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(54) Title: STERILIZATION OF SELF-ASSEMBLING PEPTIDES BY IRRADIATION

(57) Abstract: Gamma ray and e-beam irradiation provided efficient sterilization of certain self- assembling peptides (including RADA16 in solution) without substantial degradation of the major peptide, while, e.g., another self-assembly peptide, QLEL12 was significantly degraded following irradiation. Irradiation sterilization enhances the rheological property of, for example, RADA16 hydrogel once applied to tissue at a physiological pH. The rheological property increase can result in higher efficacy in a variety of biomedical applications.

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STERILIZATION OF SELF-ASSEMBLING PEPTIDES BY IRRADIATION

Priority

This application claims priority to United States provisional application No. 63/002,882, filed on March 31, 2020.

Field of Invention

This invention relates to sterilization of certain medical gels, more specifically, gels containing so-called self-assembling peptides.

Background of the Invention

Self-assembling peptides (sometimes abbreviated as “SAPs”) are a type of peptides which assemble spontaneously into highly organized nanostructures when placed in aqueous environment and a chemical or physical change in surrounding conditions occur. One other well-known structure is a nanofibrous biopolymer structure formed by natural collagen. A class of SAPs relevant for this invention consists of alternating hydrophilic and hydrophobic amino acid residues capable of forming beta-sheets. They autonomously assemble into well-ordered nanostructures in neutral water, while they can temporarily disassemble into individual molecules when high shearing force is applied to them. SAPs can form a hydrogel (also known as SAP gels) depending on their environment such as pH and/or osmolality; for example, they are capable of forming a hydrogel, when they are placed in the body at near neutral pH,. SAP gels have been previously described as being used for a variety of medical applications, e.g., improved wound healing, inducement of homeostasis; reduction of adhesion in interior tissues, particularly, in context of surgery; as temporary tissue-void

matrix fillers, facilitating ingrowth of natural tissue into such a void. Particular SAPs are described in US Patents Nos. 5,670,483; 5,955,343; 9,724,448; 10,596, 225 and Int'l Pat. Appln. Pub. WO2014/136081; and foreign equivalents thereof.

Sterilization is a very important step in the manufacturing process for most biomaterials, including for self-assembling peptide solutions. PuraStat® (RADA16 = Ac-RADARADARADARADA-NH_2 = SEQ ID NO:1; about 2.5%) is customarily filtered for sterilization (see, e.g., Int'l. Pat. Appln. Pub. No. WO 2014/008400); however, PuraStat®'s viscosity at higher concentrations is the main obstacle to its filtration, which in addition to losses in the tubing, results in substantial peptide losses. In this method for sterilizing, a solution of SAPs is forced through a porous filter, wherein the sterilizing filter has an average pore size of 0.22 µm (US Pat. Appln. No. 2015/019735). As another method, some thermally stable self-assembling peptide solutions can be sterilized by autoclaving treatment at about 121 °C for about 25 minutes (US Pat. Appln. No. 10/369,237).

Furthermore, an additional ethylene oxide sterilization step is required for the outer part of PuraStat® products. And, it was discovered that autoclaving cannot be used for some SAPs, such as RADA16 in solution, because of its complete thermal degradation (US Pat. Appln. Pub. No. 2017/0202986). Thus, another new sterilization method has been needed to reduce losses, particularly, for RADA16 and other SAPs.

Gamma irradiation sterilization of self-assembling peptides including RADA16 was described in passing in a prior publication (US Pat. Appln. Pub. No. 2016/0317607; at paragraph [0052]); but no specific conditions or actual effect of irradiation on the structure and properties of self-assembling peptides, including RADA16, have been

described so far. In fact, peptide structure can be changed by the reactive radical species generated by irradiation such as gamma ray, X-ray, and e-beam. Such a structural change can be governed by multiple factors including the amino acid composition, reactive residue position and possibly the conformation acquired by each macromolecule (Vieira R et al, Biol. Pharm. Bull. 2013, 36(4) 664-675). For example, regardless of the different sequences of the peptides in that article, all the tested nine peptides showed a progressive degradation by gamma ray irradiation up to 15 kGy. Considering that even higher doses of irradiation than 15 kGy may be required for sterilization process, numerous peptides cannot be sterilized with gamma ray irradiation without substantial degradation. It is well known that the side chains of aromatic amino acids (i.e., Phenylalanine (F), Tyrosine (Y), Tryptophan (W), Histidine (H), and Proline (P)) and sulfur-containing amino acids (i.e., Cysteine (C)) are especially weak to attack by reactive radical species (Annu. Rev. Biochem., 62, 797–821 (1993) and Vieira R et al, Biol. Pharm. Bull. 2013, 36(4) 664-675). By way of background, the followings SAPs, RADA16 (Ac-RADARADARADARADA-NH_2 (SEQ ID NO:1)), KLD12 (Ac-KLDLKLDLKLDL-NH_2 (SEQ ID NO:2)), and IEIK13 (Ac-IEIKIEIKIEIKI-NH_2 (SEQ ID NO:3)), do not include aromatic amino acids or sulfur-containing amino acids.

On the other hand, irradiation sterilization can also affect the secondary structure of self-assembling peptides and their fibrous structure. As mentioned, RADA16, KLD12 and IEIK13 have beta-sheet conformation, and these molecules self-assemble to form an ordered nanofibrous structure. Irradiation sterilization process may potentially change the secondary structure and the nanofiber structure of peptide, which could then result in the undesirable change of its rheological properties.

Thus, there exists a need for new sterilization methods that work advantageously with self-assembling peptides. For the reasons cited above, sterilization by irradiation has been avoided so far.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Appearance of PuraStat® (RADA16 2.5%) and IEIK13 1.3% before (Figure 1A) and after gamma-irradiation at 28 kGy (Figure 1B).

Figure 2. Mass spectra of PuraStat® (RADA16 2.5%) (A) before gamma irradiation, (B) after gamma irradiation at 40 kGy, and (C) after autoclaving at 121 ºC for 20 min.

Figure 3. Mass spectra of PuraStat® (RADA16 2.5%) (A) after X-ray irradiation at 25 kGy and (B) after X-ray irradiation at 40 kGy.

Figure 4. Mass spectra of PuraStat® (RADA16 2.5%) (A) after e-beam irradiation at 25 kGy and (B) after e-beam irradiation at 40 kGy.

Figure 5. Mass spectra of IEIK13 1.3% (A) before irradiation; (B) after gamma irradiation at 40 kGy; (C) after X-ray irradiation at 25 kGy; (D) after X-ray irradiation at 40 kGy; (E) after e-beam irradiation at 25 kGy; and (E) after e-beam irradiation at 40 kGy.

Figure 6. Mass spectra of QLEL12 0.15% (A) before irradiation; (B) after gamma irradiation at 23 kGy; (C) after X-ray irradiation at 25 kGy, (D) after X-ray irradiation at 40 kGy, (E) after e-beam irradiation at 25 kGy, and (F) after e-beam irradiation at 40 kGy.

Figure 7. Frequency test of PuraStat® (RADA16 2.5%) with gamma irradiation at 0.1% of strain with 40 mm cone-plate (N=3, bars represent SD).

Figure 8. Frequency test of PuraStat® (RADA16 2.5%) with gamma irradiation after gelation at 0.1% of strain with 40 mm cone-plate. Samples were treated with DMEM for 20 min (N=3, bars represent SD).

Figure 9. Thixotropic test of PuraStat® (RADA16 2.5%) with gamma irradiation at 1 Hz of frequency and 0.1% of strain with 40 mm cone-plate. Initial storage modulus (G') was measured for 1 min before 1st shearing at 1000 s^{-1}. After the 1st shearing for 1 minute, G' recovery was recorded for 1 hour to exhibit the thixotropic behavior of PuraStat®. This test was duplicated.

Figure 10. High Resolution MS (current PuraStat® products (non-irradiated), proposed PuraStat® products (irradiated)).

SUMMARY OF THE INVENTION

In the present disclosure, the effect of sterilization by irradiation on self-assembling peptide solutions was evaluated, specifically, with the following peptides: PuraStat® (Ac-RADARADARADARADA-NH_2, RADA16 (SEQ ID NO:1), IEIK13 (Ac-IEIKIEIKIEIKI-NH_2 (SEQ ID NO:3)), and QLEL12 (Ac-QLELQLELQLEL-NH_2 (SEQ ID NO:4)). It was unexpectedly found that gamma-irradiation sterilization enhanced the rheological properties of certain self-assembling peptide solutions and hydrogels without the anticipated noteworthy degradation, while certain other peptides showed the expected significant degradation and viscosity drop after gamma irradiation (i.e., unlike

RADA16 and IEIK13), while, e.g., another self-assembly peptide, QLEL12 (Ac-QLELQLELQLEL-NH_2 (SEQ ID NO:4) was significantly degraded following irradiation. This invention is further based on the prophetic finding that the same result can be expected with other similar irradiation sterilization methods including, specifically, X-ray and e-beam, at least for the respective peptides. Thus, in some embodiments, the method of sterilizing a self-assembling peptide solution comprises:

a) placing one or more containers with a solution of self-assembling peptide into an irradiation machine, said self-assembling peptide capable of forming a hydrogel when applied to a biological tissue at about neutral pH; and
b) exposing the container to gamma ray, X-ray and/or e-beam irradiation at a predetermined dose so that the peptide solution is sterilized without substantial degradation of the peptide while its desired biological and/or rheological property(ies) is/are maintained at the same level or improved.

In some embodiments, the peptides are selected from the group consisting of RADA16, KLD12, and IEIK13. In related embodiments, such peptides are exposed to the dose is 15 – 50 kGy, preferably 15 – 40 kGy, more preferably, to a minimum dose resulting in a desired sterility assurance level (SAL), without substantial degradation and/or substantial negative change in biological properties of these peptides. In some embodiments, the peptide solution is irradiated by gamma-rays, X-rays, or e-beam. In some embodiments, the over-all degradation of the total peptides in solution after irradiation does not exceed 20%, more preferably, 10%, most preferably 5%, of the amount of peptides prior to irradiation. In some embodiments, the desired biological or physical property(ies) is/are selected from the group consisting of: hemostatic, anti-

adhesion, prevention of re-bleeding, anti-stenosis, tissue occlusion, storage modulus (e.g., in some embodiments, the storage modulus of the gelled solution is increased at least by 10%, at least by 15% or at least by 20% post-irradiation, and viscosity, and tissue void filling property are maintained within acceptable or improved parameters after irradiation. In some embodiments, irradiation dose achieves sterility assurance level (SAL) of at least 10^{-5}, preferably 10^{-6}, or less. In other embodiments, using the bioburden testing described below in the example, the acceptable level of contamination of the peptide solution pre-irradiation is 1000, 500,100, 15, 10, 9, 5, 2, 1.5, 1 CFU, or less. In some embodiments, the concentration of the degradation products of the intact ("major" or "full-length") peptide in the solution post-irradiation ranges from 0.1% to 5%. In some embodiments, the pH of the peptide solution post-irradiation ranges from about 1.8 to 3.5. In some embodiments, the solution container is a plastic syringe, with or without an adapter nozzle. In such embodiments, care is taken to ensure that the plastic and rubber parts of the packaging also maintain their desired physical properties. While some yellowing of the plastic syringes may be expected and is normal, any rubberized material must preserve its e plastic properties at an acceptable level.

In some embodiments, the gelled solution is further subjected to sheering to reduce or restore its storage modulus.

Thus, the invention provides a sterilization method for self-assembling peptides. According to the methods of the invention, in some embodiments, the solution is further applied the solution to a biological tissue, for example, during surgery, or after trauma involving bleeding.

DETAILED DESCRIPTION OF THE INVENTION

As stated above, in the present disclosure, the effect of sterilization by irradiation on self-assembling peptide solutions was evaluated, specifically, with the following peptides: PuraStat® (Ac-RADARADARADARADA-NH_2 (SEQ ID NO:1); RADA16), IEIK13 (Ac-IEIKIEIKIEIKI-NH_2 (SEQ ID NO:3)), and QLEL12 (Ac-QLELQLELQLEL-NH_2 (SEQ ID NO:4)). In some embodiments, the method of sterilizing a self-assembling peptide solution comprises:

a) placing one or more containers with a solution of self-assembling peptide into an irradiation machine, said self-assembling peptide capable of forming a hydrogel when applied to a biological tissue (e.g., *in situ*) at about neutral pH; and

b) exposing the container to gamma ray, X-ray and/or e-beam irradiation at a predetermined dose so that the peptide solution is sterilized to a pre-determined Sterility Assurance Level (SAL) without substantial degradation of the peptide while its desired biological and/or physical property(ies) is/are maintained substantially at the same level or improved.

It was unexpectedly found that gamma-irradiation sterilization enhanced the rheological properties of certain self-assembling peptide solutions and hydrogels without causing any noteworthy degradation, while certain other peptides showed significant degradation and viscosity drop after gamma irradiation, i.e., unlike RADA16 and IEIK13, another self-assembling peptide, QLEL12 (Ac-QLELQLELQLEL-NH_2 (SEQ ID NO:4)) was significantly degraded following irradiation. This invention is further based on the prophetic finding that the same result can be expected with other similar irradiation

sterilization methods including, specifically, X-ray and e-beam, at least for the respective peptides. In preferred embodiments, the composition and methods of the invention maintain or improve the desired biological property(ies) such as hemostatic, anti-adhesion, prevention of re-bleeding, anti-stenosis, tissue occlusion, storage modulus, viscosity, and tissue void filling property, etc. For example, in some embodiments, the storage modulus in increased by at least 5%, 10%, 15%, 20%, or more. If such increase is undesirable for certain applications, the gel can be thinned further by dilution or sheering by methods known in the art, turning it into the solution or otherwise reducing its storage modulus. In certain embodiments, the irradiated solution of SAPs remains clear and viscous.

In some embodiments, the SAPs comprise a sequence of amino acid residues conforming to one or more of Formulas I-IV:

$((Xaa^{neu}\text{-}Xaa^{+})_x(Xaa^{neu}\text{-}Xaa^{-})_y)_n$ (I)

$((Xaa^{neu}\text{-}Xaa^{-})_x(Xaa^{neu}\text{-}Xaa^{+})_y)_n$ (II)

$((Xaa^{+}\text{-}Xaa^{neu})_x(Xaa^{-}\text{-}Xaa^{neu})_y)_n$ (III)

$((Xaa^{-}\text{-}Xaa^{neu})_x(Xaa^{+}\text{-}Xaa^{neu})_y)_n$ (IV)

Xaa^{neu} represents an amino acid residue having a neutral charge; Xaa^{+} represents an amino acid residue having a positive charge; Xaa^{-} represents an amino acid residue having a negative charge; x and y are integers having a value of 1, 2, 3, or 4, independently; and n is an integer having a value of 1-5.

In some embodiments, the SAPs further comprise an amino acid sequence that interacts with the extracellular matrix, wherein the amino acid sequence anchors the SAPs to the extracellular matrix.

In some embodiments, the amino acid residues in the SAPs can be naturally occurring or non-naturally occurring amino acid residues. Naturally occurring amino acids can include amino acid residues encoded by the standard genetic code as well as non-standard amino acids (e.g., amino acids having the D-configuration instead of the L-configuration), as well as those amino acids that can be formed by modifications of standard amino acids (e.g., pyrolysine or selenocysteine). Suitable non-naturally occurring amino acids include, but are not limited to, D-alloisoleucine(2R,3S)-2-amino-3-methylpentanoic acid, L-cyclopentyl glycine (S)-2-amino-2-cyclopentyl acetic acid.

In other embodiments, another class of materials that can self-assemble are peptidomimetics. Peptidomimetics, as used herein, refers to molecules which mimic peptide structure. Peptidomimetics have general features analogous to their parent structures, polypeptides, such as amphiphilicity. Examples of such peptidomimetic materials are described in Moore et al., Chem. Rev. 101(12), 3893-4012 (2001). The peptidomimetic materials can be classified into four categories: α-peptides, β-peptides, γ-peptides, and δ-peptides. Copolymers of these peptides can also be used. Examples of α-peptide peptidomimetics include, but are not limited to, N,N′-linked oligoureas, oligopyrrolinones, oxazolidin-2-ones, azatides and azapeptides. Examples of β-peptides include, but are not limited to, β-peptide foldamers, α-aminoxy acids, sulfur-containing β-peptide analogues, and hydrazino peptides. Examples of γ-peptides include, but are not limited to, γ-peptide foldamers, oligoureas, oligocarbamates, and phosphodiesters. Examples of δ-peptides include, but are not limited to, alkene-based δ-amino acids and carbopeptoids, such as pyranose-based carbopeptoids and furanose-based carbopeptoids.

In certain embodiments, the SAP is AC5®, AC5-V®, AC5-G™ or TK45, also known as AC1, made by Arch Therapeutics, Inc. (see www.archtherapeutics.com).

