isethionate, lactate, malate, maleate, malonate, mesylate, methylsulfate, naphthylate, 1,5-naphthalenedisulfonate, 2-napsylate, nicotinate, nitrate, orotate, oxalate, palmitate, pamoate, phosphate/hydrogen phosphate/dihydrogen phosphate, saccharate, stearate, 35 succinate, tartrate, tosylate and trifluoroacetate salts.

[00108] Suitable base salts are formed from bases which form non-toxic salts. Examples include the aluminium, arginine, benzathine, calcium, choline, diethylamine, diolamine, glycine, lysine, magnesium, meglumine, olamine, potassium, sodium, tromethamine and zinc salts. Hemisalts of acids and bases may also be formed, for example, hemisulfate and hemicalcium salts. For a review on suitable salts, see "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, Weinheim, Germany, 2002).

[00109] Compounds that have the same molecular formula but differ in the nature or sequence of bonding of their atoms or the arrangement of their atoms in space are termed "isomers". Isomers that differ in the arrangement of their atoms in space are termed "stereoisomers". Stereoisomers that are not mirror images of one another are termed "diastereomers" and those that are non-superimposable mirror images of each other are termed "enantiomers". When a compound has an asymmetric centre, for example, it is bonded to four different groups, a pair of enantiomers is possible. An enantiomer can be characterised by the absolute configuration of its asymmetric centre and is described by the R- and S-sequencing rules of Cahn and Prelog, or by the manner in which the molecule rotates the plane of polarized light and designated as dextrorotatory or levorotatory (i.e., as (+) or (-)-isomers respectively). A chiral compound can exist as either individual enantiomer or as a mixture thereof. A mixture containing equal proportions of the enantiomers is called a "racemic mixture". Where a compound of the invention has two or more stereo centres any combination of (*R*) and (*S*) stereoisomers is contemplated. The combination of (*R*) and (*S*) stereoisomers may result in a diastereomeric mixture or a single diastereoisomer. The compounds of the invention may be present as a single stereoisomer or may be mixtures of stereoisomers, for example racemic mixtures and other enantiomeric mixtures, and diastereomeric mixtures. Where the mixture is a mixture of enantiomers the enantiomeric excess may be any of those disclosed above. Where the compound is a single stereoisomer, the compounds may still contain other diastereoisomers or enantiomers as impurities. Hence a single stereoisomer does not necessarily have an enantiomeric excess (e.e.) or diastereomeric excess (d.e.) of 100% but could have an e.e. or d.e. of about at least 85%, for example at least 90%, at least 95%, at least 99%, or at least 99.9%.

[00110] The compounds described herein may possess one or more asymmetric centres; such compounds can therefore be produced as individual (*R*) or (*S*) stereoisomers or as mixtures thereof. Unless indicated otherwise, the description or naming of a particular compound in the specification and claims is intended to include both individual enantiomers and mixtures, racemic or otherwise, thereof. The methods for the determination of stereochemistry and the separation of stereoisomers are well known in the art (see

discussion in Chapter 4 of "Advanced Organic Chemistry", 4th edition J. March, John Wiley and Sons, New York, 2001), for example by synthesis from optically active starting materials or by resolution of a racemic form. Some of the compounds of the invention may have geometric isomeric centres (E and Z isomers). It is to be understood that the present invention encompasses all optical, diastereoisomers and geometric isomers and mixtures thereof

[00111] Certain compounds disclosed herein may exist in solvated as well as unsolvated forms such as, for example, hydrated forms. It is to be understood that the invention encompasses all such solvated forms.

[00112] It is also to be understood that certain compounds described herein may exhibit polymorphism, and that the invention encompasses all such forms.

[00113] Compounds may exist in a number of different tautomeric forms and references to compounds of the invention include all such forms.

[00114] The term "optionally substituted" includes either groups, structures, or molecules that are substituted and those that are not substituted.

[00115] Reference to a compound (e.g. a hydrogen sulphide donor) or moiety being "optionally substituted" includes substitution by one or more "optional substituent" selected from, for example:

halo, -CN, -NO₂, =O, C₁₋₆ alkyl, C₁₋₆ haloalkyl, 2 to 8 membered heteroalkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, Q¹, -OR¹, -S(O)_xR¹, -NR¹R², -C(O)R¹, -OC(O)R¹, -C(O)OR¹, -NR¹C(O)R¹, -C(O)NR¹R², -NR¹C(O)OR², -OC(O)NR¹R², -NR¹SO₂R², and -SO₂NR¹R²,

wherein said C₁₋₆ alkyl, 2 to 8 membered heteroalkyl, C₂₋₆ alkenyl and C₂₋₆ alkynyl is optionally substituted by one or more R³;

R¹ and R² are each independently selected from: H, C₁₋₆ alkyl, C₁₋₆ haloalkyl and Q¹,

wherein said C₁₋₆ alkyl is optionally substituted by one or more R⁴;

each R³ and R⁴ is independently selected from: halo, -CN, -OR^{1A}, -S(O)_xR^{1A}, -NR^{1A}R^{1B}, C(O)R^{1A}, -OC(O)R^{1A}, -C(O)OR^{1A}, -NR^{1A}C(O)R^{1B}, -C(O)NR^{1A}R^{1B} and Q²;

each Q¹ and Q² is independently selected from: C₃₋₆ cycloalkyl, 4- to 7-membered heterocyclyl, phenyl and 5- or 6-membered heteroaryl,

wherein said C₃₋₆ cycloalkyl, 4- to 7-membered heterocyclyl, phenyl and 5- or 6-membered heteroaryl is optionally substituted by one or more R⁵;

each R^5 is independently selected from: halo, =O, -CN, -NO₂, C₁₋₄ alkyl, C₁₋₄ haloalkyl, -OR^{2A}, -S(O)₂R^{2A}, -NR^{2A}R^{2B}, -C(O)R^{9A}, -OC(O)R^{2A}, -C(O)OR^{2A}, -NR^{2B}C(O)R^{2A}, -C(O)NR^{2A}R^{2B}, -NR^{2B}C(O)OR^{9A}, -OC(O)NR^{2A}R^{2B}, -NR^{2B}SO₂R^{2A} and -SO₂NR^{2A}R^{2B},

wherein said C₁₋₄ alkyl is optionally substituted by 1 or 2 substituents selected
 5 from: halo, -CN, -OR^{9C}, -NR^{3C}R^{3D} and -SO₂R^{3C};

R^{1A}, R^{1B}, R^{2A}, R^{2B}, R^{3C} and R^{3D} are at each occurrence independently selected from: H, C₁₋₄ alkyl and C₁₋₄ haloalkyl;

and wherein any -NR^{1R2}, -NR^{1A}R^{1B}, -NR^{2A}R^{2B} and -NR^{3C}R^{3D} within a substituent may form a 4- to 6-membered heterocyclyl, wherein said 4- to 6-membered heterocyclyl is
 10 optionally substituted by one or more substituents selected from: halo, =O, C₁₋₄ alkyl and C₁₋₄ haloalkyl;

each x is independently 0, 1, or 2.

[00116] Where optional substituents are chosen from “one or more” groups it is to be understood that this definition includes all substituents being chosen from one of the
 15 specified groups or the substituents being chosen from two or more of the specified groups, which may be the same or different. For example, “one or more optional substituents” may refer to 1 or 2 or 3 substituents (e.g. 1 substituent or 2 substituents).

[00117] Where a moiety is substituted, it may be substituted at any point on the moiety where chemically possible and consistent with atomic valency requirements. The moiety
 20 may be substituted by one or more substituents, e.g., 1, 2, 3 or 4 substituents; optionally there are 1 or 2 substituents on a group. Where there are two or more substituents, the substituents may be the same or different.

[00118] Substituents are only present at positions where they are chemically possible, the person skilled in the art being able to decide (either experimentally or theoretically) without
 25 undue effort which substitutions are chemically possible and which are not.

[00119] The term “analogue” or “derivative” includes variants of the compound referred to. For example one or more functional group or moiety in the original compound may be removed or be replaced by a different substituent; the core structure of the original compound may be substituted by one or more additional substituent; a substituent on a
 30 ring may be moved to a different position on the ring; an atom in a ring may be moved to a different position in the ring; and or a ring in the original compound may be fused with another ring (e.g. an aromatic, heteroaromatic, heterocyclic or cycloalkyl ring). Suitably the substituents added to the original compound is one or more of the “optional substituents” described above.

[00120] The various functional groups and substituents making up the compounds described herein are typically chosen such that the molecular weight of the compound does not exceed 1000. More usually, the molecular weight of the compound will be less than 750, for example less than 700, or less than 650, or less than 600, or less than 550.

5 [00121] Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of them mean “including but not limited to”, and they are not intended to (and do not) exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is
10 used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

[00122] Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example
15 described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The invention is not restricted to the details of any foregoing embodiments.
20 The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

[00123] The reader's attention is directed to all papers and documents which are filed
25 concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

Topical Source of Hydrogen Sulphide

[00124] Application of a topical source of hydrogen sulphide to the surface of a nail results
30 in rapid permeation of hydrogen sulphide into and through the nail plate resulting in high local concentrations which exceed the MIC values for bacteria and fungi responsible for nail infections.

[00125] The topical source of hydrogen sulphide is selected such that it is non-toxic (e.g. not corrosive or caustic) to the skin and nail after topical application (see, for example,

Example 8 and Figure 13). Thus, the topical source of hydrogen sulphide only permeates the nail plate, but does not permeate the skin (e.g. nail bed) or surrounding tissues.

Solutions Comprising Hydrogen Sulphide

[00126] In some embodiments the topical source of hydrogen sulphide comprises a solution comprising hydrogen sulphide. The concentration of hydrogen sulphide in a solution may be varied by, for example, the use of co-solvents and/or altering the pH of the solution. The topical source of hydrogen sulphide is selected such that application of the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide) to the surface of a nail results in rapid permeation of hydrogen sulphide into and through the nail plate.

[00127] In some embodiments the hydrogen sulphide solution comprises hydrogen sulphide at a concentration of at least about 0.1%, 1%, 5% 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% of the saturation concentration. In some embodiments the solution comprising hydrogen sulphide comprises at least about 0.001 µg/mL hydrogen sulphide, for example, at least about 0.001 µg/mL, 0.003 µg/mL, 0.005 µg/mL, 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL, 3 µg/mL, 5 µg/mL 0.1 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2 mg/mL, 2.5 mg/mL, 3 mg/mL, 3.5 mg/mL, 4 mg/mL, 4.5 mg/mL, 5 mg/mL, 5.5 mg/mL, 6 mg/mL, 6.5 mg/mL, 7 mg/mL, 7.5 mg/mL, 8 mg/mL, 8.5 mg/mL, 9 mg/mL, 9.5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 25 mg/mL, 30 mg/mL, 35 mg/mL, 40 mg/mL, 45 mg/mL, 50 mg/mL, 55 mg/mL, 60 mg/mL, 65 mg/mL, 70 mg/mL, 75 mg/mL, 80 mg/mL, 85 mg/mL 90 mg/mL, 95 mg/mL, 95 mg/mL or 100 mg/mL hydrogen sulphide. In some embodiments the solution comprising hydrogen sulphide comprises about 0.003 µg/mL to about 100 mg/mL hydrogen sulphide. For example, the solution may comprise about 0.003 µg/mL to about 95 mg/mL, about 0.005 µg/mL to about 95 mg/mL, about 0.01 µg/mL to about 95 mg/mL, about 0.1 µg/mL to about 95 mg/mL, about 1 µg/mL to about 95 mg/mL, about 3 µg/mL to about 95 mg/mL, about 5 µg/mL to about 95 mg/mL, about 1 µg/mL to about 95 mg/mL, about 0.01 µg/mL to about 95 mg/mL, about 0.1 mg/mL to about 95 mg/mL about 0.1 mg/mL to about 70 mg/mL, about 0.1 mg/mL to about 65 mg/mL, about 0.1 mg/mL to about 60 mg/mL, about 0.1 mg/mL to about 55 mg/mL, about 0.1 mg/mL to about 50 mg/mL, about 0.1 mg/mL to about 45 mg/mL, about 0.1 mg/mL to about 40 mg/mL, about 0.1 mg/mL to about 35 mg/mL, about 0.1 mg/mL to about 30 mg/mL about 0.1 mg/mL to about 25 mg/mL, about 0.1 mg/mL to about 20 mg/mL, about 0.1 mg/mL to about 15 mg/mL, about 0.1 mg/mL to about 10 mg/ml, about 0.1 mg/mL to about 9 mg/mL, about 0.1 mg/mL to about 8 mg/mL, about 0.1 mg/mL to about 7 mg/mL, about 0.1 mg/mL to about 6 mg/mL, about 0.1 mg/mL to about 5 mg/mL, about 0.1 mg/mL to about 4 mg/mL, about 0.1 mg/mL to about 3 mg/mL, about 0.1 mg/mL to about 2 mg/mL, about 0.1 mg/mL

to about 1 mg/mL, about 0.5 mg/mL to about 80 mg/mL, about 0.5 mg/mL to about 50 mg/mL, about 0.5 mg/mL to about 25 mg/mL about 0.5 mg/mL to about 20 mg/mL about 0.5 mg/mL to about 15 mg/mL, about 0.5 mg/mL to about 10 mg/ml, about 0.5 mg/mL to about 9 mg/mL, about 0.5 mg/mL to about 8 mg/mL, about 0.5 mg/mL to about 7 mg/mL, about 0.5 mg/mL to about 6 mg/mL, or about 0.5 mg/mL to about 5 mg/mL hydrogen sulphide. In a particular embodiment the solution comprises about 0.1 mg/mL to about 8 mg/mL hydrogen sulphide.

[00128] The hydrogen sulphide may be dissolved in any suitable solvent. For example a solvent selected from water, a hydrocarbon solvent, a halogenated hydrocarbon solvent, and water-soluble organic solvent (e.g. an alcohol, an ether, a ketone, a glycol, or a glycol ether). In preferred embodiments the solution comprising hydrogen sulphide is an aqueous solution comprising hydrogen sulphide. In some embodiments the solution comprises hydrogen sulphide, water and optionally one or more additional water-soluble solvents. In some embodiments the solution comprises hydrogen sulphide, water and one or more water-soluble polar organic solvents. In some embodiments the solution comprises water and one or more solvents selected from an alcohol, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile and a sulfoxide. In some embodiments the solution comprises water and one or more solvents selected from methanol, ethanol, glycerol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone. In some embodiments the solution comprises water and methanol, for example a 1:1 v/v mixture of water and ethanol.

[00129] In certain embodiments the solution comprising hydrogen sulphide is a non-aqueous solution comprising hydrogen sulphide. For example, the solution may comprise hydrogen sulphide and a non-aqueous hydrogen sulphide solvent. In certain embodiments the solution is a non-aqueous solution comprising hydrogen sulphide and one or more solvents selected from a hydrocarbon solvent, a halogenated hydrocarbon solvent, an alcohol, an ether, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile and a sulfoxide. In certain embodiments the solution is a non-aqueous solution comprising hydrogen sulphide and one or more solvents selected an alcohol, an ether, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile and a sulfoxide.

[00130] In certain embodiments the solution has a pH in the range of about 1 to about 12, for example from about 2 to about 11, from about 2 to about 10. Suitably the pH of the solution is greater than about 7, for example a pH of about 8.5, because this favours the

presence of the hydrosulphide anion (HS^-) in the solution. Without being bound by theory, it is thought that the hydrosulphide ion more readily permeates into the nail plate than H_2S in solution.

- [00131]** Accordingly, in certain embodiments the pH of the solution comprising hydrogen sulphide is greater than about 7.0. For example the pH of the solution is greater than about 7.5, greater than about 8.0, greater than about 9.0 greater than about 10 or greater than about 11. In certain embodiments the solution comprising hydrogen sulphide has a pH of about 7.2 to about 11. In certain embodiments the solution comprising hydrogen sulphide has a pH of about 7.5 to about 11. In certain embodiments the solution comprising hydrogen sulphide has a pH of about 7.5 to about 10.5. In certain embodiments the solution comprising hydrogen sulphide has a pH of about 8.0 to about 9.0. In certain embodiments the solution comprising hydrogen sulphide has a pH of about 8.0. In preferred embodiments the solution comprising hydrogen sulphide has a pH of about 8.5.
- [00132]** The solution comprising hydrogen sulphide is selected such that it is non-toxic (e.g. not corrosive or caustic) to the skin and nail after topical application (see, for example, Example 8 and Figure 13). Thus, in preferred embodiments, the solution comprising hydrogen sulphide has a pH of about 7.0 to about 9.0, for example, a pH of about 8.5.

Compositions Comprising a Hydrogen Sulphide Donor

- [00133]** In certain embodiments the topical source of hydrogen sulphide is a composition comprising a hydrogen sulphide donor. The hydrogen sulphide donor generates hydrogen sulphide in the composition applied to the nail.

- [00134]** The hydrogen sulphide donor may be any agent which is capable of generating or releasing hydrogen sulphide into the composition. Hydrogen sulphide donors are well-known to the skilled person. Preferred hydrogen sulphide donors are water-soluble. However, also contemplated are donors which are insoluble or partially soluble in water, provided the donor is capable of releasing hydrogen sulphide into the composition when the composition is applied to the nail. The composition comprising a hydrogen sulphide donor is selected such that application of the composition to the surface of a nail results in rapid permeation of hydrogen sulphide into and through the nail plate.

- [00135]** In certain embodiments the hydrogen sulphide donor is selected from an inorganic sulphide salt (e.g. CaS , KHS , NaHS , Na_2S , MgS , SrS , BaS , SiS_2 , preferably NaHS), ammonium tetrathiomolybdate, an allyl-substituted polysulphide (e.g. diallyl sulphide, diallyl disulphide, diallyl trisulphide or $\text{S}-(\text{prop-2-en-1-yl}) \text{ prop-2-ene-1-}$

sulfinothioate (allicin)), an isothiocyanate (e.g. allyl isothiocyanate (AITC), erucin, benzyl isothiocyanate, 4-hydroxybenzyl isothiocyanate or an aryl isothiocyanate), Lawesson's Reagent and analogues thereof, a phosphonamidodithioate derivative (e.g. GYY4137), a phosphonodithioate derivative, a phosphonamidothioate derivative (e.g. a JK donor), a dithiolthione derivative (e.g. a 1,2-dithiole-3-thione derivative), an N-mercapto derivative (e.g. an N-benzoylthiobenzamide derivative), an S-arylothiooxime derivative, an acyl perthiol derivative, a dithioperoxy-anhydride, a tetrasulphide derivative, a thioamide derivative (e.g. an aryl thioamide derivative such as 4-hydroxybenzothioamide), a gem dithiol derivative, a N-thiocarboxyanhydride derivative, a thiocarbamate derivative, a phosphoramidodithioate derivative, thioamino acid and a thioester prodrug.

[00136] In certain embodiments the hydrogen sulphide donor is selected from an inorganic sulphide salt. The inorganic sulphide salt may be, for example, a metal sulphide salt, for example an alkali metal sulphide or an alkaline earth metal sulphide. In certain embodiments the hydrogen sulphide donor is a water-soluble inorganic sulphide salt. In certain embodiments the hydrogen sulphide donor is selected from CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS and SiS₂. In certain embodiments the hydrogen sulphide donor is selected from CaS, NaHS, and Na₂S. In certain embodiments the hydrogen sulphide donor is NaHS, or Na₂S. In a preferred embodiment the hydrogen sulphide donor is sodium hydrosulphide (NaHS).

[00137] In certain embodiments, the hydrogen sulphide donor is selected from CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS and SiS₂, and the pH of the composition comprising the hydrogen sulphide donor has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5. In certain embodiments, the hydrogen sulphide donor is selected from CaS, NaHS, and Na₂S, and the pH of the composition comprising the hydrogen sulphide donor has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5. In certain embodiments the hydrogen sulphide donor is NaHS, or Na₂S, and the pH of the composition comprising the hydrogen sulphide donor has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5. In certain embodiments, the hydrogen sulphide donor is Na₂S, and the pH of the composition comprising Na₂S has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5.

[00138] In other embodiments, the hydrogen sulphide donor is not Na₂S. Thus, it may be that the hydrogen sulphide donor is selected from CaS, KHS, NaHS, MgS, SrS, BaS and SiS₂. It may be that the hydrogen sulphide donor is selected from CaS, and NaHS. Preferably, the hydrogen sulphide donor is NaHS.

[00139] In certain embodiments the hydrogen sulphide donor is ammonium tetrathiomolybdate (ATTM).

[00140] In certain embodiments the hydrogen sulphide donor is an allyl-substituted polysulphide. It may be that the allyl substituted polysulphide is selected from diallyl sulphide, diallyl disulphide, diallyl trisulphide or S-(prop-2-en-1-yl) prop-2-ene-1-sulfinothioate (allicin).

5 [00141] In certain embodiments, the hydrogen sulphide donor is not allicin (i.e. not S-(prop-2-en-1-yl) prop-2-ene-1-sulfinothioate). Thus, it may be that the hydrogen sulphide donor is an allyl-substituted polysulphide selected from diallyl sulphide, diallyl disulphide, or diallyl trisulphide. Preferably, it may be that the hydrogen sulphide donor is diallyl trisulphide (DATS).

10 [00142] In certain embodiments, the hydrogen sulphide donor is not a polysulphide (such as potassium polysulphide).

[00143] In preferred embodiments, the hydrogen sulphide donor is selected from the group consisting of: sodium hydrosulphide (NaHS), ammonium tetrathiomolybdate (ATTM) and diallyl trisulphide (DATS). More preferably, the hydrogen sulphide donor is NaHS. As
15 shown in Example 8, NaHS is advantageously non-toxic (e.g. not corrosive or caustic) to the skin and nail after topical application. Thus, NaHS only permeates the nail plate, but does not permeate the skin (e.g. nail bed) or surrounding tissues.

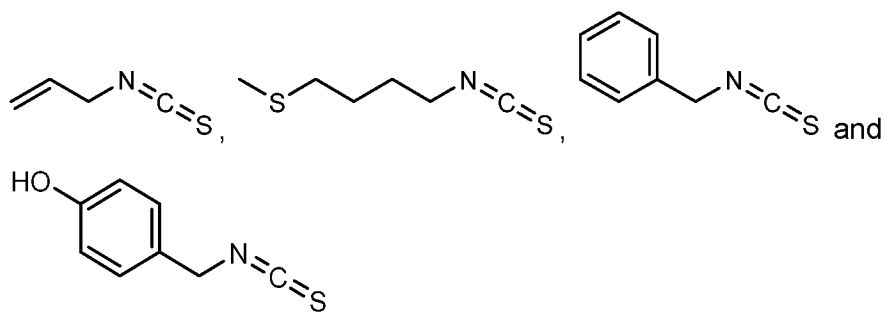
[00144] In certain embodiments the hydrogen sulphide donor is an isothiocyanate derivative, for example a compound of the formula (I):

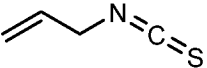


wherein R¹ is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl or optionally substituted
25 heteroaryl.

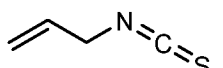
[00145] In certain embodiments R¹ is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted heteroalkyl, optionally substituted phenyl or optionally substituted 5- or 6-membered heteroaryl.

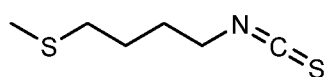
[00146] In some embodiments the compound of formula (I) is selected from:



[00147] In some embodiments, the compound of formula (I) is not:  and

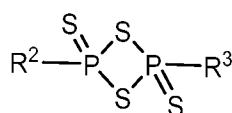


5 . In some embodiments, the compound of formula (I) is not



[00148] In some embodiments, the hydrogen sulphide donor is not an isothiocyanate derivative. In some embodiments, the hydrogen sulphide donor is not allyl isothiocyanate (AITC) and erucin. In some embodiments, the hydrogen sulphide donor is not AITC. In
10 some embodiments, the hydrogen sulphide donor is not erucin.

[00149] In certain embodiments the hydrogen sulphide donor is Lawesson's Reagent or an analogue thereof. In some embodiments the hydrogen sulphide donor is an Lawesson's reagent analogue of the formula (II):

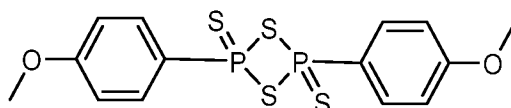


15 (II)

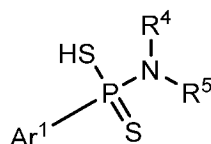
wherein R^2 and R^3 are independently selected from optionally substituted aryl and optionally substituted heteroaryl.

[00150] In certain embodiments R^2 and R^3 are independently selected from optionally substituted phenyl and optionally substituted 5- or 6-membered heteroaryl.

20 [00151] In certain embodiments the hydrogen sulphide donor is Lawesson's Reagent of the formula :



[00152] In certain embodiments the hydrogen sulphide donor is a phosphonamidodithioate derivative. For example the hydrogen sulphide donor is a compound of the formula (II), or a pharmaceutically acceptable salt thereof:



(III)

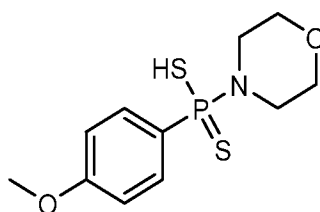
wherein

Ar¹ is optionally substituted aryl or optionally substituted heteroaryl;

R⁴ and R⁵ are independently selected from hydrogen, optionally substituted alkyl and optionally substituted heteroalkyl; or

- 10 R⁴ and R⁵ together with the nitrogen to which they are attached form a 4- to 9-membered optionally substituted heterocyclyl.

[00153] In certain embodiments Ar¹ is optionally substituted phenyl or optionally substituted 5- or 6-membered heteroaryl. In certain embodiments R⁴ and R⁵ are independently selected from hydrogen, optionally substituted alkyl; or R⁴ and R⁵ together
 15 with the nitrogen to which they are attached form a 4- to 7-membered optionally substituted heterocyclyl. In certain embodiments the compound of formula (III) is the compound of formula (IIIa), or a pharmaceutically acceptable salt thereof:



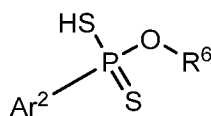
(IIIa),

- 20 or a pharmaceutically acceptable salt thereof.

[00154] In certain embodiments the compound of formula (IIIa) is an addition salt with an organic amine, for example a morpholine addition salt. The morpholine salt of the compound of formula (IIIa) is also known as GYY4137.

- [00155] In certain embodiments the hydrogen sulphide donor is a phosphonodithioate derivative. In some embodiments the phosphonodithioate derivative is a compound of the
 25 formula (IV), or a pharmaceutically acceptable salt thereof:

43



(IV)

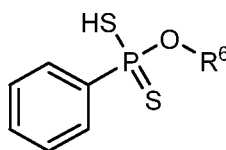
wherein

Ar² is optionally substituted aryl or optionally substituted heteroaryl; and

- 5 R⁶ is selected from hydrogen, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl.

[00156] In certain embodiments Ar² is optionally substituted phenyl or optionally substituted 5- or 6-membered heteroaryl. In certain embodiments R⁶ is optionally substituted alkyl.

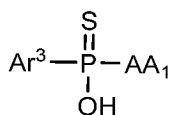
[00157] In certain embodiments the compound of formula (IV) is of the formula (IVa), or a pharmaceutically acceptable salt thereof:



(IVa)

- 15 [00158] Suitably R⁶ is C₁₋₄ alkyl or substituted C₁₋₄ alkyl.

[00159] In certain embodiments the hydrogen sulphide donor is a phosphonamidothioate derivative. In some embodiments the phosphonamidothioate derivative is a compound of the formula (V), or a pharmaceutically acceptable salt thereof:



(V)

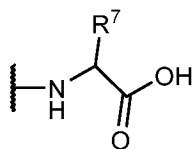
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wherein

Ar³ optionally substituted aryl or optionally substituted heteroaryl; and

AA₁ is a nitrogen linked amino acid.

[00160] AA₁ in formula (V) is a nitrogen-linked amino acid is of the formula:



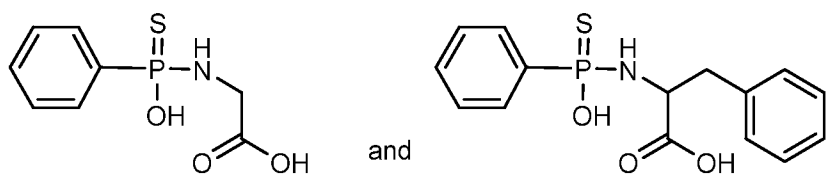
wherein R^7 is an amino acid side-chain.

[00161] The amino acid side-chain represented by R^7 may be a natural or non-natural amino acid sidechain. The amino acid AA_1 may have the D- or L- configuration.

Examples of AA_1 include: an N-linked amino acid selected from alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, proline, pyroglutamic acid, serine, threonine, tryptophan, tyrosine and valine.

[00162] In certain embodiments Ar^3 is optionally substituted phenyl or optionally substituted 5- or 6-membered heteroaryl.

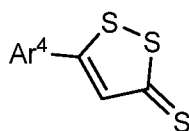
[00163] Examples of compounds of the formula (V) include:



or a pharmaceutically acceptable salt thereof, for example an alkali metal salt (e.g. a lithium salt).

[00164] In certain embodiments the hydrogen sulphide donor is a dithiolthione derivative.

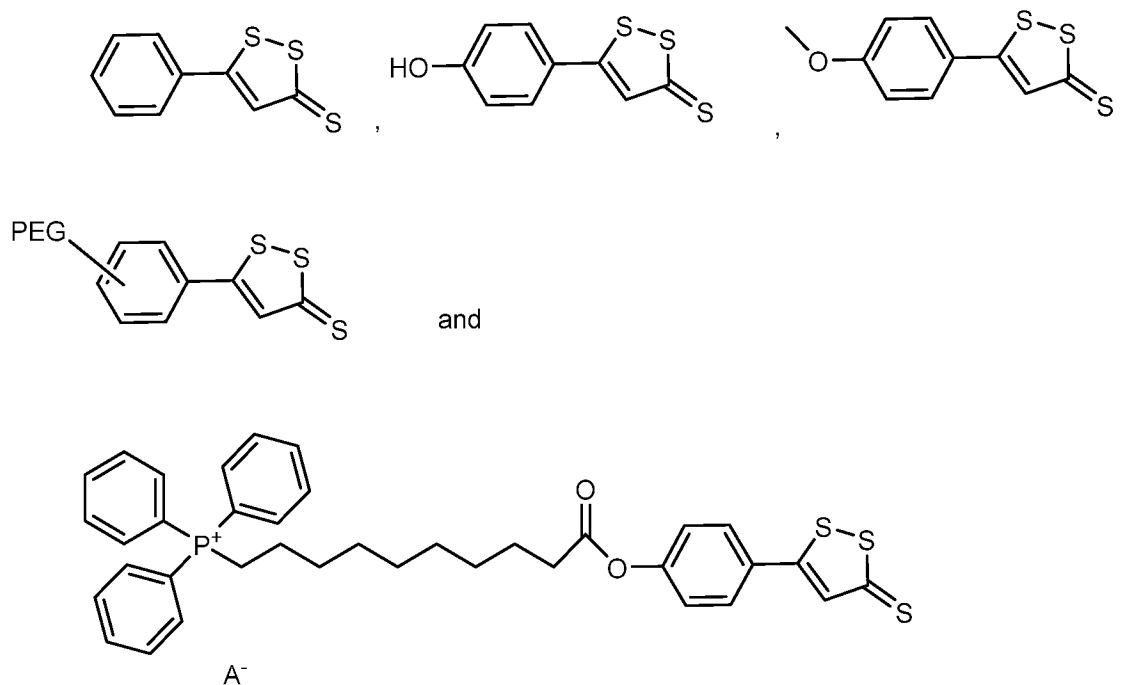
In some embodiments the dithiolthione derivative is a compound of the formula (VI), or a pharmaceutically acceptable salt thereof:



(VI)

wherein Ar^4 is optionally substituted aryl or optionally substituted heteroaryl.

[00165] In some embodiments Ar^4 is optionally substituted phenyl or optionally substituted 5- or 6-membered heteroaryl. Examples of compounds of the formula (VI) include:

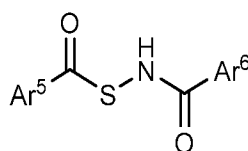


wherein PEG is a polyethylene glycol and A⁻ is an anion (e.g. a halide such as Br⁻).

[00166] In certain embodiments the hydrogen sulphide donor is an N-mercapto derivative.

In some embodiments the N-mercapto derivative is a compound of the formula (VII), or a pharmaceutically acceptable salt thereof:

5



(VII)

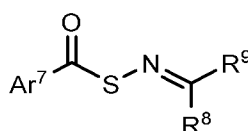
wherein Ar⁵ and Ar⁶ are independently optionally substituted aryl or optionally substituted heteroaryl.

[00167] In some embodiments Ar⁵ and Ar⁶ are independently optionally substituted phenyl or optionally substituted 5- or 6-membered heteroaryl. In some embodiments Ar⁵ and Ar⁶ are independently phenyl or substituted phenyl.

10

[00168] In certain embodiments the hydrogen sulphide donor is an S-aroylthiooxime derivative. In some embodiments the S-aroylthiooxime derivative is a compound of the formula (VIII), or a pharmaceutically acceptable salt thereof:

15



(VIII)

wherein

Ar⁷ is optionally substituted aryl or optionally substituted heteroaryl;

R⁸ is hydrogen or optionally substituted alkyl; and

R⁹ is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted
5 heteroalkyl, optionally substituted aryl or optionally substituted heteroaryl.

[00169] In some embodiments Ar⁷ is selected from optionally substituted phenyl and
optionally substituted 5- or 6- membered heteroaryl. For example Ar⁷ is phenyl or
substituted phenyl. In some embodiments R⁸ is hydrogen or C₁₋₆ alkyl. In some
embodiments R⁹ is selected from optionally substituted C₁₋₆ alkyl, optionally substituted C₂₋₆
10 alkenyl, optionally substituted phenyl and optionally substituted heteroaryl.

[00170] In some embodiments Ar⁷ is phenyl or substituted phenyl; R⁸ is hydrogen or C₁₋₆
alkyl and R⁹ is selected from phenyl, substituted phenyl, furanyl and cinnamyl. Further
specific compounds of the formula (VIII) are disclosed in Foster J., C et al., (2014). S-
aroylthiooximes: a facile route to hydrogen sulfide releasing compounds with structure-
15 dependent release kinetics Org Lett. 2014;16(6):1558-1561, which is incorporated herein
by reference.

[00171] In certain embodiments the hydrogen sulphide donor is an acyl perthiol derivative.
In some embodiments the an acyl perthiol derivative is a compound of the formula (IX), or
a pharmaceutically acceptable salt thereof:

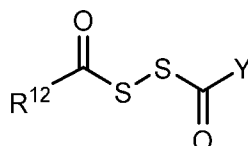


wherein R¹⁰ is selected from optionally substituted alkyl, optionally substituted heteroalkyl,
optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted
aryl and optionally substituted heteroaryl; and

25 R¹¹ is selected from hydrogen, optionally substituted alkyl, optionally substituted
heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally
substituted aryl and optionally substituted heteroaryl.

[00172] In some embodiments R¹⁰ is selected from optionally substituted alkyl, optionally
substituted phenyl and optionally substituted 5- or 6- membered heteroaryl. In some
30 embodiments R¹¹ is selected from hydrogen and optionally substituted alkyl.

[00173] In certain embodiments the hydrogen sulphide donor is a dithioperoxy-anhydride. In some embodiments the dithioperoxy-anhydride is a compound of the formula (X), or a pharmaceutically acceptable salt thereof:



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(X)

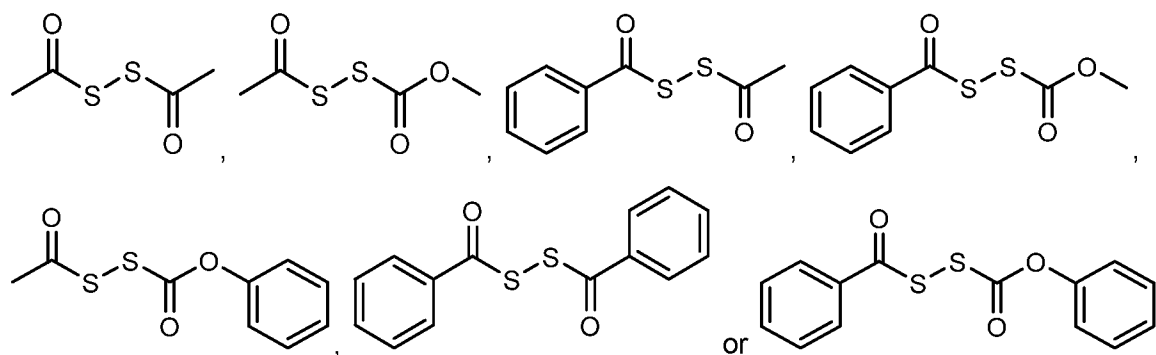
wherein R^{12} is selected from optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl; and

Y is R^{13} or $-\text{OR}^{13}$, wherein R^{13} is selected from hydrogen, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl.