In some embodiments, the SAP solution is contained in "storage and/or drug delivery system", such as, for example, storage and/or delivery systems suitable for peptide compositions described herein, for example, vials, bottles, beakers, bags, syringes, ampules, cartridges, reservoirs, or LYO-JECTS®. Storage and/or delivery systems need not be one in the same and can be separate. In specific embodiments, SAPs is provided in plastic syringe, containing, about 10 ml, about 7.5 ml, about 5, about 2.5., about 1, or about 0.5 ml of a SAP solution. In certain embodiments, the plastics (e.g., plastic syringe) may acquire a yellowish tint after irradiation, which is normal, and does not affect biomedical characteristics of the therein contained SAP solution.

In some embodiments, such storage and delivery system may further contain a "nozzle" which refers to a generally thin, cylindrical object, often with a narrow end and a wide end, which is adapted for fixing onto a delivery device described herein. In some embodiments, the terms "nozzle" and "cannula" are used interchangeably. Nozzles are composed of two connection points or ends, a first connection point or end to connect to a delivery system (e.g., a syringe) and a second connection point which may serve as the point where delivery of pharmaceutical composition is administered or as a point to connect to a secondary device (e.g., a catheter).

Thus, the invention provides methods of making sterilized solutions of the self-assembling peptides. The invention also provide use of such sterilized solutions can be applied to a biological tissue, e.g., *in situ*, for example, during surgery or after trauma

involving bleeding, or use of so-sterilized solutions in treatment or prevention of other diseases or conditions; for example, as described in as described in Int'l Pat. Appln. WO2014/133027, or as described in US Pat. Appln. Pub. Nos. 2011/02101541 and occlude site of tissue damage; or as described US Pat. Appln. Pub. No. 2016/0287744 for vascular embolization; or as described in US Pat. Appln. No. 16/085,803 for occlusion of cerebrospinal fluid leakage, or as described in as described in Int'l Pat. Pub. Appln. No. WO2013/133414 as mucosa elevating agent; or as described in as described in Int'l Pat. Pub. Appln. No. WO2014/141160 for bile leakage occlusion; or as described in US Pat. Appln. No. 16/885,753 for anti-adhesion of tissues; or as described in US Pat. Appln. No. 16/085,804 for pancreatic fistula occlusion; or as described in US Pat. Appln. No. 15/124,639 for bronchial obstruction; or as described in as described in Int'l Pat. Appln. Pub. No. WO2015/138,478 for treating pulmonary bulla collapse; or as described in Int'l Pat. Appln. Pub. No. WO2015/019,738 for treatment of pulmonary leakage; or as described in Int'l Pat. Appln. Pub. No. WO2015/196020 for filling dental bone voids; or as described US Pat. Appln. Pub. No. 2017/0128622 for filling bone voids; or as described in US Pat. Appln. No. 16/312,878 for the prevention of esophageal structure after endoscopic dissection, and foreign equivalents of any of the aforementioned publications, and other methods known in the art. Accordingly, in some embodiments, the invention provides the use of a sterilized solution of the self-assembling peptide for treating or preventing aforementioned disease or condition, wherein the sterilized solution is obtained by the methods of the invention. In certain such embodiments, the self-assembling peptide solution exhibits a post-irradiation mass spectrometric (MS) profile substantially as shown in corresponding post-irradiation

profiles of FIGS. 2-6 and/or as described in the Examples. For example, in the case of PuraStat®, the additional major M_z peaks at are observed at 836/1670, 1100, and 1513 m/z.

An average bioburden <1,000 CFU is the typical sterility of PuraStat® before sterilization. In this case, to achieve a sterility assurance level, SAL, of 10^{-6}, the range of irradiation dose should be between 25 kGy and 40 kGy. With gamma and X-ray methods, about up to 20% of the over-all peptides may degrade during sterilization process. With e-beam method, about up to 10% of the over-all peptides may degrade during sterilization process. PuraStat® rheology increases after sterilization by gamma-ray, X-ray, which can alter its hemostatic efficacy positively or negatively. However, PuraStat® rheology does not change after e-beam sterilization.

In certain embodiments, using the bioburden testing described below in the Examples, the acceptable level of contamination of the peptide solution pre-irradiation may be <1000, <500, <100, <15, <10, <9, <5, <2, <1.5, <1 CFU, or less per product unit. The preferred bioburden pre-sterilization is < 9 CFU. Thus, under 9 CFU, the range of irradiation dose can be selected between 15 kGy and 24 kGy. While the low end of the irradiation dose range may be determined by the bioburden of the pre-irradiated product, the high end of the range is chosen based on the configuration and choice of the irradiator machine and set at a minimum required to achieve desirable sterility assurance level (SAL).

With gamma and X-ray methods, up to 10% of the peptides may degrade during sterilization process. With e-beam method, the peptides may not significantly degrade during sterilization process. Even in this case, PuraStat® rheology increases after

gamma-ray and X-ray sterilization, which may somewhat change its hemostatic efficacy positively or negatively. Thus, PuraStat® rheology does not change after e-beam sterilization and thus may be preferred, if no or minimal change in rheological properties and its hemostatic properties is desirable. Therefore, e-beam irradiation may be particularly preferred for PuraStat®.

In some embodiments, the concentration of degraded full-length peptide ("major peptide") in the solution post-irradiation ranges from 0.1% to 5%, 0.1% to 4%, 0.1% to 3%, 0.1% to 2.5%, 0.1% to 2%, or 0.1% or 1.5% or less. In some embodiment, , for RADA16, KLD12, or IEIK13 solution irradiated with a total dose, which may depend on its pre-irradiation bioburden, including, for example, 15-50 kGy, 25 kGy +/- 15 kGy, 40 kGy - 10 kGy, 35 kGy -10 kGy, 30 kGy -10 kGy, 15 kGy – 24 KGy, 25 kGy -10 kGy, 20 kGy -10 kGy, 10 kGy -15 kGy, and 12 kGy -14 kGy. Doses around 12-14 kGy appear to be optimal for gamma irradiation, however, the peptide solution may also be irradiated with similar doses with X-ray or e-beam.

In some embodiments, the "storage and/or drug delivery system", for example, a plastic syringe contained in a blister pack, is irradiated in batches of at least: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000 units or more at a time. Generally, it is preferred that the total exposure of any sample in an irradiated batch does not exceed 100 kGy, more preferably 60 kGy, most preferably, 50 kGy, or less as described herein.

In some embodiments, the major peptide's degradation (also referred to as "full-length peptide's degradation") after the irradiation does not exceed 20% of the amount of the peptide prior to the irradiation and, preferably, does not exceed 18%, 16%, 14%,

12%, 10%, 8%, 5%, 3%, or 1%. In some embodiments, the total dose is achieved over a period of time sufficient to achieve necessary sterility of the solution. For example, the dose of 40 kGy can be delivered by radiation intensity 6.3 kGy/hr for about 6 hrs and 21 min. Other combination can be found in Example 1. In some embodiments, a constant dose was delivered over a number of hours, e.g., about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 hours.

In some embodiments, samples are irradiated with gamma ray doses, for example, of about 23, about 25, about 28 or about 40 kGy, or other doses indicated above with an instrument, for example, such as Gammacell 220® High Dose Rate Co-60 Irradiator in VPTrad (www.vptrad.com; MDS Nordion, Ottawa, Canada).

In other embodiments, samples are irradiated with X-ray with doses of about 25 kGy to about 40 kGy, or the doses as indicated above, with a Mevex accelerator (with the following settings: 10 MeV, 20 kW).

In general, X-ray frequency range is $3x10^{16}$-$3x10^{19}$ Hz, while the frequency range of gamma-rays frequency is $3x10^{19}$ or higher.

In other embodiments, samples are irradiated with e-beam at about 25 to about 40 kGy with a Mevex accelerator (with the following settings: 10 MeV, 20 kW) (see mevex.com/linacs/10mev-system-e-beam-sterilization-for-medical-devices/).

In some embodiments, the desired biological and physical property(ies) is/are selected from the group consisting of: hemostatic, anti-adhesion, prevention of re-bleeding, anti-stenosis, tissue occlusion, storage modulus, viscosity, tissue void filling property, mucosa elevation, and wound healing,

In preferred embodiments, the irradiation dose achieves sterility assurance level (SAL) of at least 10^{-5}, 10^{-6}, or less.

The invention also provide the sterilized solution of self-assembling peptide made by the methods described herein. Such comprising applying the solution to a biological tissue, for example, during surgery or after trauma involving bleeding, to a site with substantially neutral pH, resulting in gelling. In some embodiments, such site is internal, while in other embodiment the site can be external such surface sutures, cuts, or scrapes.

Other aspects of the invention would be apparent to those of skill in the art based on the present description, including the Examples and the appended claims.

Example 1: Irradiation conditions

Samples were irradiated with gamma rays at 23, 25, 28 and 40 kGy with VPTrad Gammacell 220® High Dose Rate Co-60 Irradiator. In some embodiments, the run dose rate and duration time for 40 kGy irradiation were 6.30 kGy/hr and 6 hours 20 minutes 58 seconds, respectively. In other embodiments, the run dose rate and duration time for 28 kGy irradiation were 4.40 kGy/hr and 6 hours 22 minutes 31 seconds, respectively. In other embodiments, the run dose rate and duration time for 25 kGy irradiation were 6.58 kGy/hr and 3 hours 47 minutes 42 seconds, respectively. In other embodiments, the run dose rate and duration time for 23 kGy irradiation were 6.58 kGy/hr and 3 hours 29 minutes 29 seconds, respectively.

In other embodiments, samples were irradiated with X-ray at 25 kGy and 40 kGy with a Mevex accelerator (10 MeV, 20 kW).

In other embodiments, samples were irradiated with e-beam at 25 and 40 kGy with a Mevex accelerator (10 MeV, 20 kW).

Example 2: HPLC conditions

HPLC tests were performed to evaluate the major peptide content after irradiation tests. An Agilent HPLC 1100 (Agilent Technologies) was used for this study. The column temperature was kept at 25 °C.

For RADA16 samples, solvent A was water with 0.1% TFA and solvent B was 80% acetonitrile with 0.1% TFA. Gradient of solvent B was controlled from 10% to 40% in 20 min and 40% for another 5 min at 25 °C. Agilent Zorbax 300SB-C18 column (4.6 mm X 250 mm, 5μm, 300 Å) was used for this test. PuraStat® (RADA16 2.5%) (40 mg) was mixed with 10 μL of DH_2O and the mixture were vortexed. The mixture was further mixed with 500 μL of formic acid and vortexed. This mixture was then mixed with DH_2O (4,450 μL) and vortexed. 20 μL samples were injected using an Agilent autosampler.

For IEIK13 samples, solvent A was water with 0.1% TFA and solvent B was 90% Acetonitrile with 0.1% TFA. Gradient of solvent B was controlled from 20% to 43% in 8 min, from 43% to 70% between 8 and 9.5 min, and from 70% to 95% between 9.5 and 30 min, and 95% for another 5 min at 25°C. An Agilent PLRP-S column (4.6 mm X 250 mm, 8 μm, 300 Å) was used for this test. A 1.3% solution of IEIK13 was diluted with water with 0.1% TFA to 0.075% and the mixture was vortexed. 35 μL samples were injected using an Agilent autosampler.

For QLEL12 samples, solvent A was water and solvent B was 80% acetonitrile. Gradient of solvent B was controlled from 20% to 80% in 15 min and 80% for another 7

min at 25°C. An Agilent PLRP-S column (4.6 mm X 250 mm, 8μm, 300 Å) was used for this test. 0.15% w/v of QLEL12 solution was diluted to 0.01% with DH_2O and vortexed. 50 μL samples were injected using an Agilent autosampler.

Example 3: Mass spectrometry conditions

Mass spectrometry tests were carried out to investigate the degradation of the peptides after irradiation sterilization. An Agilent LC/MSD ion trap mass spectrometer was used for this study. The sample solutions were prepared as described above for the HPLC samples. Each sample was injected at 9 μL/min with a syringe pump. Mass spectrum was recorded for 1 minute.

Example 4: Rheological properties of irradiated self-assembling peptides

The rheological properties of samples were evaluated using a rheometer (DHR1, TA Instruments) with 40 mm cone and plate. Peptide solution (700 μL) was placed on the rheometer plate and excess solution was gently removed by a metal spatula. Measurements were performed after 2 minutes of relaxation time at 37 °C.

Frequency tests were performed at 0.1% of strain from 0.1 Hz to 10 Hz. Frequency tests after gelation were performed under the same conditions for 20 minutes after 3 mL of DMEM was gently added around the cone and the plate.

Thixotropic tests were carried out with the following method. A shear rate of 1000 s^{-1} was applied for 1 minute to reset all rheological properties, then 1 Hz of frequency at 0.1% of strain was applied for 60 minutes to record thixotropic behaviors. This sequence was repeated, and the thixotropic property were then analyzed.

Example 5: Appearance and pH

The appearance and pH of PuraStat® after gamma irradiation are shown in Figure 1 and Table 1 (N=3).

Table 1. Appearance and pH of PuraStat® (RADA16 2.5%) after gamma irradiation.

Testing conditions	Appearance	pH
PuraStat® control	Clear and viscous	2.2
PuraStat® irradiated (gamma) at 23 kGy	Clear and viscous	2.3
PuraStat® irradiated (gamma) at 25 kGy	Clear and viscous	2.3
PuraStat® irradiated (gamma) at 28 kGy	Clear and viscous	2.3
PuraStat® irradiated (gamma) at 40 kGy	Clear and viscous	2.3

The appearance and pH of PuraStat® after gamma irradiation were only slightly altered. The pH of the PuraStat® control was 2.2. The pH of PuraStat® after gamma irradiation at 23, 25, 28, and 40 kGy were 2.3.

The appearance and pH of IEIK13 1.3% after gamma irradiation are also shown in Figure 1 and Table 2.

Table 2. Appearance and pH of IEIK13 1.3%, KLD12 1.3% and QLEL12 0.15% after gamma irradiation. (N=3)

Testing conditions	Appearance	pH
IEIK13 control	Clear and viscous	3.0
IEIK13 irradiated (gamma) at 28 kGy	Clear and viscous	3.0
IEIK13 irradiated (gamma) at 40 kGy	Clear and viscous	3.0

KLD12 control	Clear and viscous	2.2
KLD12 irradiated (gamma) at 40 kGy	Clear and viscous	2.2
QLEL12 control	Clear and viscous	7.0
QLEL12 irradiated (gamma) at 23 kGy	Clear and watery	6.7
QLEL12 irradiated (gamma) at 25 kGy	Clear and watery	6.5
QLEL12 irradiated (gamma) at 40 kGy	Clear and watery	5.9

The appearance and pH of IEIK13 (1.3%) after gamma irradiation were not changed. The pH of IEIK13 control was 3.0. The pH of IEIK13 (1.3%) after gamma irradiation at 28 kGy and at 40 kGy was 3.0.

The appearance and pH of KLD12 (1.3%) and QLEL12 (0.15%) after gamma irradiation were also shown in Table 2. The appearance and pH of KLD12 (1.3%) after gamma irradiation were not changed. The pH of KLD12 control was 2.2. The pH of KLD12 (1.3%) after gamma irradiation at 40 kGy were 2.2.

However, the appearance and pH of QLEL12 after gamma irradiation were significantly altered. The pH of the QLEL12 control was 7.0. The pH of QLEL12 after gamma irradiation at 23, 25 and 40 kGy were 6.7, 6.5 and 5.9, respectively, and the samples became watery after gamma irradiation.

The appearance and pH of PuraStat® after X-ray and e-beam irradiation are also shown in Table 3 (N=3).

Table 3. Appearance and pH of PuraStat® (RADA16 2.5%) after X-ray and e-beam irradiation test. (N=3)

Testing conditions	Appearance	pH
PuraStat® control	Clear and viscous	2.2
PuraStat® irradiated (X-ray) at 25 kGy	Clear and viscous	2.3
PuraStat® irradiated (X-ray) at 40 kGy	Clear and viscous	2.3
PuraStat® irradiated (e-beam) at 25 kGy	Clear and viscous	2.2
PuraStat® irradiated (e-beam) at 40 kGy	Clear and viscous	2.2

The appearance and pH of PuraStat® after X-ray or e-beam irradiation were only slightly changed or remained unchanged. The pH of PuraStat® control was 2.2. The pH of PuraStat® after X-ray irradiation at 25 kGy and at 40 kGy were 2.3. The pH of PuraStat® after e-beam irradiation at 25 kGy and at 40 kGy were 2.2.

The appearance and pH of IEIK13 1.3% after X-ray or e-beam irradiation are also shown in Table 4.

Table 4. Appearance and pH of IEIK13 1.3% after X-ray or e-beam irradiation (N=3).

Testing conditions	Appearance	pH
IEIK13 control	Clear and viscous	3.0
IEIK13 irradiated (X-ray) at 25 kGy	Clear and viscous	3.0
IEIK13 irradiated (X-ray) at 40 kGy	Clear and viscous	3.0
IEIK13 irradiated (e-beam) at 25 kGy	Clear and viscous	3.0
IEIK13 irradiated (e-beam) at 40 kGy	Clear and viscous	3.0

The appearance and pH of IEIK13 1.3% after X-ray or e-beam irradiation were not changed. The pH of IEIK13 1.3% control was 3.0. The pH of IEIK13 1.3% after X-ray or e-beam irradiation at 28 kGy and 40 kGy were 3.0.

The appearance and pH of KLD12 1.3% after X-ray or e-beam irradiation are also shown in Table 5.

Table 5. Appearance and pH of KLD12 1.3% after X-ray or e-beam irradiation (N=3).

Testing conditions	**Appearance**	**pH**
KLD12 control	Clear and viscous	2.2
KLD12 irradiated (X-ray) at 25 kGy	Clear and viscous	2.2
KLD12 irradiated (X-ray) at 40 kGy	Clear and viscous	2.2
KLD12 irradiated (e-beam) at 25 kGy	Clear and viscous	2.2
KLD12 irradiated (e-beam) at 40 kGy	Clear and viscous	2.2

The appearance and pH of KLD12 1.3% after X-ray or e-beam irradiation were not changed. The pH of KLD12 1.3% control was 2.2. The pH of KLD12 1.3% after X-ray or e-beam irradiation at 25 kGy and 40 kGy were 2.2.

The appearance and pH of QLEL12 0.15% after X-ray or e-beam irradiation are also shown in Table 6.

Table 6. Appearance and pH of QLEL12 0.15% after X-ray and e-beam irradiation (N=3).

Testing conditions	**Appearance**	**pH**
QLEL12 control	Clear and viscous	7.0
QLEL12 irradiated (Gamma) at 25 kGy	Clear and watery	6.5

QLEL12 irradiated (Gamma) at 40 kGy	Clear and watery	5.9
QLEL12 irradiated (X-ray) at 25 kGy	Clear and watery	6.3
QLEL12 irradiated (X-ray) at 40 kGy	Clear and watery	5.9
QLEL12 irradiated (e-beam) at 25 kGy	Clear and watery	6.6
QLEL12 irradiated (e-beam) at 40 kGy	Clear and watery	6.5

The appearance of QLEL12 after gamma, X-ray or e-beam irradiation was significantly altered. The pH of the QLEL12 control was 7.0. The pH's of QLEL12 after gamma irradiation at 25 and 40 kGy were 6.5 and 5.9, respectively, those after X-ray irradiation at 25 and 40 kGy were 6.3 and 6.5, respectively, and those after e-beam irradiation at 25 and 40 kGy were 6.6 and 6.5, respectively. QLEL12 solutions became watery after gamma, X-ray or e-beam irradiation.

In summary, RADA16, IEIK13 and KLD12 remained unchanged after gamma, X-ray and e-beam irradiation at around 25~40 kGy, but QLEL12 was not. Therefore, unlike QLEL12, RADA16, IEIK13, and KLD12 can be sterilized using a gamma, X-ray and e-beam irradiation techniques.

Example 7: Characterization by HPLC and Mass Spectrometry

The HPLC tests for RADA16, IEIK13, and QLEL12 were performed before and after gamma, X-ray and e-beam irradiation, and the results for their major peptide content were listed in Tables 7-10.

Table 7. HPLC test for major peptide content of PuraStat® (RADA16 2.5%) before and after gamma-irradiation (N=3, mean ± SD).

Testing conditions	Major peptide content (%)
PuraStat® control	78.3 ± 1.7
PuraStat® irradiated (gamma) at 23 kGy	75.5 ± 1.4*
PuraStat® irradiated (gamma) at 25 kGy	74.6 ± 0.7*
PuraStat® irradiated (gamma) at 28 kGy	68.5 ± 0.5*
PuraStat® irradiated (gamma) at 40 kGy	69.7 ± 0.5*

*: denotes if significantly lower than the data of PuraStat® control (if $p < 0.05$, two tailed Student's *t*-test).

Table 8. HPLC test for major peptide contents of PuraStat® (RADA16 2.5%) before and after X-ray and e-beam irradiation (N=3, mean ± SD).

Testing conditions	Major peptide content (%)
PuraStat® control	78.3 ± 1.7
PuraStat® irradiated (X-ray) at 25 kGy	73.8 ± 1.7*
PuraStat® irradiated (X-ray) at 40 kGy	71.9 ± 0.8*
PuraStat® irradiated (e-beam) at 25 kGy	76.0 ± 0.9*
PuraStat® irradiated (e-beam) at 40 kGy	70.0 ± 2.4*

*: denotes if significantly lower than the data of PuraStat® control (if $p < 0.05$, two tailed Student's *t*-test).

Table 9. HPLC test for major peptide contents of IEIK13 1.3% before and after gamma, X-ray, and e-beam irradiation (N=3, t mean ± SD).

Testing conditions	Major peptide content (%)
IEIK13 **control**	99.6 ± 0.0

IEIK13 **irradiated (gamma) at 40 kGy**	99.8 ± 0.2
IEIK13 **irradiated (X-ray) at 25 kGy**	99.8 ± 0.1
IEIK13 **irradiated (X-ray) at 40 kGy**	99.9 ± 0.1
IEIK13 **irradiated (e-beam) at 25 kGy**	99.6 ± 0.2
IEIK13 **irradiated (e-beam) at 40 kGy**	99.8 ± 0.1

Table 10. HPLC test for major peptide contents of QLEL12 0.15% before and after gamma, X-ray, and e-beam irradiation (N=3, mean ± SD).

Testing conditions	Major peptide content (%)
QLEL12 control	90.5 ± 1.3
QLEL12 irradiated (gamma) at 40 kGy	40.7 ± 3.1*
QLEL12 irradiated (X-ray) at 25 kGy	57.0 ± 4.9*
QLEL12 irradiated (X-ray) at 40 kGy	47.3 ± 1.3*
QLEL12 irradiated (e-beam) at 25 kGy	51.9 ± 7.3*
QLEL12 irradiated (e-beam) at 40 kGy	55.6 ± 3.0*

*: denotes if significantly lower than the data of QLEL12 control (if $p < 0.05$, two tailed Student's *t*-test).

The major peptide content of RADA16 control was 78.3%. The major peptide contents of RADA16 after gamma irradiation at 23, 25, 28 and 40 kGy were 75.5, 74.6, 68.5 and 69.7%, respectively. The major peptide contents of RADA16 after X-ray irradiation at 25 and 40 kGy were 73.8 and 71.9%, respectively. The major peptide contents of RADA16 after e-beam irradiation at 25 and 40 kGy were 76.0 and 70.0%, respectively.

The major peptide content of IEIK13 control was 99.6%. The major peptide content of IEIK13 after gamma irradiation at 40 kGy was 99.8%. The major peptide contents of IEIK13 after X-ray irradiation at 25 and 40 kGy were 99.8 and 99.9%, respectively. The major peptide contents of IEIK13 after e-beam irradiation at 25 and 40 kGy were 99.6 and 99.8%, respectively.

The major peptide content of QLEL12 control was 90.5%. However, QLEL12 showed significant decrease in its major peptide content after irradiation sterilization. The major peptide content of QLEL12 after gamma irradiation at 40 kGy was 40.7%. The major peptide contents of QLEL12 after X-ray irradiation at 25 and 40 kGy were 57.0 and 47.3%, respectively. The major peptide contents of QLEL12 after e-beam irradiation at 25 and 40 kGy were 51.9 and 55.6%, respectively. These results demonstrate that RADA16 and IEIK13 remain relatively unchanged after gamma, X-ray and e-beam irradiation compared to QLEL12.

The measured molecular weight of RADA16 was 1712, which matches its calculated molecular weight (Figure 2). The mass spec analysis demonstrated that RADA16 was not degraded after gamma, X-ray and e-beam irradiation (Figures 2-4). However, RADA16 completely degraded during autoclave treatment.

Irradiation is a cold temperature sterilization technique unlike autoclaving. Although both sterilization techniques provide high energy (i.e., radiation and heat) to self-assembling peptides such as RADA16 during sterilization, irradiation sterilization did not induce substantial degradation of RADA16 molecules.

However, all gamma, X-ray, and e-beam irradiated PuraStat® mass spectra exhibited some peaks that were not observed before irradiation, while no notable difference was observed among them. The summary of the mass spectra is listed in Table 11.

Table 11. Mass-spectrometry data of PuraStat® after irradiation sterilization process.

Mz	**Mw at n=1**	**Mw at n=2**	**Mw at n=3**	**Estimated component**	**Control PuraStat®***	**Irradiated PuraStat®#**
502	501			ARADA-NH_2 (SEQ ID NO:5)	Yes	Yes
572			1712	Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1)	Yes	Yes
665		1328		ARADARADARADA-NH_2 (SEQ ID NO:6)	Yes	Yes
836		1670		$(RADA)_4$-NH_2 (SEQ ID NO:7)	No	Yes
857			1712	Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1)	Yes	Yes
916	915			ARADARADA-NH_2 (SEQ ID NO:8)	Yes	Yes
1100	1100			ADARADARADA-NH_2 (SEQ ID NO:9)	No	Yes

1143	1142			ARADARADARA (SEQ ID NO:10)	Yes	Yes
1229	1228			Ac-RADARADARAD (SEQ ID NO:11)	Yes	Yes
1329	1328			ARADARADARADA-NH_2 (SEQ ID NO:6)	Yes	Yes
1513	1513			ADARADARADARADA-NH_2 (SEQ ID NO:12)	No	Yes
1643	1642			Ac-RADARADARADARAD (SEQ ID NO:13)	Yes	Yes
1670	1670			$(RADA)_4$-NH_2 (SEQ ID NO:7)	No	Yes
1713	1712			Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1)	Yes	Yes

*: stored at 2-8 °C for about 4 years.
#: all PuraStat® samples ware irradiated at 40 kGy (with gamma rays, X-rays, and e-beam)

The control PuraStat® showed M_z peaks at 572, 857, and 1713, which are assigned to the major peptide, Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1). Control PuraStat® also exhibited other M_z peaks at 502, 665/1329, 916, 1143, and 1229, which are estimated as ARADA-NH_2 (SEQ ID NO:5), ARADARADARADA-NH_2 (SEQ ID NO:6), ARADARADA-NH_2 (SEQ ID NO:8), ARADARADARA (SEQ ID NO:10), Ac-RADARADARAD (SEQ ID NO:11), respectively.

Table 12 shows the pattern of degradation of Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1) when PuraStat® is stored at 2-8 °C for about 4 years. From the pattern, we found that degradation mainly occurs at the points between ~RAD and A~.

On the other hand, the irradiated PuraStat® showed additional M_z peaks at 836/1670, 1100, and 1513, which are estimated as $(RADA)_4$-NH_2 (SEQ ID NO:7), ADARADARADA-NH_2 (SEQ ID NO:9), and ADARADARADARADA-NH_2 (SEQ ID NO:12), respectively.

Table 13 shows the additional pattern of degradation of Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1) when PuraStat® is sterilized with irradiation. Especially, the peaks at 836 and 1670 are ones of major additional peaks, which represent RADARADARADARADA-NH_2 (SEQ ID NO:7). This means that irradiation can cause additional degradation of PuraStat® at the point between acetyl group (Ac) and RAD~. The peaks at 1100 and 1513 are also ones of major additional peaks, which represent ADARADARADA-NH_2 (SEQ ID NO:9), and ADARADARADARADA-NH_2 (SEQ ID NO:12). This means that irradiation can cause additional degradation of PuraStat® at the point between ~R and AD~.

Table 12. Pattern of degradation of Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1) (PuraStat® was stored at 2-8 °C for about 4 years)

Ac-RADARADARADARADA-NH_2 (SEQ ID NO:1) (before degradation)
Ac-RAD / ARADARADARADA-NH_2 (SEQ ID NO:6)
Ac-RADARAD (SEQ ID NO:14) / ARADARADA-NH_2 (SEQ ID NO:8)
Ac-RADARADARAD (SEQ ID NO:11) / ARADA-NH_2 (SEQ ID NO:5)

Ac-RAD / ARADARADARA (SEQ ID NO:10) / DA-NH_2

Ac-RADARADARADARAD (SEQ ID NO:13) / A-NH_2

Table 13. Additional pattern of degradation of Ac-$(RADA)_4$-NH_2 when PuraStat® is sterilized with irradiation

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Ac-RADARADARADARADA-NH_2 (SEQ ID NO:1) (before degradation)

Ac / RADARADARADARADA-NH_2 (SE ID NO:7)

Ac-R / ADARADARADARADA-NH_2 (SEQ ID NO:12)

Ac-RADAR (SEQ ID NO:14) / ADARADARADA-NH_2 (SEQ ID NO:9)

Also, the molecular weight of IEIK13 was measured at 1622, which matches its calculated molecular weight (Figure 5). The mass spec analysis demonstrated that IEIK13 was only insubstantially degraded after gamma, X-ray and e-beam irradiation.

Furthermore, the molar mass of QLEL12 was measured at 1506, which matches its calculated molar mass (Figure 6). However, the mass spec analysis demonstrated that QLEL12 was significantly degraded after gamma, X-ray and e-beam irradiation.

Example 7: Rheological properties

Based on ISO 11137 (Sterilization of health care products - Radiation), radiation sterilization methods can be used with 25 kGy or 15 kGy irradiation as the sterilization dose to achieve a sterility assurance level, SAL, of 10^{-6}.

The rheology results are shown in Figures 7 and 8 for PuraStat® with gamma irradiation sterilization before and after gelation, respectively. The determined rheological results are listed in Tables 14-15.

Table 14. Results from frequency tests of PuraStat® (RADA16 2.5%) with gamma irradiation at 0.1% of strain with 40 mm cone-plate

Sample #	Storage Modulus G' at 1 Hz (Pa)			
	PuraStat® control	Gamma-irradiated PuraStat® at 23 kGy	Gamma-irradiated PuraStat® at 25 kGy	Gamma-irradiated PuraStat® at 40 kGy
1	343.7	514.1	546.0	551.0
2	323.3	449.7	490.2	607.7
3	301.7	449.5	467.0	618.4
Mean	322.9	471.1*	501.0*	592.4*,$
SD	21.0	37.2	40.6	36.2

*: denotes if $p < 0.05$ compared to control (two tailed Student's *t*-test).
$: denotes if $p < 0.05$ compared to the others (two tailed Student's *t*-test).

PuraStat® gamma-irradiated at 23 kGy, 25 kGy, and 40 kGy showed higher storage modulus than PuraStat® control (Figure 7 and Table 14). PuraStat® gamma-

irradiated at 40 kGy showed significantly higher storage modulus than PuraStat® gamma-irradiated at 23 and 25 kGy. Also, PuraStat® gamma-irradiated at 25 kGy showed slightly higher storage modulus than PuraStat® gamma-irradiated at 23 kGy. This indicates that gamma-irradiation positively affected the rheological properties of PuraStat®. PuraStat® gamma-irradiated at 23 kGy (471.1 ± 37.2 Pa), 25 kGy (501.0 ± 40.6 Pa) and 40 kGy (592.4 ± 36.2 Pa) exhibited 46%, 55%, and 83% increases in their storage moduli, respectively, compared to PuraStat® control (322.9 ± 21.0 Pa).

Table 15. Results from frequency tests of PuraStat® (RADA16 2.5%) with gamma irradiation after gelation. Samples were treated with DMEM for 20 min at 0.1% of strain with a 40 mm cone-plate

Sample #	Storage Modulus G' at 1 Hz (Pa)			
	PuraStat® control	Gamma-irradiated PuraStat® at 23 kGy	Gamma-irradiated PuraStat® at 25 kGy	Gamma-irradiated PuraStat® at 40 kGy
1	4454	8866	6885	14568
2	5280	8845	10395	10286
3	5183	8622	10148	12134
Mean	4972	8711*	9143*	12330*
SD	451	135	1959	2148

*: denotes if $p < 0.05$ compared to control (two tailed Student's *t*-test).
$: denotes if $p < 0.05$ compared to the others (two tailed Student's *t*-test).

Furthermore, after gelation triggered by simulated body fluid (i.e., DMEM buffer) for 20 min), PuraStat® gamma-irradiated at 23 kGy (8711 ± 135 Pa), 25 kGy (9143 ±

1959 Pa) and 40 kGy (12330 ± 2148 Pa) exhibited 75%, 84%, and 148% increases in their storage moduli, respectively, compared to PuraStat® control (4972 ± 451 Pa) (Figure 8 and Table 11).

The rheology results are shown in Tables 16 and 17 for PuraStat® with X-ray irradiation sterilization before and after gelation, respectively.