10

[00174] In some embodiments R^{12} and R^{13} are each independently selected from optionally substituted alkyl, optionally substituted phenyl and optionally substituted 5- or 6-membered heteroaryl. In some embodiments the compound of formula (X) is:

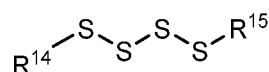
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[00175] Further examples of dithioperoxy-anhydrides are disclosed in Roger T et al., New biologically active hydrogen sulfide donors. *Chembiochem.* 2013;14(17):2268-2271, incorporated herein by reference.

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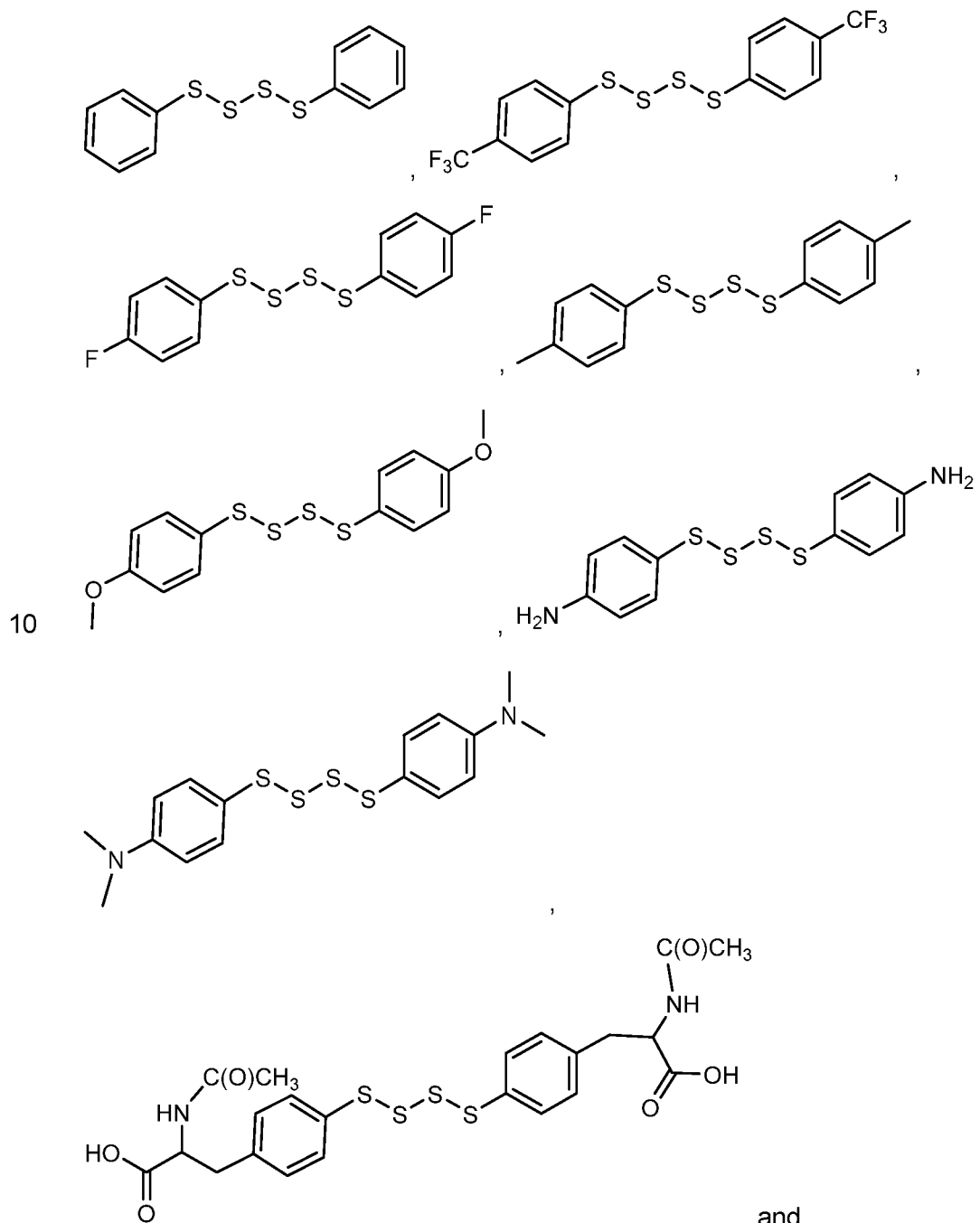
[00176] In certain embodiments the hydrogen sulphide donor is a tetrasulphide derivative. In some embodiments the tetrasulphide derivative is a compound of the formula (XI), or a pharmaceutically acceptable salt thereof:

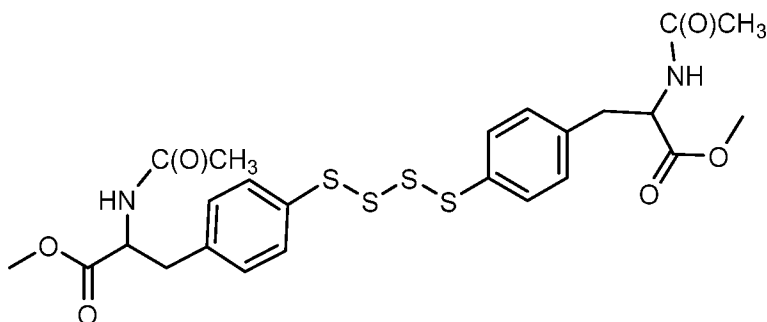


(XI)

wherein R^{14} and R^{15} are each independently selected from hydrogen, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl.

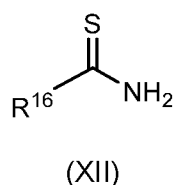
- [00177] In certain embodiments R^{14} and R^{15} are each independently selected from optionally substituted alkyl, optionally substituted phenyl and optionally substituted 5- or 6-membered heteroaryl. In some embodiments the compound of formula (XI) is selected from:





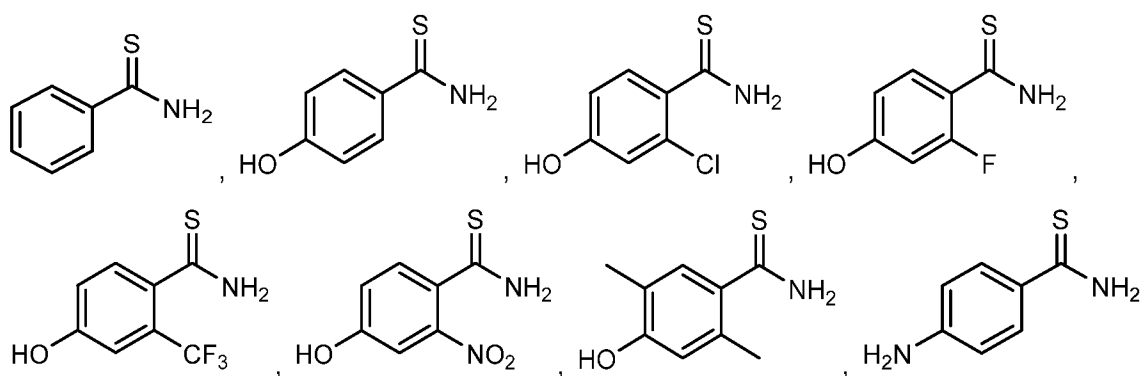
[00178] Tetrasulphide hydrogen sulphide donors are also described in Cerda MM et al., Applications of Synthetic Organic Tetrasulfides as H₂S Donors. Org Lett. 2017; 19(9):2314–2317, which are incorporated herein by reference.

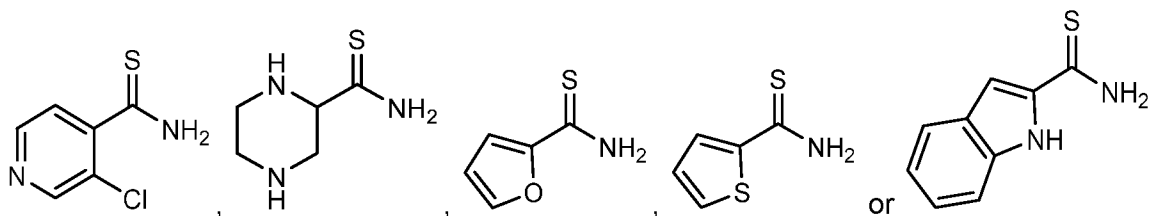
- 5 [00179] In certain embodiments the hydrogen sulphide donor is a thioamide derivative. In some embodiments the thioamide derivative is a compound of the formula (XII), or a pharmaceutically acceptable salt thereof:



- 10 wherein R¹⁶ is selected from hydrogen, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl.

- [00180] In some embodiments R¹⁶ is selected from optionally substituted alkyl, optionally substituted 4-7-membered heterocyclyl, optionally substituted phenyl and optionally substituted 5- to 9- membered heteroaryl. In some embodiments the compound of formula (XII) is:





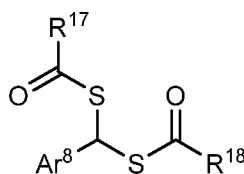
or a pharmaceutically acceptable salt thereof.

[00181] In a particular embodiment the thioamide hydrogen sulphide donor is 4-hydroxybenzothioamide.

- 5 **[00182]** Examples of thioamide hydrogen sulphide donors are disclosed in Martelli A et al., Arylthioamides as H₂S Donors: L-Cysteine-Activated Releasing Properties and Vascular Effects in Vitro and in Vivo. ACS Med Chem Lett. 2013;4(10):904-908, which is incorporated herein by reference.

[00183] In certain embodiments the hydrogen sulphide donor is a gem dithiol derivative.

- 10 In some embodiments the gem dithiol derivative is a compound of the formula (XIII), or a pharmaceutically acceptable salt thereof:



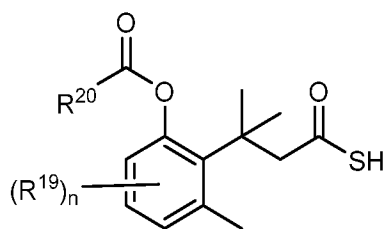
- 15 (XIII)

wherein Ar⁸ is optionally substituted aryl or optionally substituted heteroaryl; and

R¹⁷ and R¹⁸ are independently selected from hydrogen, optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl.

- 20 **[00184]** In some embodiments R¹⁷ and R¹⁸ are independently selected from optionally substituted alkyl, optionally substituted phenyl and optionally substituted 5- or 6-membered heteroaryl. In some embodiments R¹⁷ and R¹⁸ are independently selected from optionally substituted C₁₋₆ alkyl. In some embodiments Ar⁸ is selected from optionally substituted phenyl and optionally substituted 5- or 6- membered heteroaryl. Examples of
25 gem dithiol hydrogen sulphide donors are disclosed in Cairns, T.L. et al., gem-Dithiols. J. Am. Chem. Soc. 1952, 74, 3982–3989, which is incorporated herein by reference.

[00185] In certain embodiments the hydrogen sulphide donor is a thioester prodrug. In some embodiments the thioester prodrug is a compound of the formula (XIV), or a pharmaceutical salt thereof:



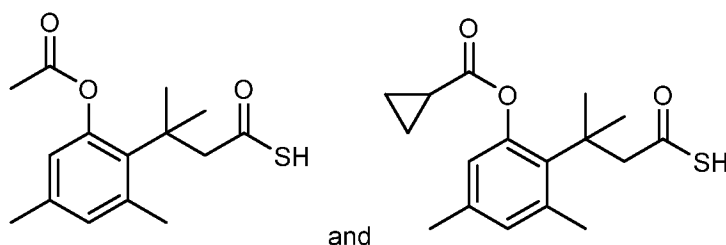
(XIV)

wherein R^{20} is selected from optionally substituted alkyl, optionally substituted heteroalkyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, optionally substituted aryl and optionally substituted heteroaryl;

R^{19} is an optional substituent; and

n is 0, 1, 2 or 3.

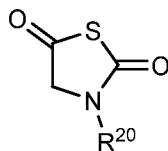
[00186] In some embodiments R^{20} is selected from optionally substituted alkyl, optionally substituted cycloalkyl, optionally substituted 4- to 7-membered heterocyclyl. Examples of thioester prodrugs include:



or a pharmaceutically acceptable salt thereof. For example the compound may be in the form of an alkali metal salt, such as a sodium salt. Examples of thioester prodrug hydrogen sulphide donors are disclosed in Zheng Y et al., Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angew Chem Int Ed Engl.* 2016 Mar 24;55(14):4514-8, which is incorporated herein by reference.

[00187] In certain embodiments the hydrogen sulphide donor is an N-thiocarboxyanhydride derivative. In some embodiments the N-thiocarboxyanhydride derivative is a compound of the formula (XV):

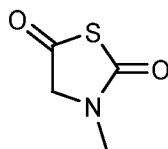
52



(XV)

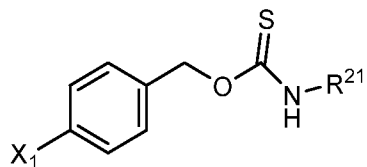
wherein R^{20} is hydrogen or optionally substituted alkyl.

- 5 [00188] In some embodiments R^{20} is optionally substituted C_{1-6} alkyl. In some embodiments the N-thiocarboxyanhydride hydrogen sulphide donor is:



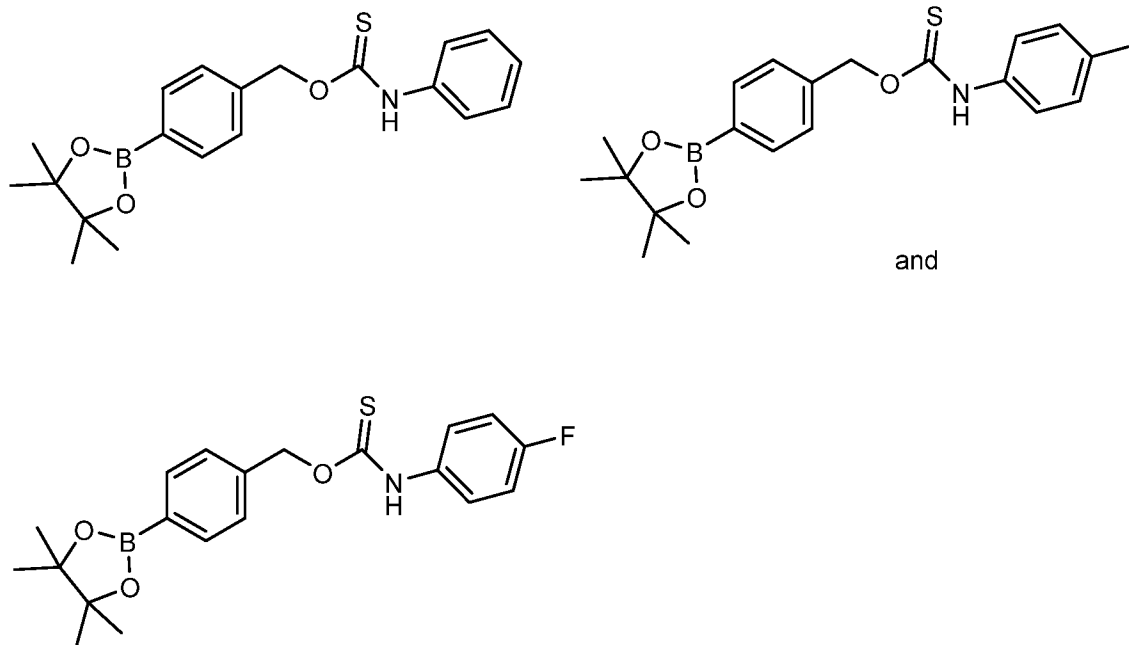
[00189] Further examples of N-thiocarboxyanhydride hydrogen sulphide donors are disclosed in Chadwick R. et al., Journal of the American Chemical Society 2016 138 (41), 13477-13480, which is incorporated herein by reference.

- 10 [00190] In certain embodiments the hydrogen sulphide donor is a thiocarbamate derivative. In some embodiments the thiocarbamate derivative is a compound of the formula (XVI), or a pharmaceutically acceptable salt thereof:



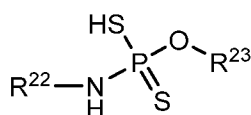
(XVI)

- 15 wherein X_1 is boronate or a boronate ester; and R^{21} is optionally substituted aryl or optionally substituted heteroaryl. Examples of thiocarbamate hydrogen sulphide donors include:



[00191] Further examples of thiocarbamate hydrogen sulphide donors are disclosed in Zhao Y et al., Hydrogen Sulfide Donors Activated by Reactive Oxygen Species. *Angew Chem Int Ed Engl.* 2016 Nov 14;55(47):14638-14642, which are incorporated herein by reference.

[00192] In certain embodiments the hydrogen sulphide donor is a phosphoramidodithioate derivative. In some embodiments the phosphoramidodithioate derivative is a compound of the formula (XVII), or a pharmaceutically acceptable salt thereof:



(XVII)

wherein R^{22} and R^{23} are each independently selected from optionally substituted aryl and optionally substituted heteroaryl.

[00193] In certain embodiments R^{22} and R^{23} are independently selected from optionally substituted alkyl, optionally substituted phenyl and optionally substituted 5- or 6-membered heteroaryl.

[00194] In certain embodiments the hydrogen sulphide donor is a thioamino acid, for example S-allylcysteine, S-propargyl-cysteine, thioglycine or thiovaline. Further examples of thioamino acid hydrogen sulphide donors are disclosed in Zhou, Z. M et al, Thioglycine and L-thiovaline: Biologically active H_2S donors. *Bioorganic & Medicinal Chemistry* 2012, 20 (8), 2675-2678, which is incorporated herein by reference.

[00195] In some embodiments the hydrogen sulphide donor is not acetylcysteine, cysteine or mercaptoethanol.

[00196] In certain embodiments the hydrogen sulphide donor is present in the composition in an amount of about 0.1% to about 40% by weight of the composition. For example the hydrogen sulphide donor is present in the composition in an amount from about 0.1% to about 35%, about 0.1% to about 30%, about 0.1% to about 25%, about 0.1% to about 20%, about 0.1% to about 15%, about 0.1% to about 10%, about 0.1% to about 8%, about 0.1% to about 5%, about 0.1% to about 3%, about 1% to about 35%, about 1% to about 30%, about 1% to about 25%, about 1% to about 20%, about 1% to about 15%, about 1% to about 10%, about 1% to about 8%, about 1% to about 5% or about 0.1% to about 3%, wherein the % is by weight based on the weight of the composition.

[00197] In certain embodiments the hydrogen sulphide donor releases hydrogen sulphide into the composition to provide a solution comprising hydrogen sulphide. Accordingly, the composition may comprise the hydrogen sulphide donor and a liquid, wherein the liquid is a solvent for hydrogen sulphide. Suitable solvents include any of the solvents described herein in relation to solutions comprising hydrogen sulphide. The solution comprising hydrogen sulphide generated by the hydrogen sulphide donor is topically applied to the nail. Thus applying a composition comprising a hydrogen sulphide to the nail enables a solution comprising hydrogen sulphide to be generated in-situ thereby providing delivery of hydrogen sulphide into and through the nail plate.

[00198] In certain embodiments the composition comprising the hydrogen sulphide donor is an aqueous composition. In certain embodiments the composition comprises a hydrogen sulphide donor, water and optionally one or more water-soluble organic solvents. In some embodiments the composition comprises a hydrogen sulphide donor, water and one or more solvents selected from an alcohol, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile and a sulfoxide. In some embodiments the composition comprises a hydrogen sulphide donor, water and one or more solvents selected from methanol, ethanol, glycerol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone.

[00199] In certain embodiments the composition comprising the hydrogen sulphide donor is a non-aqueous composition comprising a hydrogen sulphide donor, and an organic solvent. Thus it may be that the non-aqueous composition comprises the hydrogen sulphide donor and one or more solvents selected from an alcohol, a ketone, a carbonate ester, an alkyl sulphone, an alkyl phosphate, an alkyl amide, a lactam, a glycol, a glycol ether, acetonitrile and a sulfoxide. In some embodiments the composition comprises a

hydrogen sulphide donor, water and one or more solvents selected from methanol, ethanol, glycerol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone.

[00200] The liquid (e.g. water and/or organic solvent(s)) may be present in an amount of, for example about 1% to about 99.9% by weight of the composition. For example, the liquid may be present in the composition an amount of about 5% to about 99%, about 10% to about 99%, about 20% to about 99%, about 30% to about 99%, about 40% to about 99%, about 50% to about 99%, about 60% to about 99%, about 70% to about 99%, about 80% to about 99%, about 90% to about 99%, about 40% to about 90%, about 50% to about 90%, about 40% to about 80%, about 50% to about 80%, about 5% to about 40%, about 5% to about 30% or about 5% to about 20%, wherein the % is by weight based on the weight of the composition.

[00201] In some embodiments the hydrogen sulphide donor is dissolved or dispersed in the composition. In some embodiments the hydrogen sulphide donor is dissolved in the composition. In some embodiments the composition comprises a solution of a hydrogen sulphide donor and a hydrogen sulphide solution. In some embodiments the hydrogen sulphide donor is dispersed in the composition. In some embodiments the composition comprises a dispersion of a hydrogen sulphide donor and a solution of hydrogen sulphide.

[00202] In certain embodiments the hydrogen sulphide donor releases hydrogen sulphide by hydrolysis. Accordingly, in some embodiments the composition comprises water and one or more hydrogen sulphide donors selected from an inorganic sulphide salt (e.g. CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS, SiS₂, preferably NaHS), ammonium tetrathiomolybdate, Lawesson's Reagent and analogues thereof, a phosphonamidodithioate derivative (e.g. a compound of the formula (III), formula (IIIA) or GYY4137), a phosphonodithioate derivative (e.g. a compound of the formula (IV) or formula (IVa)), a phosphonamidothioate derivative (e.g. a compound of the formula (V)), a phosphoramidodithioate derivative (e.g. a compound of the formula (XVII)), a gem dithiol derivative (e.g. a compound of the formula (XIII)) and a dithiolthione derivative (e.g. a compound of the formula (VI)). In this embodiment the composition optionally further comprises one or more additional solvents (e.g. one or more water-soluble organic solvents described herein).

[00203] In certain embodiments the composition further comprises an agent to activate or enhance release of hydrogen sulphide from the hydrogen sulphide donor. For example, the composition further comprises a thiol (e.g. glutathione, cysteine, homocysteine or N-acetylcysteine), a catalyst, an enzyme, a pH adjusting agent, a base (e.g. a bicarbonate), or an oxidising agent (e.g. hydrogen peroxide).

[00204] In certain embodiments the composition comprises a hydrogen sulphide donor wherein release of hydrogen sulphide is activated or enhanced by a thiol (a “thiol activator/release enhancer”). Accordingly, in some embodiments the composition comprises a thiol and a hydrogen sulphide donor selected from In certain embodiments the hydrogen sulphide donor is selected from ammonium tetrathiomolybdate, an N-mercapto derivative (e.g. a compound of the formula (VII), an S-arylthiooxime derivative (e.g. a compound of the formula (VIII), an acyl perthiol derivative (e.g. a compound of the formula (IX)) , a dithioperoxy-anhydride (e.g. a compound of the formula (X), a tetrasulphide derivative (e.g. a compound of the formula (XI)), a thioamide derivative (e.g. a compound of the formula (XII), such as 4-hydroxybenzothioamide) an isothiocyanate derivative (e.g. a compound of formula (I)) , and a gem dithiol derivative (e.g. a compound of the formula (XIII).

[00205] The thiol activator/release enhancer may be any thiol containing compound, for example an amino acid or a protein that comprises one or more thiol moieties. For example, the thiol may be glutathione, cysteine, homocysteine or N-acetylcysteine.

[00206] In some embodiments the thiol activator/release enhancer is present in an approximate equimolar amount, or preferably in a molar excess relative to the hydrogen sulphide donor. In some embodiments the molar ratio of hydrogen sulphide donor to thiol activator/release enhancer is about 1:1 to about 1:10. For example the molar ratio of hydrogen sulphide donor to thiol activator/release enhancer is about 1:1 to about 1:5, or about 1:2 to 1:5. In some embodiments the molar ratio of hydrogen sulphide donor to thiol activator/release enhancer is about 1:4.

[00207] In certain embodiments the composition comprises a hydrogen sulphide donor wherein release of hydrogen sulphide is activated or enhanced by a bicarbonate. In some embodiments the composition comprises a thioamino acid and a bicarbonate. The thioamino acid may be, for example S-allylcysteine, S-propargyl-cysteine, thioglycine or thiovaline.

[00208] In certain embodiments the composition comprises a thiocarbamate hydrogen sulphide donor and an oxidising agent. The thiocarbamate hydrogen sulphide donor may be, for example, a compound of the formula (XVI). The oxidising agent may be, for example hydrogen peroxide.

[00209] The pH of the composition comprising a hydrogen sulphide donor can affect the rate of release of hydrogen sulphide from the donor and also the equilibrium between hydrogen sulphide in solution in the composition and hydrogen sulphide in the gaseous state. In some embodiments the composition comprising a hydrogen sulphide donor has a pH in the range of about 1 to about 12, for example from about 2 to about 11, from about 2

to about 10. In certain embodiments the pH of the solution comprising hydrogen sulphide is greater than about 7.0. For example, a pH in the range of about 7.4 to about 11. It may be that the pH of the composition comprising a hydrogen sulphide donor has a pH of about 8.5. In some embodiments the composition has a pH of less than 7, for example a pH in the range of from about 1 to about 7.4, or about 1.5 to about 7.0.

[00210] In certain embodiments the topical source of hydrogen sulphide is a composition comprising:

an inorganic sulphide salt (e.g. CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS, and SiS₂, preferably NaHS); and water; wherein the composition has a pH in the range of about 7.5 to about 11.0. Preferably, the composition has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5.

[00211] Suitably in this embodiment the inorganic sulphide salt is selected from CaS, KHS, Na₂S, MgS, SrS, BaS, SiS₂ and NaHS. Preferably the inorganic sulphide salt is NaHS or Na₂S. More preferably the inorganic sulphide salt is NaHS.

[00212] Thus, in embodiments, the inorganic sulphide salt is selected from CaS, KHS, Na₂S, MgS, SrS, BaS, SiS₂ and NaHS, and the composition comprising the inorganic sulphide salt has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5. Preferably the inorganic sulphide salt is NaHS or Na₂S, and the composition comprising the inorganic sulphide salt (NaHS or Na₂S) has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5. More preferably, the inorganic sulphide salt is NaHS, and the composition comprising the NaHS has a pH in the range of about 7.0 to about 9.0, for example, a pH of about 8.5.

[00213] In other embodiments, the inorganic sulphide salt is not Na₂S. Thus, it may be that the inorganic sulphide salt is selected from CaS, KHS, MgS, SrS, BaS, SiS₂ and NaHS. Preferably, the inorganic sulphide salt is NaHS.

[00214] In some embodiments the inorganic sulphide salt (e.g. NaHS) is present in the composition at a concentration of about 0.003 µg/mL to about 95 mg/mL. For example, the inorganic sulphide salt (e.g. NaHS) is present in the composition at a concentration of about 0.003 µg/mL to about 95 mg/mL hydrogen sulphide. For example, the solution may comprise about 0.005 µg/mL to about 95 mg/mL, about 0.01 µg/mL to about 95 mg/mL, about 0.1 µg/mL to about 95 mg/mL, about 1 µg/mL to about 95 mg/mL, about 3 µg/mL to about 95 mg/mL, about 5 µg/mL to about 95 mg/mL, about 1 µg/mL to about 95 mg/mL, about 0.01 µg/mL to about 95 mg/mL, about 0.1 mg/mL to about 95 mg/mL about 0.1 mg/mL to about 70 mg/mL, about 0.1 mg/mL to about 65 mg/mL, about 0.1 mg/mL to about 60 mg/mL, about 0.1 mg/mL to about 55 mg/mL, about 0.1 mg/mL to about 50

- mg/mL, about 0.1 mg/mL to about 45 mg/mL, about 0.1 mg/mL to about 40 mg/mL, about 0.1 mg/mL to about 35 mg/mL, about 0.1 mg/mL to about 30 mg/mL about 0.1 mg/mL to about 25 mg/mL, about 0.1 mg/mL to about 20 mg/mL, about 0.1 mg/mL to about 15 mg/mL, about 0.1 mg/mL to about 10 mg/ml, about 0.1 mg/mL to about 9 mg/mL, about 0.1 mg/mL to about 8 mg/mL, about 0.1 mg/mL to about 7 mg/mL, about 0.1 mg/mL to about 6 mg/mL, about 0.1 mg/mL to about 5 mg/mL, about 0.1 mg/mL to about 4 mg/mL, about 0.1 mg/mL to about 3 mg/mL, about 0.1 mg/mL to about 2 mg/mL, about 0.1 mg/mL to about 1 mg/mL, about 0.5 mg/mL to about 80 mg/mL, about 0.5 mg/mL to about 50 mg/mL, about 0.5 mg/mL to about 25 mg/mL about 0.5 mg/mL to about 20 mg/mL about 0.5 mg/mL to about 15 mg/mL, about 0.5 mg/mL to about 10 mg/ml, about 0.5 mg/mL to about 9 mg/mL, about 0.5 mg/mL to about 8 mg/mL, about 0.5 mg/mL to about 7 mg/mL, about 0.5 mg/mL to about 6 mg/mL, or about 0.5 mg/mL to about 5 mg/mL. In a particular embodiment the solution comprises about 0.1 mg/mL to about 8 mg/mL of the inorganic sulphide salt (e.g. NaHS).
- 15 **[00215]** Suitably the composition comprising a the inorganic sulphide salt (e.g. NaHS) has a pH in the range of about 7.5 to about 11.0. Thus it may be that the composition has a pH of about 7.5 to about 10.5. For example the composition has a pH of about 8.0 to about 9.0. Thus it may be that the composition has a pH of about 8.0. It may be that the composition has a pH of about 8.5.
- 20 **[00216]** Suitably in this embodiment at least a proportion of the inorganic sulphide salt is dissolved in the composition, preferably substantially all of the inorganic sulphide salt is in solution in the composition. Dissolution of the inorganic sulphide salt in the water present in the composition will form a solution comprising hydrogen sulphide. Accordingly, the composition may comprise the inorganic sulphide salt, a hydrogen sulphide solution and
- 25 water. Suitably in this embodiment the inorganic sulphide salt (e.g. NaHS) is dissolved in the composition and is present at a concentration of from about 0.05 μ M to about 1.7 M. For example, inorganic sulphide salt (e.g. NaHS) is dissolved in the composition and is present at a concentration of for example, about 0.1 μ M to about 1.7M, about 0.1 mM to about 1.7 M, about 0.1 mM to about 1 M, about 0.1 mM to about 100 mM, about 0.1 mM to about 50 mM, about 0.5 mM to about 50 mM, about 0.5mM to about 40 mM, about 0.5 mM to about 30 mM, about 0.5 mM to about 20 mM, about 0.5 mM to about 10 mM, about 0.2 mM to about 1 mM, about 0.4 mM to about 1 mM, or about 0.5 mM to about 1 mM. Thus it may be that the inorganic sulphide salt (e.g. NaHS) is dissolved in the composition and is present at a concentration of about 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM
- 30 about 1 mM. In a preferred embodiment the inorganic sulphide salt (e.g. NaHS) is dissolved in the composition and is present at a concentration of about 0.6 mM.

[00217] Suitably the composition comprising the inorganic sulphide salt (e.g. NaHS) is an aqueous solution. In some embodiments, the composition comprising the inorganic sulphide salt (e.g. NaHS) is a non-aqueous solution.

5 [00218] In certain embodiments the topical source of hydrogen sulphide is a composition comprising:

ammonium tetrathiomolybdate (ATTM) and water, wherein the composition has a pH in the range of about 1 to about 5.

10 [00219] Suitably in this embodiment the ATTM is present in the composition at a concentration of from about 0.1 to about 50 mM, for example a concentration of about 0.5 mM to about 50 mM, about 0.5mM to about 40 mM, about 0.5 mM to about 30 mM, about 0.5 mM to about 20 mM, about 0.5 mM to about 10 mM, about 0.2 mM to about 1 mM or about 0.4 mM to about 1 mM. For example, the ATTM is dissolved in the composition and is present at a concentration of about 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM or 1 mM, preferably at a concentration of about 0.6 mM.

15 [00220] Suitably in this embodiment the composition has a pH in the range of about 1 to about 4. For example the composition has a pH of about 1.5 to about 3.0. Thus it may be that the composition has a pH of about 2.0.

[00221] Suitably the composition comprising ATTM is an aqueous solution. In some embodiments, the composition comprising the ATTM is a non-aqueous solution.

20 [00222] Suitably in this embodiment the composition further comprises a thiol to enhance release of hydrogen sulphide from the ammonium tetrathiomolybdate. The thiol may be, for example glutathione, cysteine, homocysteine or N-acetylcysteine. Suitably the thiol is present in a molar excess relative to the ammonium tetrathiomolybdate. For example the molar ratio of ammonium tetrathiomolybdate to thiol is from about 1:1 to about 1:10 (e.g. about 1:1 to about 1:5, or about 1:2 to 1:5, suitably about 1:4.

25 [00223] Release of hydrogen sulphide from the ammonium tetrathiomolybdate provides a hydrogen sulphide solution in the composition. Accordingly the composition comprises ammonium tetrathiomolybdate, water, a hydrogen sulphide solution and optionally a thiol compound to enhance release of hydrogen sulphide from the ammonium
30 tetrathiomolybdate.

[00224] In certain embodiments the topical source of hydrogen sulphide is a composition comprising:

diallyl trisulphide (DATS), water and a thiol to enhance release of hydrogen sulphide from the diallyl trisulphide; wherein the composition has a pH in the range of about 3 to about 9.0.

5 [00225] Suitably in this embodiment the composition has a pH in the range of about 4.0 to about 8.5. For example the composition has a pH of about 6.5 to about 8.0. Thus it may be that the composition has a pH of about 7.4. In some embodiments the pH of the composition is about 7.0.

10 [00226] Suitably in this embodiment the DATS is present in the composition at a concentration of about 0.1 mM to about 10 mM, for example about 0.1 mM to about 5 mM, or about 0.1 to about 1 mM. In a preferred embodiment the DATS is present in the composition at a concentration of about 0.2 mM.

[00227] Suitably the composition comprising the DATS is an aqueous solution. In some embodiments, the composition comprising the DATS is a non-aqueous solution.

15 [00228] Suitably in this embodiment the thiol is, for example, glutathione, cysteine, homocysteine or N-acetylcysteine. Suitably the thiol is present in a molar excess relative to the diallyl trisulphide. For example the molar ratio of diallyl trisulphide to thiol is from about 1:1 to about 1:10 (e.g. about 1:1 to about 1:5, or about 1:2 to 1:5, suitably about 1:4).

20 [00229] The topical source of hydrogen sulphide may be formulated as any for suitable for topical administration to the surface of an infected nail. Accordingly, in certain embodiments the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) is in the form of a solution, a dispersion, an emulsion, a foam, a lotion, a cream or a gel.

25 [00230] In some embodiments the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) is in the form of a gel. Suitably the gel is a hydrogel when the topical source of hydrogen sulphide comprises water. Also contemplated are anhydrous gels, wherein the topical source of hydrogen sulphide is substantially free from water (e.g. wherein the topical source of hydrogen sulphide contains less than about 10%, less than 5%, less than 2%, less than 1%, or less than 0.5% water).