Table 16. Results from frequency tests of PuraStat® (RADA16 2.5%) with X-ray irradiation at 0.1% of strain with 40 mm cone-plate

Sample #	Storage Modulus G' at 1 Hz (Pa)		
	PuraStat® control	X-ray-irradiated PuraStat® at 25 kGy	X-ray-irradiated PuraStat® at 40 kGy
1	343.7	448.3	610.2
2	323.3	429.3	632.5
3	301.7	471.7	578.9
Mean	322.9	449.8*	607.2*,$
SD	21.0	21.2	27.0

*: denotes if $p < 0.05$ compared to control (two tailed Student's *t*-test).
$: denoted if $p < 0.05$ compared to the others (two tailed Student's *t*-test).

PuraStat® X-ray-irradiated at 25 kGy and 40 kGy also showed higher storage modulus than PuraStat® control. PuraStat® X-ray-irradiated at 40 kGy showed significantly higher storage modulus that PuraStat® X-ray-irradiated at 25 kGy. This indicates X-ray irradiation positively affected the rheological properties of PuraStat®.

PuraStat® irradiated at 25 kGy (449.8 ± 21.2) and 40 kGy (607.2 ± 27.0 Pa) exhibited 39% and 88% increases in their storage moduli, respectively, compared to PuraStat® control (322.9 ± 21.0 Pa) (Table 17).

Table 17. Results from frequency tests of PuraStat® (RADA16 2.5%) with X-ray irradiation after gelation. Samples were treated with DMEM for 20 min. at 0.1% of strain with a 40 mm cone-plate.

Sample #	Storage Modulus G' at 1 Hz (Pa)		
	PuraStat® control	X-ray-irradiated PuraStat® at 25 kGy	X-ray-irradiated PuraStat® at 40 kGy
1	4454	9563	12832
2	5280	6404	10611
3	5183	9522	10294
Average	4972	8497*	11246*
SD	451	1812	1383

*: denotes if $p < 0.05$ compared to control (two tailed Student's *t*-test).

Furthermore, after gelation triggered by simulated body fluid (i.e., DMEM buffer) for 20 min, PuraStat® X-ray-irradiated at 25 kGy (8497 ± 1812 Pa) and 40 kGy (11246 ± 1383 Pa) exhibited 71% and 126% increases in their storage moduli, respectively, compared to PuraStat® control (4972 ± 451 Pa) (Table 17).

The rheology results are shown in Tables 18 and 19 for PuraStat® with e-beam irradiation sterilization before and after gelation, respectively.

Table 18. Results from frequency tests of PuraStat® (RADA16 2.5%) with e-beam irradiation at 0.1% of strain with 40 mm cone-plate.

Sample #	Storage Modulus G' at 1 Hz (Pa)		
	PuraStat® control	e-beam-irradiated PuraStat® at 25 kGy	e-beam-irradiated PuraStat® at 40 kGy
1	343.7	348.1	349.6
2	323.3	356.1	371.5
3	301.7	324.0	338.4
Mean	322.9	342.7	353.2
SD	21.0	16.7	16.8

PuraStat® e-beam-irradiated at 25 kGy and 40 kGy, however, did not show a significant change in storage modulus compared to PuraStat® control. This indicates e-beam irradiation sterilization does not have a major effect on the rheological properties of PuraStat®. However, although the p values did not show statistical significance (i.e., $p > 0.05$), PuraStat® X-ray-irradiated at 25 kGy (342.7 ± 16.7 Pa) and 40 kGy (353.2 ± 16.8 Pa) exhibited 6% and 9% increases in their storage moduli, respectively, compared to PuraStat® control (322.9 ± 21.0 Pa) (Table 18).

Table 19. Results from frequency tests of PuraStat® (RADA16 2.5%) with e-beam irradiation after gelation. Samples were treated with DMEM for 20 min at 0.1% of strain with 40 mm cone-plate.

Sample #	Storage Modulus G' at 1 Hz (Pa)		
	PuraStat® control	e-beam-irradiated PuraStat® at 25 kGy	e-beam-irradiated PuraStat® at 40 kGy
1	4454	3623	4918
2	5280	4830	9327
3	5183	3542	7875
Average	4972	4007	7373
SD	451	715	2247

After gelation triggered by simulated body fluid (i.e., DMEM buffer) for 20 min), PuraStat® e-beam-irradiated at 25 kGy and 40 kGy did not show significant difference (i.e., the *p* values were higher than 0.05) in their storage moduli compared to PuraStat® control (Table 19).

PuraStat® demonstrated shear thinning property at high shear rate and thixotropic behavior suggesting slow rheological property recovery when high shearing stopped (Figure 9). From these properties of PuraStat® , the stiffness of PuraStat® can be lowered for easier handling during application to patients and stiffness can then slowly recover to initial values after application. These intrinsic thixotropic properties of

PuraStat® were not changed, even after gamma irradiation at 23 and 25 kGy, while irradiated PuraStat® showed higher storage modulus than PuraStat® control.

Because the peptides' molecular structure was not substantially changed considering the results of HPLC and Mass Spectrometry, the assembled nanofibrous structure of the peptides could be a factor to increase their rheological properties. The rheological properties of self-assembling peptide solution might increase when self-assembled peptide structure is more organized. By way of a non-binding theory, high energy from irradiation can make peptide molecules move slightly to have more organized nanofibrous structure resulting in improved rheological properties.

The increased rheological properties were at least partially reversed by repeated high shearing and returned back close to the original levels. After thoroughly shearing them twice at 1000 s^{-1} for 1 minute, PuraStat® samples irradiated at 23 kGy and 25 kGy showed gradual decrease in their storage modulus from 484.5 Pa to 410.9 Pa and 514.3 Pa to 426.5 Pa, respectively, while PuraStat® control did not show significant change in its storage modulus (Figure 9). It could be expected that the rheological properties of irradiated PuraStat® become closer to those of PuraStat® control with more shearing. Therefore, gamma irradiation enhances the structure of self-assembled nanofibers to increase their rheological properties without a detectable change in peptide molecular structure degradation or crosslinking.

Example 8: Bioburden Testing

A. Collection of Samples. Upon irradiation, 50 samples were collected as follows: 10 samples for bioburden tests inside the packages (collecting 4 at the beginning, each

3 at the middle and the end of the packaging operation); 10 samples for bioburden tests of the filling liquid in the syringe (collecting 4 at the beginning, each 3 at the middle and the end of the packaging operation); spare samples, e.g. 30 samples, may be collected in case extra testing may be required.

B. Viable Bacteria Count Test for Sterilization Validation. The sample was aseptically prepared in a clean bench, and all instruments and solvents to be used were sterilized.

For blister-packaged products, the following procedure was repeated twice with one sealed package to make a total 100 mL of sample solution. A syringe was used to inject 50 mL of rinsing fluid for the sterilization test (USP Fluid D) into the inside of the package. A sample were shaken thoroughly and allowed to stand to reduce bubbles. Then, the outside opening of the package was sterilized by lightly passing the opening through a flame without heating the contents. Then, a syringe or another suitable method was used to extract all the injected solution from the package, which was then collected in a heat-resistant bottle. With a sealed blister package containing about 100 CPU of spores purified from a standard strain such as *Bacillus subtilis* (NBRC3134), a recovery rate and a correction coefficient based on the recovery rate were calculated in advance from the viable bacteria count and the added bacteria count from a sample which was prepared in the same manner as above. A correction coefficient was calculated as 1/recovery rate (%) x 100. This correction coefficient was recalculated when the sample preparation procedure was changed.

For testing content fluid, 1 mL of the content solution was mixed with 9 mL of Soybean-Casein Digest Medium agar medium (SCD agar medium), and the gel finely

dispersed to make 10 mL of sample solution.

For blister package products, 100 mL of the sample solution per culture medium was used and the test was conducted using the membrane filter method of the Japanese Pharmacopoeia Microbial Limit Test.

For testing content fluid, 1 mL of the sample solution per culture medium and conduct the test using the agar plate dilution method of the Japanese Pharmacopoeia Microbial Limit Test. This test was repeated 10 times to obtain 10 culture media plates corresponding to 10 mL of sample solution.

The cultures were maintained at 30°C–35°C for 3–5 days (or longer) on SCD agar medium. As a general rule, the cultures were observed at least once every operating day during the culture period and on the final measurement day.

After completion of the culture, the actual measured values of the colonies of SCD agar medium were converted with the following calculation:

(1) For blister package products

Viable bacteria count in a blister package = Viable bacteria count in 100 mL of sample liquid x Correction coefficient

(2) For Content fluid

Viable bacteria count in 1 mL of content liquid = Total viable bacteria count in 10 mL equivalent to sample solution (10 culture media plates)

Remarks: Among the test method for the content fluid, the value of the SIP (aliquot) used for calculation were determined to be 1/5 = 0.2, not the total amount when testing with a 5-mL product by the test method equivalent to 1 mL of content liquid. * SIP (Sample Item Portion) is equivalent to an aliquot (defined portion of the healthcare

product used for testing).

Example 9: Experimental design of PuraStat® sterilization by Gamma irradiation

Irradiation conditions--PuraStat® samples (Lot# 17C09A30) were irradiated with gamma rays at 25, 28 and 40 kGy with Gammacell 220® High Dose Rate Co-60 Irradiator (MDS Nordion, Ottawa, Canada). The run dose rate and duration time for 40 kGy irradiation were 6.30 kGy/hr and 6 hours 20 minutes 58 seconds, respectively. The run dose rate and duration time for 28 kGy irradiation were 4.40 kGy/hr and 6 hours 22 minutes 31 seconds, respectively. The run dose rate and duration time for 25 kGy irradiation were 6.58 kGy/hr and 3 hours 47 minutes 42 seconds, respectively.

Methods and test results: The appearance of PuraStat® was observed after each test. The pH of PuraStat® was tested using an Accumet AB15 pH meter (Fisher Scientific). HPLC tests were performed to evaluate the major peptide content after irradiation tests. Agilent HPLC 1100 (Agilent Technologies) was used for this study. Column temperature was kept at 25 °C.

Solvent A was water with 0.1% TFA and solvent B was 80% Acetonitrile with 0.1% TFA. Gradient of solvent B was controlled from 10% to 40% in 20 min and 40% for another 5 min at 25 °C. Agilent Zorbax 300SB-C18 column (4.6 mm X 250 mm, 5 μm, 300 Å) was used for this test. PuraStat® (RADA16 2.5%) (40 mg) were mixed with 10 μL of DH_2O and 500 μL of formic acid and vortexed. And the mixture was mixed with DH_2O (4,450 μL) and vortexed. 20 μL of samples were injected using an Agilent autosampler.

Mass spectrometry tests were carried out to investigate the degradation of the peptides after irradiation sterilization. Agilent LC/MSD ion trap mass spectrometer was used for this study. The sample solutions were prepared as described above for the HPLC samples. Each sample was injected at 9 µL/min with a syringe pump. Mass spectrum was recorded for 1 minutes. The MS-trace is shown in Fig. 10. The molecular weight of PuraStat® (Ac-$(RADA)_4$-NH_2) (SEQ ID NO:1) is 1712, which matches its calculated molecular weight from the major three peaks at m/3=572, m/2=857, and m=1713 in all the spectra of control and irradiated PuraStat® samples.

The assigned peaks to Ac-RADARADARADARADA-NH_2 (SEQ ID NO:1) are,

M/z= 572 = (Mw +3)/3, so the calculated Mw = 1713

M/z= 857 = (Mw +2)/2, so the calculated Mw = 1712

M/z= 1713 = Mw +1, so the calculated Mw = 1712

The rheological properties of PuraStat® samples before and after gamma sterilization were evaluated using a rheometer (Discovery HR 1, TA Instruments) at 37°C. Flow tests were carried out with 20 mm plate-plate geometry and 800 µm of gap distance at a shear rate range of 0.001 1/sec to 3,000 1/sec at 37°C. Sample solution (350 µL) was placed on the rheometer plate and excess solution was gently removed; measurements were performed after 2 minutes of relaxation time at 37°C. The viscosity (h) were recorded from very low shear rate (0.001 1/sec) to high shear rate (3000 1/sec).

L929 neutral red uptake tests were performed to investigate the cytotoxicity of the irradiated PuraStat® . These tests were performed by Toxikon located in Bedford

MA, USA. The study was done based upon ISO 10993-5, 2009, Biological Evaluation of Medical Devices – Part 5: Tests for in Vitro Cytotoxicity and ISO 10993-12, 2012, Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials. The biological reactivity of a mammalian cell monolayer, L929 mouse fibroblast, in response to the test article extract was determined. The test article extract was obtained with serum-supplemented Minimum Essential Medium (MEM) at the ratio 0.2 g of article per mL. Extraction was done for 24 ± 2 hours at 37 ± 1 °C. Positive control (Natural Rubber) and negative control (Negative Control Plastic) articles and an untreated control (blank) were prepared to verify the proper functioning of the test system. The test article and control article extracts were used to replace the maintenance medium of the cell culture. The test article extract was tested at the 100% (neat) concentration. All cultures were incubated in at least 6 replicates for 24 to 26 hours. At 37 ± 1 °C, in a humidified atmosphere containing 5 ± 1 % carbon dioxide (CO_2). The viability of cells following the exposure to the extracts was measured via their capacity to uptake a vital dye, Neutral Red. This dye was added to the cells to be actively incorporated in viable cells. The number of viable cells correlates to the color intensity determined by photometric measurements at 540 nm after extraction.

The viability of cells exposed to the negative control article and positive control article extracts needs to be greater than or equal to 70% and less than 70% of the untreated control, respectively, to confirm the validity of the assay. The test article meets the requirements of the test if the viability % is greater than or equal to 70% of the untreated control.

We tested only PuraStat® samples irradiated at the highest dose (i.e., 40 kGy) considering they represent all the irradiated samples, because they should have more effects, if yes, on cytotoxicity than those irradiated at lower doses.

Table 20. Summary Table of Irradiation Sterilization Tests

Testing conditions	Appearance	pH	HPLC and MS	Rheology	Cytotoxicity
PuraStat® (Control)	Clear and viscous	2.2	Control	Control	Not toxic
PuraStat® with Gamma at 25kGy	Clear and viscous	2.3	~5% degrade	-	-
PuraStat® with Gamma at 28k or 40 kGy	Clear and viscous	2.3	~15% degrade	equivalent	Not toxic

The amount of peptide prior to the irradiation. After up to 25 kGy of irradiation, PuraStat®'s degradation did not exceed 5% of the amount of peptide prior to the irradiation.

From the pattern of degradation of control PuraStat®, we found that degradation mainly occurs at the points between ~RAD and A~. From the additional pattern of degradation of Ac-$(RADA)_4$-NH_2 (SEQ ID NO:1) when PuraStat® is sterilized with irradiation, we found that irradiation can cause additional degradation at the point between ~R and AD~.

Overall, the rheological property of PuraStat® with gamma irradiation was equivalent to PuraStat® control. All the irradiated PuraStat® and PuraStat® control samples meet the requirements of the test and are not considered to have a cytotoxic potential.

Table 21. The HPLC and LC-MS analysis for two Product Forms

HPLC and LCMS results in each peak									
PuraStat® current product (non-irradiated)					PuraStat® proposed product (irradiated)				
HPLC				LC-MS	HPLC				LC-MS
Peak	**t_R**	**Area**	**Area %**	**Representative m/z**	**Peak**	**t_R**	**Area**	**Area %**	**Representative m/z**
1	6.37	25.70	4.39	1329(666)	1	6.35	8.20	1.90	1329(666)
2	6.73	8.49	1.44	1229(615)	2	6.73	2.90	0.67	1229(615)
3	7.15	9.98	1.71	1300(651)	3	7.27	12.00	2.78	1143(572)
4	7.30	9.29	1.59	1143(572) 893	4	7.56	2.29	0.53	1671(836)
5	7.75	4.75	0.81	1671(836), 1713(857)	5	7.71	2.34	0.54	1671(836)
6	8.01	7.49	1.28	1713(857), 750	6	7.90	7.62	1.77	1671(836), 1713(857)
7	8.18	6.95	1.19	1643(823), 1713(857)	7	8.16	4.06	0.94	1643(823), 1713(557), 1513
8	8.29	2.15	0.36	1643(823), 1713(857)	8	8.27	7.95	1.84	1643(823), 1713(557), 1513
9	8.45	473.23	80.80	1713(857)	9	8.47	348.78	80.89	1713(857)
10	8.80	11.89	2.03	1557, 783, 857	10	8.77	12.95	3.00	1557, 783
11	8.93	4.54	0.77	783, 857	11				
12	9.06	4.85	0.82	839, 1713(857)	12	9.00	10.46	2.43	839, 1713(857)
13	10.00	16.26	2.77	482, 839, 891	13	9.96	11.61	2.69	839, 482

Table 21 below shows results of HPLC tests was performed to evaluate the major peptide content before (control) and after Gamma irradiation.

Agilent HPLC 1100 (Agilent Technologies) was used for this study. Column temperature was kept at 25 °C. Agilent Zorbax 300SB-C18 column (4.6 mm X 250 mm, 5mm, 300 Å) was used for this test. Solvent A was water with 0.1% TFA and solvent B was 80% Acetonitrile with 0.1% TFA. Gradient of solvent B was controlled from 10% to 40% in 20 min and 40% for another 5 min at 25 °C. PuraStat® (40 mg) were mixed with 10 µL of DH2O and 500 µL of formic acid and vortexed. And the mixture was mixed with DH_2O (4,450 µL) and vortexed. 20 mL of samples were injected using an Agilent autosampler. Results are shown in following Table 23.

Table 23.

	PuraStat® control	PuraStat® irradiated above 25 kGy
Major Peptide Content (n=3)	83,8 +/- 1%	81,3 +/- 0,7%
Manufacturing Specification	75% and more	

Findings--Overall, the major peptide content of PuraStat® decreased with gamma irradiation methods and the extent of decrease was more significant with higher dose. After up to 40 kGy of irradiation, PuraStat®'s degradation did not exceed 15% of the

Overall, the major peptide content of PuraStat® decreased with gamma irradiation methods and the extent of decrease was more significant with higher dose. After up to 40 kGy of irradiation, PuraStat®'s degradation did not exceed 15% of the amount of peptide prior to the irradiation. After up to 25 kGy of irradiation, PuraStat®'s degradation did not exceed 5% of the amount of peptide prior to the irradiation.

From the pattern of degradation of control PuraStat®, we found that degradation mainly occurs at the points between ~RAD and A~. From the additional pattern of degradation of Ac-$(RADA)_4$-NH_2 when PuraStat® is sterilized with irradiation, we found that irradiation can cause additional degradation at the point between ~R and AD~.

Overall, the rheological property of PuraStat® with gamma irradiation was equivalent to PuraStat® control. All the irradiated PuraStat® and PuraStat® control samples met the requirements of the test and WERE not considered to have a cytotoxic potential.

CLAIMS

1. A method of sterilizing a self-assembling peptide (SAP) solution, the method comprising:

a) placing one or more containers with the solution of self-assembling peptide into an irradiation machine, said self-assembling peptide capable of forming a hydrogel when applied to a biological tissue at about neutral pH; and

(b) exposing the container to gamma ray, X-ray and/or e-beam irradiation at a predetermined dose so that the peptide solution is sterilized to a pre-determined Sterility Assurance Level (SAL) without substantial degradation of the peptide while its desired biological or physical property(ies) is/are maintained substantially at the same level or improved.

2. The method of claim 1, wherein the peptide is selected from the group consisting of RADA16, KLD12, and IEIK13.

3. The method of claim 2, wherein the dose is 15 - 50 kGy .

4. The method of claims 1-3, wherein the solution is irradiated by X-ray or e-beam.

5. The method of claims 2-4, wherein the peptide solution contains about 2.5% of RADA16, and the dose is 15-24 kGy.

6. The method of claims 1-5, wherein the amount of total ("over-all") peptides that are degraded after the irradiation does not exceed 20% of the amount of the peptides prior to the irradiation.

7. The method of claims 1-6, wherein the desired biological and physical property(ies) is/are selected from the group consisting of: hemostatic, anti-adhesion,

prevention of re-bleeding, anti-stenosis, tissue occlusion, mucosa elevation, wound healing, storage modulus, viscosity, and tissue void filling property.

8. The method of claim 7, wherein the pre-irradiation bioburden of the self-assembling peptide solution is 9CFU per product unit or less.

9. The method of claims 1-8, wherein irradiation dose achieves sterility assurance level (SAL) of at least 10^{-6}.

10. The method of claims 1-9, wherein the concentration of the degradation products of the full-length peptide ("major peptide") in the solution post-irradiation ranges from 0.1% to 5%.

11. The method of claim 1-10, wherein the pH of the solution pre- and post-irradiation ranges from about 1.8 to 3.5.

12. The method of claim 1-11, wherein the solution container is a plastic syringe.

13. The method of claim 1-12, wherein the storage modulus of the gelled solution is increased at least by 10% post-irradiation.

14. The method of claim 1-13, further comprising shearing the solution to reduce or restore its storage modulus.

15. A sterilized solution of self-assembling peptide made by the method of any one of claims 1-14.

16. A method of using the sterilized solution of claim 15, comprising applying the solution to a biological tissue.

17. The method of claim 16, wherein the sterilized solution is applied during surgery or after trauma involving bleeding.

18. The method of claim 17, wherein the sterilized solution is applied to an internal site in a human subject.

19. The method of claim 1, wherein the self-assembling peptide solution exhibits a post-irradiation mass spectrometric (MS) profile substantially as shown in corresponding post-irradiation profiles of FIGS. 2-6 and/or as described in the Examples (e.g., in the case of PuraStat®, the additional major M_z peaks at are observed at 836/1670, 1100, and 1513 m/z).

20. Use of a sterilized solution of the self-assembling peptide for treating or preventing a disease or condition, wherein the sterilized solution is obtained by the method of any one of claims 1-19.

Doc ID 966D2228

Figure 1A

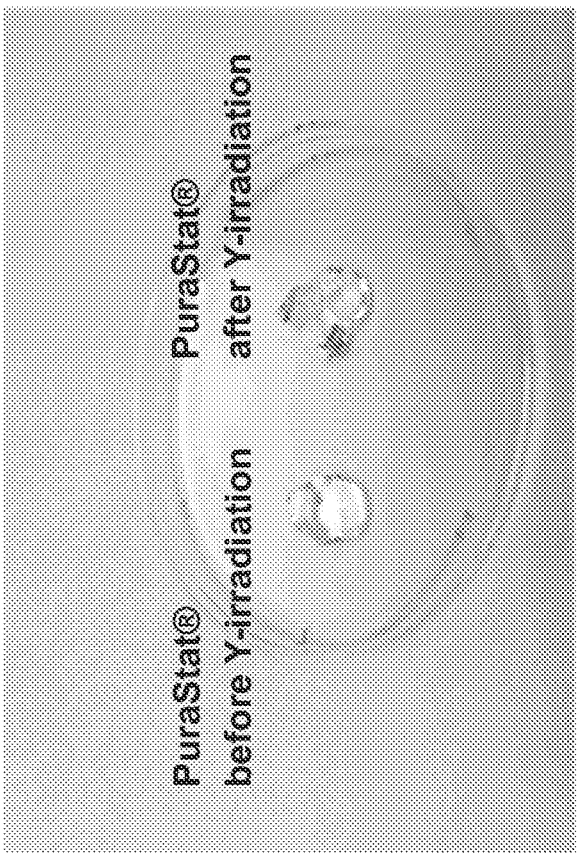


Figure 1B

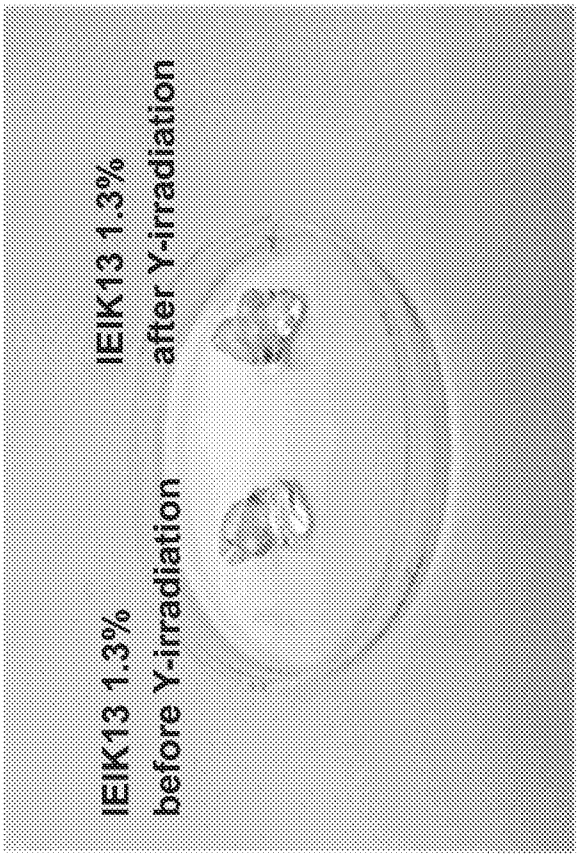


Figure 2A

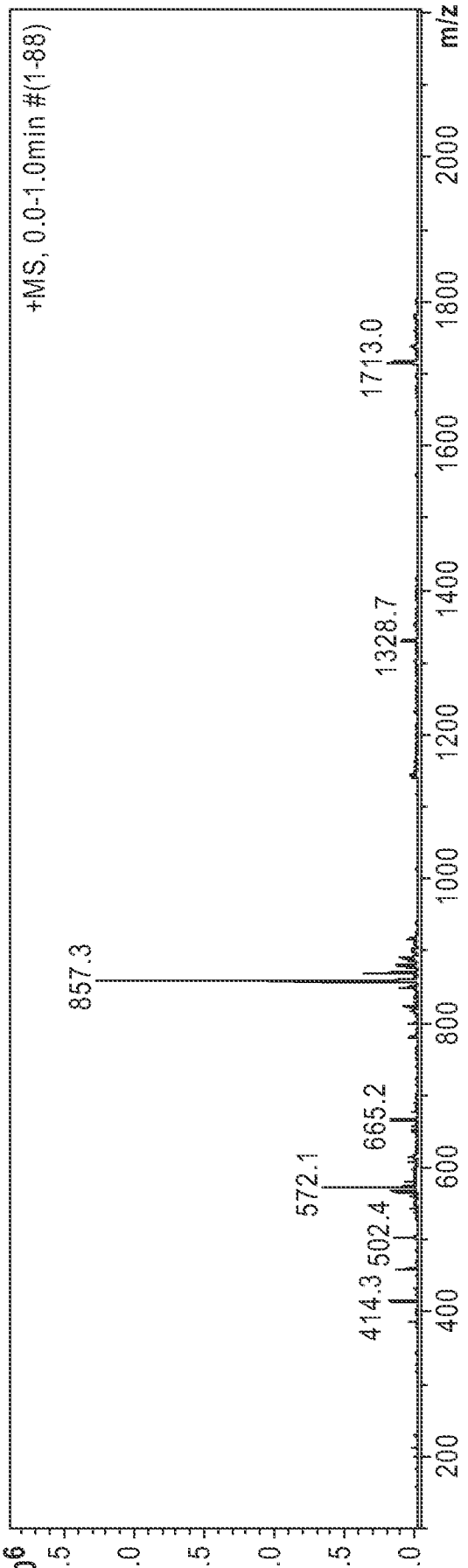


Figure 2B

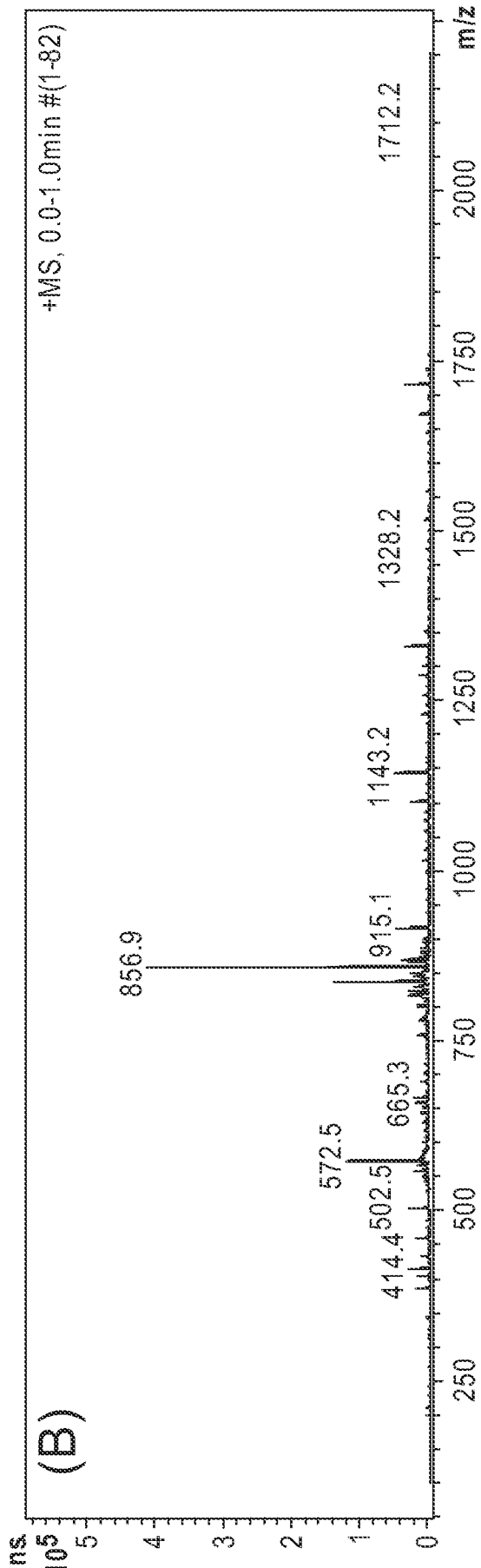


Figure 2C

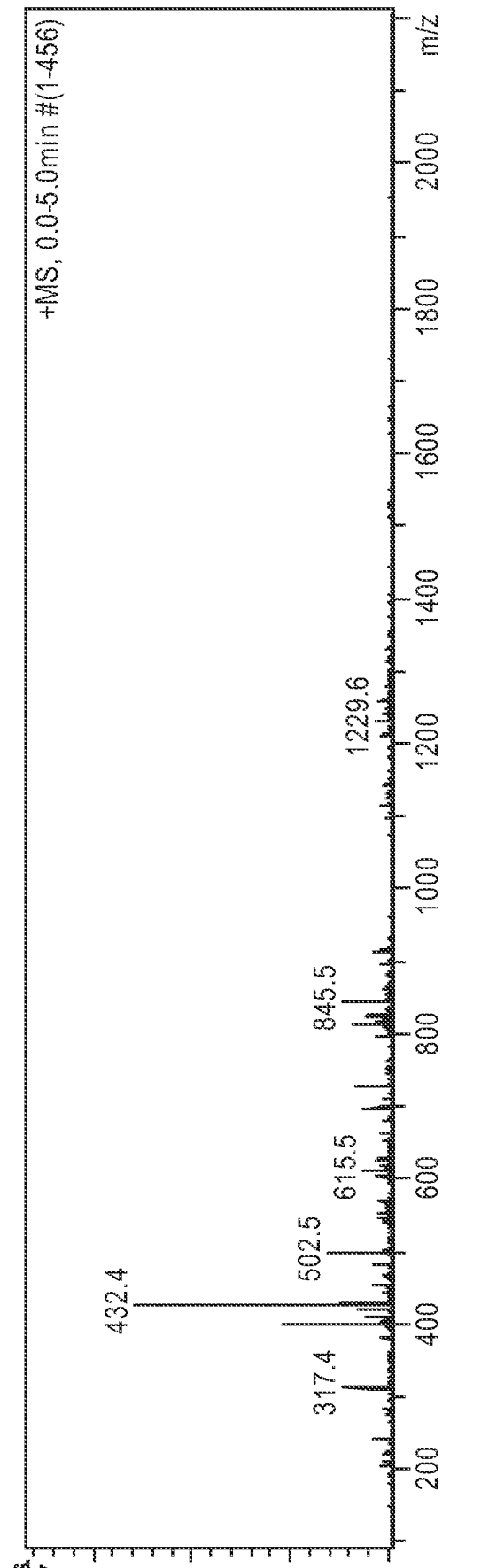

+MS, 0.0-5.0min #(1-456)
Intens.
x10^7
3
2
1
0
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z
317.4
432.4
502.5
615.5
845.5
1229.6