30 [00231] Accordingly in some embodiments the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide, or a composition comprising a hydrogen sulphide donor) further comprises a gel-forming agent. Suitable gel-forming agents are well known to the skilled person and include, for example, a carboxypolymethylene; a polyacrylic polymer and/or copolymer such as polyacrylic acid, a polyacrylate polymer, a cross-linked polyacrylate polymer, a cross-linked polyacrylic acid polymer and/or copolymer, and

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- mixtures thereof; a cellulose ether such as hydroxyalkyl cellulose polymers such as one or more gel-forming agent selected from hydroxypropyl methyl cellulose (HPMC), hydroxypropyl cellulose, hydroxyethyl cellulose, methyl cellulose and carboxymethyl cellulose, or a salt thereof (preferably sodium carboxymethyl cellulose), a methacrylate, a
- 5 polyvinylpyrrolidone, a cross-linked polyvinyl pyrrolidone, polyvinylalcohol, polyethylene oxide, polyethylene glycol, a carboxy vinyl polymer, a polysaccharide, an alginate, carrageenan, xanthan gum, gum acacia, arabic gum, guar gum, gellan gum, pullulan, agar, chitin, chitosan, pectin, locust bean gum, tragacanth gum; collagen, gelatin, a starch (e.g. maltodextrin, amylose, corn starch, potato starch, rice starch, tapioca starch, pea starch), a
- 10 modified starch, dextrin, bentonite, calcium stearate, colloidal silicon dioxide, dextrin or a phospholipid. In some embodiments the gel-forming agent is selected from carboxymethyl cellulose, or a salt thereof (preferably sodium carboxymethyl cellulose), hydroxypropyl methyl cellulose, chitosan, pectin, gelatin, a polyacrylamide and a cross-linked acrylic acid polymer (e.g. a carbomer).
- 15 **[00232]** In some embodiments the gel-forming agent is a carbomer. Carbomers are high molecular weight cross-linked poly(acrylic acid) polymers. The polymers may be cross-linked by polyalcohol allyl ethers, for example, allyl sucrose or allyl pentaerythritol. The carbomer may be a homopolymer, for example 910, 934P, 940GE, 941GE, 971P, 974P, wherein "GE" refers to medical grade and "P" oral grade. Derivatives of Carbomer polymers
- 20 may also be used, for example Carbopol interpolymers comprising a carbomer polymer comprising a block copolymer of polyethylene glycol and a long chain alkyl acid ester, such derivatives are commercially available as ETD 2020 NF and Ultrez 10 NF from Lubrizol. Carbomers (also known as Carbopols) are well known and are characterised in the United States Pharmacopeia/National Formulary (USP/NF) monograph for Carbomers and the
- 25 European Pharmacopeia (Ph. Eur.) monograph for Carbomers, reference to which is incorporated herein. The carbomer may have a viscosity of from about 4,000 to about 70,000, for example about 10,000 to about 60,000, for about 20,000 to about 50,000, about 25,000 to about 45,000 or about 29,400 to about 39,400 cP, wherein the viscosity is that of a 0.5 wt.% solution of the carbomer in water, neutralised to pH 7.3 - 7.8 at 25 °C, measured
- 30 using a Brookfield RVT, 20 rpm, spindle #6. Suitably the carbomer comprises from about 56% to about 68.0% by weight carboxylic acid (–COOH) groups, measured by titrating an aqueous solution or dispersion of the polymer against NaOH.
- [00233]** In a preferred embodiment, the gel-forming agent is a hydroxyethyl cellulose. As illustrated in the Examples, compositions (particularly aqueous compositions) comprising
- 35 hydroxyethyl cellulose and hydrogen sulphide and/or a hydrogen sulphide donor such as NaHS form stable gels which are storage stable. In some embodiments, the hydroxyethyl

cellulose has an average molecular weight of approximately 500,000 Da to 850,000 Da, for example about 720,000 Da. In some embodiments, a 2% solution of the hydroxyethyl cellulose has a viscosity of from about 4,500 mPa.s to 6,500 mPa.s, preferably from about 4,500 mPa.s to 6,500 mPa.s when measured using a Brookfield viscometer at 25°C and a shear rate of 10^{s⁻¹}.

[00234] The amount of gel-forming agent present in the topical source of hydrogen sulphide may be selected so as to provide a gel composition having the required rheological properties, for example a viscosity suitable for topical application. Generally the gel will be of a viscosity such that it can be readily dispensed and spread over the nail to be treated. The rheology of the gel composition will depend upon the particular gelling agent used, as well as, for example the nature of the hydrogen sulphide donor and amount of liquid (e.g. water present). Generally the gel-forming agent (for example any of the gel-forming agents disclosed herein such as hydroxyethylcellulose), will be present in the gel composition is an amount of up to about 20% by weight, for example up to about 1%, 2%, 3%, 4%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 9.5%, 10%, 12%, 14%, 16%, 18% or 20% by weight of the gel composition. Suitably the gelling-forming agent (e.g. hydroxyethylcellulose), may be present in an amount of from about 0.01% to about 10% by weight of the gel composition, for example about 0.01% to about 8%, about 0.05% to about 7%, about 0.05% to about 6%, about 0.05% to about 5%, about 0.05% to about 4%, about 1% to about 6%, about 1% to about 5%, about 1% to about 4%, about 2% to about 5%, about 2% to about 4%, or about 2% to about 3%, wherein the % is by weight based on the weight of the gel composition.

[00235] When the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) is formulated as a hydrogel, the hydrogel suitably comprises at least about 40% water, for example at least 50% water such as, for example, about 50% to about 75%, about 50% to about 70%, about 50% to about 60%, or about 70% to about 80% by weight of the hydrogel.

[00236] The topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) may have a viscosity in the range of, for example, about 5,000 cP to about 25,000 cP. For example a viscosity of, about 5,000 cP to about 20,000 cP, or about 7,000 cP to about 15,000 cP. The viscosity may be measured using well-known methods, for example wherein the viscosity is measured at 25°C, measured using a Brookfield RVT, 20 rpm, spindle #6.

Other Ingredients

[00237] The topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) may further comprise one or more additional excipients selected from viscosity modifying agents, emulsifiers,

surfactants, humectants, oils, waxes, additional solvents, preservatives, pH modifying agents (for example a suitable acid or base, for example an organic acid or organic amine base), buffers, antioxidants (for example butylated hydroxyanisole or butylated hydroxytoluene), preservatives, colorants or fragrances. Representative examples of such additional excipients are well known, for example as listed in the Handbook of
5 Pharmaceutical Excipients, 7th Edition, Rowe et al.

[00238] In some embodiments the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) comprises a buffer. Buffering the topical source of hydrogen sulphide may be used to, for
10 example, maintain a more constant release of hydrogen sulphide from a hydrogen sulphide donor present in the topical source of hydrogen sulphide and/or maximise the concentration of hydrogen sulphide in solution in the topical source of hydrogen sulphide. Buffers that may be present in the topical source of hydrogen sulphide include, for example one or more borate, carbonate, citrate, glycine, a phosphate (e.g. phosphate
15 buffered saline), or Tris (tromethamine) buffer.

[00239] When present, the buffer is present in an amount of, for example about 0.01% to about 20% by weight of the topical source of hydrogen sulphide (e.g. a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor), such as, for example about 0.01% to about 10% (e.g. about 0.1 to about 5%) weight of the topical
20 source of hydrogen sulphide

[00240] A buffer may be present in the topical source of hydrogen sulphide in an amount sufficient to provide a desired pH for the topical source of hydrogen sulphide. For example, the buffering agent may be present in an amount sufficient to provide the topical source of hydrogen sulphide with a pH of about 2 to about 11. For example a pH of about
25 3 to about 11, about 7 to about 11, about 7 to about 9, about 7 to about 8.5, about 1.5 to about 5, about 1.5 to about 2.5, about 4 to about 7, about 5 to about 7, or about 6 to about 7.

Manufacture

[00241] The topical source of hydrogen sulphide may be prepared using conventional
30 methods. For example when the topical source of hydrogen sulphide is a gel composition comprising a hydrogen sulphide donor, the composition may be prepared by dissolving or dispersing the hydrogen sulphide donor in a liquid medium (e.g. an aqueous medium) and mixing the solution or dispersion with a gel-forming agent to provide a gel comprising the liquid medium and hydrogen sulphide donor.

35 Plasma comprising ionised hydrogen sulphide

[00242] In some embodiments the topical source of hydrogen sulphide comprises a plasma comprising ionised hydrogen sulphide as described above in the brief summary of the disclosure. Suitably the plasma is a non-thermal or “cold” plasma formed by ionising gaseous hydrogen sulphide or a mixture of hydrogen sulphide and one or more gases, for example nitrogen and/or an inert gas such as helium, argon, krypton or neon.

[00243] Plasmas comprising hydrogen sulphide can be generated using known methods. For example non-thermal plasmas may be generated using gliding arc discharge, rotating gliding arc discharge, low current arc discharge, corona discharge, glow discharge, dielectric barrier discharge, spark discharge, pulsed corona, radio-frequency capacitively or inductively coupled discharge, or microwave discharge. Examples of systems for generating non-thermal hydrogen sulphide plasmas are known, for example as described in K. Gutsol et al., Plasma assisted dissociation of hydrogen sulfide, International Journal of Hydrogen Energy, 37(2), 2012, 1335-1347, and US 7,572,998, both incorporated herein by reference. The hydrogen sulphide plasma may be generated using a system described in for example WO 2016/020407 or WO 2018/175327.

[00244] In embodiments, the plasma comprising ionised hydrogen sulphide is formed from a gaseous mixture comprising hydrogen sulphide and a carrier gas. It may be that the carrier gas comprises one or more inert gases (e.g. helium, argon, krypton and/or neon). Thus, it may be that the plasma comprising ionised hydrogen sulphide is formed from a gaseous mixture comprising hydrogen sulphide and one or more inert gases (e.g. helium, argon, krypton and/or neon). Preferably, it may be that the plasma comprising ionised hydrogen sulphide is formed from a gaseous mixture comprising hydrogen sulphide and argon.

[00245] In embodiments, the gaseous mixture comprises from about 0.1% to about 20% of hydrogen sulphide by volume. It may be that the gaseous mixture comprises from about 0.5% to about 10% of hydrogen sulphide by volume. It may be that the gaseous mixture comprises from about 1% to about 5% of hydrogen sulphide by volume. For example, it may be that the gaseous mixture comprises about 2% of hydrogen sulphide by volume. Thus, it may be that the gaseous mixture comprises about 2% of hydrogen sulphide and about 98% of argon by volume.

[00246] In embodiments, the plasma comprising ionised hydrogen sulphide is applied to an infected nail without a substantially airtight cover during treatment (see, for example, Example 21).

[00247] In other embodiments, the plasma comprising ionised hydrogen sulphide is applied to an infected nail, wherein the plasma is maintained under a substantially airtight cover during treatment.

[00248] In embodiments, the cover is a chamber. It may be that the hand or foot of the subject (with the infected nail) may be exposed to the plasma comprising ionised hydrogen sulphide by placing the hand or foot into the chamber containing the plasma. It may be that the chamber is sealed around the wrist or lower leg (e.g. ankle) of the subject, thereby exposing the infected nail to the plasma.

[00249] In other embodiments, the cover is a chamber which is placed over the infected nail.

[00250] Alternatively, upon generation the plasma may be directed to the surface of the nail using a suitable conduit. Thus, it may be that the cover is formed by a conduit in gaseous communication with the source of the plasma and the surface of the nail.

Cover

[00251] Maintaining the topical source of hydrogen sulphide under a substantially airtight cover during treatment maximises the permeation of hydrogen sulphide into and through the nail plate. The cover may have any form, provided it acts to maintain the topical source of hydrogen sulphide on the surface of the nail in an airtight environment.

[00252] Suitably the cover provides an airtight chamber over the topical source of hydrogen sulphide on the dorsal surface of the nail. As will be recognised by the skilled person the nail plate itself may not be air-impermeable. The cover therefore acts to enhance transmission of a solution comprising hydrogen sulphide (i.e. hydrogen sulphide in liquid form) present in the topical source of hydrogen sulphide into and through the nail plate. Once the solution comprising hydrogen sulphide has penetrated into and through the nail plate, the hydrogen solution provides an anti-infective effect against the pathogen(s) causing the nail infection. In particular embodiments hydrogen sulphide gas is also produced from the topical source of hydrogen sulphide that has penetrated into and through the nail plate thereby providing hydrogen sulphide gas in the nail plate, the nail bed and optionally the tissues surrounding the nail bed. As illustrated in the examples both hydrogen sulphide solutions and gaseous hydrogen sulphide provide an anti-infective (e.g. anti-fungal) effect. It may be that the topical source of hydrogen sulphide (e.g. a liquid composition comprising NaHS) penetrates into and through the nail plate, then hydrogen sulphide gas produced by the topical source of hydrogen sulphide once it has penetrated the nail (see, Example 3), penetrates back through the nail plate and out of the nail. It may be that the topical source of hydrogen sulphide (e.g. a liquid composition comprising NaHS) does not permeate the skin under (or around) the nail plate (e.g. the nail bed).

[00253] The patch may comprise any material provided it is substantially air-impermeable. In some embodiments the cover comprises one or more polymeric materials. For example the cover may comprise one or more air-impermeable polymer layers. Thus it may be that the cover comprises a polymeric material selected from a polyester, a polyurethane, a polytetrafluoroethylene, a polyethylene, a polysiloxane, a polyisocyanate, a polycarbonate poly(ethylene terephthalate), an acrylic polymer (e.g. a poly(methyl methacrylate), a poly(ethyl methacrylate) or a polyacrylonitrile)), a polyvinylchloride, or an epoxy-resin, or a co-polymers of two or more thereof. Polytetrafluoroethylene (PTFE) is particularly stable in the presence of hydrogen sulphide and is a preferred polymer comprising the cover.

5 **[00254]** In some embodiments the cover comprises an inner surface (ventral surface) which is in contact with, or is exposed to, the topical source of hydrogen sulphide on the nail when the cover is placed over the topical source of hydrogen sulphide, wherein the inner surface comprises an air-impermeable layer of PTFE. In this embodiment the cover may further comprise one or more additional polymeric layers over the PTFE layer. The polymer layer(s) comprising the cover may be, for example about 2.5 μm to about 2.5 mm thick, or about 0.1 mm to 1 mm thick. In some embodiments the cover is about 1 mm to about 3 mm thick.

[00255] In some embodiments the cover is in the form of an occlusive dressing or occlusive patch. Reference to the dressing or cover being occlusive means that the dressing or patch provides a substantially airtight barrier between the air and topical source of hydrogen sulphide on the nail. Accordingly known occlusive wound dressing or patches (e.g. occlusive transdermal patches) may be used to provide the cover, provided the patch or dressing provides an air-impermeable barrier over the topical source of hydrogen sulphide on the nail. Suitably the patch or dressing provides a substantially airtight chamber over the topical source of hydrogen sulphide.

[00256] In embodiments the cover is sealably attached to the subject being treated so as to provide a substantially airtight seal between the cover and the subject thereby maintaining the topical source of hydrogen sulphide on the dorsal surface of the nail in a substantially airtight chamber. Accordingly reference to "sealably attached" includes forming a seal between the cover to, for example the surface of the nail plate and/or the skin surrounding the nail plate.

[00257] The cover may be sealably attached to the subject using a suitable adhesive. The adhesive used will depend upon the nature of the material used for the cover and the location where the cover is attached to the subject (e.g. the surface of the nail plate or the skin surrounding the nail). In some embodiments the adhesive is a cyanoacrylate adhesive. In some embodiments the adhesive is a pressure sensitive adhesive. For example, the

pressure-sensitive adhesive may comprise a polymer selected from a silicone polymer (e.g. a polysiloxane), polyisobutylene, polyacrylate and copolymers or mixtures thereof. In some embodiments the adhesive comprises a polysiloxane or polyisobutylene.

5 [00258] In certain embodiments the pressure-sensitive adhesive comprises a polyisobutylene adhesive. Suitably the polyisobutylene comprises a blend of a high molecular weight polyisobutylene (about 450,000 to 4,000,000 viscosity average molecular weight) and a low molecular weight polyisobutylene (about 40,000 to 450,000 viscosity average molecular weight).

10 [00259] In certain embodiments the pressure-sensitive adhesive comprises a polyacrylate adhesive. The polyacrylate adhesive is suitably obtained by copolymerizing one or more acrylate monomers (e.g. acrylates, acrylamides and methacrylates), one or more modifying monomers, and one or more functional group-containing monomers in an organic solvent. Suitably the acrylate monomers comprise alkyl acrylates of 4-17 carbon atoms (e.g. 2-ethylhexyl acrylate, butyl acrylate, and isooctyl acrylate). The modifying monomers alter
15 the glass transition temperature of the resulting polymer and include vinyl acetate, ethyl acrylate and methacrylate, and methyl methacrylate. Such monomers may be further functionalized to provide functional group containing monomers comprising e.g. one or more carboxy or hydroxy functional groups (e.g. acrylic acid, methacrylic acid and hydroxyethyl acrylate). In certain embodiments the pressure-sensitive adhesive is a polyacrylate based
20 pressure-sensitive adhesive. Polyacrylate based pressure-sensitive adhesives are commercially available, e.g. under the trademark DURO-TAK®, especially the 87 series such as DURO-TAK 87-2051. It may be that the polyacrylate based pressure-sensitive adhesive is DURO-TAK 87-2051.

25 [00260] In certain embodiments the pressure-sensitive adhesive comprises a silicone adhesive, for example pressure sensitive adhesives prepared from silicone polymer and resin.

[00261] Also contemplated are UV-curable adhesives. In these embodiments the cover is placed over the topical source of hydrogen sulphide and is bonded to the subject by exposing the adhesive to a source of UV radiation.

30 [00262] In those embodiments described herein where the cover is in the form of an artificial nail a preferred adhesive is a cyanoacrylate adhesive or a UV-curable adhesive. More preferably the adhesive is a cyanoacrylate adhesive.

[00263] The adhesive may be applied to the subject to form a perimeter of adhesive around the topical source of hydrogen sulphide on the nail and then sealing the cover to
35 the adhesive to provide an airtight chamber over the topical source of hydrogen sulphide.

[00264] In other embodiments the cover comprises an adhesive and is sealably attached to the subject by placing the cover over the topical source of hydrogen sulphide on the nail so as to bring the adhesive on cover into contact with the subject (e.g. the nail surface) thereby forming a substantially airtight seal between the cover and the subject.

5 [00265] In certain embodiments the cover comprises an adhesive layer on the ventral surface of the cover, wherein in use the adhesive layer contacts the subject (e.g. the nail plate and/or surrounding skin) to provide a substantially airtight seal between the cover and the subject. In certain embodiments the cover may be sealably attached to the subject using an adhesive tape or dressing placed over the cover and sealed to the nail
10 bed and or skin in the vicinity of the cover. For example an adhesive tape or dressing may be wrapped around the finger or toe over the cover to provide a substantially airtight chamber over the topical source of hydrogen sulphide on the nail plate.

[00266] In some embodiments the inner ventral surface of the cover is shaped to provide one or more chambers in the cover, wherein in use the chamber(s) in the cover are located
15 over the topical source of hydrogen sulphide on the nail plate to provide a substantially airtight chamber containing at least a portion of the topical source of hydrogen sulphide on the nail plate during treatment. In some embodiments the cover comprises a single chamber. In other embodiments the cover comprises two or more chambers. In some embodiments the two or more chambers in the cover may be in fluid communication with
20 one another. This configuration may provide more even distribution of the topical source of hydrogen sulphide between the chambers and thus onto the surface of the nail to be treated. The chamber(s) present in the cover may provide a small headspace over the topical source of hydrogen sulphide when in position in the nail. The head space above the topical source of hydrogen sulphide may be, for example in the range of 0.1 to 50 μL .

25 [00267] In some embodiments the topical source of hydrogen sulphide is applied to the dorsal surface of the nail plate and the cover is placed over the topical source of hydrogen sulphide on the nail bed surface. In other embodiments the cover comprises the topical source of hydrogen sulphide. In this embodiment the topical source of hydrogen sulphide is conveniently brought into fluid contact with the nail plate when the cover is placed over
30 the nail to be treated.

[00268] In some embodiments the cover comprises one or more reservoirs containing the topical source of hydrogen sulphide, wherein the one or more reservoirs is in fluid communication with the ventral surface of the cover. In use the topical source of hydrogen sulphide is thereby in fluid contact with the dorsal surface of the nail plate when the cover
35 is in place on the nail. In some embodiments the reservoir is in the form of one or more chambers present in the cover and the topical source of hydrogen sulphide (e.g. a solution

comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor) is contained within the chamber(s). In some embodiments the chamber or layer comprises a porous polymer or sponge. In this embodiment the topical source of hydrogen sulphide is distributed within the porous layer or sponge. The presence of a porous polymer of
5 sponge can act to control the rate at which the topical source of hydrogen sulphide is applied to the surface of the nail. This configuration is particularly useful when the topical source of hydrogen sulphide is of a low viscosity to prevent or minimise loss or leakage of the topical source of hydrogen sulphide during handling when the cover is placed onto and secured in place on the nail.

- 10 **[00269]** In certain embodiments the cover is in the form of a patch comprising a dorsal surface and a ventral surface;

wherein the dorsal surface comprises a substantially air impermeable layer;

the ventral surface comprises an adhesive layer;

- 15 the patch comprises one or more layer or reservoir comprising the topical source of hydrogen sulphide in fluid communication with the ventral surface; and

wherein the patch is sealably attached to the subject by means of the adhesive layer to provide a substantially air-tight chamber over at least a portion of the infected nail.

- [00270]** In use, the adhesive layer in the cover in this embodiment is contacted with the subject (e.g. to the nail plate) to provide a substantially airtight seal between the cover and
20 the subject. When in place on the nail to be treated the topical source of hydrogen sulphide is in fluid contact with the dorsal surface of the nail.

- [00271]** In this embodiment the layer or reservoir may, for example, comprise a porous polymer or sponge as described above. The ventral surface of the patch may be contoured to complement the dorsal surface of the nail to be treated. This configuration is
25 particularly suitable when the cover comprises a relatively rigid or inflexible structure, so as to provide good contact between the ventral surface of the cover and the dorsal surface of the nail, thereby facilitating the sealing of the cover and the subject. In some embodiments the cover is made from a flexible material thereby enabling a seal to be formed by simply pressing the adhesive layer of the cover onto the nail to be treated.

- 30 **[00272]** In some embodiments the adhesive layer is present around the perimeter of the ventral surface of the cover. In some embodiments the adhesive layer may extend over substantially the whole ventral surface of the cover, provided that the adhesive layer allows fluid contact between the topical source of hydrogen sulphide and the dorsal surface of the nail. For example, the adhesive layer covering the layer(s) or reservoir(s) containing the

topical source of hydrogen sulphide may be present as a porous layer which allows the topical source of hydrogen sulphide to permeate through the pores in the adhesive layer and contact the surface of the nail. In an alternative embodiment the adhesive layer may be comprise one or more windows such that the topical source of hydrogen sulphide is in
5 direct fluid contact with the surface of the nail plate when the cover is in place on the subject. In some embodiments the adhesive layer is present around the perimeter of the ventral surface of the cover, wherein the width of the adhesive layer around the perimeter is at least 1 mm, for example at least 1.5 mm, at least 2 mm, at least 3 mm or at least 4 mm, for example the adhesive layer is 1 to 4 mm wide, preferably 1.5 to 3 mm wide so as
10 to provide a good seal between the cover and the subject, thereby reducing or preventing the leakage of hydrogen sulphide gas to the atmosphere during treatment.

[00273] In certain embodiments the cover or patch is in the form of an artificial nail. The artificial nail has an outer dorsal surface that can be adapted to resemble the dorsal surface and convex shape of the nail (toenail or fingernail) being treated. The inner
15 surface of the artificial nail (ventral surface) can be contoured to provide one or more chambers which sit over the topical source of hydrogen sulphide on the nail plate during treatment. The inner surface of the artificial nail can include additional structural features to incorporate the topical source of hydrogen sulphide within the artificial nail. For example one or more reservoirs and/or porous layers which can be filled with the topical source of
20 hydrogen sulphide prior to attaching the cover over the nail to be treated. The inner surface of the artificial nail may also include one or more posts to provide a contact surface with the dorsal surface of the subject's nail to be treated, thereby providing improved contact between the artificial nail and the surface of the nail to be treated. For example the posts can be used as a surface to attach an adhesive to when securing the artificial nail to
25 the subject's nail during treatment. In a further embodiment the inner surface of the artificial nail may further comprise an adhesive layer, for example in the form of a porous adhesive mesh or webbing which enables the artificial nail to be secured to the infected nail, whilst maintaining the topical source of hydrogen sulphide in fluid contact with the dorsal surface of the infected nail. Alternatively the adhesive layer may be provided with
30 one or more window in the adhesive layer, through which the topical source of hydrogen sulphide contacts the dorsal surface of the infected nail. Alternatively the nail may be attached to the subject by applying adhesive to the nail plate and contacting the ventral surface of the artificial nail to the dorsal surface of the nail to be treated. In some embodiments the ventral surface of the artificial nail is provided with a perimeter surface
35 which is shaped to contact the dorsal surface of the nail of a subject. Suitably the perimeter surface surrounds the chamber(s) present in the artificial nail when it is applied to the subject, thereby facilitating sealing of the artificial nail to the dorsal surface of a nail

of the subject. The perimeter surface suitably has a width of at least at least 1 mm, for example at least 1.5 mm, at least 2 mm, at least 3 mm or at least 4 mm, for the perimeter surface is 1 to 4 mm wide, preferably 1.5 mm to 3 mm wide so as to provide a good seal between the cover and the subject and thereby prevent or minimise the leakage of H₂S from the cover during treatment. The artificial nail may be secured to the dorsal surface of the subject's nail by, for example, an adhesive applied to the perimeter surface of the artificial nail. Alternatively, adhesive can be applied to the nail in the shape of the perimeter surface of the artificial nail and the artificial nail is placed onto the subjects nail to seal the artificial nail in place. In a particular embodiment the perimeter surface is provided with an adhesive layer. Suitably the adhesive layer is provided with a removable backing layer that is removed prior to fixing the artificial to the nail to be treated. The adhesive in any of the embodiments of the artificial nail may be any of the adhesives disclosed herein. For example the adhesive may be a cyanoacrylate adhesive.

[00274] Certain embodiments wherein the cover is in the form of an artificial nail are illustrated in Figures 16 and 18. The artificial nail comprises a dorsal surface (101) and a ventral surface (102). The dorsal surface may be shaped to match the convex shape and appearance of a healthy nail. In some embodiments the dorsal surface of the artificial nails can be coloured using, for example nail varnish or another suitable colorant to mask or disguise the topical source of hydrogen sulphide during use and/or to hide disfiguration of the subject's nails caused by for example a fungal infection. Alternatively, the dorsal surface of the artificial nail may be translucent to resemble the appearance of a healthy human nail. In some embodiments the dorsal surface of the artificial nail is provided with one or more chambers (104). The chamber(s) (104) are adapted to house the topical source of hydrogen sulphide during use, and also provide an airtight chamber over the topical source of hydrogen sulphide when the artificial nail is attached to the subject during treatment. The chamber(s) (104) may independently have any suitable configuration, for example cylindrical, triangular, square, rectangular, trapezoidal or hexagonal. In embodiments where there are more than one chamber (104) the chambers may be isolated from one another. However, also contemplated are embodiments wherein one or more chambers (104) are in fluid contact, thereby enhancing even distribution of the topical source of hydrogen sulphide to the dorsal surface of the nail to be treated. In some embodiments the artificial nail is provided with a sealing surface (103) which is shaped to complement the shape of the dorsal surface of the nail of the subject to be treated. Suitably the sealing surface (103) forms a perimeter surface which surrounds the chamber(s) (104) such that when the artificial nail is placed on the nail to be treated the sealing surface (103) contacts the dorsal surface of the nail to be treated around substantially the whole perimeter of the artificial nail. Suitably the sealing surface (103)

has a width (105) of at least 1 mm around the perimeter surface, for example a width of 1.5 to 5 mm, or 1.5 mm to 4 mm. This provides a good surface contact with the nail to be treated and facilitates sealing of the artificial nail to the nail to be treated. The artificial nail may be sealed to the nail to be treated using, for example, a suitable adhesive. Thus in
5 some embodiments adhesive is applied to the sealing surface (103) around the perimeter of the artificial nail such that the artificial nail forms a substantially airtight cover over the topical source of hydrogen sulphide in the chamber (104) when the artificial nail is adhered to the dorsal surface of the nail to be treated. Alternatively, adhesive may be applied to the dorsal surface of the nail to be treated and the sealing surface (103) of the artificial nail
10 is brought into contact with the adhesive to seal the artificial nail to the dorsal surface of the nail to be treated, thereby providing a substantially airtight cover over the topical source of hydrogen sulphide. Also contemplated are artificial nails wherein at least the sealing surface (103) is formed from a suitable elastomeric material, wherein the artificial nails can be sealed directly to the nail to be treated. For example, such artificial nails may
15 be secured to the nail to be treated using an adhesive tape or adhesive dressing (e.g. an adhesive wrapped around the fingernail or toenail to be treated).

[00275] In some embodiments of the artificial nails described herein, the artificial nail is pre-filled with the topical source of hydrogen sulphide. The topical source of hydrogen sulphide may be located in one or more chambers, reservoirs, or layers in the ventral
20 surface of the artificial nail, wherein the chambers, reservoirs or layers are adapted to provide fluid contact between the topical source of hydrogen sulphide and the dorsal surface of the nail to be treated when the artificial nail is in place on the subject. The reservoir(s), chamber(s) or layer(s) present in the artificial nail may comprise a porous polymer or sponge material as hereinbefore defined.

[00276] In some embodiments the artificial nail further comprises a removable substantially air-impermeable backing layer adapted to maintain the topical source of hydrogen sulphide in a substantially airtight environment inside the artificial nail prior to use. In this embodiment the backing layer is removed prior to applying the artificial nail to the subject, thereby bringing the topical source of hydrogen sulphide into fluid
30 communication with the dorsal surface of the infected nail.

[00277] In some embodiments the artificial nail is made by 3D printing. The use of 3D printing enables the profile of the ventral surface of the artificial nail to correspond accurately with the dorsal surface of the nail to be treated. This is advantageous for the treatment of some nail infections when the infection causes damage to the structure of the
35 nail bed. Accurately profiling the ventral surface of the artificial nail therefore facilitates the formation of a good seal between the artificial nail and the nail to be treated. 3D printing is

also convenient for incorporating other structural features in the artificial nail, for example one or more chambers, layers, or reservoirs to incorporate the topical source of hydrogen sulphide. 3D printing methods are known and include for example obtaining a scan of the surface of the nail to be treated and forming the artificial nail using a suitable 3D printer and, for example, a polymer-based 3D-printing ink.

[00278] In some embodiments the artificial nail comprises a polymeric material. The artificial nail may comprise any of the polymers described herein in relation to the covers, provided the artificial nail is substantially air impermeable. In some embodiments the artificial nail comprises an acrylic polymer or co-polymer. In some embodiments the artificial nail comprises an epoxy resin. In some embodiments the artificial nail comprises a PTFE polymer. In some embodiments the ventral surface of the artificial nail that is in contact with the topical source of hydrogen sulphide comprises PTFE, for example as a PTFE layer on the ventral surface of the artificial nail. In some embodiments the artificial nail is of a thickness sufficient to prevent or inhibit the permeation of hydrogen sulphide gas through the artificial nail. For example the artificial may be from 0.5 mm to 3 mm thick. Typically the artificial nail is about 1 mm to about 2 mm thick.

[00279] The covers described herein (for example a patch, dressing or artificial nail) may comprise a removable backing layer on the ventral surface of the cover. The backing layer provides a removable protective or impermeable layer. The backing layer serves to protect the cover during storage and transit, and is intended to be removed prior to application of the cover to the nail to be treated. The backing layer may, for example, be formed from the same materials used for the cover. In some embodiments the backing layer may be formed from metal foils, Mylar®, polyethylene terephthalate, siliconised polyester, fumed silica in silicone rubber, polytetrafluoroethylene, cellophane, siliconised paper, aluminised paper, polyvinyl chloride film, composite foils or films containing polyester such as polyester terephthalate, polyester or aluminised polyester, polytetrafluoroethylene, polyether block amide copolymers, polyethylene methyl methacrylate block copolymers, polyurethanes, polyvinylidene chloride, nylon, silicone elastomers, rubber-based polyisobutylene, styrene, styrene-butadiene, and styrene-isoprene copolymers, polyethylene, and polypropylene.

[00280] When the cover comprises the topical source of hydrogen sulphide the backing layer is an air impermeable layer. The use of an air impermeable material as the backing layer prevents or minimises loss of hydrogen sulphide from the topical source of hydrogen sulphide during storage and prior to attaching the cover to the nail to be treated. The backing layer may be of any thickness, but is suitably between about 2.5 µm to 2.5 mm thick.

Treatment of Nail Infections

- [00281] The topical source of hydrogen sulphide provides a method for treating a nail infection in a subject, the method comprising applying an effective amount of a topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.
- 5 [00282] Reference to a method of treatment herein is intended to encompass a topical source of hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.
- 10 [00283] The methods of treatment are also intended to encompass the use of a topical source of hydrogen sulphide for the manufacture of a medicament for the treatment of a nail infection in a subject, wherein the topical source of hydrogen sulphide is applied to an infected nail, and wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.
- 15 [00284] In some embodiments the method for treating the nail infection is a therapeutic treatment. In some embodiments the method for treating the nail infection is a non-therapeutic or cosmetic method for the treatment of a nail infection.
- [00285] In certain embodiments the nail infection is a fungal, yeast and/or bacterial infection.
- 20 [00286] In certain embodiments the nail infection is a dermatophyte fungal nail infection, for example a *Trichophyton Spp.* (e.g. *T. rubrum*, *T. mentagrophyte*, *T. verrucosum*, *T. violaceum*, *T. krajdennii*, *T. tonsurans*, *T. soundanense*, *T. equinum*, *Epidermophyton floccosum*, *Arthroderma spp.* or *Microsporum spp.* (e.g. *microsporum canis*) infection.
- [00287] In certain embodiments the nail infection is a non-dermatophyte fungal nail
- 25 infection, for example an *Aspergillus spp.*, *Fusarium spp.* (e.g. *F. oxysporum*), *Acremonium spp.*, *Scopulariopsis spp.* (e.g. *Scopulariopsis brevicaulis*), *Alternaria spp.*, (e.g. *alternata*), *Syncephalastrum spp.*, *Scytalidium spp.*, *Paecilomyces spp.*, *Chaetomium spp.*, *Onychocola spp.* or *Neoscytalidium spp.* infection.
- [00288] In certain embodiments the nail infection is a yeast nail infection, for example an
- 30 *Candida spp.* nail infection (e.g. a *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata* or *C. tropicalis* nail infection).
- [00289] In certain embodiments the nail infection is onychomycosis. In certain
- embodiments the nail infection is distal lateral subungual onychomycosis, white superficial onychomycosis, proximal subungual onychomycosis, endonyx onychomycosis or total
- 35 dystrophic onychomycosis.

[00290] In certain embodiments the infection is a fungal nail infection (e.g. onychomycosis) and the treatment prevents, inhibits or reverses one or more symptoms or effects of the fungal nail infection for example one or more of subungual hyperkeratosis, inflammation of the nail bed and surrounding tissues, paresthesia, onychauxis, spongiosis, acanthosis, papillomatosis and associated edema, hyperkeratosis, crumbling of the nail plate, deformity of the nail plate, ridging of the nail bed, onycholysis, onychauxis or secondary bacterial infections.

[00291] Certain subjects with underlying diseases are prone to contracting nail infections and also suffering serious pathological effects arising from a fungal or bacterial nail infection. Accordingly, in some embodiments the subject treated is a subject with one or more of an autoimmune disease, an immunodeficiency (e.g. HIV) or a compromised immune system (e.g. subject undergoing treatment for cancer), diabetes or peripheral vascular disease.

[00292] In certain embodiments the nail infection is a bacterial nail infection. For example the nail infection is a Gram positive bacterial infection (e.g. *Staphylococcus aureus*) or a Gram negative bacterial infection (e.g. *Pseudomonas aeruginosa* or *Klebsiella spp.*).

[00293] In certain embodiments the nail infection is paronychia.

[00294] The method described herein provides high concentrations of hydrogen sulphide in the nail plate and nail bed which exceed the MIC of pathogens causing the infection. The high concentration of active at the site of infection may enable the topical source of hydrogen sulphide to treat pathogens in the form of a biofilm, which are difficult to treat using conventional nail infection treatments. Accordingly, in certain embodiments the nail infection is in the form of a biofilm, for example a fungal and/or bacterial biofilm. In some embodiments the biofilm is in the nail bed. In some embodiments the biofilm is in the keratin matrix of the nail plate. It may be that the topical source of hydrogen sulphide disrupts and/or eradicates the biofilm (e.g. the fungal and/or bacterial biofilm).

[00295] Bacteria and fungi are prone to developing resistance to conventional antibiotic and anti-fungal agents. This is particularly the case for fungal and bacterial nail infections because conventional treatments require prolonged treatment times and are often unsuccessful thereby increasing the risk of resistance developing. In certain embodiments the pathogen responsible for the nail infection is resistant to one or more conventional anti-pathogenic treatments. For example, the nail infection may be a bacterial strain that is resistant to one or more conventional antibacterial agent (e.g. a bacteria that is resistant to any of the antibacterial agents disclosed herein, such as MRSA), or a fungal strain that is resistant to one or more conventional antifungal agent (e.g. a fungus that is resistant to one or more triazole antifungal agents, tavaborole or ciclopirox or terbinafine).

[00296] The topical source of hydrogen sulphide may be used to treat a nail with an existing infection (e.g. a bacterial or fungal infection). Also contemplated are methods for preventing or reducing the risk of acquiring a nail infection. In this embodiment the topical source of hydrogen sulphide is applied to a healthy nail so as to reduce or prevent
5 infection (e.g. bacterial or fungal infection) of the nail.

[00297] Also contemplated is the use of the topical source of hydrogen sulphide to prevent recurrence of a nail infection. In this embodiments following the initial treatment of a nail infection, the topical source of hydrogen sulphide is applied to the nail to prevent or reduce the risk of recurrence of the nail infection.

10 [00298] Applying the topical source of hydrogen sulphide to a nail as described herein results in rapid penetration of high concentrations of hydrogen sulphide into and through the nail plate to the nailbed and surrounding tissues. Accordingly, the methods of treatment described herein are expected to be effective in the treatment of infections (e.g. fungal infections) within the nail plate and/or in the nailbed and surrounding tissues.

15 [00299] The nature of the nail infection may be diagnosed using known methods. For example a fungal nail infection may be diagnosed using, for example, direct microscopy, fungal culture, histopathology, PCR, flow cytometry, and dermoscopy. The presence of fungal elements such as hyphae, mycelium, and arthrospores can be detected through direct microscopic examination using potassium or sodium hydroxide (KOH or NaOH),
20 sodium sulphide, or Parker's blue-black permanent ink (see for example Westerberg DP, Voyack MJ et al., Onychomycosis: Current trends in diagnosis and treatment. Am. Fam. Physician 2013; 88(11): 762-70).

Subject

[00300] The subject treated with the topical source of hydrogen sulphide is preferably a
25 human and the nail is a fingernail or toenail. However, the topical source of hydrogen sulphide may also be used to treat infections in, or associated with, a keratinous tissue (e.g. hooves, claws, horns and beaks) in other animals such as cats, dogs, horses, cattle, sheep, goats, pigs and birds. Accordingly in certain embodiments reference herein to the treatment of a "nail infection" encompasses the treatment of infections (e.g. bacterial or
30 fungal infections) in, or associated with, the hooves, claws, horns or beaks of non-human animals.

Combination Therapy

[00301] The topical source of hydrogen sulphide may be used alone as a monotherapy to treat a nail infection. However, in some embodiments the topical source of hydrogen
35 sulphide is used together with one or more antifungal or antibacterial agents.

[00302] In some embodiments the topical source of hydrogen sulphide is used in combination with one or more antifungal agents selected from a triazole (e.g. terbinafine, itraconazole, fluconazole, efinaconazole, voriconazole, posaconazole, miconazole, albaconazole, ravuconazole, fosravuconazole, lanconazole or luliconazole), tavaborole
5 or ciclopirox, terbinafine or amphotericin B or 5-fluorocytosin, VT-1161, P-3051, tazarotene (Tazorac), NCV-422, ME1111 or NP213 (Novexatin).

[00303] In some embodiments the topical source of hydrogen sulphide is used in combination with one or more antibacterial agents, for example, gentamicin, neomycin, streptomycin, cefpodoxime proxetil, clindamycin, lincomycin, erythromycin, bacitracin,
10 gramicidin, vancomycin, doxycycline, minocycline, oxytetracycline, tetracycline, fosfomycin, fusidic acid, mupirocin, sulfacetamide, metronidazole, dapsone, triclosan, quaternary ammonium salts, silver sulfadiazine.

[00304] In some embodiments the topical source of hydrogen sulphide is used in combination with photodynamic therapy.

15 [00305] Combination treatment may be achieved by way of the simultaneous, sequential or separate dosing of the individual components of the treatment. Such combination products employ the topical source of hydrogen sulphide within a therapeutically effective dosage range described hereinbefore and the other active agent within its approved dosage range.

20 [00306] Herein, where the term “combination” is used it is to be understood that this refers to simultaneous, separate or sequential administration. In one aspect of the invention “combination” refers to simultaneous administration. In another aspect of the invention “combination” refers to separate administration. In a further aspect of the invention “combination” refers to sequential administration. Where the administration is sequential or
25 separate, the delay in administering the second component should not be such as to lose the beneficial effect of the combination.

Dosages and Dosage Regimens

[00307] The topical source of hydrogen sulphide rapidly delivers high concentrations into and through the nail plate. This may enable the nail infection to be treated with a single
30 application of the topical source of hydrogen sulphide. However, also contemplated are multiple administrations of the topical source of hydrogen sulphide to the nail. In certain embodiments the topical source of hydrogen sulphide is applied to the nail once per day, once every 2 days, once every week, once every 2 weeks, once every 4 weeks, once every 8 weeks, once every 12 weeks or once every 24 weeks. The frequency of administration of
35 the topical source of hydrogen sulphide may be readily determined by a physician. The

cover is suitably left in place over the topical source of hydrogen sulphide throughout each treatment period. However, also contemplated is removal of the cover after an initial period, for example after substantially all of the hydrogen sulphide has been delivered into the nail plate. One or more further doses of the topical source of hydrogen sulphide may then be applied in subsequent treatment periods.

[00308] The dosage of the compound of the invention will vary depending upon a number of factors including, for example, the extent and severity of the nail infection.

[00309] Suitably the topical source of hydrogen sulphide is delivered in an amount sufficient to provide a concentration of hydrogen sulphide in the nail plate and/or nail bed that exceed the MIC of the pathogen(s) responsible for the nail infection. For example the topical source of hydrogen sulphide delivers a concentration of hydrogen sulphide in the nail plate and/or nail bed which is at least 1x, 2x, 3x, 4x, 5x or 10x the MIC.

[00310] In some embodiments the topical source of hydrogen sulphide transmits at least 100 $\mu\text{g}/\text{cm}^2$ of hydrogen sulphide through the nail plate in the first 24 hours after applying the topical source of hydrogen sulphide to the surface of the nail. In some embodiments the topical source of hydrogen sulphide transmits at least 500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000 or 10,000 $\mu\text{g}/\text{cm}^2$ of hydrogen sulphide through the nail plate in the first 24 hours after application. In some embodiments the topical source of hydrogen sulphide transmits 1000 to 15,000 $\mu\text{g}/\text{cm}^2$ of hydrogen sulphide through the nail plate in the first 24 hours after application. For example 2000 to 10000 or 5000 to 10000 $\mu\text{g}/\text{cm}^2$ of hydrogen sulphide through the nail plate in the first 24 hours after application. The transmission of hydrogen sulphide provided by the topical source of hydrogen sulphide may, for example, be measured using the method described in Example 2 herein, wherein the topical source of hydrogen sulphide is applied to the surface of a human nail plate with a thickness of 30 μm in a sealed microchamber.

[00311] In some embodiments the topical source of hydrogen sulphide (e.g. solution comprising hydrogen sulphide or composition comprising as hydrogen sulphide donor) is applied to the nail in a volume of about 0.1 μL to about 500 μL , for example from about 0.1 to about 250 μL or from about 0.1 μL to about 50 μL .

30 Systems

[00312] Also provided is a system comprising a cover; and a topical source of hydrogen sulphide;

the cover being adapted to provide a substantially airtight chamber over the topical source of hydrogen when the topical source of hydrogen sulphide is applied to an infected nail in a subject.

[00313] The cover may be any of the covers described herein. For example, the cover may be in the form of an artificial nail described herein. The topical source of hydrogen sulphide may be, for example, a solution comprising hydrogen sulphide or a composition comprising a hydrogen sulphide donor as described herein.

5 [00314] In some embodiments the system comprises the cover and the topical source of hydrogen sulphide as separate elements. For example, the system may comprise the cover and a separate sealed airtight container containing the topical source of hydrogen sulphide. In this embodiment the topical source of hydrogen sulphide may be applied to the ventral side of the cover and the cover is then paced on the nail to be treated.

10 Alternatively, the topical source of hydrogen sulphide may be applied directly to the nail plate and the cover is applied over the topical source of hydrogen sulphide on the nail plate and is secured in place to the subject to provide an airtight chamber over the topical source of hydrogen sulphide on the nail plate.

[00315] In some embodiments the system comprises a cover which contains the topical source of hydrogen sulphide, for example wherein the cover comprises a patch, dressing or artificial nail that is pre-filled with the topical source of hydrogen sulphide. In this embodiment the cover (e.g. the artificial nail) will further comprise a removable air-impermeable backing layer which maintains the topical source of hydrogen sulphide in an airtight environment within the cover prior to use. The backing layer may be any of the
15
20 backing layers described herein. In use the backing layer is removed from the cover and the cover is placed over the nail to be treated thereby bringing the topical source of hydrogen sulphide into fluid contact with the nail plate.

[00316] In some embodiments the system comprises two or more covers (e.g. artificial nails) of different sizes. The user is then able to select a patch of an appropriate size for
25 the nail to be treated with the topical source of hydrogen sulphide.

[00317] In some embodiments the system further comprises a means for sealing the cover to the subject during treatment. For example the system may comprise an adhesive or an adhesive tape or dressing for sealing the cover to the subject so as to provide a substantially airtight chamber over the topical source of hydrogen sulphide on the nail
30 being treated. In some embodiments cover comprises an adhesive, for example the cover comprises an adhesive layer which is used to seal the cover to the subject. The adhesive may be any suitable adhesive, including those described herein in the section describing the covers.

FURTHER EMBODIMENTS

35 [00318] The invention is further illustrated by the following numbered paragraphs.

1. A topical source of hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.
- 5 2. The topical source of hydrogen sulphide for use of Embodiment 1, wherein the topical source of hydrogen sulphide is a plasma comprising ionised hydrogen sulphide, a solution comprising hydrogen sulphide, or a composition comprising a hydrogen sulphide donor.
3. The topical source of hydrogen sulphide for use of Embodiment 1 or Embodiment 2, wherein the topical source of hydrogen sulphide comprises a solution comprising hydrogen
10 sulphide;
optionally wherein the topical source of hydrogen sulphide comprises an aqueous hydrogen sulphide solution.
4. The topical source of hydrogen sulphide for use of any one of Embodiments 1 to 3, wherein the topical source of hydrogen sulphide comprises a composition comprising a
15 hydrogen sulphide donor.
5. The topical source of hydrogen sulphide for use of Embodiment 4, wherein the hydrogen sulphide donor is selected from: an inorganic sulphide salt (e.g. CaS, KHS, NaHS, Na₂S, MgS, SrS, BaS or SiS₂), ammonium tetrathiomolybdate, an allyl-substituted polysulphide (e.g. S-(prop-2-en-1-yl) prop-2-ene-1-sulfinothioate (allicin), diallyl sulphide,
20 diallyl disulphide or diallyl trisulphide), an isothiocyanate derivative (e.g. allyl isothiocyanate, erucin, or an aryl isothiocyanate (e.g. benzyl isothiocyanate or 4-hydroxybenzyl isothiocyanate)), Lawessonn's Reagent and analogues thereof, a phosphonamidodithioate derivative (e.g. GYY4137), a phosphonodithioate derivative, a phosphonamidothioate derivative, a dithiolthione derivative (e.g. a 1,2-dithiole-3-thione derivative), an N-mercapto
25 derivative (e.g. an N-benzoylthiobenzamide derivative), an S-arylothiooxime derivative, an acyl perthiol derivative, a dithioperoxy-anhydride, a tetrasulphide derivative, a thioamide derivative (e.g. an aryl thioamide derivative such as 4-hydroxybenzothioamide), a gem dithiol derivative, a N-thiocarboxyanhydride derivative, a thiocarbamate derivative, a phosphoramidodithioate derivative, thioamino acid and a thioester prodrug
- 30 6. The topical source of hydrogen sulphide for use of Embodiment 4, wherein the hydrogen sulphide donor is selected from sodium hydrosulphide (NaHS), disodium sulphide, ammonium tetrathiomolybdate, diallyl trisulphide and 4-hydroxybenzothioamide.

7. The topical source of hydrogen sulphide for use of Embodiment 4, wherein the hydrogen sulphide donor is NaHS or Na₂S, preferably wherein the hydrogen sulphide donor is NaHS.
8. The topical source of hydrogen sulphide for use of any one of Embodiments 2 to 6,
5 wherein the composition comprises an agent to activate or enhance release of hydrogen sulphide from the hydrogen sulphide donor, for example wherein the agent is a thiol (e.g. glutathione, cysteine, homocysteine or N-acetylcysteine), a catalyst, an enzyme, a pH adjusting agent, a base (e.g. a bicarbonate) or an oxidising agent.
9. The topical source of hydrogen sulphide for use of any one of Embodiments 2 to 8,
10 wherein the composition comprises a liquid selected from water and a polar organic solvent,
optionally wherein the liquid is selected from one or more of water, methanol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone.
10. The topical source of hydrogen sulphide for use of any one of Embodiments 2 to 9,
15 wherein the composition is an aqueous composition.
11. The topical source of hydrogen sulphide for use of any one of Embodiments 2 to 10, wherein the composition comprises a hydrogen sulphide solution.
12. The topical source of hydrogen sulphide for use of any one of Embodiments 1 to 11,
20 wherein the composition is in the form of a solution, a dispersion, an emulsion, a foam, a lotion, a cream or a gel.
13. The topical source of hydrogen sulphide for use according to any one of Embodiments 2 to 12, wherein the hydrogen sulphide donor is present in the composition at a concentration of from about 0.003 µg/mL to about 100 mg/mL.
14. The topical source of hydrogen sulphide for use according to Embodiment 13,
25 wherein the hydrogen sulphide donor is present in the composition at a concentration of from 0.05 mg/mL to 50 mg/mL, preferably 0.1 mg/mL to 10 mg/mL.
15. The topical source of hydrogen sulphide for use according to any one of Embodiments 2 to 14, wherein the composition has a pH in the range of about 4 to about
30 10.
16. The topical source of hydrogen sulphide for use according to Embodiment 15, wherein the composition has a pH in the range of about 7.5 to about 9.5, for example about 8.0 to about 9.0, suitably wherein the composition has a pH of about 8.5.

17. The topical source of hydrogen sulphide for use according to any one of Embodiments 2 to 16, wherein the composition is formulated as a gel composition,
optionally wherein the composition is formulated as a hydrogel composition.
- 5 18. The topical source of hydrogen sulphide for use according to Embodiment 17, wherein the composition comprises a gel-forming agent.
19. The topical source of hydrogen sulphide for use according to Embodiment 18, wherein the gel-forming agent is a gel-forming polymer, optionally wherein the gel-forming
10 polymer comprises hydroxyethylcellulose.
20. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 19, wherein the topical source of hydrogen sulphide is applied to the dorsal surface of the infected nail.
- 15 21. The topical source of hydrogen sulphide for use according to Embodiment 1 or Embodiment 2, wherein the topical source of hydrogen sulphide is a plasma comprising ionised hydrogen sulphide, wherein the plasma is formed from gaseous mixture comprising hydrogen sulphide and one or more inert gases.
- 20 22. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 20, wherein the cover is an occlusive dressing or occlusive patch;
optionally wherein the cover is in the form of an artificial nail.
23. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 22, wherein the cover is or comprises, an air-impermeable polymer,
25 optionally wherein the cover is or comprises polytetrafluoroethylene.
24. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 23, wherein the cover is adapted to provide a substantially airtight chamber over the topical source of hydrogen sulphide on the infected nail.
25. The topical source of hydrogen sulphide for use according to any one of
30 Embodiments 1 to 24, wherein the cover is sealably attached to the subject over the topical source of hydrogen sulphide.
26. The topical source of hydrogen sulphide for use according to Embodiment 25, wherein the cover is located over the topical source of hydrogen sulphide and is sealed onto the dorsal surface of the infected nail.

27. The topical source of hydrogen sulphide according to Embodiment 25 or 26, wherein the cover is sealed to the subject by an adhesive.
28. The topical source of hydrogen sulphide for use according to any one of Embodiments 3 to 27, wherein the cover comprises the topical source of hydrogen sulphide and the topical source of hydrogen sulphide is applied to the infected nail by contacting the cover with at least a portion of the surface of the infected nail.
29. The topical source of hydrogen sulphide for use according to Embodiment 28, wherein the cover comprises one or more layer or reservoir containing the topical source of hydrogen sulphide, wherein the one or more layer or reservoir is in fluid communication with the dorsal surface of the infected nail when the cover is placed on the infected nail.
30. The topical source of hydrogen sulphide for use according to any one of Embodiments 3 to 29, wherein the cover is in the form of a patch comprising a dorsal surface and a ventral surface;
- wherein the dorsal surface comprises a substantially air impermeable layer;
- the ventral surface comprises an adhesive layer;
- the patch comprises one or more layer or reservoir comprising the topical source of hydrogen sulphide in fluid communication with the ventral surface; and
- wherein the patch is sealably attached to the subject by means of the adhesive layer to provide a substantially air-tight chamber over at least a portion of the infected nail.
31. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 30, wherein the nail infection is a fungal, yeast and/or bacterial infection.
32. The topical source of hydrogen sulphide for use according to Embodiment 31, wherein the nail infection is a dermatophyte fungal nail infection, for example a *Trichophyton* Spp. (e.g. *T. rubrum*, *T. mentagrophyte*, *T. verrucosum*, *T. violaceum*, *T. kraidenii*, *T. tonsurans*, *T. soundanense*, *T. equinum*, *Epidermophyton floccosum*, *Arthroderma* spp. or *Microsporum* spp. (e.g. *microsporum canis*) infection.
33. The topical source of hydrogen sulphide for use according to Embodiment 31, wherein the nail infection is a non-dermatophyte fungal nail infection, for example an *Aspergillus* spp., *Fusarium* spp. (e.g. *F. oxysporum*), *Acremonium* spp., *Scopulariopsis* spp. (e.g. *Scopulariopsis brevicaulis*), *Alternaria* spp., (e.g. *alternata*), *Syncephalastrum* spp., *Scytalidium* spp., *Paecilomyces* spp., *Chaetomium* spp., *Onychocola* spp or *Neoscytalidium* spp. infection.

34. The topical source of hydrogen sulphide for use according to Embodiment 31, wherein the nail infection is a yeast nail infection, for example an *Candida spp.* nail infection (e.g. a *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata* or *C. tropicalis* nail infection).

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35. The composition for use according to any one of Embodiments 1 to 34, wherein the nail infection is onychomycosis, for example distal lateral subungual onychomycosis, white superficial onychomycosis, proximal subungual onychomycosis, endonyx onychomycosis or total dystrophic onychomycosis.

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36. The topical source of hydrogen sulphide for use according to Embodiment 31, wherein the nail infection is a bacterial nail infection, for example a Gram positive bacterial infection (e.g. *Staphylococcus aureus*) or a Gram negative bacterial infection (e.g. *Pseudomonas aeruginosa* or *Klebsiella spp.*).

15

37. The topical source of hydrogen sulphide for use according to Embodiment 31 or Embodiment 36, wherein the nail infection is paronychia.

38. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 39, wherein the nail infection comprises a biofilm.

20

39. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 38, wherein the nail infection is caused by a pathogen which is a treatment-resistant pathogen, for example a bacterial strain that is resistant to one or more conventional antibacterial agent, or a fungal strain that is resistant to one or more conventional antifungal agent.

25

40. The topical source of hydrogen sulphide for use according to any one of Embodiments 1 to 39, wherein the nail infection is a toe nail infection.

30

41. A method for treating a nail infection in a subject, the method comprising applying an effective amount of a topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.

35

42. The method according to Embodiment 41, wherein the treatment is a cosmetic treatment.

43. A system comprising:
a cover; and
a topical source of hydrogen sulphide;
the cover being adapted to provide a substantially airtight chamber over the topical
5 source of hydrogen when the topical source of hydrogen sulphide is applied to an
infected nail in a subject.
44. The system according to Embodiment 43, wherein the cover comprises the topical
10 source of hydrogen sulphide.
45. The system according to Embodiment 45, wherein the cover comprises one or more
layer or reservoir containing the topical source of hydrogen sulphide.
46. The system according to any one of Embodiments 43 to 45, further comprising a
15 means for sealably attaching the cover to a surface tissue of a subject.
- 47 The system according to Embodiment 46, wherein the means for sealably attaching
the cover to a surface tissue of a subject is an adhesive,
optionally wherein the adhesive is an acrylic adhesive.
- 20 48. The system according to Embodiment 43, wherein the cover is in the form of a patch
comprising a dorsal surface and a ventral surface;
wherein the dorsal surface comprises a substantially air impermeable layer;
the ventral surface comprises an adhesive layer; and
the patch comprises one or more layer or reservoir comprising the topical source of
25 hydrogen sulphide in fluid communication with the ventral surface.
49. The system according to any one of Embodiments 43 to Embodiment 48, wherein
the cover is an occlusive dressing or occlusive patch,
optionally wherein the cover is in the form of an artificial nail.
30
50. The system according to any one of Embodiments 43 to 49, wherein the cover is, or
comprises, an air-impermeable polymer, for example PTFE.
51. The system according to any one of Embodiments 43 to 50, wherein the cover further
35 comprises a removable, substantially air impermeable backing layer which is adapted to

maintain the topical source of hydrogen sulphide in the cover in a substantially airtight environment prior to use.

5 EXAMPLES

The following abbreviations are used in the Examples:

AN: *Aspergillus niger*

ATTM: ammonium tetra thiomolybdate

AUC: area under the curve

10 CA: *Candida albicans*

DATS: diallyl trisulphide

FO: *Fusarium oxysporum*

h: hour

HBTA: hydroxybenzothioamide

15 LOD: limit of detection

MC: *Microsporum canis*

MIC: minimum inhibitory concentration

min: minute

NaHS: sodium hydrosulphide

20 N,N-dpd: N,N-dimethyl-p-phenylenediamine

OD: optical density

PBS: phosphate buffered saline

SD: Sabouraud Dextrose

TSA: tryptone soy agar

25 TSB: tryptone soy broth

Example 1: H₂S gas release from H₂S donors and effects of donor solution pH

Methods: H₂S analysis using T101 gas analyser

30 [00319] The release of H₂S gas from a H₂S donor under a constant airflow was measured using a Teledyne T101 gas analyser. The T101 measured H₂S gas in an airflow rate of 650 mL/min, with a LOD of 0.4 ppb, a 10 V analogue output range, and a data output once every 1 min (note in the display of some data only the data at every 15 min was displayed to reduce the number of data points and increase the result clarity).

35 [00320] Four H₂S donor compounds were selected, and their concentrations were matched according to their molar concentrations. NaHS (1 mM, 0.2 mM, and 0.1 mM) 4-hydroxybenzothioamide (HBTA) (0.2 mM) with 0.8 mM of L-glutathione, diallyl trisulphide

(DATS) (0.2 mM) with 0.8 mM of L-glutathione all at pH 7.4 and ammonium tetrathiomolybdate (ATTM) (1 mM, 0.2 mM, and 0.1 mM) with (4 mM 0.8 mM and 0.4 mM) of L-glutathione at pH 2 were added to a 120 mm petri dish (VWR, Poole, UK). The NaHS, DATS and ATTM solutions were prepared in phosphate buffered saline (PBS, 10 mM).

- 5 The HBTA solution was prepared in a 1:1 mixture of water and methanol. Each solution was placed inside an airtight container with a volume of 2.1 L. The pH was selected to support both the reaction that was to release the gas optimally and the final speciation of H_2S . For NaHS, HBTA, and DATS, the reaction pH was 7.4 and the final speciation was 50% H_2S and 50 % for HS^- . For ATTM the pH was 2 the final speciation was 99.9% H_2S and 0.1 % for HS^- . To regulate the “constant air flow” the experiments were conducted in vacuum-sealed food containers that were then attached to the T101 analyser for 24 h and H_2S measurements were made every minute for 24 h. The data were processed using the pKSolver add-on in Excel. The concentrations of the gas were calculated based on the volume of the container (2.1 L). The $C_{\max}$, $T_{\max}$, and AUC were calculated from the
- 10
- 15 PKsolver software and were obtained as the average of three replicates of the release for each donor.

H_2S release and detection using an agar trap

- [00321] To understand the gas-liquid equilibrium of H_2S under no-air flow conditions a zinc agar trap method was used. To detect H_2S in the liquid state the following methylene blue assay was used. An H_2S calibration curve was generated using a series of standard concentrations. To produce the stock solution, a 20 ml solution of 1 mM of NaHS in PBS at pH 7.4 was produced. A 40 mL solution of N,N-dimethyl-p-phenylenediamine (N,N-dpd) solution (40 mM in 7.2 M HCl) and a 40 ml FeCl_3 solution (30 mM in 1.2 M HCl) were added to the NaHS solution (56 $\mu\text{g/mL}$ NaHS or 34 $\mu\text{g/mL}$ H_2S final stock concentration).
- 20
- 25 The final solution was left for 30 min until methylene blue was formed. The NaHS stock solution was then diluted with PBS to achieve concentrations in the range of 8.5 $\mu\text{g/mL}$ –0.17 $\mu\text{g/mL}$ from which a calibration curve was constructed. A 200 μL aliquot from each concentration was taken and the absorbance was measured using a Tecan Spark plate reader (Tecan, Reading, UK) at 670 nm in a flat bottom 96-well plate (Greiner, Stonehouse, UK). The assay was verified using the international council of harmonization (ICH) guidelines for linearity and precision and accuracy. Interday and intraday calibration curves were produced by running the same method three times on one day and two other times on two different days. Limits of detection and quantification were calculated. The zinc agar trap was formed using 100 mL of 1%w/v agar powder to which 1% w/v zinc acetate
- 30
- 35 solution and 12% w/v NaOH solution were added. The mixture was autoclaved at 120 °C

for 2 h. The agar solution was then added to one side of a 250 mL tissue culture flask (Greiner, Stonehouse, UK) and the other side of the tissue culture flask was removed.

[00322] NaHS (0.2 mM), 4-hydroxybenzothioamide (HBTA) (0.2 mM) with 0.8 mM of L-glutathione, diallyl trisulphide (DATS) (0.2 mM) with 0.8 mM mg of L-glutathione all at pH 7.4 and ammonium tetra thiomolybdate (ATTM) (0.2 mM) with 0.8 mM of L-glutathione at pH 2 were the test samples. The NaHS, DATS and ATTM solutions were prepared in phosphate buffered saline (PBS, 10 mM). The HBTA solution was prepared in a 1:1 mixture of water and methanol. These solutions relocated the test samples used in the airflow gas analyser experiments described above. The solutions were added to two 25 mL glass vials, and then the Petri dishes were immediately placed inside the airtight containers with a volume of 2.1 L. The agar tissue culture flask was placed with the agar side facing upwards next to the Petri dishes in the airtight container and left at room temperature. After 24 h, the donor solution was removed and assayed with the methylene blue method and the agar was removed from the flasks and homogenised in glass bottles. N, N-dpd, and FeCl₃ in the ratio of 2:2:1 (N,N-dpd: FeCl₃: agar or solution) were mixed for 30 min. Samples (200 µL) were then measured at 670 nm using a Tecan Spark plate reader and concentration was calculated from the H₂S assay regression line equation generated using the standard concentrations detailed above.

Results:

[00323] The release profiles of H₂S from the 4 different donors under a constant airflow of 650 ml/min using the T101 gas analyser were very different (Figure 2). NaHS was the fastest-releasing H₂S donor with the shortest T_{max}, followed by HBTA, ATTM, and DATS (ANOVA, p < 0.05). The maximum concentration (C_{max}) of the H₂S released for NaHS was significantly higher than the DATS and HBTA (ANOVA, p < 0.05), but not ATTM. The C_{max} for ATTM was higher than HBTA (P < 0.05), but not DATS. The C_{max} for DATS and HBTA were not statistically different to each other. The amount of H₂S gas release indicated by the total area under the curve (AUC) for NaHS was higher than HBTA (ANOVA, p < 0.05), but not DATS or ATTM. The AUC for both DATS and ATTM had an AUC higher than HBTA (ANOVA, p < 0.05). The T_{max}, C_{max} and AUC for the H₂S gas release are shown in Table 1:

Table 1:

Indices	T _{max} (min)	C _{max} ($\mu\text{g/mL}$)	AUC ($\mu\text{g/mL}\times\text{min}$)
NaHS	60	4.26 ± 1.07	596.31 ± 96.13
DATS	285	1.07 ± 0.60	603.11 ± 165.06
HBTA	75	0.10 ± 0.01	34.20 ± 6.03
ATTM	105	3.10 ± 0.95	493.60 ± 23.46

[00324] When comparing the amounts of H₂S remaining in the liquid phase over 24 h after the solid donors had been added to the liquid, NaHS had the most H₂S left in the liquid state (ANOVA, $p < 0.05$), at all time points compared to HBTA and ATTM solutions

5 (Figure 2B). As for the gaseous phase across the different donors, ATTM solutions produced the highest amount of H₂S gas (ANOVA, $P > 0.05$), followed by NaHS (ANOVA, $P < 0.05$), and release from HBTA (Figure 2A).

[00325] The overall recovery of H₂S, the total measured during the experiments as a percentage of the starting amount, from both phases across all donors was calculated and
10 these were $87.53 \pm 15.09\%$, $63.36 \pm 8.98\%$, and $6.69 \pm 2.17\%$ for NaHS, ATTM, and HBTA respectively.

Effect of pH on H₂S Release Profile

[00326] The H₂S gas release profiles from NaHS at pH 8.5 and pH 4, under constant airflow of 650 mL/min, were observed using the T101 gas analyzer (Figure 3A). The
15 release of H₂S under alkaline conditions, pH 8.5, was observed to be faster, with a maximum time (T_{max}) of 2 hours, as compared to 25 hours under acidic conditions. Furthermore, a higher maximum concentration (C_{max}) of H₂S was generated under alkaline conditions in comparison to acidic conditions. The total area under the curve (AUC) for NaHS, which is an indicator of the amount of H₂S gas release, was observed to
20 be higher under alkaline conditions than under acidic conditions as illustrated in Table 2:

Table 2

Indices	T _{max} (hour)	C _{max} ($\mu\text{g/mL}$)	AUC ($\mu\text{g/mL} \times \text{hour}$)
NaHS - pH 8.5	2	7.12 ± 0.12	35.60 ± 0.70
NaHS - pH 4	25	0.81 ± 0.10	24.16 ± 2.85

[00327] The present study also investigated the impact of varying pH levels of NaHS
25 solution on the quantity of H₂S in both the liquid and gas phases in a sealed chamber with no air flow. The findings indicate that a reduction in the pH value of the solution results in a decrease in the quantity of H₂S in its liquid phase. The NaHS solution with a pH value of 8.5 demonstrated the highest amount of H₂S in its liquid phase, compared to solutions at pH 6 and pH 4 after 24 h (Figure 3B). Conversely, a lower pH value, such as pH 4,
30 resulted in the production of the highest amount of gas, as compared to pH 6 and 8.5 (Figure 3B). Hence, in the container with no airflow a low pH favoured H₂S in the gas

state, whilst in the experiments with the airflow the opposite was found (compare Figure 3B with Figure 3A). The overall recovery of H₂S from both phases across all NaHS solutions was determined and found to be $76.13 \pm 7.26 \%$, $87.73 \pm 8.83\%$, and $83.34 \pm 7.79 \%$ for pH 8.5, 6, and 4, respectively.

5

Example 2: Ionised HS⁻ presented in the liquid state is responsible for penetration through the human nail plate

Methods: H₂S permeation - A Franz cell receiver compartment was constructed using 3D printing and consisted of three components. A human nail was affixed to the base of the first component. The first component was then positioned in the center of the second component, which featured an o-ring. In this configuration, the nail rested on the rubber o-ring. The third component was a ring designed to fasten the first and second components together thereby sandwiching the nail between the first and second components. The rubber o-ring served to enhance compressibility and create a tight seal between the two parts, preventing any leakage.

[00328] The permeation of H₂S from donor solutions in phosphate-buffered saline was measured over 24 h using an open microchamber, a closed microchamber, and a microchamber with a “shelf” to hold the donor solution at a distal location over the surface of a human nail to thereby supply H₂S as a gas to the nail surface. A 12 mm magnetic stir bar was placed into the receiver compartment of each diffusion cell. The receiver compartment was loaded with 1% w/w zinc acetate in 50 mM phosphate-buffered saline (pH 7.4) to capture the permeated H₂S. After 1 h equilibration at 32 °C, 0.5 mL of receiver fluid was withdrawn (time point= 0 min). Then to initiate the experiment a NaHS solution (0.2 mL of 28 mg/mL) at pH 8.5 or an ATTM solution (0.2 mL of 21.6 mg/mL) at pH 2 was applied to the donor compartment of the diffusion cell that was then covered with a synthetic rubber seal to seal the chamber inside a fume hood for the liquid application and gas application using the shelf. For the experiments with an open microchamber, the rubber seal was omitted to leave the cell open. It was not possible to saturate the H₂S donor in the buffer due to its high water solubility. The leakage of H₂S gas from the microchamber was checked using a Teledyne T101 hydrogen sulphide gas analyser after the cell set up and sealing. One sample (0.5 mL) of the receiver solution was taken at the following time points: 15, 30, 60, 90, 120, 150, 180, 240, and 1440 min. Fresh receiver solution was replaced into the cell to keep the volume consistent and air bubbles removed. The samples were quantified using the “methylene blue” assay after removal by mixing with 0.25 mL N, N-dpd (40 mM in 7.2 M HCl), and 0.25 mL FeCl₃ (30 mM in 1.2 M HCl). A H₂S calibration curve was generated. To do this a 5.6 mg aliquot of NaHS (3.4 mg of H₂S)

was added to 50 ml 1% w/w zinc acetate in PBS at pH 7.4. A 25 ml N, N-dpd (40 mM in 7.2 M HCl) solution was produced and a 25 mL FeCl₃ (30 mM in 1.2 M HCl) solution was produced. The solutions were added to the NaHS solution (56 µg/mL NaHS or 34 µg/mL H₂S). The final solution was left for 30 min until methylene blue was formed. The stock solution was then diluted (8.5 µg/mL- 0.17 µg/mL) to produce a calibration curve. Absorbance was measured using a Tecan Spark plate reader at 670 nm. H₂S concentrations were calculated from the standard regression equation of the calibration curve.

Results

- [00329]** The cumulative H₂S mass/area (µg/cm²) permeation through a human nail was assessed over 24 h. H₂S permeated through the nail when the microchamber was sealed and the H₂S donor NaHS was applied directly to the nail surface as a liquid (12800 ± 6780 µg/cm², **Figure 4A**, upper trace). In contrast, when the microchamber was left open and the NaHS applied as a liquid under identical conditions there was no H₂S permeation through the nail plate, even when applied at twice the concentration of NaHS used in Figure 4A (**Figure 4B**). When using the sealed microchamber with the shelf to apply H₂S gas to the nail plate the H₂S gas did not penetrate the nail plate which suggests that the HS⁻ ions in the hydrogen sulphide solution is the diffusing species (**Figure 4A**, lower trace).
- [00330]** Permeation of H₂S using ATTM as a H₂S donor applied as a solution to the nail plate and maintained in the sealed microchamber also resulted in the permeation of H₂S through the nail plate. By way of comparison, topical application of ciclopirox to the nail plate resulted in a negligible transmission through the nail plate in the 24 hour measurement period (**Figure 4C**). A summary of the H₂S penetration results is shown in Table 3, together with the penetration rate for Ciclopirox and Efinaconazole from the literature, (Keita S et al., The low keratin affinity of efinaconazole contributes to its nail penetration and fungicidal activity in topical onychomycosis treatment, Antimicrob Agents Chemother, 2014; 58 (7); 3837-3842) and (Heather D et al., Assessment of the nail penetration of antifungal agents, with different physico-chemical properties, PLoS ONE, 2020; 15(2): e0229414), respectively.

Table 3

Drug	Nail plate penetration (µg/cm ² /24 h)
NaHS	12800
ATTM	2759
Ciclopirox	0.362*
Efinaconazole	0.074*

* Taken from literature:

[00331] This example shows that topical application of an H₂S solution to a nail plate results in rapid permeation of H₂S into and through the nail plate when the H₂S is maintained under an airtight cover. However, topical application of the H₂S solution in an open (i.e. not sealed) microchamber, or H₂S as a gas, resulted in no measurable H₂S permeation through the nail plate. The mass transfer of H₂S using the sealed microchamber (i.e. no airflow), was very rapid compared to the topical application of ciclopirox over the same 24 hour measurement period.