Figure 3A

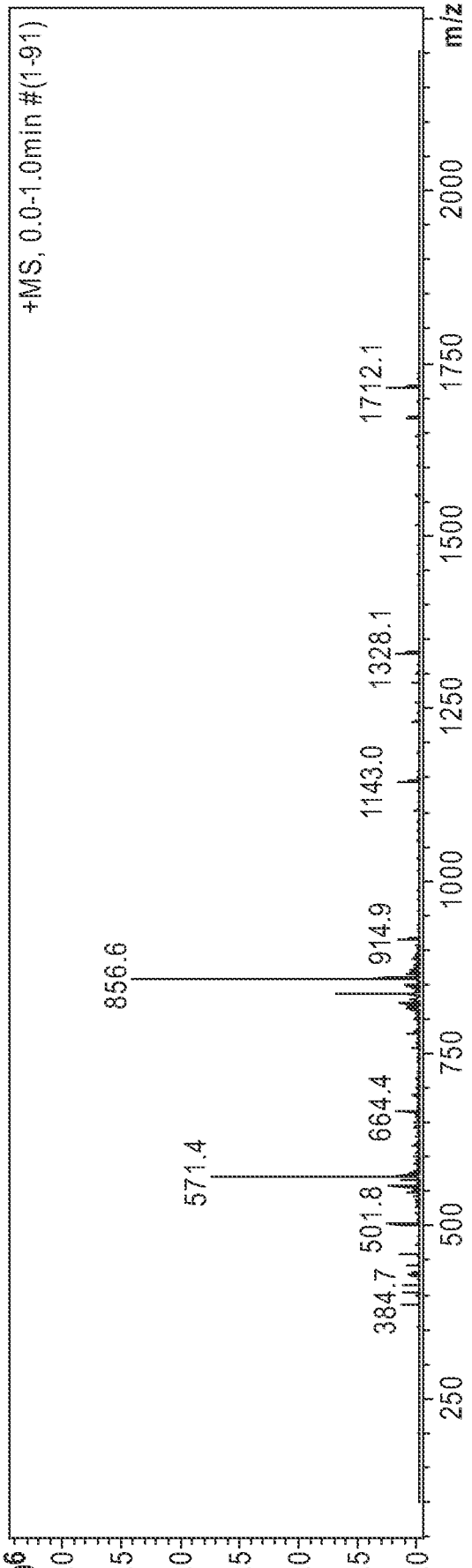


Figure 3B

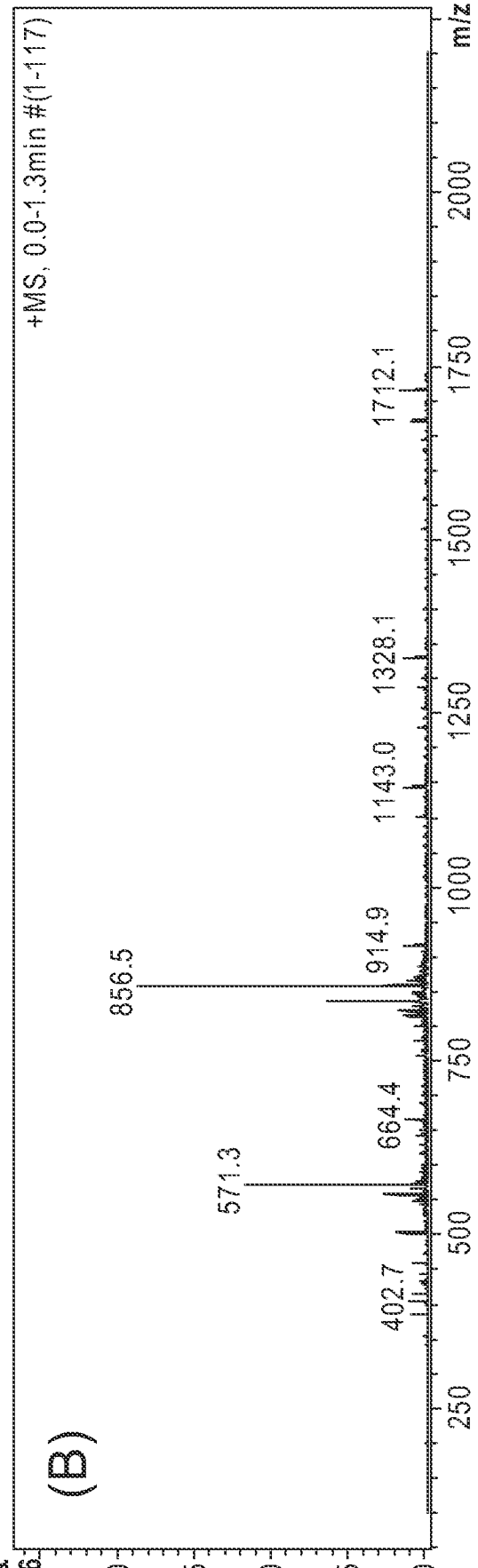


Figure 4A

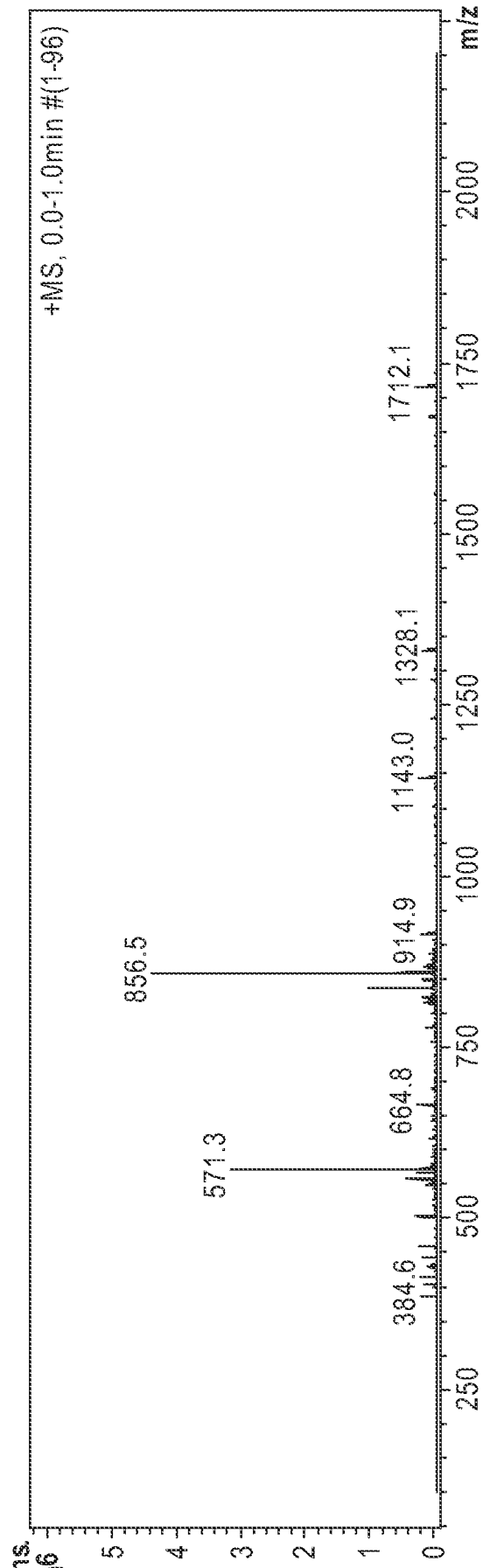


Figure 4B

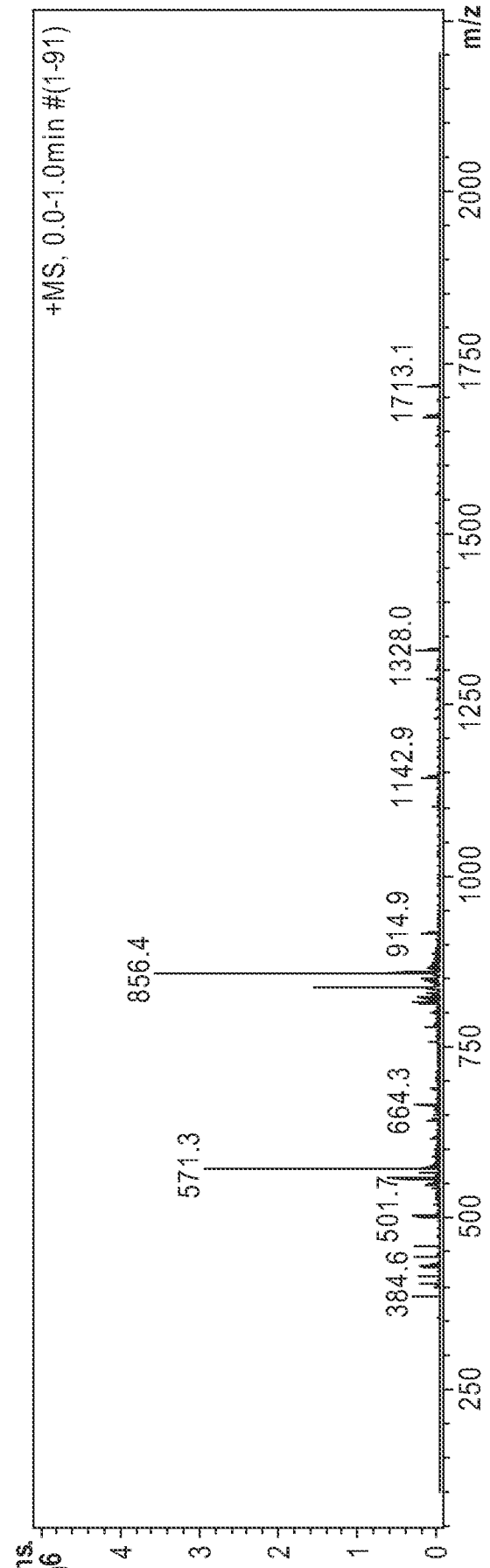


Figure 5A

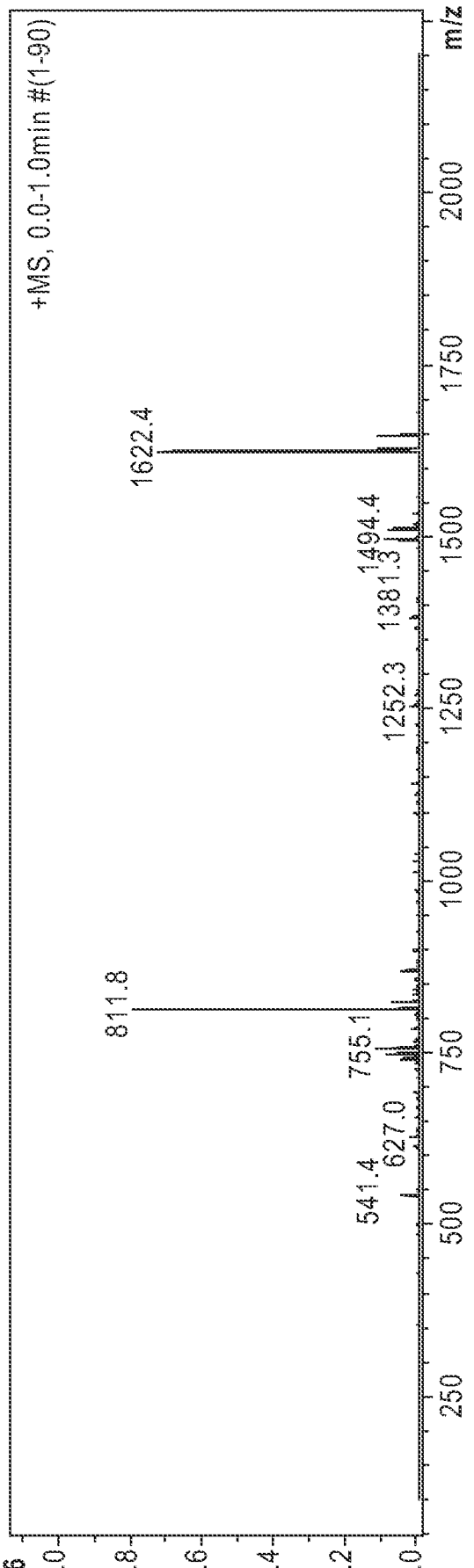


Figure 5B

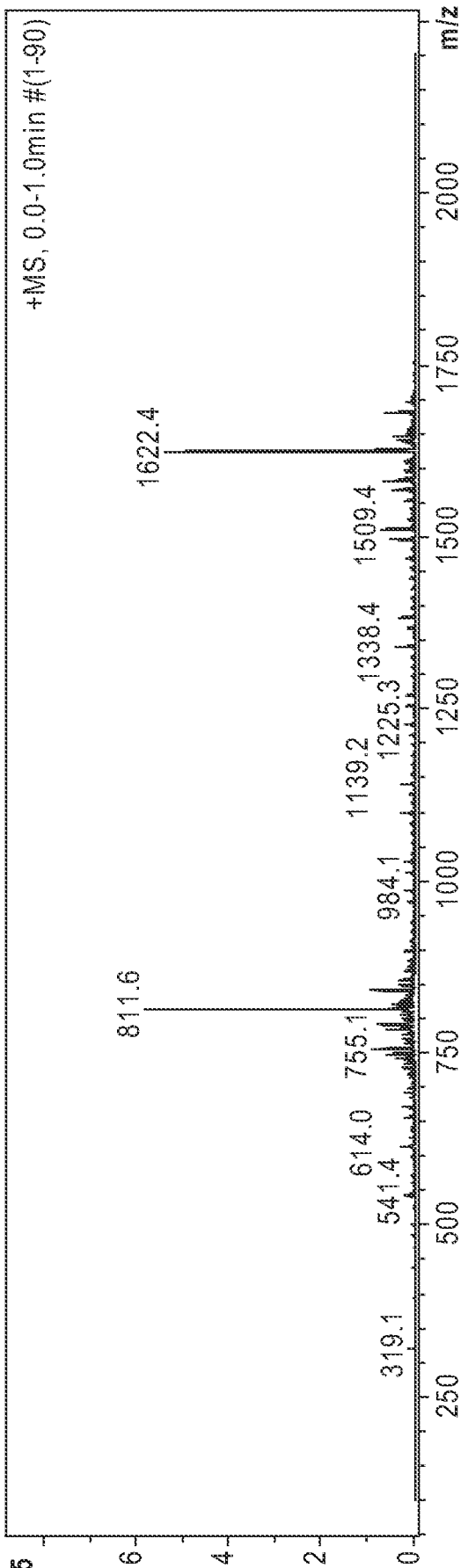


Figure 5C

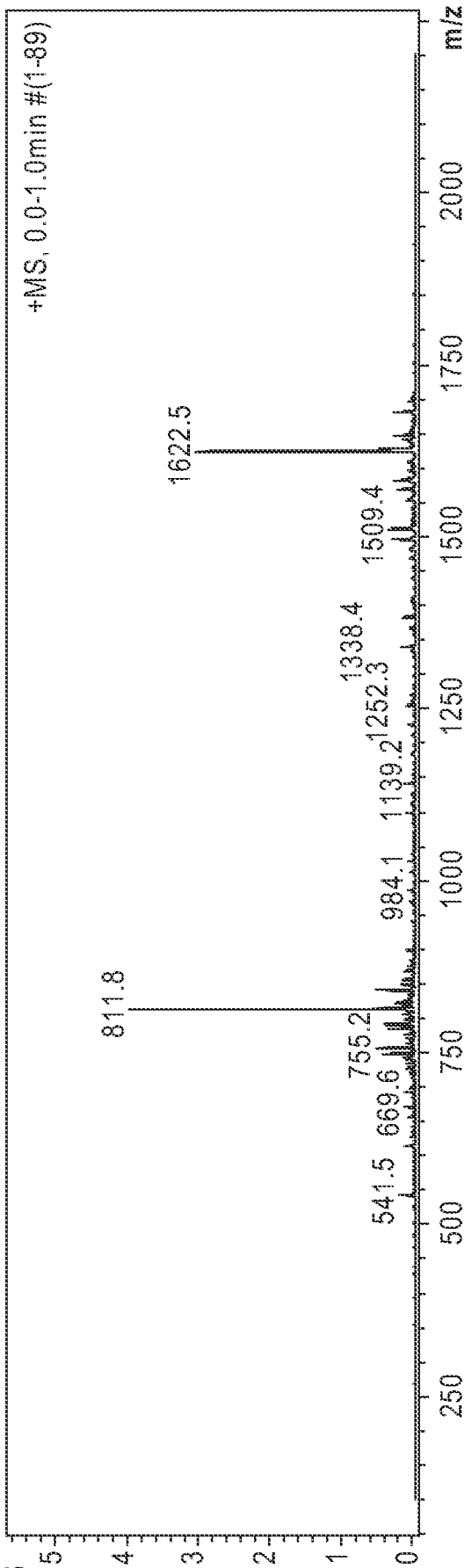