Effect of pH on H₂S permeation through a nail plate

[00332] The NaHS solution delivered more H₂S through the nail at pH 8.5 compared to pH 4. At pH 8.5 HS⁻ ion is the dominant microspecies in the administered NaHS solution (pK_a ~7), after releasing from the NaHS and again this suggests that HS⁻ was the predominant diffusing species (**Figure 5**). In contrast at pH 4 the dominant species is H₂S and much lower hydrogen sulphide mass transfer through the nail was observed (**Figure 5**, lower trace). When comparing two different donors, both NaHS (12800 ± 6780 µg/cm²) and ATTM (2759 ± 311 µg/cm²) successfully delivered the H₂S through the nail plate (**Figure 4B**).

Example 3 – The HS⁻ reverts to H₂S gas once it has penetrated the nail

Methods: H₂S gas nail deposition test - A 10 mg aliquot of nail clippings was placed in glass vials. Aliquots of 1 mL of each of the following compositions were added to the clippings: NaHS (0.2 mM); hydroxybenzothioamide (HBTA) (0.2 mM) with 0.8 mM of L-glutathione; diallyl trisulphide (DATS) (0.2 mM) with 0.8 mM of L-glutathione all at pH 7.4; and ammonium tetra thiomolybdate (ATTM) (0.2 mM) with 0.8 mM of L-glutathione at pH 2. The clippings were incubated in the solutions for 24 h at 32 °C under no airflow. After 24 h, the clippings were removed and dried then added to the zinc agar trap on the side opposite the agar in the tissue culture flask, the flasks were sealed and left at room temperature for 48 hours. After 48 h, the clippings were removed from the flask and 1 mL of N, N-dpd, and 1 mL of FeCl₃ were added to the agar for 30 min. Samples were then measured at 670 nm using a Tecan Spark plate reader and concentration was calculated from the H₂S assay regression line equation.

Results

[00333] Gaseous H₂S was generated in the nail after applying the gas donors to the nail in the liquid state. Applying NaHS to the nail in a sealed chamber gave the highest amount of gas (ANOVA, P < 0.05), whereas ATTM had the lowest (p < 0.05). Deposition from HBTA and DATS was not statistically different (ANOVA, P > 0.05). All donors deposited an

amount of H₂S that was above the MIC of *T. rubrum* (0.162 µg H₂S/mL or 0.162 µg H₂S/g nail) (Figure 6).

Example 4 – Increasing the time of H₂S exposure increases the efficiency of the pathogen growth inhibition

Methods

[00334] Petri dishes with Sabouraud dextrose agar were inoculated with *T. rubrum*, and then incubated at 30 °C for 1 week. The spores were then isolated by washing the surface of the plates with sterile distilled water with 0.05% v/v Tween 20, and the collected solution was then passed through a 40 µm cell strainer (SLS, Nottingham, UK). The spore solution was then centrifuged at 12 g for 9 min (ALC PK121 centrifuge, JENCONS, UK), and the supernatant was removed. The spores were then resuspended in 5 mL of sterile distilled water and counted using the hemacytometer (Fisher, Loughborough, UK). In a petri dish with Sabouraud dextrose agar, 10⁶ spores/mL of *T. rubrum* solution (three drops, 5 µL each) were added to the agar surface. The agar plates were stored in airtight containers with H₂S donor solutions (NaHS or ATTM) at different (0, 0.001, 0.01, 0.5, 1, and 10 mM) or (0, 0.0068, 0.068, 0.34, 0.68 and 6.8 mg H₂S/10mL) at pH 7.4 for NaHS or pH 2 for ATTM in 60 mm petri dish at the bottom of the container with no airflow. The plates were examined visually for fungal growth. Different exposure times were tested and in these experiments, the plates were incubated with the solution for different defined treatment times (1, 3, 6, and 24 h) at 30 °C. After removing the H₂S solutions, the Petri dishes were incubated at 30 °C for 1 week. The plates were examined visually for fungal growth. Each experiment had 3 technical replicates from the same spore culture and 3 biological replicates from different spore cultures. The images were taken using a USB microscope camera (Amazon, UK). The camera was secured 30 cm above the plates at 90 °. The images of the plates were taken using a white background.

Results

[00335] The inhibitory effect of constant exposure of *T. rubrum* conidia to H₂S was tested using 4 different time points at 1, 3, 6, and 24 h. After the gas exposure, the fungi were allowed to grow without the presence of the gas for 7 d. A positive inhibitory effect was seen with NaHS for 6- and 24- h treatment times (Figure 7). The minimum inhibitory concentration (MIC) of H₂S gas using the NaHS on *T. rubrum* conidia was 0.162 µg H₂S/mL (n=3) for both treatment times. No growth inhibition was seen when NaHS was used to generate the H₂S gas after 1- and 3-h treatment times. A similar effect of treatment time as NaHS was observed for ATTM for 6 and 24 h (Figure 8). The minimum inhibitory

concentration (MIC) of H₂S from ATTM on *T. rubrum* conidia was 0.162 µg H₂S/mL (n=3) for both treatment times. However, inhibition of growth was seen also with ATTM after 1- and 3-h treatment times at higher concentrations, unlike NaHS. The 1- hour treatment with ATTM had a MIC of 3.32 µg H₂S/mL, whereas the 3-h treatment had a MIC of 0.323 µg H₂S/mL.

Example 5 – H₂S inhibits fungi growth at all stages of the life cycle

Methods

[00336] In a petri dish prepared with Sabouraud dextrose agar, 10⁶ spores/mL of *T. rubrum* solution (three drops, 5 µL each) were added to the agar surface, then were left for 0, 1, 3, 6, and 24 h before treatment with H₂S. The Petri dishes were stored in airtight containers with NaHS (0, 0.001, 0.01, 0.5, 1, and 10 mM) or (0, 0.0068, 0.068, 0.34, 0.68, and 6.8 mg H₂S/10 mL) in two separate 60 mm petri dish at the bottom of the container, under no airflow. The plates were incubated for 24 h at 30 °C. After removing the H₂S solutions, the Petri dishes were incubated at 30 °C for 1 week. The plates were examined visually for fungal growth. Each experiment had 3 technical replicates from the same spore culture and 3 biological replicates from different spore cultures. The images were taken using a USB microscope camera (Amazon, UK). The camera was secured 30 cm above the plates at 90 °. The images of the plates were taken using a white background. After one week, the conidia were collected with sterile distilled water, transferred to microscopy slides, and imaged at 63x using a Leica light upright microscope DM2500 with the FLEXACAM C1 camera attachment (Leica, Germany). Images were taken using the Lecia Microscope imaging software. Each experiment had 3 biological replicates from different spore cultures. Results are displayed for images of the highest resolution and quality.

Results

[00337] *T. rubrum* was pre-grown on SD agar plates for different times (1, 3, 6, and 24 h) and it was then treated for 24 h with the H₂S released from NaHS. The time points represented different points in the growth life cycle, from the beginning of the germination process at 0 h when the conidia were added to the plate, tube elongation and hyphae formation at 6 h, and until 24 h for mycelial formation. To determine the stage of the life cycle, the fungus was recovered from the surface of the plate post-treatment and the recovered fungus was visualized using the light microscope. H₂S exhibited an inhibitory effect on conidia in all stages of the spore germination cycle. The MIC concentration of 0.162 µg H₂S/mL inhibited the growth of conidia that were pre-grown for 1-, 3-, 6-, and 24 h (Figure 9).

Example 6 – H₂S inhibits the growth of a wide range of pathogens when applied as a liquid

Methods: RPMI in 2x concentration was prepared by dissolving 20.8 grams of RPMI 1640 in 900 mL distilled water, then adding 69.09 g of MOPS and 36 g glucose; the pH was adjusted to 5.4 using HCl, and then the volume was completed to 1000 mL. RPMI was then sterilised with a 0.2 µm filter (SLS, Nottingham, UK). In sterile 1.5 mL tubes, 650 µL of the 2x concentration RPMI was added, and then 2 x 10⁵ conidia of each species were added to the tubes. Control tubes were only mixed with sterile distilled water without H₂S. For the liquid treatment, NaHS concentrations (0, 0.005, 0.05, 0.25, 5, and 5 mM) or (0, 0.0034, 0.034, 0.175, 0.34, and 3.4 mg H₂S/mL) were added into the 1.5 mL tubes with the RPMI and the fungi and the volumes were topped up to 1.3 mL with sterile distilled water. Next, 200 µL of each tube was pipetted into each well in a transparent 96-well plate (6 wells for each tube), avoiding any pipetting into the outer wells as the airtight seal on the outer wells is not as efficient as on the inner wells. All plates were sealed with a plastic adhesive cover and stored at 30 °C until visible growth in the control wells was seen (1 day for yeasts, 3 days for other species, and 5 days for dermatophytes). Plates were measured at 450 nm using a plate reader (LARIOstar Plus, BMG Labtech, Aylesbury, UK). A total of 6 technical replicates for each of the 3 biological replicates were assayed. The following organisms were tested: *Trichophyton rubrum* ATCC28188 (TR188) was purchased from Fischer Scientific (Loughborough, UK). *Trichophyton rubrum* NCPF 0719 (TR 719), *Trichophyton rubrum* NCPF 0936 (TR 936), *Trichophyton rubrum* NCPF 0420 (TR 420), *Candida albicans* SC 5314 (CA) 230, *Microsporium canis* NCPF 0179 (MC), *Aspergillus niger* IHEM 26751 (AN), and *Fusarium oxysporum* NCPF 2674 (FO) were acquired from the National Collection of Pathogenic Fungi (UK).

Results

[00338] When assessing the spectrum of activity across the different isolates of *T. rubrum* H₂S was most potent in inhibiting the growth of TR188 and TR 936, having a MIC of 34 µg H₂S/mL and 170 µg H₂S/mL, respectively. The potency against the TR 420 and TR 719 isolates was slightly higher with an MIC of 340 µg H₂S/mL (**Figure 10A**, all data was n=3). When testing the spectrum of activity across the fungal species that commonly cause onychomycosis, CA and MC were the most sensitive, having a MIC of all other pathogens had an MIC of 170 µg H₂S/mL. AN had a MIC of 340 µg H₂S/mL. Finally, FO was the most resistant, having an MIC of 3400 µg H₂S/mL (**Figure 10B**).

Example 7 – H₂S can kill both superficial infections and those within the human nail

Methods

[00339] Agar plate kill assay - An ATP vs spores/mL calibration curve was constructed as means to enumerate the spores. A Tris-acetate-EDTA buffer was made by adding 1 mM EDTA disodium salt pH 8, 40 mM Tris base, and 20 mM acetic acid, then adjusting the pH to 7.75 using HCl. This buffer was then diluted x50 times to produce the working concentration of the Tris-acetate-EDTA buffer solution. The Luciferin- Luciferase reagent was mixed with (1x) concentration Tris-acetate-EDTA buffer in a 50 mL sterile sample tube (1:4 ratio of the luciferin-luciferase reagent: Tris-acetate-EDTA buffer). A 90% v/v solution of DMSO in Tris-acetate EDTA buffer was made and used as the ATP extraction buffer. In a black 96 flat-bottomed well plate, 50 μ L of the 90%:10% v/v DMSO: Tris-acetate EDTA buffer was added, with 150 μ L of the luciferin-luciferase reagent. Finally, 50 μ L of varying concentrations of *T. rubrum conidia* were added (0, 1×10^6 , 2.5×10^6 , 5×10^6 , 7.5×10^6 , 1×10^7 spores/mL). The plate was immediately placed in a Spark-Tecan plate reader (Tecan, Reading, UK), measuring its luminescence at 20 min with an integration time of 10 s. To test the activity of the antifungal agents, in a petri dish prepared with Sabouraud dextrose agar, 10^6 spores/ml of *T. rubrum* solution (three drops, 5 μ L each) were added to the agar surface. The Petri dishes were stored at 30 °C for one week. After 1 week, the plates were treated with 1 mL of NaHS concentrations (0, 0.001, 0.01, 0.5, 1, and 10 mM) or (0, 0.0068, 0.068, 0.34, 0.68, and 6.8 mg H₂S/mL) for 24 h under either constant or no airflow. The NaHS solution was added directly onto the plates. For the ciclopirox olamine treatment, the following concentrations were used: 0.003, 0.03, 0.08, 0.2, 0.5, and 0.98 μ g/mL. The concentrations were selected based on the literature values of ciclopirox MIC. At the end of the treatment, the *T. rubrum* was harvested by applying 5 mL of 0.05% v/v Tween-20 in sterile distilled water on the plate and spread around. The conidia and hyphae were separated through a 40 μ m sterile nylon cell strainer (SLS, Nottingham, UK). The treatment was then terminated by washing the conidia with sterile distilled water twice by centrifuging (12G for 10 min) and discarding the supernatant. After the washing, 3 mL of sterile distilled water was added to the conidia, and then the conidia were counted using an improved Neubauer haemocytometer (Thermofisher, UK), and the aliquots were diluted to 2.5×10^6 spore/mL. In a black 96-well plate, 50 μ L of the 90: 10% v/v DMSO: Tris-acetate EDTA buffer was added, with 150 μ L of the luciferin-luciferase reagent. Finally, 50 μ L of the conidia (concentration 2.5×10^6) recovered from the plates were added to the wells. The plate was immediately placed in a Spark-Tecan plate reader (Tecan, Reading, UK), measuring its luminescence at 20 min with an integration time of 10 s.

[00340] Nail infection model - In a petri dish prepared with an infected nail in minimal salt agar, a 1 mL aliquot of 10^6 spores/mL of *T. rubrum* was added to nail clippings and they were then left to incubate at 30°C. After 20 days, the plates were treated with 1 mL of H₂S concentration 6.8 mg/mL (10 mM) for 24 h in either an airtight chamber or in an open

container in the fume hood. At the end of the treatment, *T. rubrum* was collected by applying 5 mL of 0.05% v/v Tween-20 in sterile distilled water on the plate and spread around. The conidia and hyphae were separated through a 40 µm sterile nylon cell strainer. The treatment was then terminated by washing the conidia with sterile distilled water twice by centrifuging and discarding the supernatant. After the washing, 3 mL of sterile distilled water was added to the conidia, and then the conidia were counted using a haemocytometer, and the aliquots were diluted to 2.5×10^6 . In a black 96-well plate, 50 µL of the 90: 10% v/v DMSO: Tris-acetate EDTA buffer was added, with 150 µL of the luciferin-luciferase reagent. Finally, 50 µL of the conidia were added after treatment with and without the microchamber, or no treatment for the control. The plate was immediately placed in a Spark-Tecan plate reader, measuring its luminescence at 20 min with an integration time of 10s. The assay was verified by producing a standard curve with different concentrations of spores, then untreated spores were recovered from the plates and were assayed.

15 Results

[00341] To determine if the H₂S could kill established fungal colonies, fungi were grown for 7 days followed by treatment with different concentrations of H₂S for 24 h. Next, the number of viable conidia that could be collected from the fungi present was determined using an ATP assay. A calibration curve for the ATP activity with different spore concentrations was linear and fit for the purpose (data not shown). The recovered untreated conidia (control) using the washing procedure was determined to be 99.8% ± 11.4% viable with a variance (% CV) < 20%. The conidia recovered from plates grown for 7 days were then compared to conidia treated with different concentrations of H₂S or ciclopirox. H₂S liquid treatment caused a gradual decrease in the number of viable conidia, 0.0068 mg/mL resulted in 31% ± 3.8% spore death, 0.068 mg/mL resulted in 47% ± 2.7% spore death, 0.35 mg/mL led to a 55.6% ± 2% reduction in the number of viable cells, finally, 0.68 and 6.8 mg/mL resulted in 65% ± 4.5% and 99% ± 2.7% dead conidia respectively (**Figure 11A**). All the concentrations were significantly different from each other and from the control (ANOVA, $P < 0.05$).

30 [00342] The ciclopirox concentrations (0.03-0.98 µg/mL) covered the reported MIC 0.3-0.98 µg/mL, but none of the concentrations showed complete kill. The lowest ciclopirox concentration resulted in 30% cell death, while all other ciclopirox concentrations resulted in a statistically similar value of viable conidia reduction of approximately 75% cell death (KW, $P > 0.05$). The effect of the ciclopirox treatment did not appear to be concentration dependent but it did show a statistically significant reduction in fungus cell viability when compared to the control (KW, $P < 0.05$) (**Figure 11B**).

[00343] After confirming the invasion of *T. rubrum* into the nail plate, the ATP assay was used to evaluate the efficacy of treating the infected nail with H₂S in an airtight chamber. The recovered untreated conidia (control) were 90% ± 14.8% viable with a % CV < 20%. This recovery value verifies that the assay is fit for purpose. H₂S treatment with an open
5 microchamber resulted in a 31% ± 7.3% reduction in the viable cells. Whereas the use of the airtight microchamber with the same concentration (6.8 mg/mL for 24 h) resulted in an 83.5% ± 3% cell death in the infected nail (ANOVA, P < 0.05) (**Figure 12**).

Example 8 – H₂S is not toxic to the skin and nail after topical application

10 Methods: Raman spectroscopy

[00344] A Raman microscope (Renishaw inVia Reflex, UK) was used to characterise the S-S and -SH groups of the keratin tissues. The spectra were recorded between 100 and 3200 cm⁻¹. A 785 nm edge laser was used and with a grating set to 600 L/mm, and the name of the detector was Master Renishaw CCD Camera. The human nails (10 mg) were
15 incubated in NaHS solution (0.2 mM or 1.12 mg/mL) for 24 h at 32°C. The pH of the solutions was controlled at pH 7.4. As the reference peak, the spectra were subjected to baseline corrections and normalisation using the amide I band, 1628-1679 cm⁻¹ (Renishaw WiRe software, UK).

ATR-FTIR

[00345] The nail was incubated in NaHS solution (0.2mM or 1.12 mg/mL) for 24 h at 32°C at pH 7.4. After 24 h, the tissue samples were dried and then secured as flat as possible inside the ATR-FTIR Spectrometer (Frontier, Perkin Elmer, USA). Measurements were taken between wavelength (550-4000 cm⁻¹) and 64 scans, and the resolution was set at 4cm⁻¹; the force gauge was maintained at 89-90 N. The amide I band was analysed by
25 applying 15 points smoothing filter, then 19 points second derivative, and baseline correction was performed between 1700 cm⁻¹ and 1600 cm⁻¹. (Spectrum 10 software, Perkin Elmer, USA).

Skin exposure testing

[00346] Surgically excised samples of human skin were obtained directly after
30 abdominoplastic surgery after acquiring patient approval from the School of Life Sciences Ethics Committee, King's College London (reference number 17/SC/0441). Epidermal sheets were prepared using heat separation. The prepared epidermal sheets were then dried with tissue paper and stored flat and wrapped in aluminum foil at -20°C until use. The skin was cut into 9 squares with dimensions of 2x2 cm, and then each square was placed
35 into a well on a 6-well plate. Into each well, 300 µL of DMEM was added. PBS (50 mM)

was used as the negative control, glycolic acid (10% w/v) was used as a positive control for skin damage, and 28 mg/mL of NaHS (0.5 M) in PBS was used to evaluate the effect of H₂S on skin integrity. Rhodamine was employed to test if the applied test agents enhanced the skin permeability, which indicated skin damage. To begin the experiment, a small stirring bar was added into a clean small Franz cell. The treated skin was mounted onto the receiver compartment of the small Franz cell and the cell was sealed. Citrate buffer at pH 4 was degassed using a vacuum for 30 min and then added to the receiver compartment of the Franz cell. The cells were left in a water bath at 37°C for 30 min to equilibrate. For every 1 mL of citrate buffer at pH 4, 1.4 mg of RhB was weighed and 200 µL of this was added to the donor compartment. The Franz cells were run without sampling for 3 h. After 3 h, the cells were opened, and the skin was removed. Finally, the surface of the skin was washed with PBS. Images were obtained with two fluorescence channels: DAPI (405 nm) and RhB (561 nm). Images were analyzed using NIS-Elements Imaging Software (Nikon). For the histology images a 200 µL aliquot of each solution was added to the corresponding skin square (n=3 each). The skin was incubated for 2 h at 37 °C. After 2 h, the skin was removed, washed with distilled water, and dried tissue paper, and then fresh DMEM and test solutions were added to the skin. This process was repeated until the 6-h time-point, thus a total of 3 repeated applications. At the end of the 6-hour time point, the skin was removed from the wells, washed with distilled water, and dried with tissue paper. The skin was then cut using a scalpel into 2 mm strips. The strips were embedded in OCT blocks and stored at -80 °C until it was time to cut them using the cryostat. A Bright model OTF cryostat (Bright instruments, Huntingdon, UK) was used to cut cross-sectional slices of 10 µm thickness onto upper frost slides. The OCT was left to dry after cutting for 30 min, then the slides were washed in PBS for 1 min, then fixed in 10% v/v formalin for 10 s. The slides were then left to dry for 10 min. After the slides dried, they were stained with Harris Haematoxylin for 2 min, and then washed in tap water for 1 min. The slides were dipped in differentiation solution for 10 s then the reaction was stopped by washing the slides in tap water for 1 min. Finally, the slides were stained with eosin for 10 seconds and washed in tap water for 1 min. After the staining was completed, the slides were dehydrated using a gradient of acetone for 1 min in each (70%, 90%, 100%, and 100% again), and then they were dipped in xylene for 2 min. The slides were then left to completely dry in the fume hood for 10 min. After the slides were completely dry, they were mounted using DPX and sealed with a cover slip, then stored at 4 °C until imaged. The slides were imaged using a Leica DM 200 Led light microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica digital camera (Model DFC 295) at a magnification of (x20). Images were processed using Las v4.4 Imaging Software (Leica Microsystems, Wetzlar, Germany).

Results

[00347] The Rhodamine permeation experiment showed no signal in the untreated, the negative control or the H₂S treated skin. However, a bright red signal in the epidermis was shown upon application of the positive control. These data indicated that the H₂S did not increase the permeation of chemicals into the skin and thus did not damage the tissue (Figure 13A-D). The histology results showed that the integrity of the skin was not compromised in untreated human skin, negative control treated skin, and skin treated with H₂S, i.e., the skin displayed an intact Stratum corneum, epidermis, and dermis. As for the skin treated with 10% w/v glycolic acid, which was a positive control known to show local tolerability problems, the skin was visibly damaged and ruptured (Figure 13E-H). Raman spectroscopy was used as a technique to evaluate the possible damage to the nail disulphide bonds after treatment with H₂S. An intact keratin displays a disulphide bond (S-S) peak between 500-550 cm⁻¹. The denaturation of the protein would result in the cleavage of the disulphide bond and the formation of the sulfhydryl group (-SH) (peak at 2560-2590). The spectra of the nail in H₂S had a prominent S-S peak and no -SH peak which suggested that after 24 h treatment with 0.68 mg/mL, H₂S did not cause keratin denaturation (Figures 13I and J). ATR-FTIR was used as a technique to evaluate the possible damage to the nail's secondary protein structure after treatment with H₂S. An intact keratin structure should display 5 peaks in the amide I region (1600-1700 nm). The control nail showed β_1 , β_2 , β_3 , β_{turn} , and α -helix peaks again typical of the human nail. There were no shifting nor significant changes ($p > 0.05$) in peak areas after treatment with H₂S (Figures 13I and J).

Example 9 – H₂S induces an increase in oxidative stress inside pathogens when administered as a liquid or a gas

Methods: Oxidative stress assay

[00348] In sterile sample vials (7 mL), 1 mL aliquots of 2×10^5 spores/mL were pipetted into the vials, then 250 μ L of 2x concentration of Sabouraud dextrose broth was added, then left for 12 h in a shaking incubator at 30 °C. After 12 h, the vials were removed, and they were ready for either H₂S gas treatment or the direct application of NaHS solution. For the gas exposure experiments, 228 μ L of sterile distilled water was added to the vials, to match the volume of the liquid treatment (detailed below). The vials labelled gas treatment were then stored without lids next to a 7 mL vial containing 1 mL of 0.34 mg H₂S/mL NaHS, to generate H₂S, in either the sealed airtight container (2.1 L) for no airflow conditions or the same container with the lid removed in the fume hood for constant air flow conditions. For the liquid treatment, either sterile water (control) or 228 μ L of NaHS

solution (0.34 mg H₂S/mL final concentration) was pipetted into the vials, and they were stored with the vial with the lid either closed for no airflow conditions or the lid open in the fume hood for constant air flow conditions. Treatment time was 4 h. The concentration chosen was based on the MIC displayed, however, the volumes used in the liquid and gaseous treatments were reduced to 1 mL so as not to dilute the conidia concentration in the liquid treatment. After 4 h, the samples in the vials were removed, the contents transferred into 1.5 mL microcentrifuge tubes and the samples centrifuged at 9g for 1 min (ALC PK121 centrifuge, JENCONS, UK). After centrifugation, the supernatant was discarded, and the remaining conidia were washed with SD broth. After washing the samples, 500 µL of the staining solution (10µM DCFH-DA + 25µM Calcofluor white) was added to each of the sample tubes and they were left for 30 min at 30 °C. After 30 min, the tubes were centrifuged again, and the supernatant was removed. The conidia were washed with 1x concentration SD broth and twice with sterile PBS. Finally, 100 µL of sterile PBS was added to the tubes, 10 µL of the stained cell solution was transferred to the super frost slides (Thermofisher, Hemel Hempstead, UK), and a standard cover slip (Thermofisher (Hemel Hempstead, UK) was added. Zeiss LSM 880 confocal microscope (Zeiss, Birmingham, UK) was used for the fluorescent imaging of conidia and hyphae and in visualising the accumulation of ROS. Calcofluor white was imaged at 405 nm, while DCFH-DA was imaged at 488 nm at magnification (x20). Each experiment had 3 biological replicates from different spore cultures.

Results: The fungi were imaged after both H₂S gas and liquid treatment using fluorescent dyes to visualize the fungal cell wall and any oxidative stress that occurred as a response to the treatments. During the confocal imaging, Calcofluor white stained the fungal cell with calcofluor white (showing up in the images as blue) while DCFH-DA, a redox-sensitive probe, resulted in a more intense green colour when there was an accumulation of ROS in the fungal cells. When the ROS accumulation was visualised in *T. rubrum* conidia, the treatment with H₂S in either the gaseous or the liquid form under no airflow was found to produce a strong fluorescent signal for ROS, whereas the same treatment under a constant air flow did not produce ROS (**Figure 14**). Furthermore, ROS was produced and accumulated when the hyphae were subjected to the liquid treatment of H₂S under no airflow (**Figure 15**).

Example 10 - Design and 3D-printing of artificial nails

Method: Twelve participants took part in this study where their thumb fingernail dimensions were measured. The dimensions measured included nail length, width, and curvature, with each measurement taken using a digital calliper and radius gauge. The resulting data was then used to design a 3D artificial nail that mimicked the smallest,

median, and largest nail dimensions. The design process utilized 3Ds Max software 2022 and involved initially copying the measured dimensions. A pocket for hydrogen sulphide gel loading was created at the centre of the nail, leaving a margin of at least 1 mm for acrylic glue application to seal the system. Formlabs 3+ 3D printer was used to print the artificial nail designs with clear resin (An Epoxy based resin supplied by Formlabs).

Results: There were variations in nail width, length, and curvature between the 12 participants, with differences of 4 mm, 8.2 mm, and 4 mm, respectively. The three different sizes of artificial nails were designed with the dimensions shown in **Figure 16A**. The artificial nails were 3D printed as illustrated in **Figure 16B**. One of the significant advantages of 3D printing of artificial nails is the ability to customize the design to meet individual needs. With 3D printing technology, it is possible to create artificial nails that mimic the natural nails' size, shape, and curvature. This technology also allows for precise placement of pockets for the application of the topical source of hydrogen sulphide, leading to improved therapeutic outcomes. 3D printing of artificial nails offers a promising solution for personalized and efficient nail replacement therapy. Therefore, To evaluate the fitting quality of the median size artificial nail without gel application, it was fitted on the participant, as shown in **Figure 16C**.

Example 11 – Preparation of hydrogen sulphide gel

[00349] Method: Four different polymers; 3% (w/v) of hydroxyethylcellulose (Natrasol M (NM)), 2% (w/v) of methylcellulose E4M (MC E4M), 5% (w/v) of methylcellulose E15 (MC E15), and 0.5% (w/v) of Carbopol 934 (CP 934) were investigated for forming a gel with a NaHS solution. To create the gel, each the polymer was dissolved in PBS (phosphate-buffered saline) at the required weight percentage to give a NaHS concentration of 56 mg/mL. Once the polymers had dissolved, the pH of the solution was adjusted to 8.5. The mixture was then left overnight to allow for gel formation. The weight percentages of each polymer used was chosen to achieve a gel with the desired consistency and stability. Finally, the pH, appearance, and stability of the gel were checked over 7 days at 45°C. This step ensures that the gel is stable and suitable for its intended use. The pH and appearance of the gel should remain relatively constant over time, while the stability test assesses the gel's ability to withstand temperature changes and other stressors.

[00350] Results: Of the four polymers tested, only the hydroxyethylcellulose gel (Natrosol M) was able to successfully form a stable gel with sodium hydrogen sulphide that was stable to storage at elevated temperature. The pH of this gel was well maintained, and the appearance and gel consistency remained consistent over the seven-day testing period. In contrast, when Methylcellulose E4M and E14 were used, precipitation occurred,

suggesting instability of the gel. Furthermore, when Carbopol 934 was used, the colour of the gel turned yellow, which indicates a chemical reaction occurred, and it was unable to form a stable gel with sodium hydrogen sulphide (**Figure 17**). These findings suggest that hydroxyethylcellulose (Natrosol M) is a preferred polymer for creating hydrogen sulphide gel.

Example 12 – Measurement of H₂S leakage from artificial nails

[00351] Method: To assess the H₂S gas release from a simple solution 11.2 mg sodium hydrogen sulphide was dissolved in phosphate buffered saline (PBS) at pH 8.5. This solution was loaded onto the surface of the artificial nail without sealing onto a finger. The nail loaded with the solution was transferred to a food container box that was sealed airtight and connected to a H₂S gas analyser. The analyser recorded the H₂S concentration in the food box every minute for 2.8 days . The experiment was repeated 3 times. A gel with the same concentration of sodium hydrogen sulphide as the PBS solution was created using a Natrosol M polymer and the release H₂S experiment, with gel on the surface of the artificial nail and not sealed to a finger, was repeated, three times using the same analyser setup. The gel was prepared following the exact procedure detailed in Example 11 above.

[00352] A third group of experiments were conducted using the same gel but placed inside two different artificial nails, one with a narrow adhesive boarder and one with a thick adhesive boarder (**Figure 18**). The 3D-finger model used in the experiments was 3D printed that employed FormLabs “Dental Model Resin” (a methyacrylate-based resin), simulating the properties of human skin. The artificial nail, key for leakage assessments, was fabricated using 3D printing that employed the Formlabs “Clear Resin” (an epoxy-based resin) and this was applied to the 3D-printed finger. These materials provided a realistic platform for the leakage tests, aiding in accurately determining whether the artificial nail maintains an airtight seal. Both the nails were glued to a 3D printed finger using an acrylic nail glue (Boots UK artificial nail glue). The H₂S release was again tested from the two types of artificial nails glued onto the 3D printed fingers in food container boxes that were seal airtight and connected to a H₂S gas analyser for a duration of 2.8 days To test if the two types of nails were sealed after gluing them to the 3D printed finger the gel incorporated a methylene blue dye and leakage was visually recoded by the leakage of the dye after the nails had been glued to the 3D printed finger. In (**Figure 19A**), gas sampling was intentionally conducted over approximately 2.8 days (4000 minutes) instead of the standard 24 hours. This extended duration was needed to capture the

complete 'bell' shaped leakage profile, ensuring a more accurate representation of the results.

[00353] Results: The solution and gel systems that were placed on the artificial nail but not sealed to the 3D printed finger released the H₂S gas rapidly with AUC values of 3981.58 ± 930.78 µg/ml*h and 3666.86 ± 410.47 µg/ml*h, respectively (**Figure 19A**). The experiment with the methylene blue dye showed that the artificial nail with the small adhesive boarder did not fully seal when glued on the 3D printed finger as evidenced by the release of the methylene blue dye (**Figure 19C**). The artificial nails with the large adhesive boarder did seal onto the nail and appeared to remain sealed with no leakage of the dye was observed (**Figure 19D**). The poorly sealed "Leaking artificial nail" allowed 33% of the H₂S available in the gel to leak as gas into the chamber. In contrast the "Non-leaking artificial nail" did not allow any measurable H₂S gas release (**Figure 19 B**).

Example 13 - Determination of Gas Minimum Inhibitory Concentration (MIC) of Hydrogen Sulphide Against *C.albicans* on Agar Plates

[00354] Method: To assess the MIC of hydrogen sulphide against *C.albicans* using sodium hydrogen sulphide (NaHS) as an H₂S donor, a loopful of *Candida albicans* from a growth culture was added to 10 mL of Sabouraud Dextrose (SD) broth. The culture was incubated overnight at 30 °C with constant shaking. Following incubation, the culture was centrifuged at 12 g for 10 min, the supernatant discarded, and the pellet resuspended in 10 mL of phosphate-buffered saline (PBS). This washing step was repeated, and the pellet was finally resuspended in 5 mL of PBS. The cell suspension was then counted using a hemacytometer to achieve a baseline concentration of 10⁶ cells/mL (OD: optical density). Serial dilutions were prepared from this stock solution, resulting in 10⁵, 10⁴, 10³, and 10² dilutions (using PBS). Target concentrations of NaHS (5.6 µg/mL, 14 µg/mL, 28 µg/mL, 56 µg/mL, 112 µg/mL, 280 µg/mL and 560 µg/mL) were prepared, from a stock solution of 1120 µg/mL NaHS, made by dissolving 28 mg of NaHS in 25 mL of distilled water. Closed chambers were set up to contain four SD agar plates inoculated with 10 µL of the respective dilution and two small plates containing the target NaHS concentrations, allowing exposure to H₂S gas released from NaHS. These closed chambers were incubated at 30 °C for 24 h. After the incubation period, the plates were removed, and the growth on the agar plates was evaluated by performing a viable count analysis. This involved counting the colonies formed on each plate to determine the inhibitory effect of the H₂S gas on *Candida albicans* growth. The MIC was defined as the lowest concentration of NaHS at which no visible growth was observed, providing insights into the antifungal properties of H₂S.

[00355] **Results:** The results showed a distinct inhibitory effect was evident following 24 h treatment with NaHS. The Minimum Inhibitory Concentration (MIC) of H₂S gas, from NaHS, against *C.albicans* was established at 14 µg/mL on infected SD agar plates (Figure 20). This suggested that NaHS has a good spectrum of activity against fungi.

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Example 14 - Determination of Gas Minimum Inhibitory Concentration (MIC) of Hydrogen Sulphide against *Staphylococcus aureus* on Agar Plates

[00356] **Method:** To evaluate the minimum inhibitory concentration (MIC) of H₂S against *Staphylococcus aureus* using sodium hydrogen sulphide (NaHS) as an H₂S donor, a loopful of *S. aureus* ATCC 9144 from an actively growing culture was added to 10 mL of tryptone soy broth (TSB). The culture was incubated overnight at 37 °C. After incubation, the culture was centrifuged at 12 g for 10 minutes, the supernatant was discarded, and the cell pellet was resuspended in 5 mL of phosphate-buffered saline (PBS). The optical density of the cell suspension was then adjusted to OD_{600nm} = 0.001 using a plate reader to achieve a baseline concentration of 10⁶ cells/mL. Serial dilutions were prepared from this stock solution, resulting in 10⁵, 10⁴, 10³, and 10² dilutions using PBS. Target concentrations of NaHS (5.6 µg/mL, 14 µg/mL, 28 µg/mL, 56 µg/mL, 112 µg/mL, 280 µg/mL, and 560 µg/mL) were prepared using a stock solution of 1120 µg/mL NaHS created by dissolving 28 mg of NaHS in 25 mL of distilled water. Closed chambers were then prepared, each containing four tryptone soy agar (TSA) plates inoculated with 10 µL of the respective dilution and two small plates containing the target NaHS concentrations. This setup allowed the plates to be exposed to H₂S gas released from NaHS. The chambers were incubated at 37 °C for 24 h. After the incubation period, the plates were removed, and the growth on the agar plates was assessed by performing a viable count analysis. This process involved counting the colonies on each plate to determine the inhibitory effect of H₂S gas on *S. aureus* growth. The MIC was defined as the lowest concentration of NaHS at which no visible growth was observed, providing valuable insights into the antimicrobial properties of H₂S.

[00357] **Results:** The results showed a distinct inhibitory effect was evident following 24-hour treatment with NaHS. The Minimum Inhibitory Concentration (MIC) of H₂S gas, sourced from NaHS, against *S.aureus* was established at 5.6 µg/mL on infected TSA agar plates (Figure 21). This demonstrated the efficacy of NaHS as an antimicrobial agent.

Example 15 - Liquid Minimum Inhibitory Concentration (MIC) Determination of Hydrogen Sulphide, Amorolfine, and Ciclopirox against *T.rubrum* in liquid culture

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- [00358] **Method:** The determination of the liquid MIC for NaHS was compared to commonly employed agents amorolfine, and ciclopirox using *T. rubrum* agar plates to evaluate the antifungal activity of each compound. Initially, *T. rubrum* spores were harvested, and their concentration was adjusted to 1×10^6 spores/mL, with a hemacytometer. For the growth mediums, Sabouraud Dextrose (SD) broth was prepared by dissolving 30 g of SD broth powder in 1 L of distilled water and sterilised by autoclaving. RPMI-1640 broth was prepared by dissolving 10.4 g of RPMI-1640 powder in 900 mL of distilled water, followed by the addition of 34.5 g MOPS and 18 g glucose, with the pH adjusted to 5.0 before sterilisation via filtration through a 0.22-micron membrane filter. To evaluate the antifungal compounds, seven tubes for each treatment medium were filled with 11 ml of the respective broth. Stock solutions were prepared by dissolving 329 mg of sodium NaHS, 518 mg of ciclopirox, and 446 mg of amorolfine in suitable solvents. From these stock solutions, serial dilutions were made to achieve concentrations of 0, 0.6, 1.3, 2.6, 5.3, 10.6, and 21.3 $\mu\text{g/mL}$. One millilitre of each concentration was then added to the corresponding tubes, creating a total of 14 tubes for each treatment. For the tubes designated for inoculation, 50 μL of the *T. rubrum* spore suspension (1×10^6 spores/mL) was added to each of the 7 tubes. The tubes were then incubated at 30 °C for 5-7 days, allowing for observation of fungal growth inhibition at different concentrations, thus determining the MIC of hydrogen sulphide, amorolfine, and ciclopirox against *T. rubrum*.
- [00359] **Results:** The liquid MIC determination of NaHS, amorolfine, and ciclopirox revealed differential response of the tested treatments against the organisms in the two growth mediums, with ciclopirox and NaHS exhibiting heightened inhibitory effects in the SD broth medium compared to the RPMI medium. Conversely, amorolfine displayed a more pronounced inhibitory effect in the RPMI medium relative to the SD broth. The results demonstrated that the inhibitory effect of ciclopirox and NaHS in the SD broth medium was 5.3 $\mu\text{g/mL}$ and 10.6 $\mu\text{g/mL}$, respectively. This inhibitory effect was found to be twice as potent as that observed in the RPMI medium, with values of 2.6 $\mu\text{g/mL}$ and 5.3 $\mu\text{g/mL}$, respectively. In amorolfine's case, the inhibitory effect in the SD broth and RPMI mediums was seen as 1.3 and 5.3 $\mu\text{g/mL}$, respectively. RPMI is the standard media to use for clinical comparison and these results highlighted the excellent efficacy of NaHS compared to the commercial treatments. These liquid MIC results of NaHS, amorolfine, and ciclopirox against *T. rubrum* are shown in Table 4 below.

Table 4

Target con. ($\mu\text{g/mL}$)	Liquid MIC results for <i>T.rubrum</i> with SD broth			Liquid MIC results for <i>T.rubrum</i> with RPMI broth		
	Ciclopirox ($\mu\text{g/mL}$)	Amorolfine ($\mu\text{g/mL}$)	NaHS ($\mu\text{g/mL}$)	Ciclopirox ($\mu\text{g/mL}$)	Amorolfine ($\mu\text{g/mL}$)	NaHS ($\mu\text{g/mL}$)
	Growth of <i>T.rubrum</i>	Growth of <i>T.rubrum</i>	Growth of <i>T.rubrum</i>	Growth of <i>T.rubrum</i>	Growth of <i>T.rubrum</i>	Growth of <i>T.rubrum</i>
0	Yes	Yes	Yes	Yes	Yes	Yes
0.6	Yes	Yes	Yes	Yes	Yes	Yes
1.3	Yes	No	Yes	Yes	Yes	Yes
2.6	Yes	No	Yes	No	Yes	Yes
5.3	No	No	Yes	No	No	No
10.6	No	No	No	No	No	No
21.3	No	No	No	No	No	No

Example 16 - Liquid Minimum Inhibitory Concentration (MIC) Determination of Hydrogen Sulfide, Amorolfine, and Ciclopirox Using *C.albicans* in Liquid Culture

- 5 **[00360] Method:** The liquid MIC for NaHS was compared with amorolfine, and ciclopirox was determined using *Candida albicans*. First, *C. albicans* cells were collected and adjusted to a concentration of 1×10^6 cells/mL using a hemacytometer. Sabouraud Dextrose (SD) broth was made by dissolving 30 g of SD broth powder in 1 L of distilled water and sterilising it through autoclaving. RPMI-1640 broth was prepared by dissolving
- 10 10.4 g of RPMI-1640 powder in 900 mL of distilled water, adding 34.5 g MOPS and 18 g glucose, adjusting the pH to 5.0, and filtering with a 0.22-micron membrane filter for sterilisation. To test the antifungal compounds, seven tubes for each treatment were filled with 11 mL of the prepared broths. Stock solutions were made by dissolving 329 mg of NaHS, 518 mg of ciclopirox, and 446 mg of amorolfine in appropriate solvents. Serial
- 15 dilutions were then made to get concentrations of 0, 0.6, 1.3, 2.6, 5.3, 10.6, and 21.3 $\mu\text{g/mL}$. Each concentration (1 mL) was added to the respective tubes, making 14 tubes per treatment. For the inoculation, 50 μL of the *C. albicans* cell suspension (1×10^6 cells/mL) was added to each of the 7 designated tubes. The tubes were incubated at 30 °C for 24 h to observe fungal growth inhibition at different concentrations, determining the MIC of
- 20 NaHS, amorolfine, and ciclopirox against *C. albicans*.

- [00361] Results:** The liquid MIC determination of hydrogen sulphide NaHS, amorolfine, and ciclopirox, conducted using *Candida albicans* agar plates with Sabouraud Dextrose (SD) broth and RPMI-1640 broth mediums, revealed differing responses to the two growth mediums. Ciclopirox exhibited the same inhibitory effects in both SD broth and RPMI
- 25 mediums. In contrast, amorolfine and NaHS showed higher inhibitory effects in the RPMI medium compared to the SD broth medium. Specifically, ciclopirox displayed an inhibitory

effect of 1.3 µg/mL in both SD and RPMI mediums. NaHS exhibited inhibitory effects of 10.6 µg/mL in SD broth and 1.3 µg/mL in RPMI medium. For amorolfine, the inhibitory effect was observed at 5.3 µg/mL in SD broth and 2.6 µg/mL in RPMI medium. RPMI is the standard media to use for clinical comparison and these results highlighted the excellent efficacy of NaHS compared to the commercial treatments. These liquid MIC results of hydrogen sulfide (H₂S), Amorolfine, and Ciclopirox against *C.albicans* are shown in Table 5 below.

Table 5

Target con. (µg/ml)	Liquid MIC results for <i>C.albicans</i> with SD broth			Liquid MIC results for <i>C.albicans</i> with RPMI broth		
	Ciclopirox (µg/mL)	Amorolfine (µg/mL)	NaHS (µg/mL)	Ciclopirox (µg/mL)	Amorolfine (µg/mL)	NaHS (µg/mL)
	Growth of <i>C. albicans</i>	Growth of <i>C. albicans</i>	Growth of <i>C. albicans</i>	Growth of <i>C. albicans</i>	Growth of <i>C. albicans</i>	Growth of <i>C. albicans</i>
0	Yes	Yes	Yes	Yes	Yes	Yes
0.6	Yes	Yes	Yes	Yes	Yes	Yes
1.3	No	Yes	Yes	No	Yes	No
2.6	No	Yes	Yes	No	No	No
5.3	No	No	Yes	No	No	No
10.6	No	No	No	No	No	No
21.3	No	No	No	No	No	No

Example 17 - Liquid Minimum Inhibitory Concentration (MIC) Determination of Hydrogen Sulfide Using *Staphylococcus aureus* in Liquid Culture

[00362] Method: The liquid MIC for NaHS was determined using *Staphylococcus aureus* (ATCC 9144) cultured on Trypton Soy Agar to evaluate the antibacterial effectiveness of NaHS. *S. aureus* cells were collected and a loop full of the bacterial growth were inoculated in TSB broth and incubated at 37 °C for 24 hours, then the concentration was adjusted to 1×10^6 cells/mL by achieving an OD_{600nm} measurement of 0.001 nm. Muller Hinton Broth (MHB) was prepared by dissolving 21 g of MHB powder in 1 L of distilled water and sterilising it through autoclaving. Ten tubes for the NaHS treatment were filled with 11 mL of the prepared broth and the stock solutions were made by dissolving 329 mg of NaHS in PBS. Serial dilutions were then performed to obtain concentrations of 0, 0.08, 0.16, 0.3, 0.6, 1.3, 2.6, 5.3, 10.6, and 21.3 µg/mL. Each concentration (1 mL) was added to the respective tubes. For inoculation, 50 µL of the *S. aureus* cell suspension (1×10^6 cells/mL) was added to each of the 10 designated tubes. The tubes were incubated at 37 °C for 24 h to observe bacterial growth inhibition at various concentrations, determining the MIC of hydrogen sulphide against *S. aureus*.

[00363] Results: The results demonstrated that the MIC of NaHS against *S.aureus* was 10.6 µg/mL which again demonstrates the broad spectrum of action of NaHS (Table 6).

Table 6

NaHS target con. ($\mu\text{g/mL}$)	<i>S.aureus</i> growth
0	Yes
0.08	Yes
0.16	Yes
0.3	Yes
0.6	Yes
1.3	Yes
2.6	Yes
5.3	Yes
10.6	No
21.3	No

Example 18 - Determination the Ability of NaHS, Amorolfine, and Ciclopirox to Kill *T.rubrum* When Grown in Human Nails

5 **[00364] Method:** To determine the concentration of *T. rubrum* on the nail after 10 days of growth, three infected nails were selected and placed in 5 mL tubes containing 3 mL of 0.05% Tween 20 water. The samples were gently shaken for 1 h, 3 h, and 24 h to recover the maximum number of spores. Following the shaking periods, the nails were removed, and the washing suspensions were filtered through a cell strainer to remove hyphal
10 fragments. The filtered suspensions were then centrifuged at 9 g for 12 min to collect the spores. The supernatant was discarded, and the spore pellets were reconstituted in 1 mL of PBS. Spores were counted using a hemacytometer to achieve a concentration of 10^6 , and ATP assay was performed. The shaking process was repeated with fresh 0.05% Tween 20 water for an additional 2 h (total 3 h) and another 23 h (total 24 h) to recover
15 spores at these time points.

[00365] To assess the effectiveness of NaHS treatments on *T. rubrum*, seven different concentrations of NaHS were prepared in 1 mL volumes 0, 0.6, 1.3, 2.6, 5.3, 10.6, and 21.3 $\mu\text{g/mL}$. Each concentration was mixed with a separate infected nail in 3 mL of RPMI, resulting in a total volume of 4 mL (1 mL treatment solution and 3 mL RPMI). The samples
20 were incubated for 24 h. After 24 h of incubation, the nails were left in their tubes and the RPMI broth was transferred to new tubes. The broth was filtered through a cell strainer to remove hyphal fragments and centrifuged at 9 g for 12 min to isolate the spores. The supernatant was removed and the pellets were reconstituted in 1 mL of PBS. Spore counting and ATP assays were then performed. To evaluate the effect of NaHS on the
25 spores remaining on the nails, the treated nails were removed from the treatment solution and submerged in 3 mL of 0.05% Tween 20 water, followed by incubation for 3 h. Spore counting and ATP assays were performed on the solution after filtration and centrifugation. For confirmation of spore kill, the remaining solution from each nail was used to inoculate

SD agar plates. These plates were observed for growth over a period of 7 days, which would indicate the presence of viable spores. The percentage kill of the treatments was calculated using the following formula:

$$\text{Kill Percentage} = \frac{\text{Bioluminescence of the control} - \text{Bioluminescence of the Treatment}}{\text{Bioluminescence of the Control}} \times 100$$

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[00366] **Results:** Both NaHS and ciclopirox demonstrated a notable reduction in the number of spores as the treatment concentrations increased, indicating their effectiveness against *T. rubrum* infected human nails killing > 90% in the 24 h experiment. Conversely, amorolfine killed less than 80% of the organisms over the same concentration range (Figure 22). These results concurred with the plate results.

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Example 19 – Determination of the Ability of NaHS, Amorolfine, and Ciclopirox to Kill *C.albicans* in Human Nails

[00367] **Method:** To determine the concentration of *Candida albicans* on the nails after 5 days of growth, three infected nails were selected and placed in 5 mL tubes containing 3 mL of PBS. The samples underwent gentle shaking for 1 h, 3 h, and 24 h to maximise organism recovery. After each shaking period, the nails were removed, and the washing suspensions were centrifuged at 9 g for 10 min to collect the cells. The supernatant was discarded, and the cell pellets were resuspended in 1 mL of PBS. ATP assays were conducted to quantify the cell concentration, aiming for 10⁶ cells/mL. This shaking procedure was repeated with fresh PBS for an additional 2 h (totally 3 h) and then another 23 h (totally 24 h) to recover cells at these time points. To assess the effectiveness of NaHS treatments on *C. albicans*, seven different concentrations of NaHS (0, 0.6, 1.3, 2.6, 5.3, 10.6, and 21.3 µg/mL) were prepared in 1 mL volumes. Each concentration was mixed with a separate infected nail in 3 mL of RPMI, resulting in a total volume of 4 mL (1 mL treatment solution and 3 mL RPMI). The samples were then incubated for 24 h. After incubation, the nails remained in their tubes while the RPMI broth was transferred to new tubes. The broth was centrifuged at 9 g for 10 min to isolate the cells, followed by removal of the supernatant and resuspension of the pellets in 1 mL of PBS. ATP assays were performed to assess cell viability. To evaluate the effect of NaHS on the cells remaining on the nails, the treated nails were removed from the solution and submerged in 3 mL of PBS, then incubated for 3 h. ATP assays were conducted on the solution after centrifugation. For confirmation of cell kill, the remaining solution from each nail was used to inoculate SD agar plates. These plates were observed for growth over 24 h to determine the presence

of viable cells. The kill percentage of the treatments was measured using the following formula:

$$\text{Kill Percentage} = \frac{\text{Bioluminescence of the control} - \text{Bioluminescence of the Treatment}}{\text{Bioluminescence of the Control}} \times 100$$

[00368] Results: Both NaHS and ciclopirox demonstrated a notable reduction in the number of cells as the treatment concentrations increased, indicating their effectiveness against *C.albicans* infected human nails with a killing percentage around 90%. Conversely, amorolfine only killed around 60% of the organisms over the same concentration range (Figure 23). These results concurred with the plate results.

10 **Example 20 - The effect of Cold Plasma Treatment at Different Times on the Growth of *T.rubrum* Agar Plates Under a Cover**

[00369] Method: To evaluate the effect of cold plasma applied under a cover to *T. rubrum* cultures by varying treatment durations, an experimental setup was established with an argon gas flow set at 5 L/min. Hydrogen sulphide was then introduced at a flow rate of 0.02 L/min, maintaining an initial ratio of 2% H₂S to 98% argon by volume. Fresh *T. rubrum* cultures, aged 10 days and approximately 1 cm in diameter, were exposed to these gas conditions. Each culture was placed inside a small glass vial (3 cm in height), which served as the plasma treatment chamber. The treatment head was lowered on top of the treatment chamber to seal the top during treatment. Three small agar plates were treated with argon only, and three with the argon + H₂S mixture, with plasma applied for 5 minutes and 10 minutes. A total of nine small *T. rubrum* agar plates were used, including three control plates that remained untreated. For spore collection, a 0.05% v/v Tween 20 solution was prepared, and 2 mL of this solution was used to reconstitute the spores from the *T. rubrum* agar plates. The solution, containing spores and hyphae, was transferred into a sterile Falcon tube equipped with a 45 µm cell strainer. The mixture was then centrifuged at 9 g for 12 min. Following centrifugation, the supernatant was removed, and the spore pellets were reconstituted with 1 mL of PBS solution. Re-inoculation of *T. rubrum* on agar plates was performed by adding three 5 µL drops of the reconstituted spore solution evenly onto the surface of Sabouraud Dextrose (SD) agar plates. All plates were incubated in a controlled temperature room at 30 °C for 5 to 7 days to assess the growth of *T. rubrum*. The impact of cold plasma treatment on the fungal cultures was then evaluated based on the observed growth patterns on the agar plates. The kill percentage of the treatments was measured using the following formula:

$$\text{Kill Percentage} = \frac{\text{Bioluminescence of the control} - \text{Bioluminescence of the Treatment}}{\text{Bioluminescence of the Control}} \times 100$$

[00370] **Results:** The results indicate that cold plasma treatment with H₂S and argon has a significant antifungal effect against *T. rubrum* after 10 minutes of exposure. The killing rate percentage demonstrates a substantial reduction in *T. rubrum*, with approximately 85% of the spores being effectively killed following the 10-minute treatment which was significantly better than the argon alone (Figure 24).

Example 21 - *C.albicans* Biofilm Eradication Study Not Under a Cover

[00371] **Method:** To assess the effect of the drugs on the biofilms, biofilms were produced by transferring 100 µL of the cell suspension (1x10⁶ cells/mL in RPMI-1640) to each well of sterilised 96-well plates. The plates were then incubated at 30 °C for 24 h to allow biofilm formation. After biofilm formation, the medium was carefully aspirated to avoid disturbing the biofilm, achieved by angling the pipette tips towards the corners of the wells to minimise contact. The plates were then washed three times with sterile PBS (200 µL per well) to remove planktonic and non-adherent cells, and inverted onto absorbent paper to remove any residual buffer. Drug stock solutions were prepared in PBS for water-soluble drugs, and in PBS with 1% DMSO (v/v) for water-insoluble drugs, before being diluted in RPMI 1640 medium. The final concentrations of the drug dilutions ranged from 50 to 200 µg/mL. A volume of 200 µL of each drug concentration, prepared in RPMI, was added to the respective wells of the microtiter plate, which was then incubated for 24 h at 30 °C. Subsequently, the XTT assay was performed to determine the viability of the cells. XTT was prepared as a supersaturated solution at a concentration of 0.5 mg/mL in sterile PBS and then filter-sterilised using a 0.22 µm pore size filter. A 10 mM stock solution of menadione in 100% acetone was also prepared and added to the XTT solution at a volume of 10 µL per 10 mL of XTT solution. 100 µL of the mixture was then distributed into the wells of the 96-well plates, which were incubated at 37 °C for 3 hours in the dark. The resulting solution was analysed at 495 nm, and the absorbance was measured using a plate reader. The percent biofilm eradication was calculated by using the following formula:

$$\text{Biofilm eradication (\%)} = \frac{\text{Absorbance of the control} - \text{Absorbance of the Treatment}}{\text{Absorbance of the Control}} \times 100$$

[00372] **Results:** The results indicate that cold plasma treatment with H₂S and argon has a significant effect against the biofilms formed by the *C.albicans* which was found to be comparable to the amphotericin-b (a potent anti-fungal agent) at all the time points, i.e., 5, 10 and 15 minutes. In terms of percent eradication of the formed biofilms the plasma H₂S and argon eradicated more than 80% of the biofilms (Figure 25).

Example 22 - *In vivo* Permeation of H₂S to Rats Nails

[00373] Method: All procedures were conducted in accordance with the U.K. Animal Scientific Procedures Act (1986) and Amendments Regulations (2012) and approved by the King's College London Animal Care and Ethics Committee. Rats (SD male rats, 10-12 weeks old, Charles River, Kent, UK) were caged in groups of 3 with free access to water and food. A temperature of 19 - 22 °C was maintained, with a relative humidity of 45 – 65%, and a 12 h light/dark cycle. Animals were acclimatised for 7 days before each experiment. The previously described fingernail design was adapted to fit a rat's paw, ensuring suitability. The device was affixed to healthy hind rat paws (see Figure 26 for the design) using a flexible bandage to maintain full mobility throughout the study. Each device was placed on 3 digits per paw, covering 6 digits per single animal. Topical application of 100 µL of 8.5 mg/mL, 17 mg/mL, and 34 mg/mL NaHS gel was performed on each rat nail and left for 1, 3 and 6 h. The whole procedure was non-recovery, where rats were anaesthetised for the whole duration due to the rats removing the covers if not under anaesthesia. Rats were humanely culled via schedule 1 method at specified timepoints, and both nails and plasma samples were collected. Control endogenous H₂S nail levels were collected from healthy rats, and plasma samples were collected from healthy rats and post exposure at each time point when the highest NaHS gel concentration was applied. The presence of NaHS gel and rat plasma H₂S levels were analysed using the established “methylene blue” quantification method for H₂S described earlier in the document in Example 2.

[00374] Results: When the NaHS gel was applied to healthy rats, a maximum of 111.5 ± 48.8 µg of H₂S was deposited per g of nail (Figure 27). A clear dose-response relationship was observed at the 1 h and 3 h time points for all three different gel concentrations. However, with longer application times of 6 h, the medium (17 mg/mL) and highest (34 mg/mL) gel concentrations resulted in the same amount of H₂S deposition in the nail. Notably, the 34 mg/mL gel reached a maximum concentration in the nail permeated in as little as one hour, indicating that this system facilitated rapid and efficient permeation of H₂S.

[00375] The animal plasma levels were consistent and showed no significant differences after H₂S topical exposure at various time points, even when the highest NaHS gel concentration was applied (Figure 28). The levels detected were approximately 440 µM in the plasma of healthy rats, aligning with reported literature values of about 300 µM. The findings from this *in vivo* study suggest that there is no systemic exposure associated with the topical application of NaHS.

CLAIMS

1. A topical source of hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the topical source of hydrogen sulphide to an infected nail, wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment,

wherein the topical source of hydrogen sulphide comprises a composition comprising a hydrogen sulphide donor,

wherein the hydrogen sulphide donor is selected from sodium hydrosulphide (NaHS), ammonium tetrathiomolybdate (ATTM), diallyl trisulphide (DATS) and 4-hydroxybenzothioamide (HBTA).

2. The topical source of hydrogen sulphide for use of claim 1, wherein the hydrogen sulphide donor is NaHS, ATTM, or DATS, preferably wherein the hydrogen sulphide donor is NaHS.

3. The topical source of hydrogen sulphide for use of claim 1 or claim 2, wherein the composition comprises a liquid selected from water and a polar organic solvent,

optionally wherein the liquid is selected from one or more of water, methanol, acetone, propylene carbonate, sulfolane, tributyl phosphate, a glycol, a glycol ether and N-methylpyrrolidone,

preferably wherein the composition is an aqueous composition.

4. The topical source of hydrogen sulphide for use of any one of claims 1 to 3, wherein the composition comprises a hydrogen sulphide solution.

5. The topical source of hydrogen sulphide for use according to any one of claims 1 to 4, wherein the hydrogen sulphide donor is present in the composition at a concentration of from 0.003 µg/mL to about 100 mg/mL.

6. The topical source of hydrogen sulphide for use according to any one of claims 1 to 5, wherein the composition has a pH in the range of about 4 to about 10;

optionally wherein the composition has a pH in the range of about 7.5 to about 9.5, for example about 8.0 to about 9.0, preferably wherein the composition has a pH of about 8.5.

7. The topical source of hydrogen sulphide for use according to any one of claims 1 to 6, wherein the composition is formulated as a gel composition,

optionally wherein:

- (i) the composition is formulated as a hydrogel composition; and/or
- (ii) the composition comprises a gel-forming agent, preferably wherein the gel-forming agent is a gel-forming polymer, more preferably wherein the gel-forming polymer comprises hydroxyethylcellulose.

8. The topical source of hydrogen sulphide for use according to any one of claims 1 to 7, wherein the topical source of hydrogen sulphide is applied to the dorsal surface of the infected nail.

9. The topical source of hydrogen sulphide for use according to any one of claims 1 to 8, wherein the cover is an occlusive dressing or occlusive patch;

optionally wherein the cover is in the form of an artificial nail.

10. The topical source of hydrogen sulphide for use according to any one of claims 1 to 9, wherein the cover is or comprises, an air-impermeable polymer, optionally wherein the cover is or comprises polytetrafluoroethylene.

11. The topical source of hydrogen sulphide for use according to any one of claims 1 to 10, wherein the cover is adapted to provide a substantially airtight chamber over the topical source of hydrogen sulphide on the infected nail.

12. The topical source of hydrogen sulphide for use according to any one of claims 1 to 11, wherein the cover is sealably attached to the subject over the topical source of hydrogen sulphide;

optionally wherein:

(i) the cover is located over the topical source of hydrogen sulphide and is sealed onto the dorsal surface of the infected nail; and/or

(ii) the cover is sealed to the subject by an adhesive.

13. The topical source of hydrogen sulphide for use according to any one of claims 1 to 12, wherein the cover comprises the topical source of hydrogen sulphide and the topical source of hydrogen sulphide is applied to the infected nail by contacting the cover with at least a portion of the surface of the infected nail;

optionally wherein the cover comprises one or more layer or reservoir containing the topical source of hydrogen sulphide, wherein the one or more layer or reservoir is in fluid communication with the dorsal surface of the infected nail when the cover is placed on the infected nail.

14. The topical source of hydrogen sulphide for use according to any one of claims 1 to 13, wherein the cover is in the form of a patch comprising a dorsal surface and a ventral surface;

wherein the dorsal surface comprises a substantially air impermeable layer;

the ventral surface comprises an adhesive layer;

the patch comprises one or more layer or reservoir comprising the topical source of hydrogen sulphide in fluid communication with the ventral surface; and

wherein the patch is sealably attached to the subject by means of the adhesive layer to provide a substantially air-tight chamber over at least a portion of the infected nail.

15. A plasma comprising ionised hydrogen sulphide for use in a method of treating a nail infection in a subject, the method comprising applying the plasma to an infected nail.

16. The plasma comprising ionised hydrogen sulphide for use according to claim 15, wherein the plasma is formed from a gaseous mixture comprising hydrogen sulphide and one or more inert gases,

optionally wherein the plasma is formed from a gaseous mixture comprising hydrogen sulphide and argon.

17. The plasma comprising ionised hydrogen sulphide for use according to claim 15 or claim 16, wherein the plasma is maintained under a substantially airtight cover during treatment.

18. The plasma comprising ionised hydrogen sulphide for use according to claim 17, wherein:

(i) the cover is a chamber placed over the infected nail; or

(ii) the cover is formed by a conduit in gaseous communication with source of the plasma and the surface of the nail; or

(iii) the cover is a chamber wherein the subjects hand or foot is placed in the chamber containing the plasma.

19. The topical source of hydrogen sulphide for use or the plasma comprising ionised hydrogen sulphide for use according to any one of claims 1 to 18, wherein the nail infection is a fungal, yeast and/or bacterial infection;

optionally wherein:

- (i) the nail infection is a dermatophyte fungal nail infection, for example a *Trichophyton Spp.* (e.g. *T. rubrum*, *T. mentagrophyte*, *T. verrucosum*, *T. violaceum*, *T. kraidenii*, *T. tonsurans*, *T. soundanense*, *T. equinum*, *Epidermophyton floccosum*, *Arthroderma spp.* or *Microsporum spp.* (e.g. *microsporum canis*) infection;
- (ii) the nail infection is a non-dermatophyte fungal nail infection, for example an *Aspergillus spp.*, *Fusarium spp.* (e.g. *F. oxysporum*), *Acremonium spp.*, *Scopulariopsis spp.* (e.g. *Scopulariopsis brevicaulis*), *Alternaria spp.*, (e.g. *alternata*), *Syncephalastrum spp.*, *Scytalidium spp.*, *Paecilomyces spp.*, *Chaetomium spp.*, *Onychocola spp.* or *Neoscytalidium spp.* infection;
- (iii) the nail infection is a yeast nail infection, for example an *Candida spp.* nail infection (e.g. a *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata* or *C. tropicalis* nail infection);
- (iv) the nail infection is a bacterial nail infection, for example a Gram positive bacterial infection (e.g. *Staphylococcus aureus*) or a Gram negative bacterial infection (e.g. *Pseudomonas aeruginosa* or *Klebsiella spp.*);
- (v) the nail infection is paronychia; and/or
- (vi) the nail infection comprises a biofilm.

20. The topical source of hydrogen sulphide for use or the plasma comprising ionised hydrogen sulphide for use according to any one of claims 1 to 19, wherein the nail infection is onychomycosis, for example distal lateral subungual onychomycosis, white superficial onychomycosis, proximal subungual onychomycosis, endonyx onychomycosis or total dystrophic onychomycosis.

21. The topical source of hydrogen sulphide for use or the plasma comprising ionised hydrogen sulphide for use according to any one of claims 1 to 20, wherein the nail infection is caused by a pathogen which is a treatment-resistant pathogen, for example a bacterial strain that is resistant to one or more conventional antibacterial agent, or a fungal strain that is resistant to one or more conventional antifungal agent.

22. The topical source of hydrogen sulphide for use or the plasma comprising ionised hydrogen sulphide for use according to any one of claims 1 to 21, wherein the nail infection is a toe nail infection.

23. A cosmetic method for treating a nail infection in a subject, the method comprising applying an effective amount of a topical source of hydrogen sulphide to an infected nail,

wherein the topical source of hydrogen sulphide is maintained under a substantially airtight cover during treatment.

24. A system comprising:
a cover; and
a topical source of hydrogen sulphide;
the cover being adapted to provide a substantially airtight chamber over the topical source of hydrogen when the topical source of hydrogen sulphide is applied to an infected nail in a subject;

optionally wherein:

- (i) the cover comprises the topical source of hydrogen sulphide;
- (ii) the cover comprises one or more layer or reservoir containing the topical source of hydrogen sulphide;
- (iii) the system further comprises a means for sealably attaching the cover to a surface tissue of a subject; optionally wherein the means for sealably attaching the cover to a surface tissue of a subject is an adhesive, preferably wherein the adhesive is an acrylic adhesive;
- (iv) wherein the cover is in the form of a patch comprising a dorsal surface and a ventral surface;
wherein the dorsal surface comprises a substantially air impermeable layer;
the ventral surface comprises an adhesive layer; and
the patch comprises one or more layer or reservoir comprising the topical source of hydrogen sulphide in fluid communication with the ventral surface;
- (v) the cover is an occlusive dressing or occlusive patch,
optionally wherein the cover is in the form of an artificial nail;
- (vi) the cover is, or comprises, an air-impermeable polymer, for example PTFE;

and/or

- (vii) the cover further comprises a removable, substantially air impermeable backing layer which is adapted to maintain the topical source of hydrogen sulphide in the cover in a substantially airtight environment prior to use.