Figure 5D

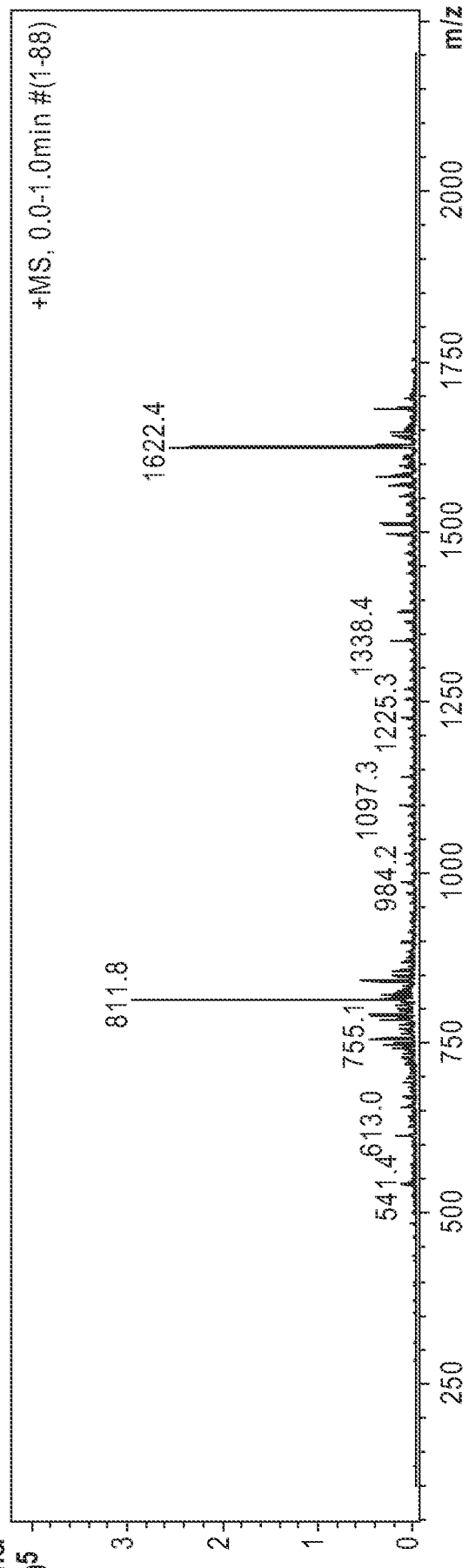


Figure 5E

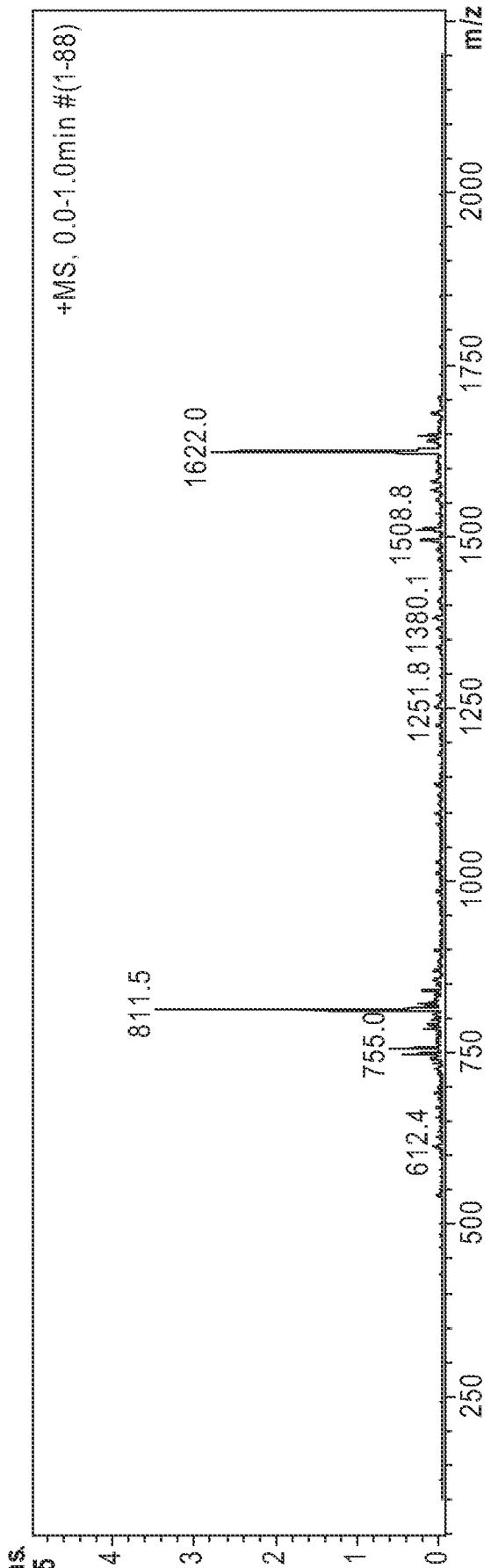


Figure 5F

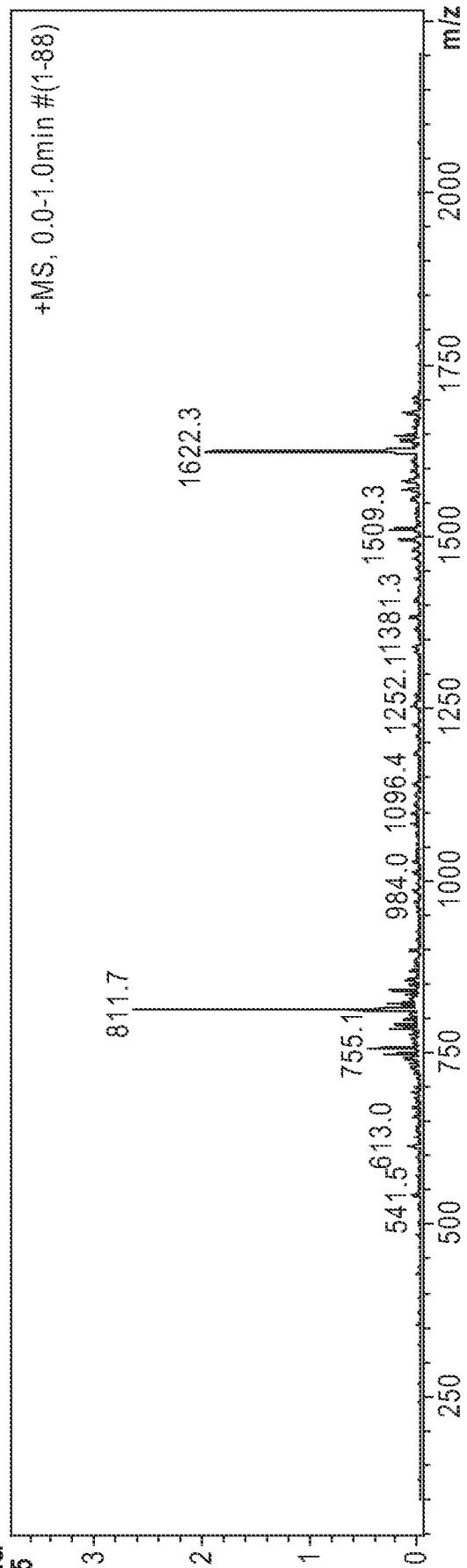


Figure 6A

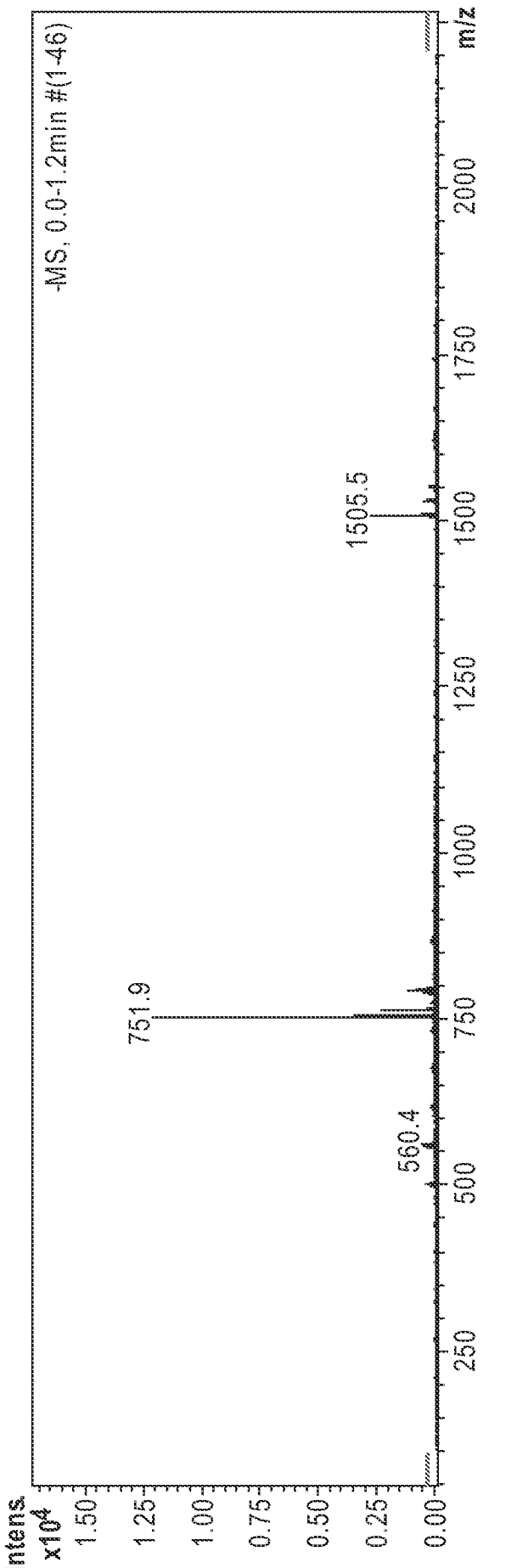

-MS, 0.0-1.2min #(1-46)
Intens.
x10^4
1.50
1.25
1.00
0.75
0.50
0.25
0.00
250
500
750
1000
1250
1500
1750
2000
m/z
751.9
560.4
1505.5