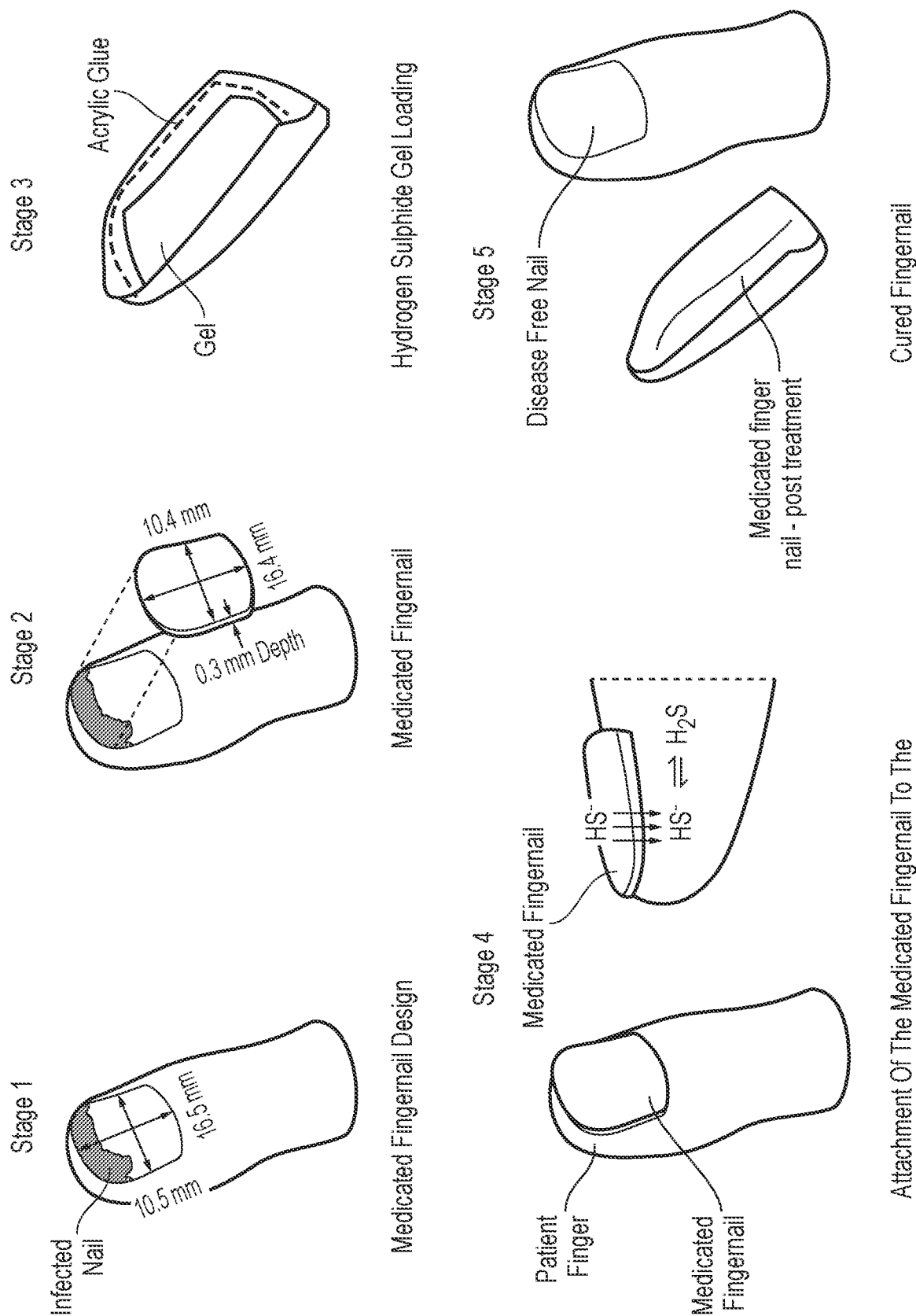


FIG. 1

2/20

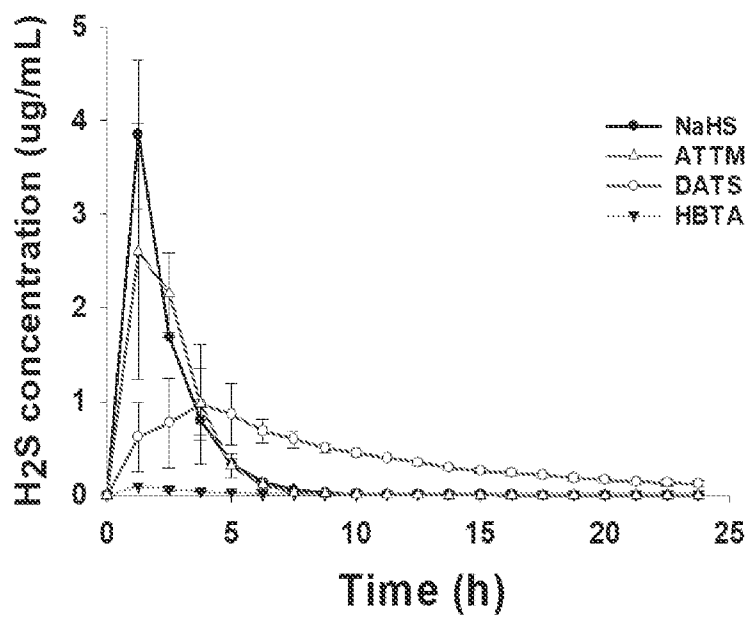


FIG. 2A

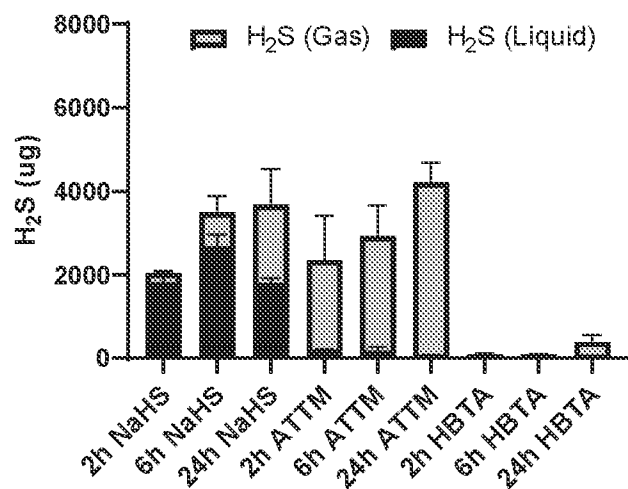


FIG. 2B

3/20

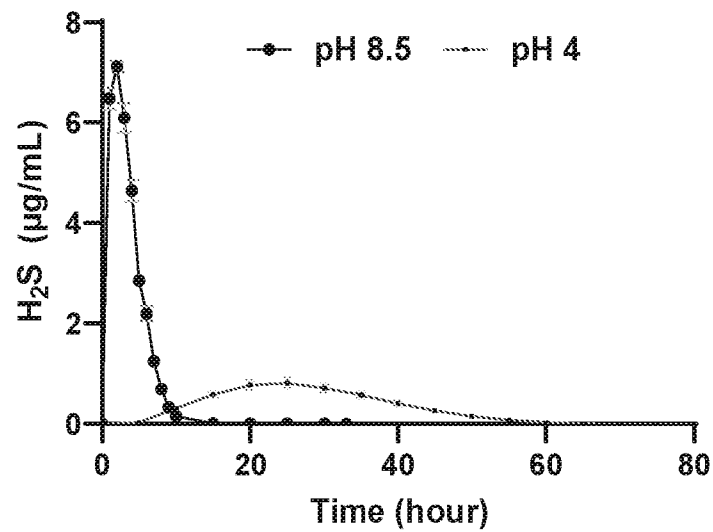


FIG. 3A

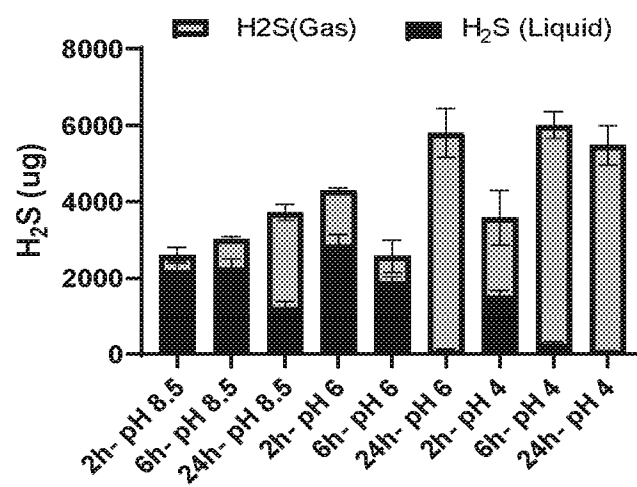


FIG. 3B

4/20

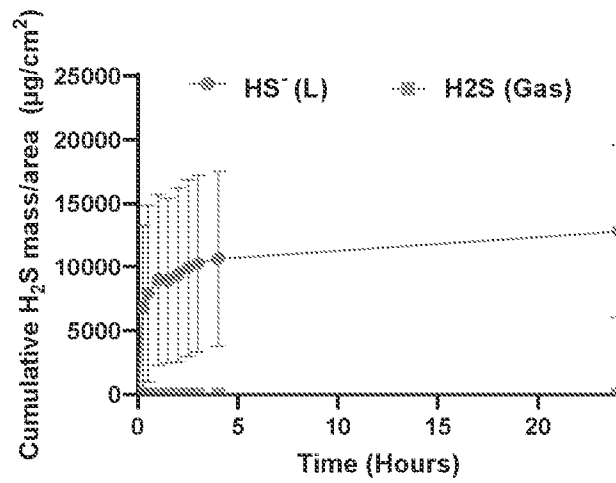


FIG. 4A

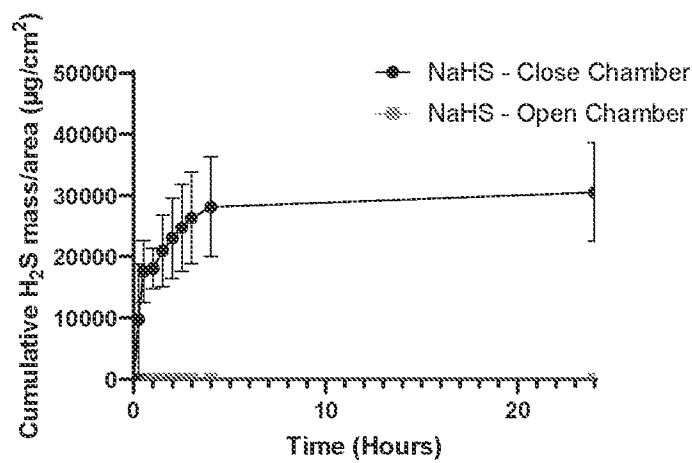


FIG. 4B

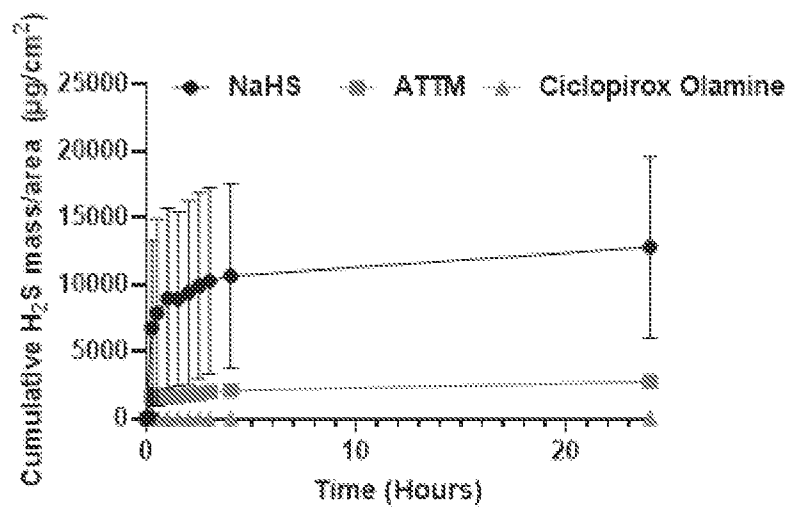


FIG. 4C

5/20

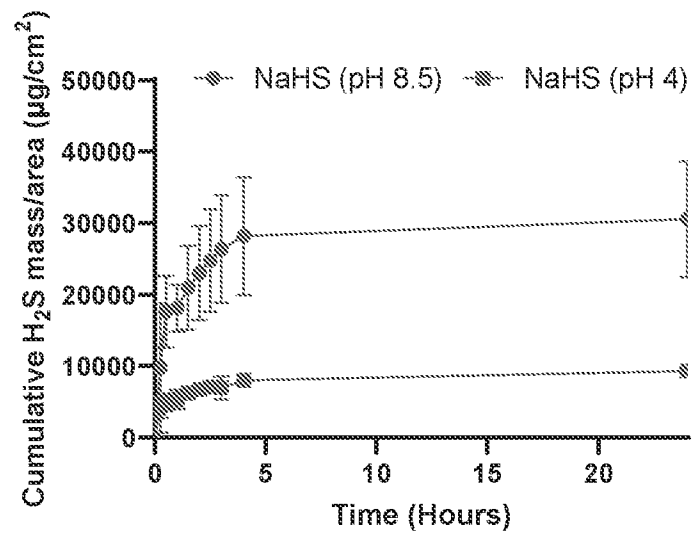


FIG. 5

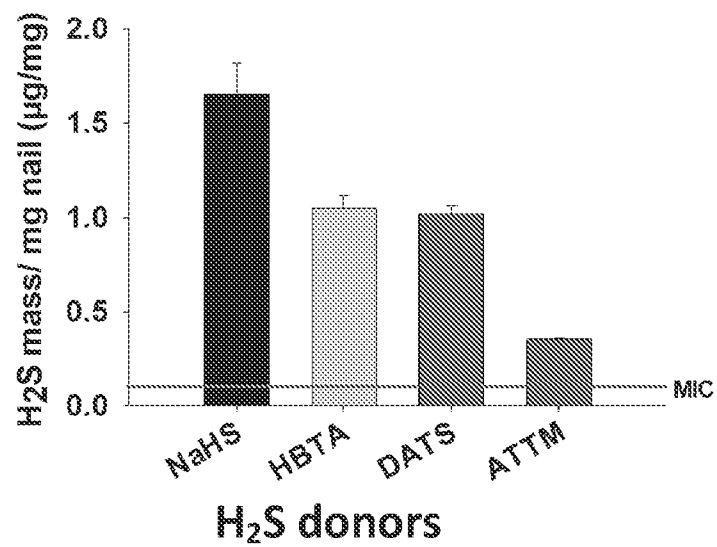


FIG. 6

6/20

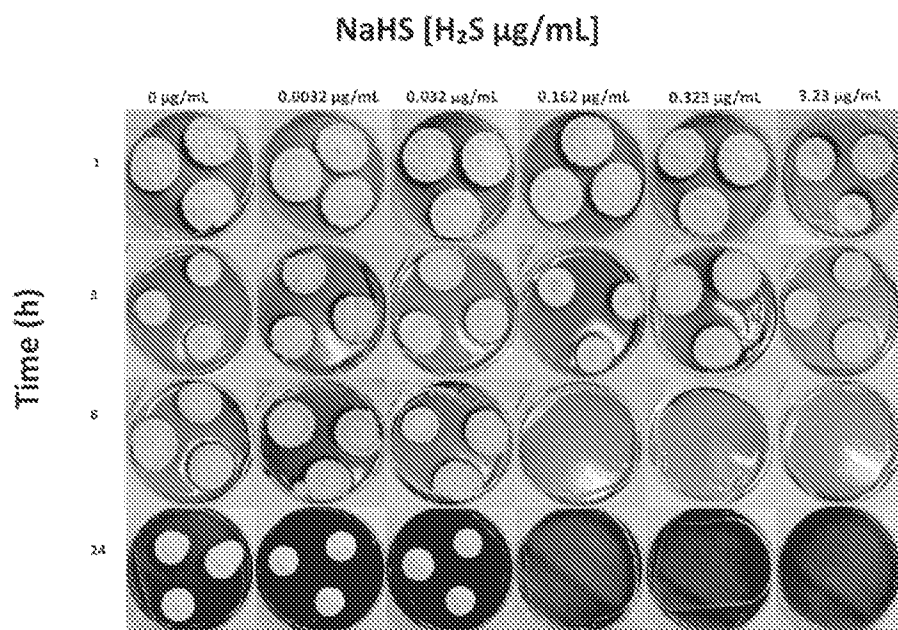


FIG. 7

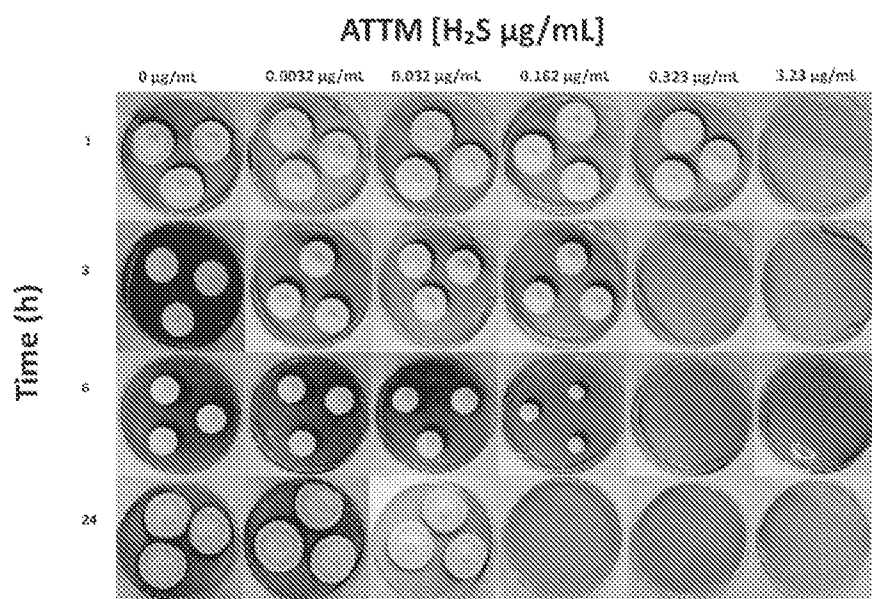


FIG. 8

7/20

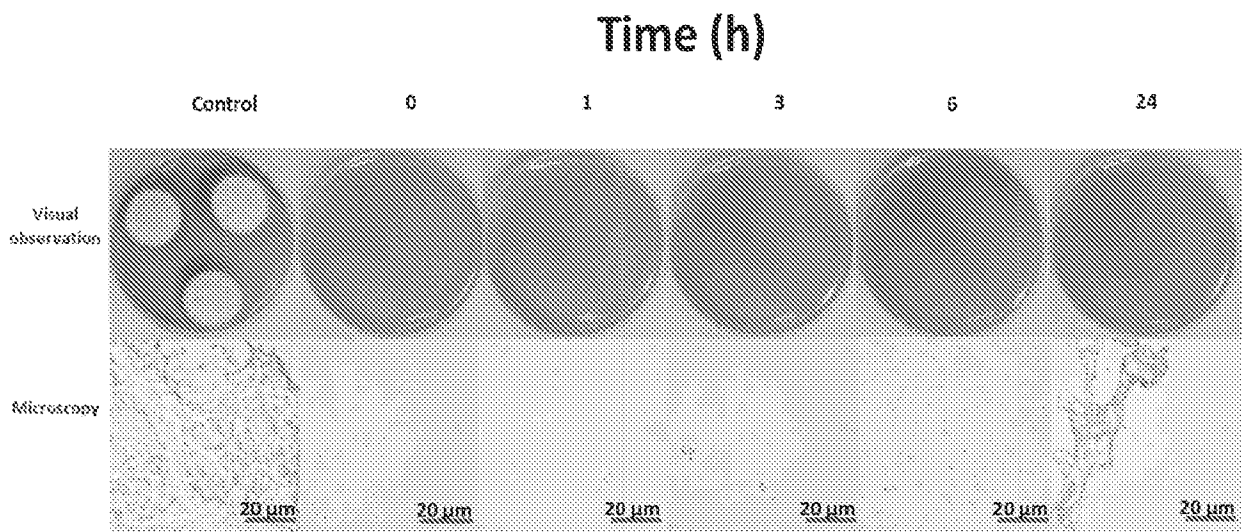


FIG. 9

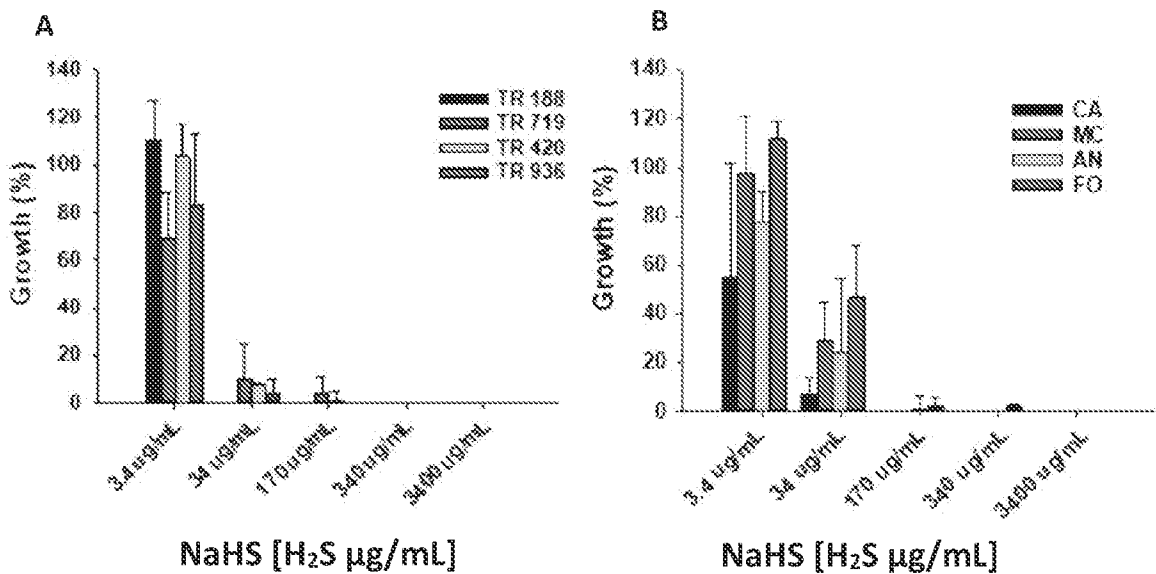


FIG. 10

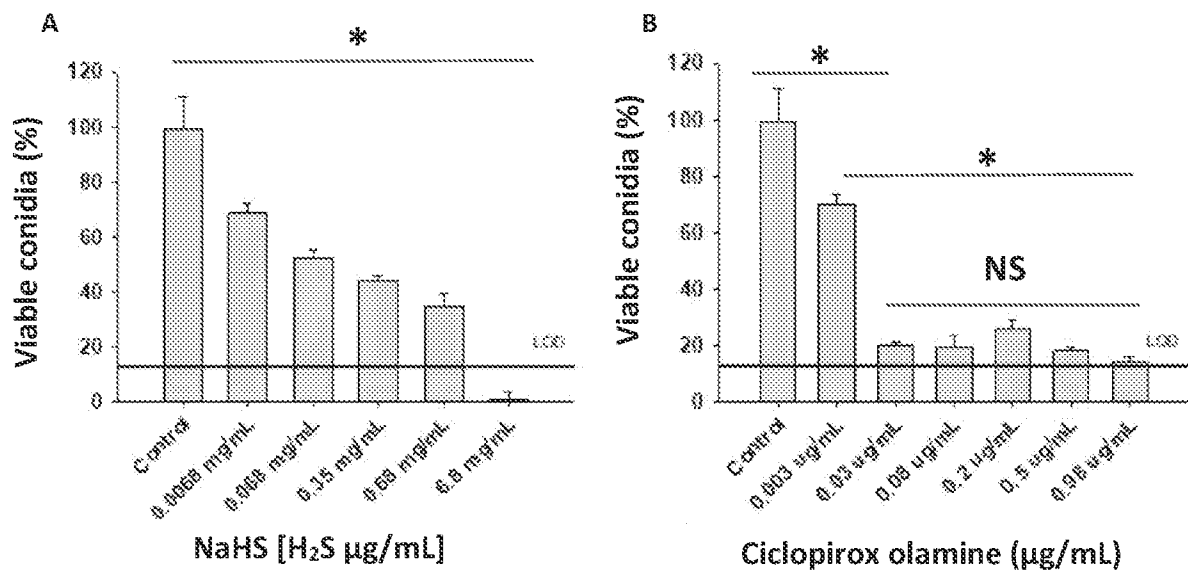


FIG. 11

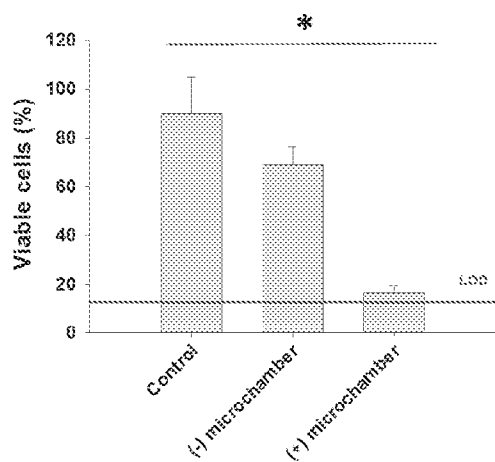


FIG. 12

9/20

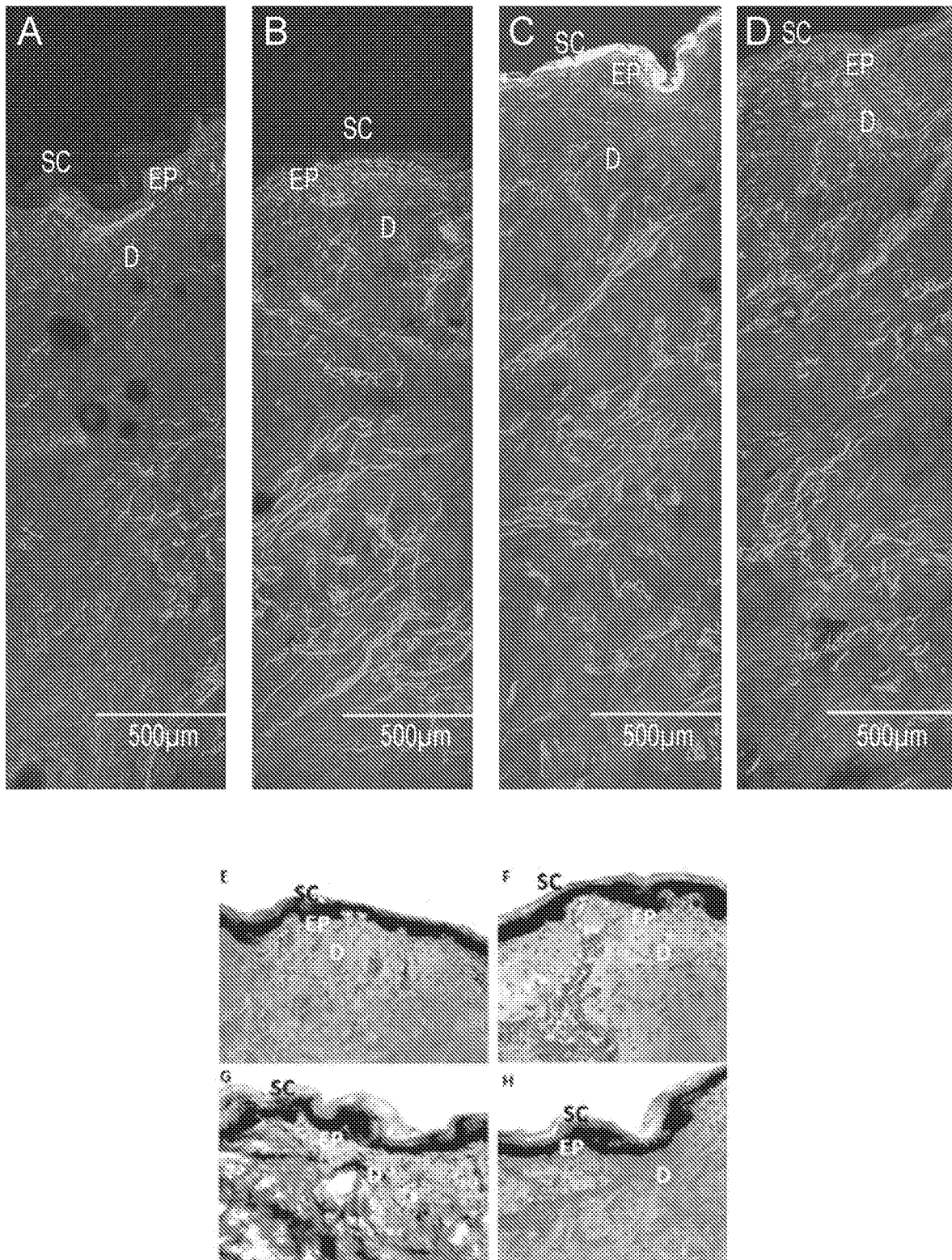


FIG. 13

10/20

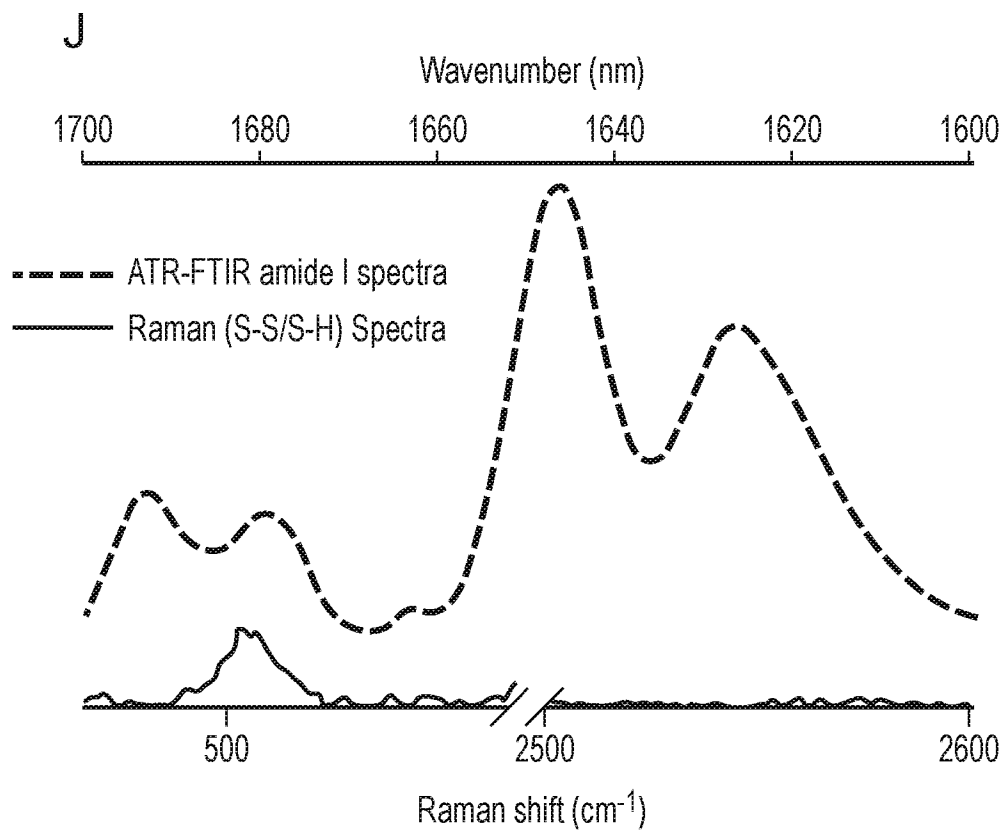
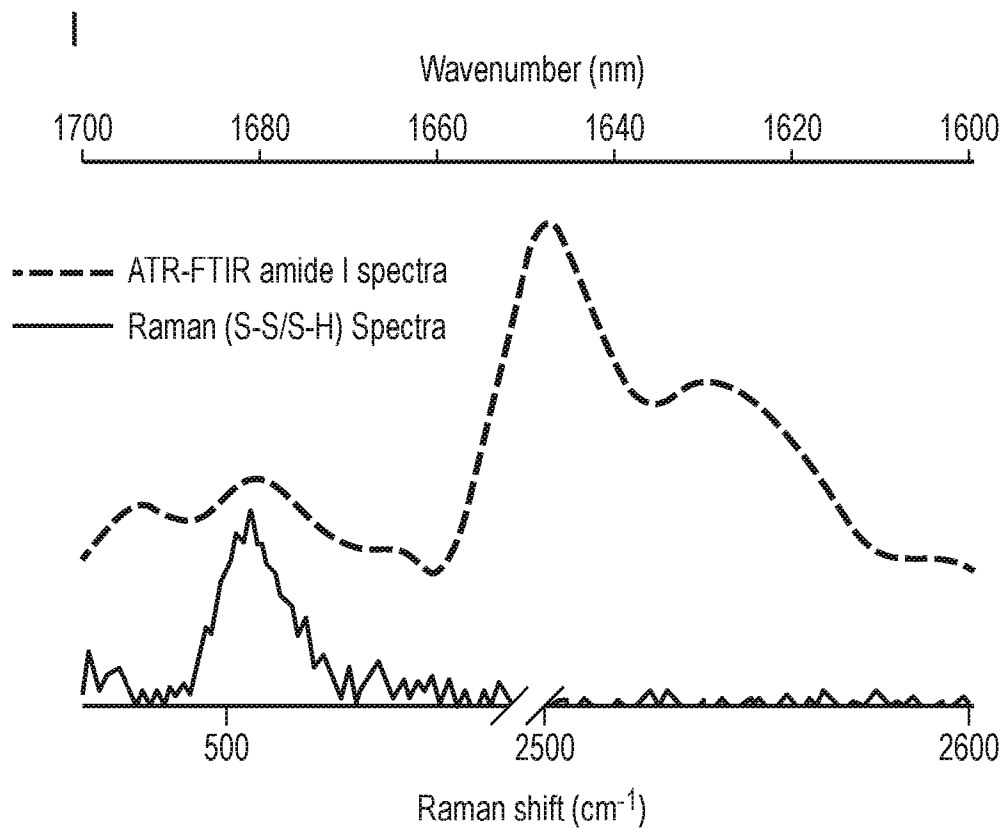


FIG. 13 (Continued)

11/20

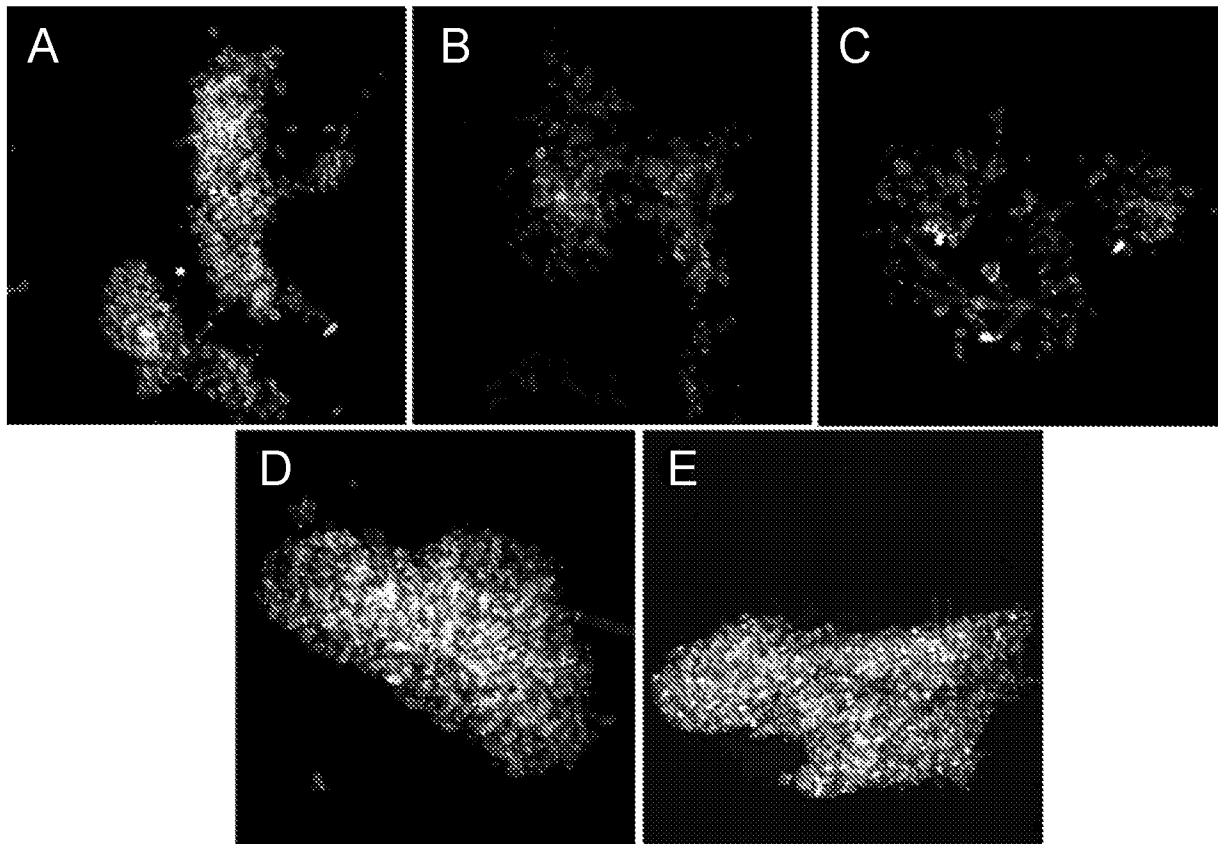


FIG. 14

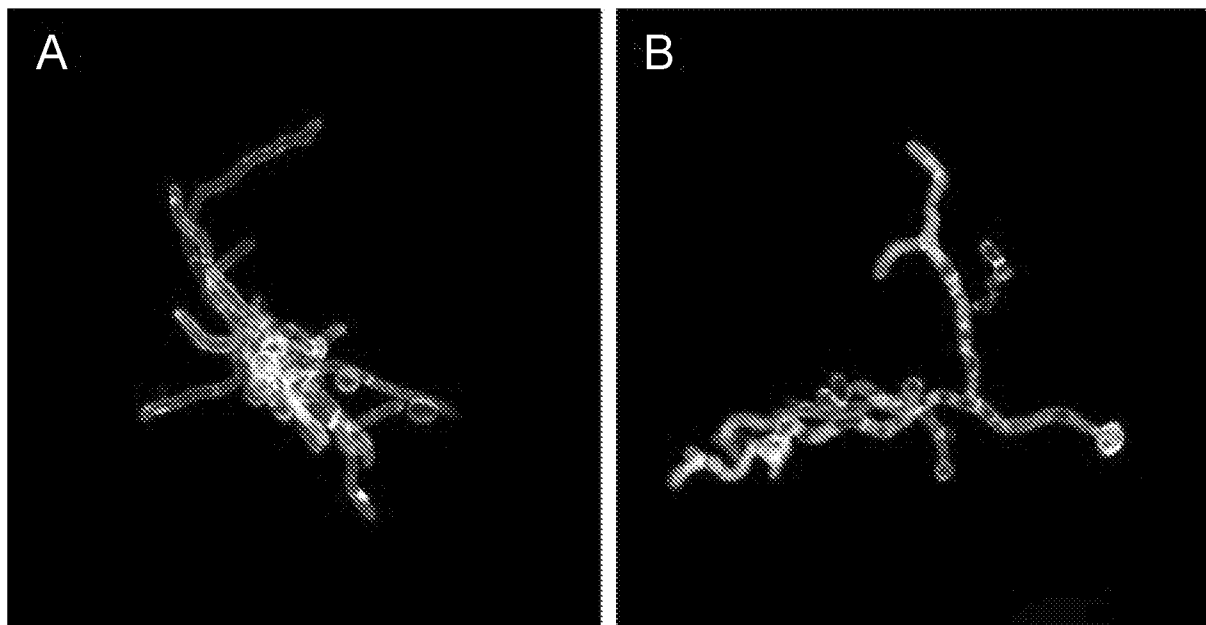


FIG. 15

12/20

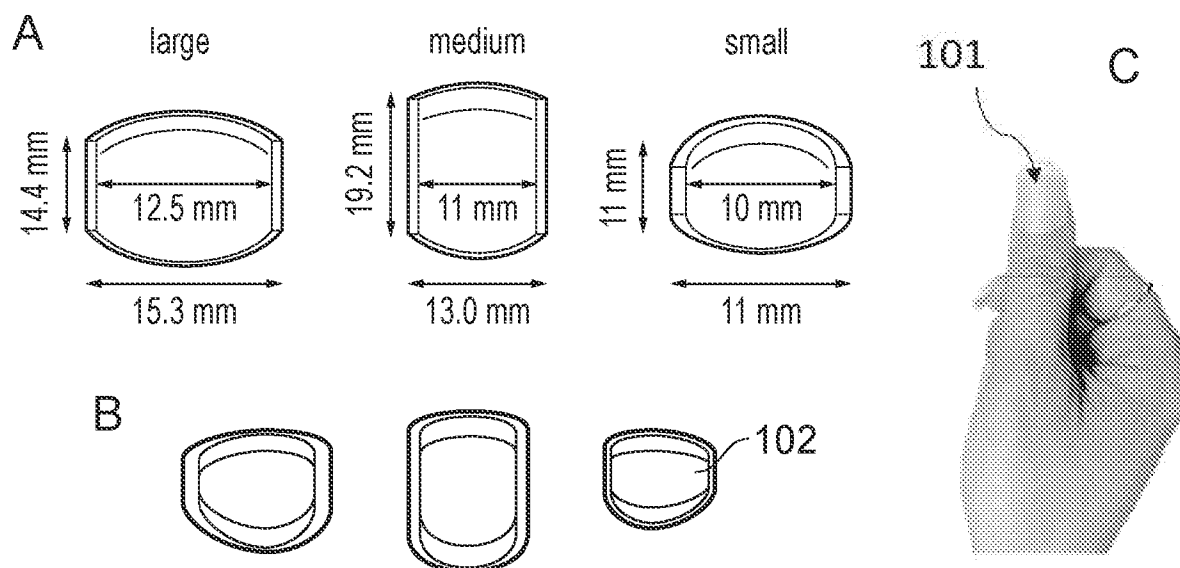


FIG. 16

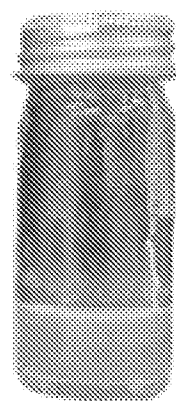
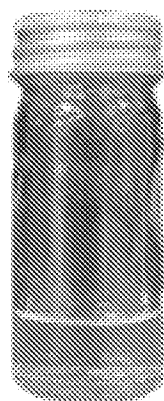
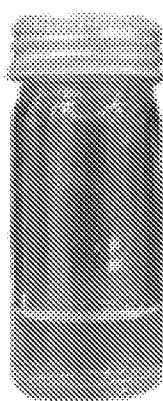
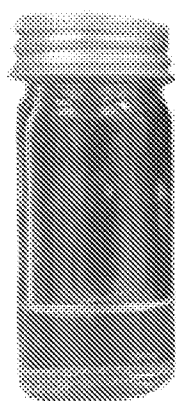
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NM

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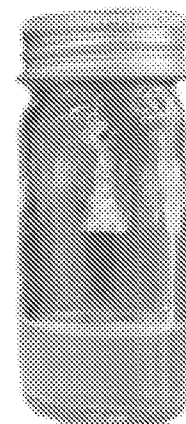
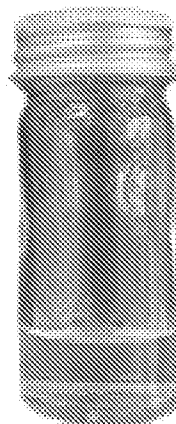
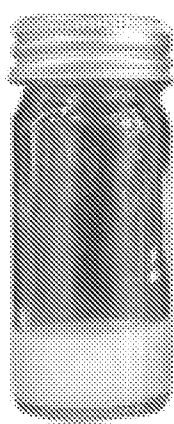
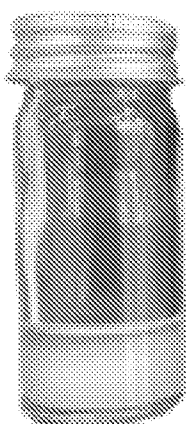


FIG. 17

13/20

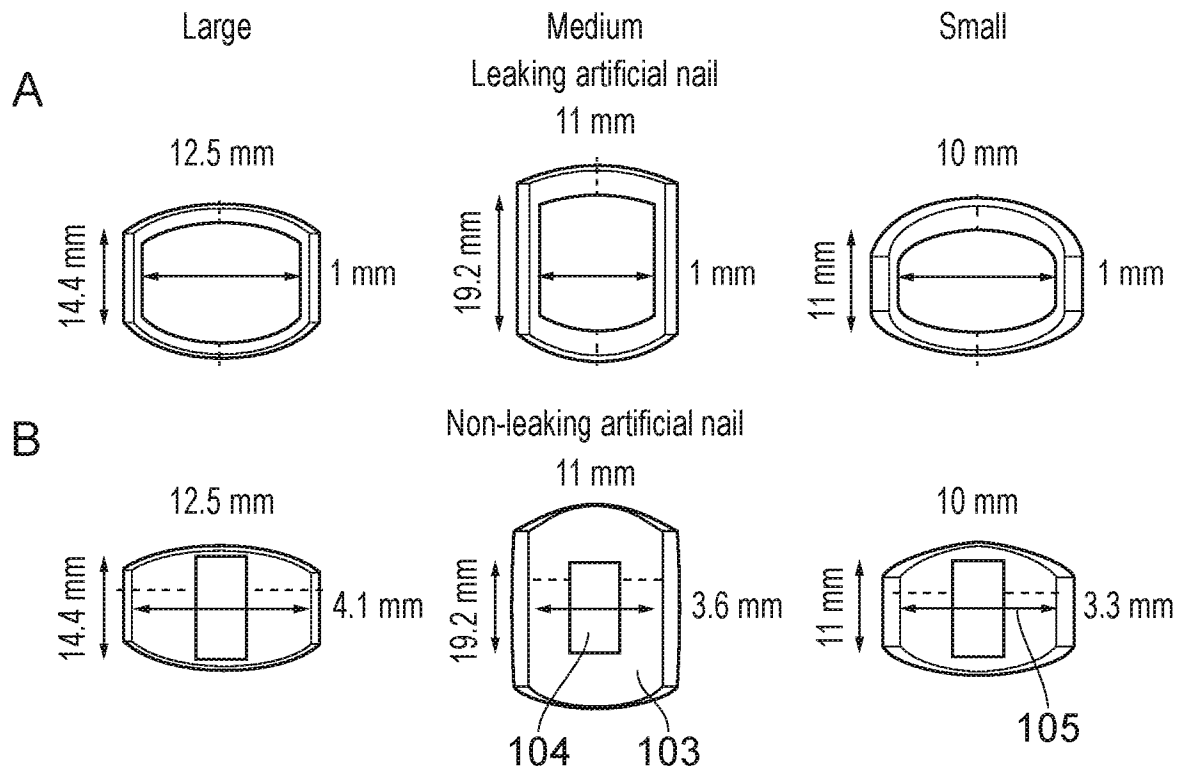


FIG. 18

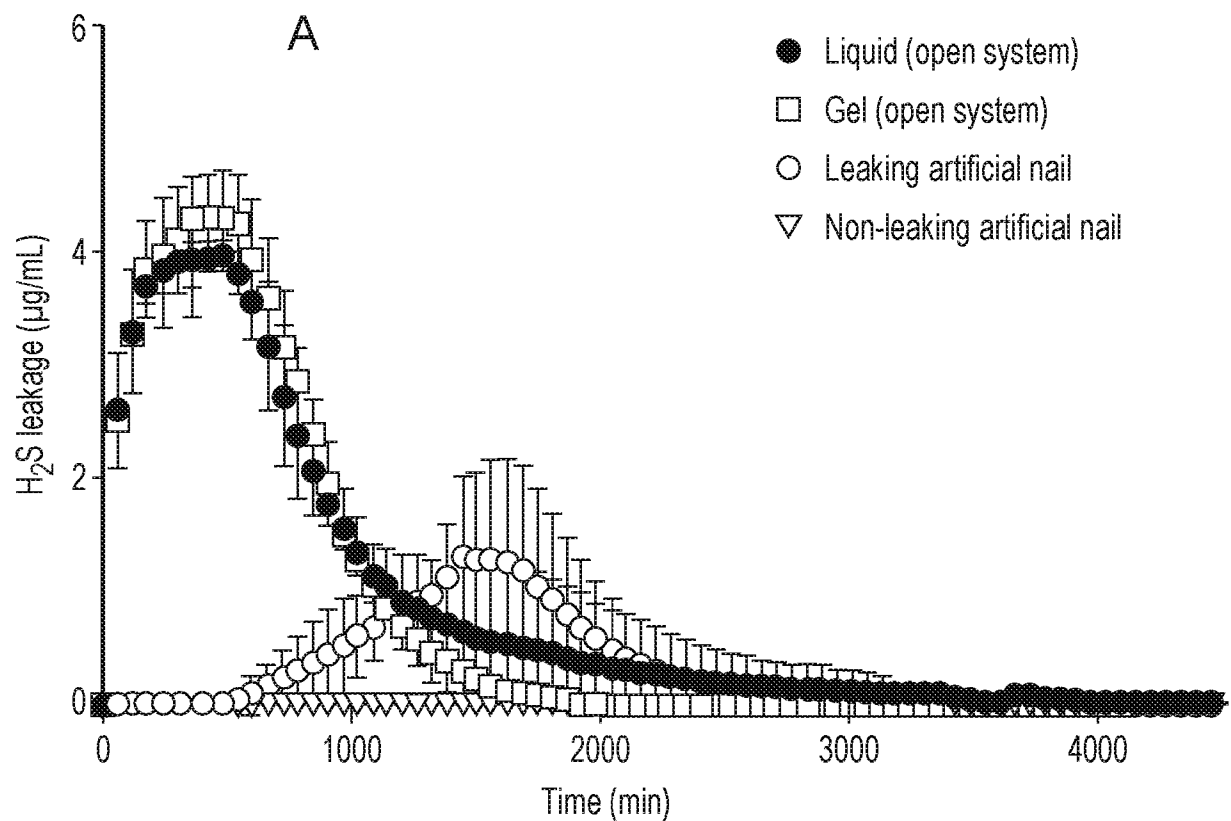


FIG. 19

14/20

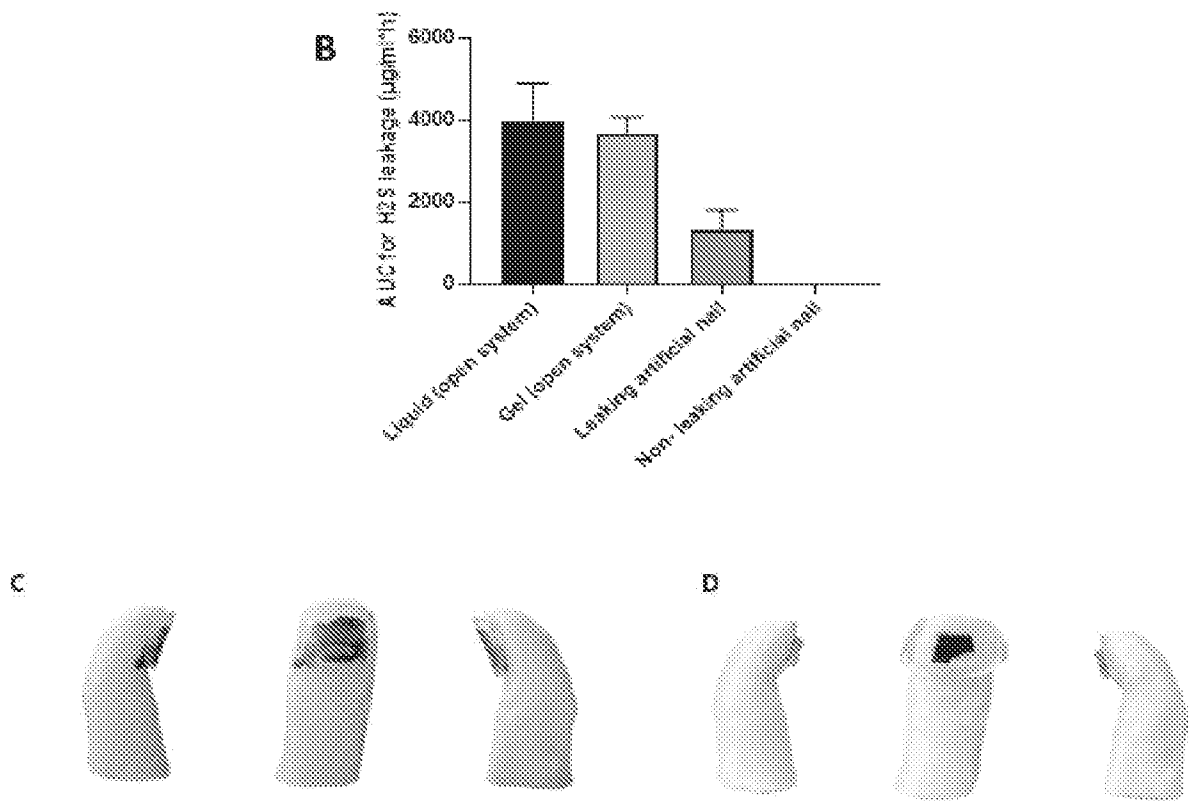


FIG. 19 (Continued)

15/20

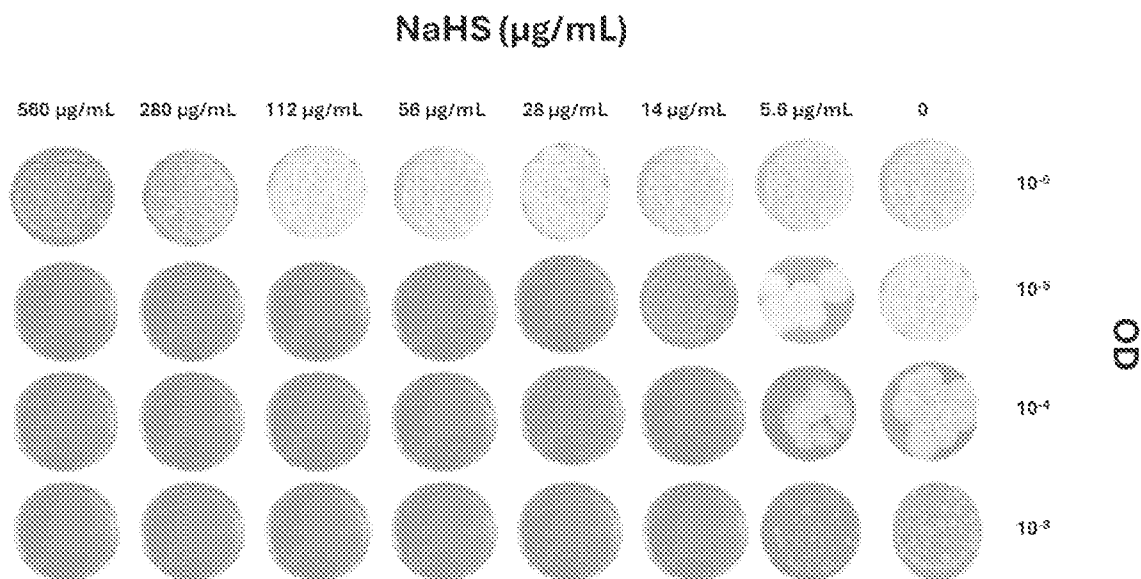


FIG. 20

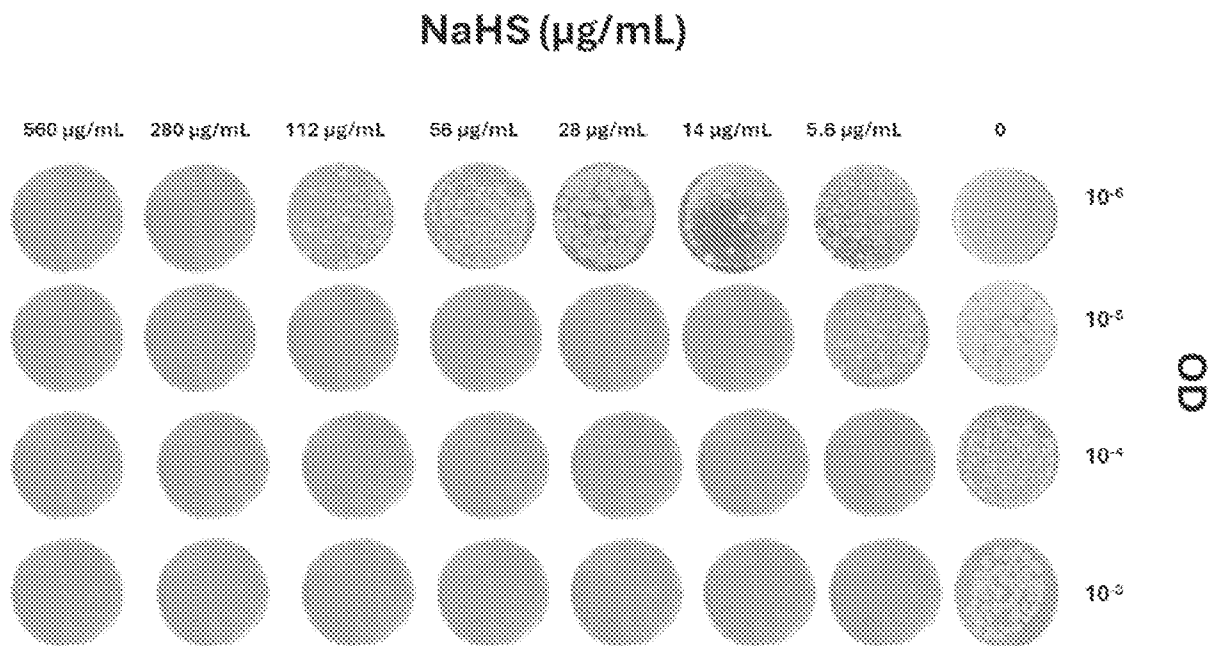


FIG. 21

16/20

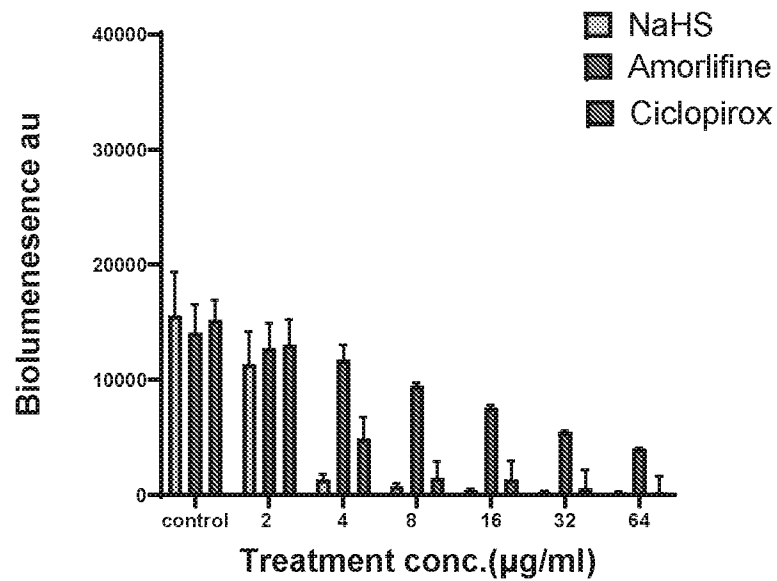


FIG. 22A

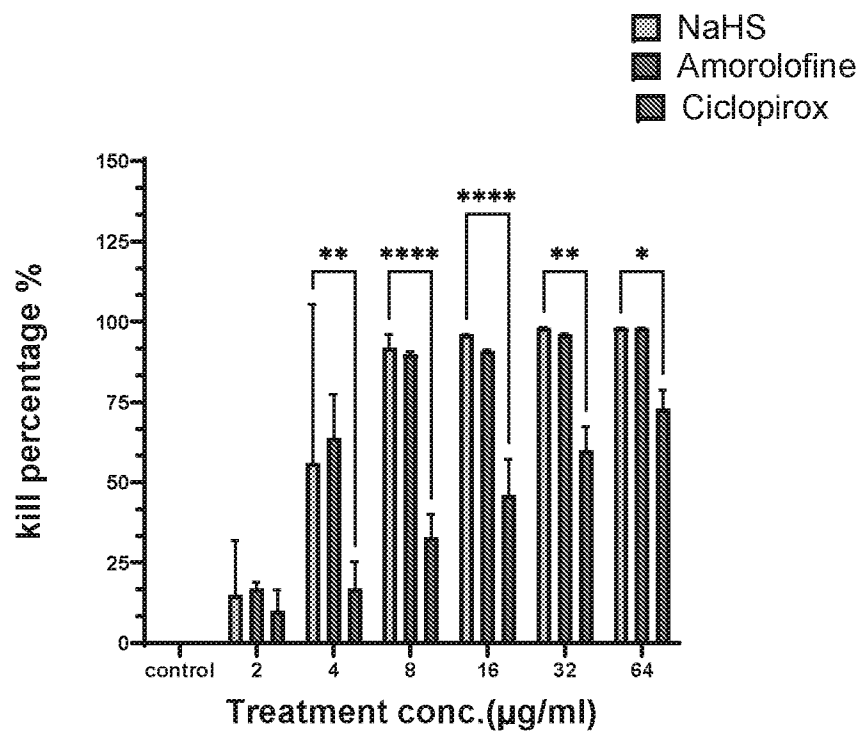


FIG. 22B

17/20

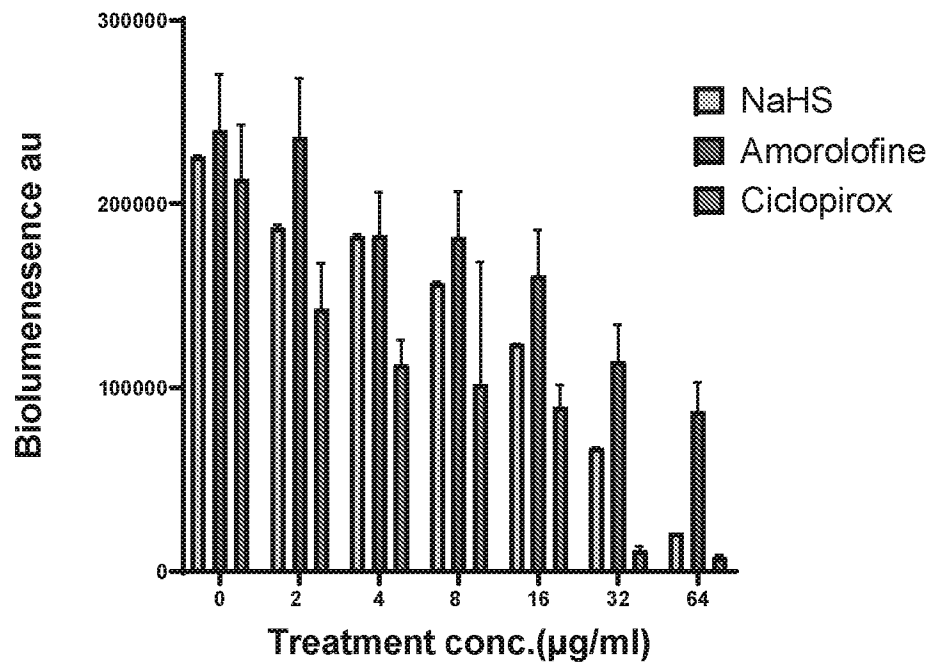


FIG. 23A

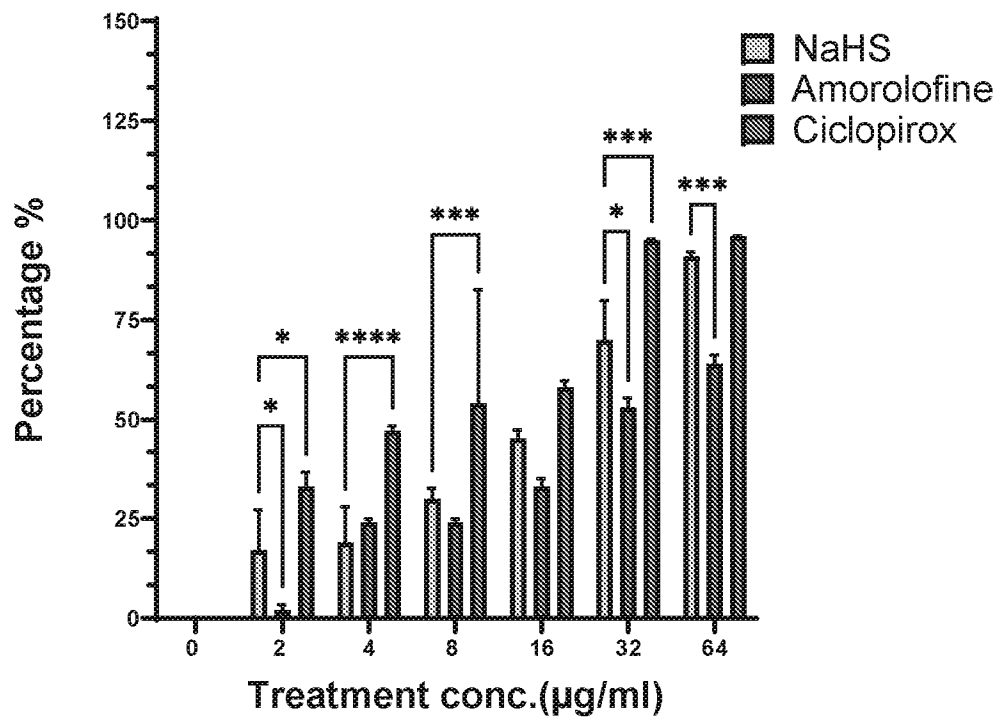


FIG. 23B

18/20

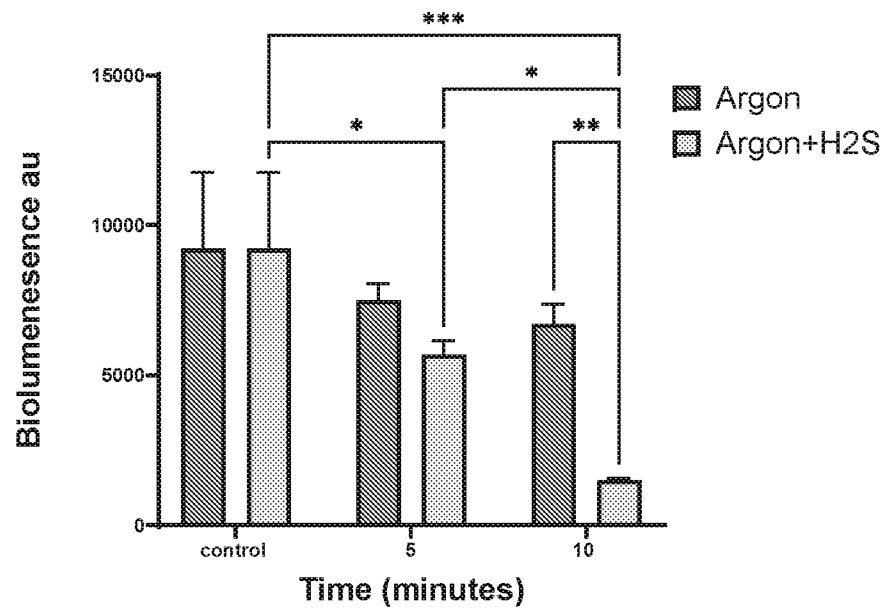


FIG. 24A

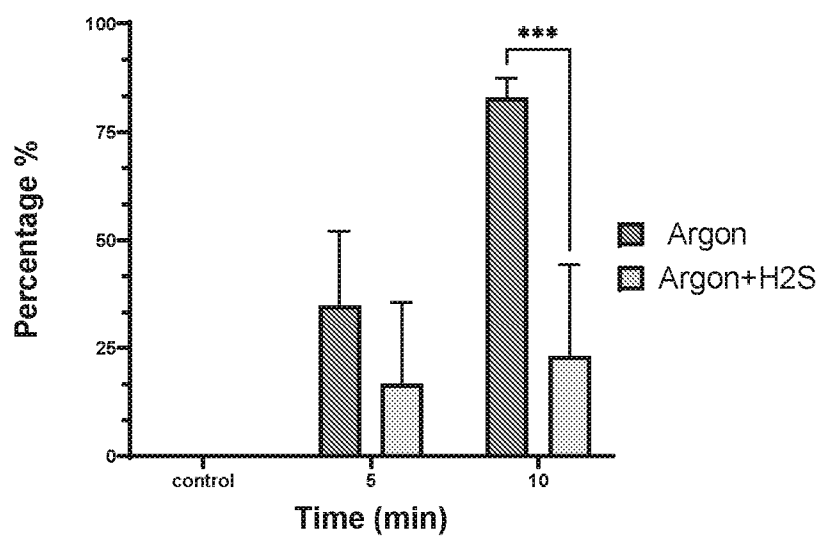


FIG. 24B

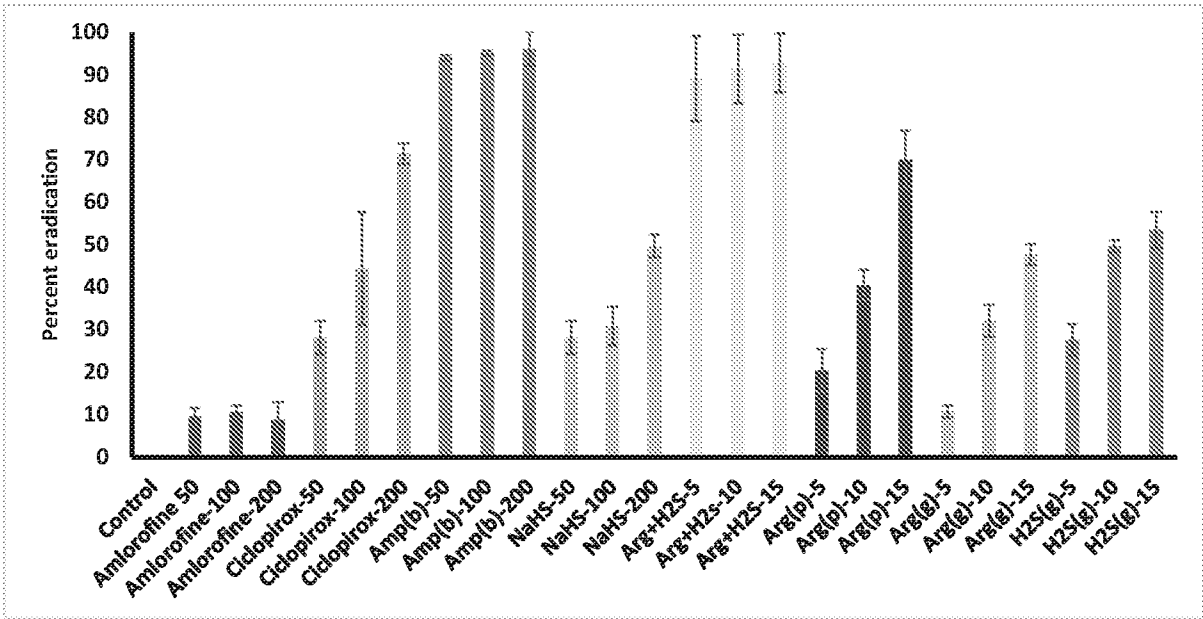


FIG. 25

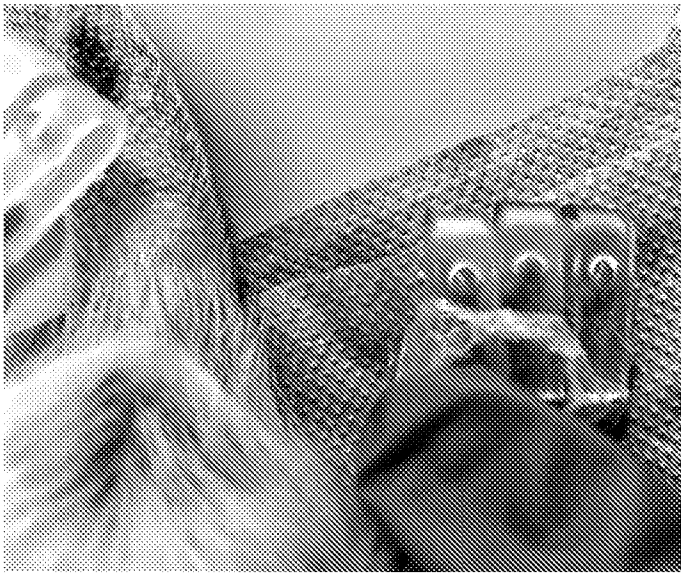
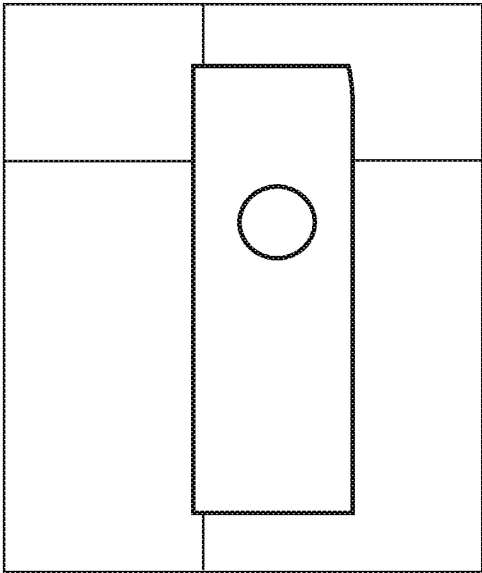


FIG. 26

20/20

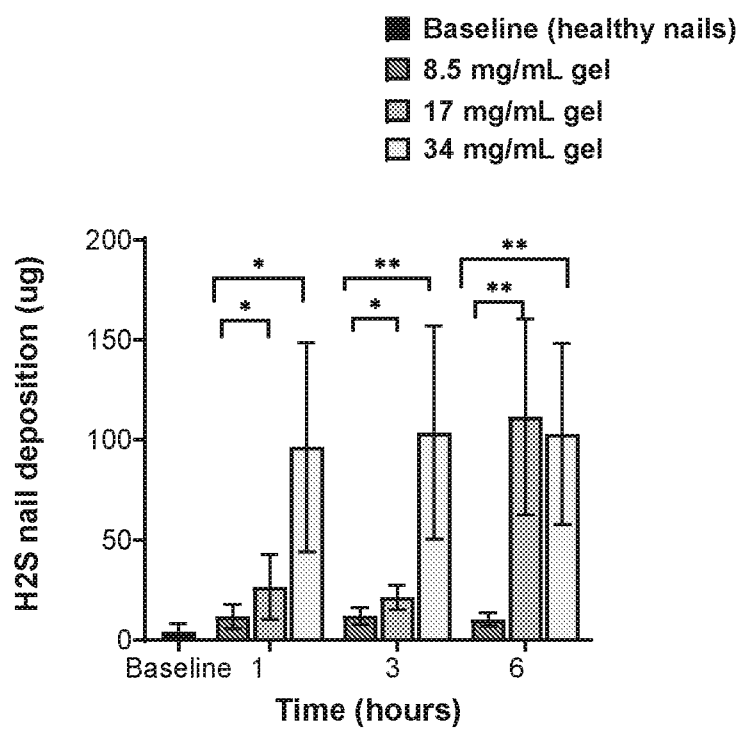


FIG. 27

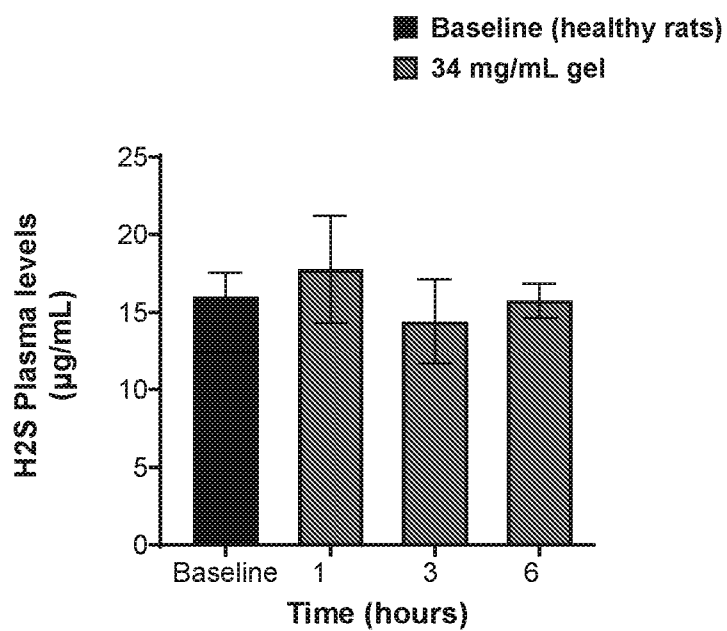


FIG. 28

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2024/051864

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/10 A61K33/04 A61P31/02 A61P31/04 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K A61P Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, CHEM ABS Data, EMBASE, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2017/062311 A1 (HUIZENGA JOEL [US]) 13 April 2017 (2017-04-13) -----	1
A	WO 2014/132083 A2 (UNIV ASTON [GB]) 4 September 2014 (2014-09-04) -----	1
A	MING-JIE WANG ET AL: "Mechanisms of angiogenesis: Role of hydrogen sulphide", CLINICAL AND EXPERIMENTAL PHARMACOLOGY AND PHYSIOLOGY, WILEY-BLACKWELL PUBLISHING ASIA, AU, vol. 37, no. 7, 10 February 2010 (2010-02-10), pages 764-771, XP071594487, ISSN: 0305-1870, DOI: 10.1111/J.1440-1681.2010.05371.X ----- - / - -	1 - 24
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 28 November 2024		Date of mailing of the international search report 09/12/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Bonzano, Camilla

INTERNATIONAL SEARCH REPORT

International application No

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T	MIAOMIAO HE ET AL: "A H₂S-Evolving Alternately-Catalytic Enzyme Bio-Heterojunction with Antibacterial and Macrophage-Reprogramming Activity for All-Stage Infectious Wound Regeneration", ADVANCED MATERIALS, VCH PUBLISHERS, DE, vol. 36, no. 35, 6 July 2024 (2024-07-06), page n/a, XP072693901, ISSN: 0935-9648, DOI: 10.1002/ADMA.202405659 -----	
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International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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