Figure 6B

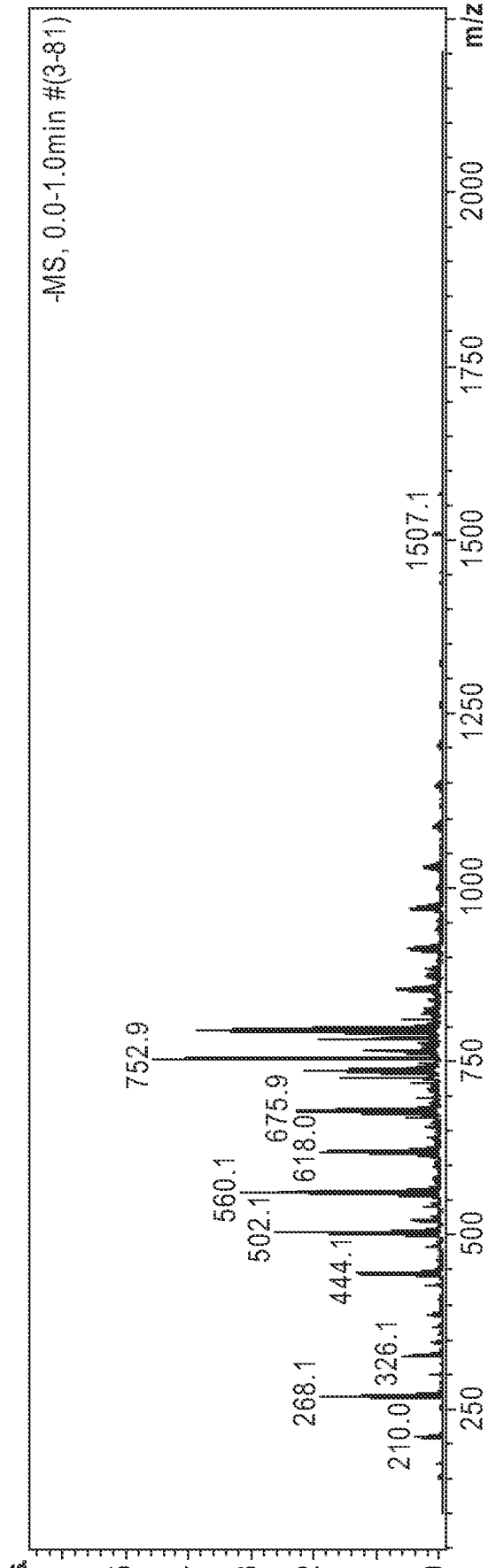


Figure 6C

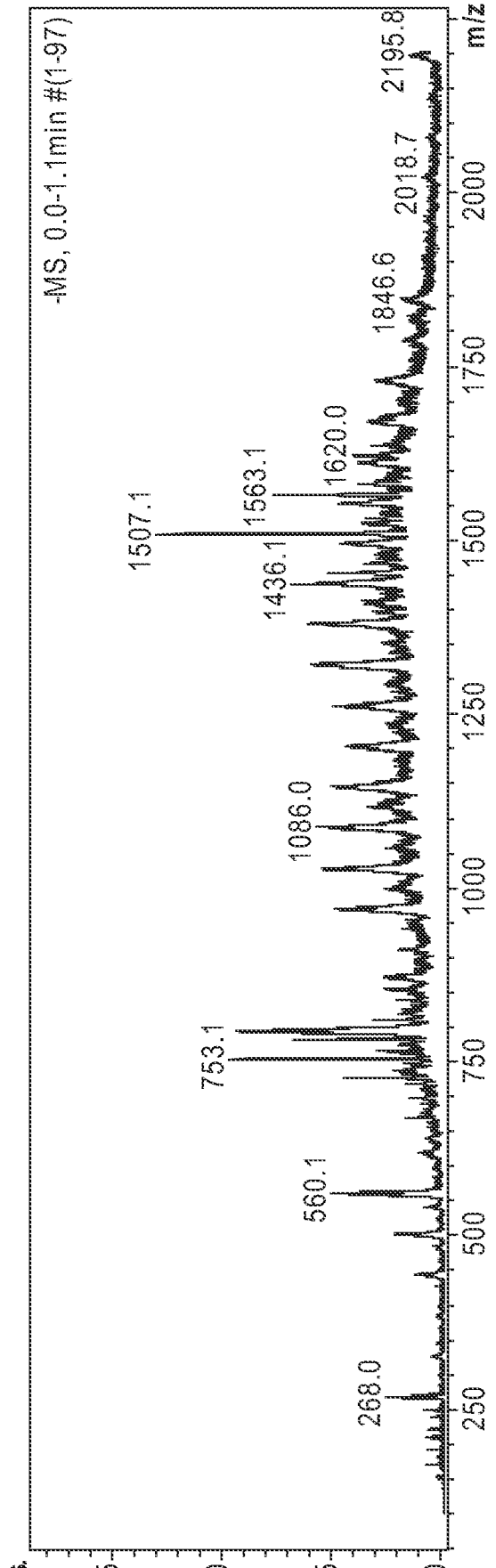


Figure 6D

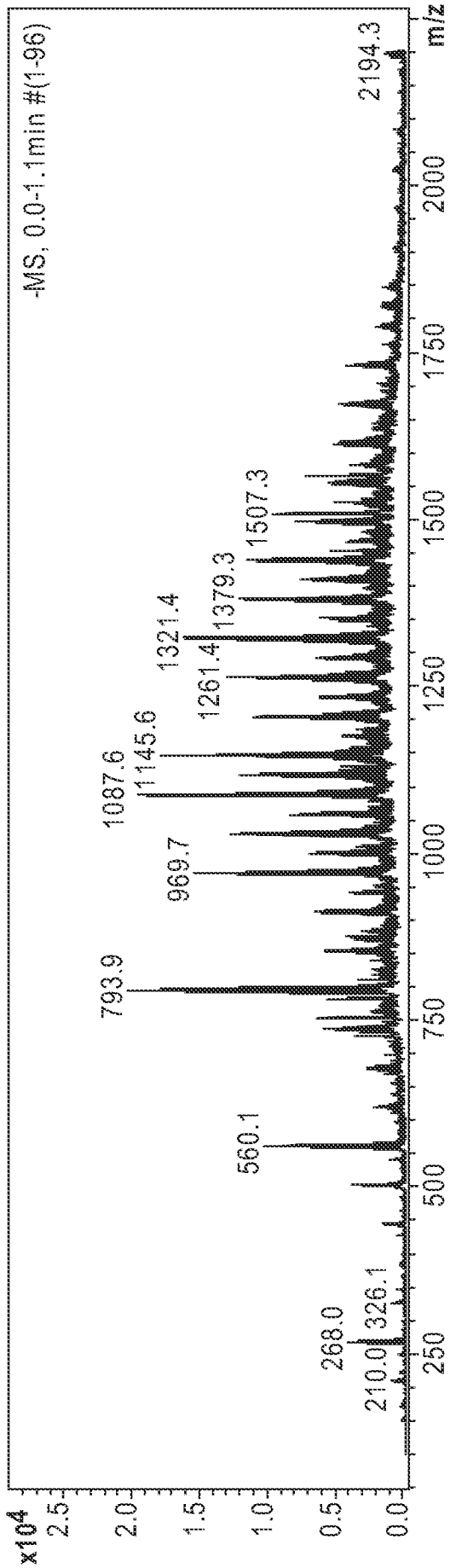


Figure 6E

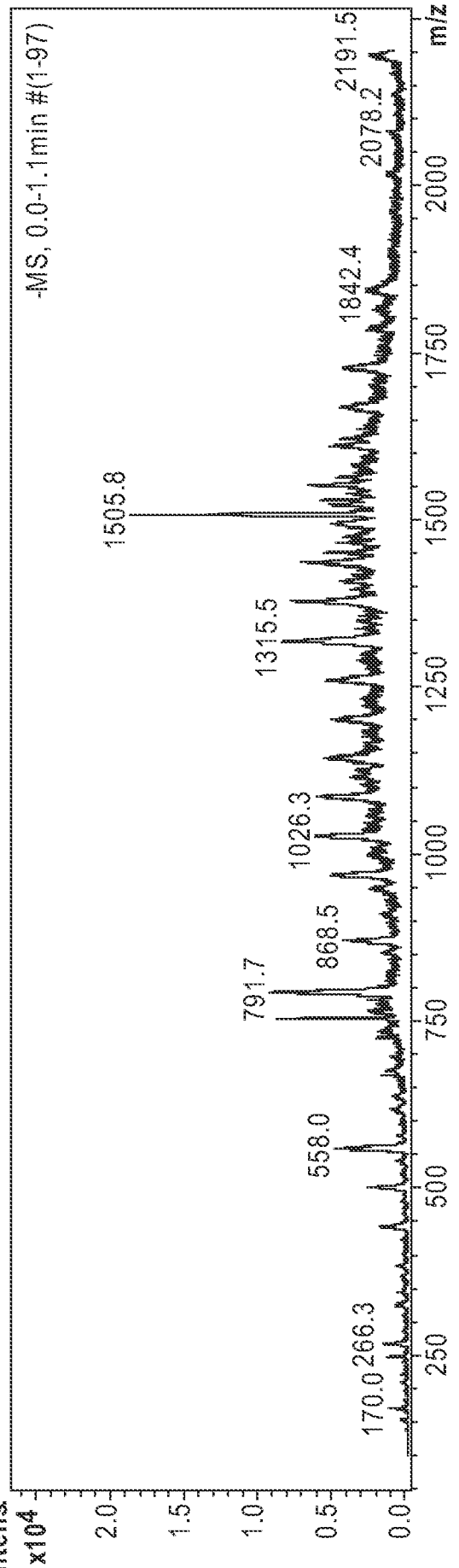
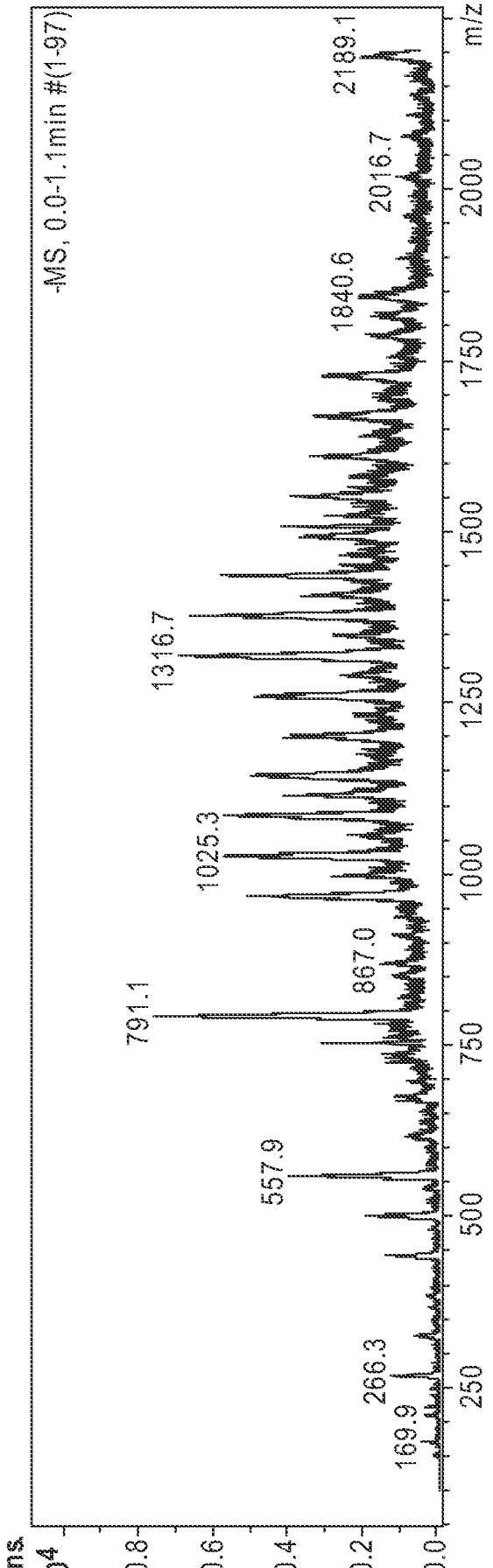


Figure 6F


-MS, 0.0-1.1min #(1-97)
Intens.
x10^4
0.8
0.6
0.4
0.2
0.0
250
500
750
1000
1250
1500
1750
2000
m/z
169.9
266.3
557.9
791.1
867.0
1025.3
1316.7
1840.6
2016.7
2189.1

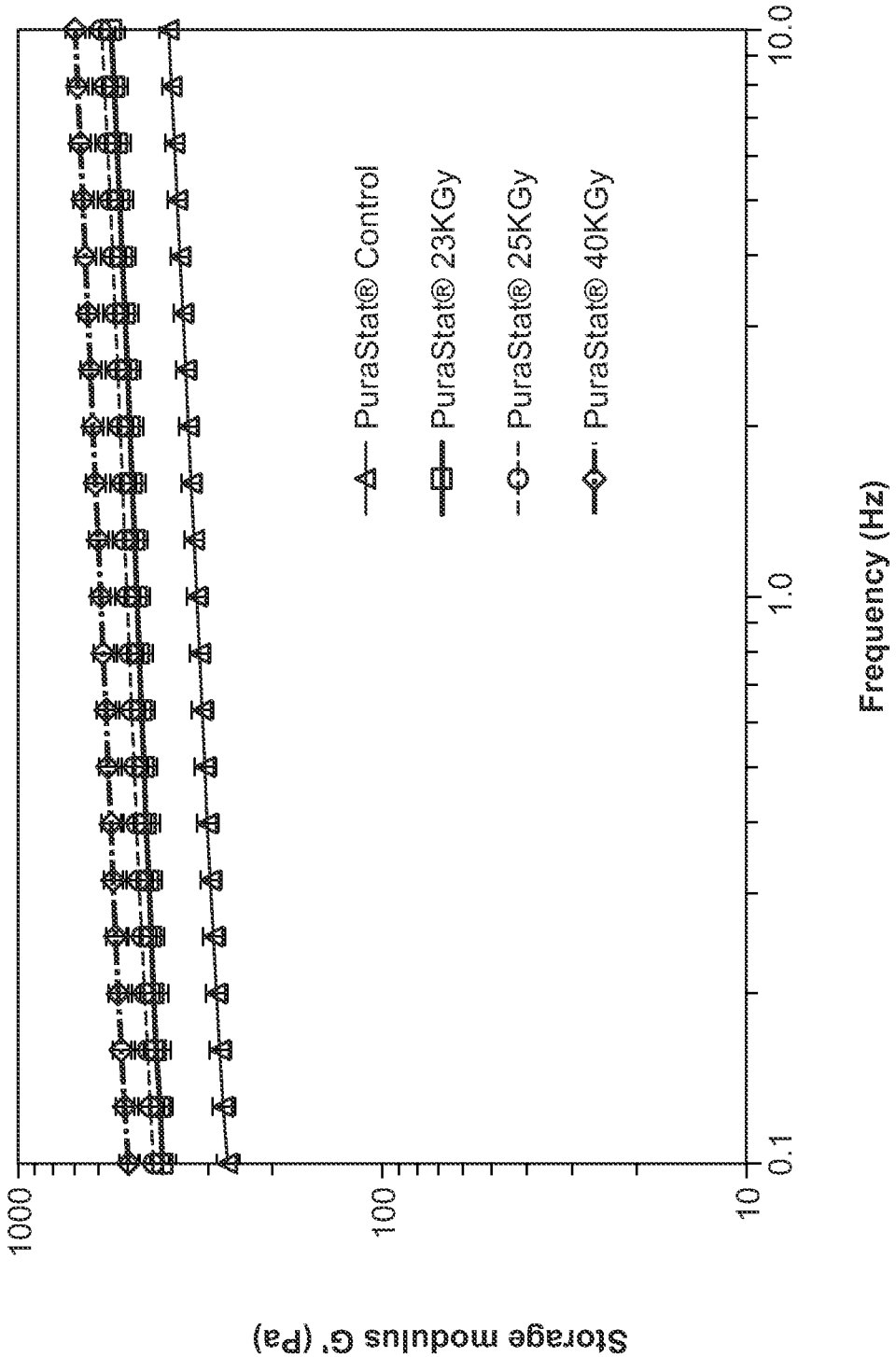

Storage modulus G' (Pa)
1000
100
10
0.1
1.0
10.0
Frequency (Hz)
PuraStat® Control
PuraStat® 23KGy
PuraStat® 25KGy
PuraStat® 40KGy

Figure 7

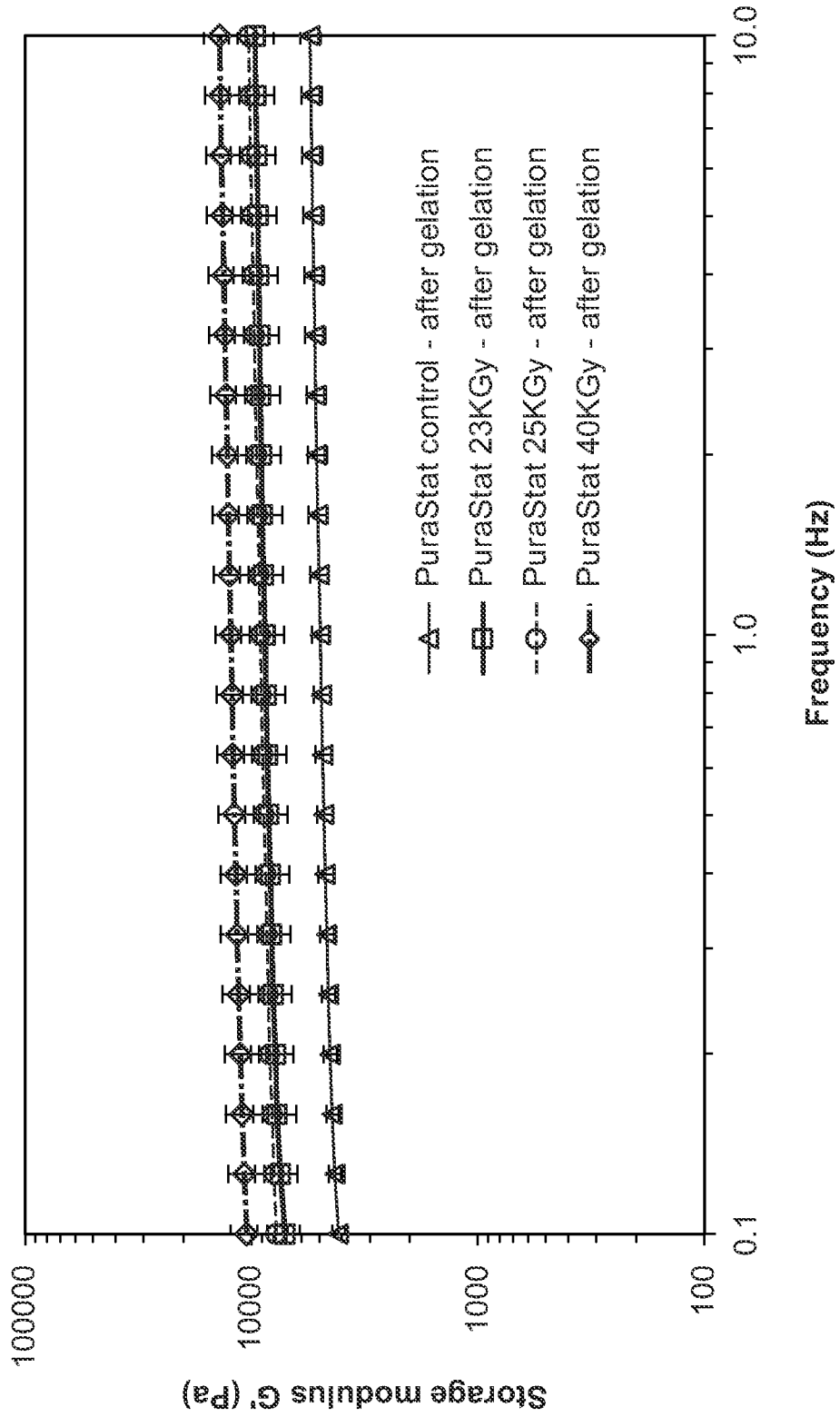
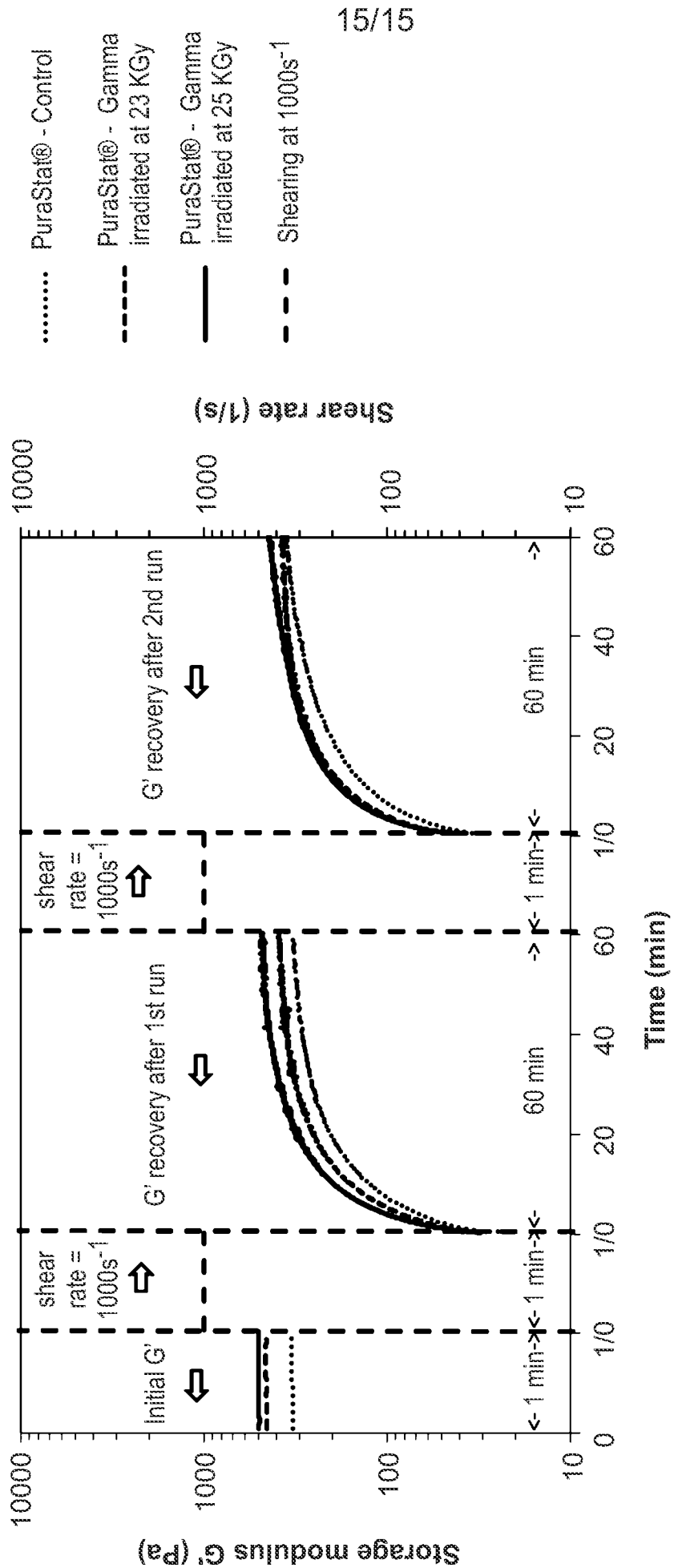

Storage modulus G' (Pa)
100000
10000
1000
100
0.1
1.0
10.0
Frequency (Hz)
PuraStat control - after gelation
PuraStat 23KGy - after gelation
PuraStat 25KGy - after gelation
PuraStat 40KGy - after gelation

Figure 8

Figure 9


PuraStat® - Control
PuraStat® - Gamma irradiated at 23 KGy
PuraStat® - Gamma irradiated at 25 KGy
Shearing at 1000s-1
Shear rate (1/s)
10000
1000
100
10
Storage modulus G' (Pa)
Initial G'
shear rate = 1000s-1
G' recovery after 1st run
G' recovery after 2nd run
1 min
60 min
0
1/0
20
40
60
Time (min)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/24954

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/16, A61K 38/00, A61L 27/52, A61L 27/50 (2021.01)

CPC - C07K 7/08, A61L2/081, C07K 2319/735, A61L 29/145

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2019/0111165 A1 (3-D MATRIX, LTD.) 18 April 2019 (18.04.2019) abstract; para [0024], [0036], [0051], [0054], [0101]-[0102]	1-3, 19
Y	KANG et al., The Influence of Electron Beam Sterilization on In Vivo Degradation of TCP/PCL of Different Composite Ratios for Bone Tissue Engineering. Micromachines. 6 March 2020, Vol. 11, no 3, article 273, pp1-12; pg 3, para 3-4; pg 4, para 1; pg 6, para 3	1-3, 19

☐ 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
"D" document cited by the applicant in the international application
"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	Date of mailing of the international search report
21 June 2021	AUG 12 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Kari Rodriquez Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/24954

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☒ on paper or in the form of an image file.

   b. ☒ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☒ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/24954

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-18, 20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.