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FIG. 1A

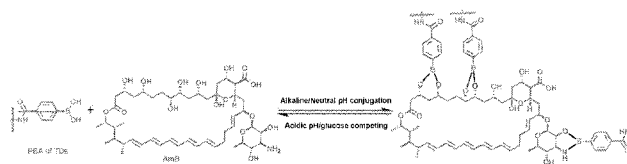


FIG. 1B

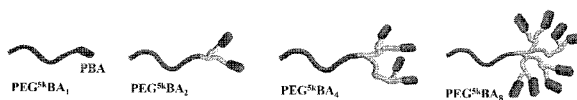
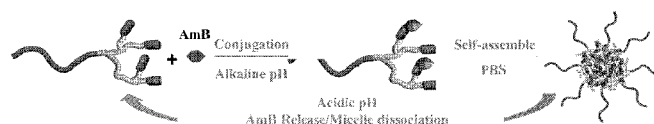


FIG. 1C



(57) **Abstract:** Amphotericin B (AmB) is the gold standard for treatment of life-threatening systemic fungal infections. To develop cost-effective AmB formulations with reduced toxicity, embodiments disclosed introduce controlled number of phenylboronic acid (PBA) moieties into telodendrimer (TD) nanopatform for optimizing AmB conjugation via reversible boronate ester strategy. This multivalent flexible boronate TD nanopatform can also be applied in developing prodrugs for different cis-diol containing or α - or β -hydroxyl ketone-containing drug molecules.

SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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BORONATE PRODRUG NANOFORMULATIONS IN DISEASE TREATMENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to US Provisional Application No. 63/471,669, filed on June 7, 2023, the entire disclosures of which are incorporated
5 herein by reference as though set forth in full.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant number GM130941 awarded by the National Institute of Health. The government has certain rights in the invention.

10 BACKGROUND OF THE INVENTION

[0003] The immunocompromised patients suffering dialysis, organ transplant, catheterization, HIV infection, chemotherapy or under intensive care medication are at higher risk for lethal fungal infection, commonly by Candida, Aspergillus, Cryptococcus, and Pneumocystis. The challenge of fungal infections and the
15 development of drug resistance demand highly efficacious antifungal therapeutics. Polyenes and azoles play the critical role for invasive fungal infections treatment of immunocompromised patients. Among them, Amphotericin B (AmB) is the gold standard for the treatment of life-threatening systemic fungal infections for over sixty-years in the clinic due to its broad activity spectrum, low resistant rate, and outstanding
20 pharmacological and clinical efficacy. The antifungal activity of AmB is attributed with its high binding affinity with ergosterol in fungal membrane, creating an ion permeable transmembrane channel and causing cell destruction. However, the clinical use of AmB is associated with severe nephrotoxicity and infusion-related complication syndrome due to the proinflammatory cytokine production and hyperkalemia, causing
25 cardiac arrhythmias and even death. Especially, the conventional AmB sodium deoxycholate formulation, i.e. Fungizone®, has very narrow therapeutic window limited by its dose-dependent toxicity. Lipid-based formulations of AmB, such as AmBisome®, ABELCET®, and Amphotin LIP® have been developed to significantly lower the incidence of infusion-related reactions and increase the renal tolerance.
30 However, Lipid based AmB has been reported to cause idiosyncratic reactions such as chest pain, flushing, and abdomen pain due to liposome formulation other than AmB itself. In addition, prohibitive cost and limited manufacturer of Lipid AmB

drastically compromise its clinic application. Much effort have been made to develop cost-effective formulations with reduced toxicity.

BRIEF SUMMARY OF EMBODIMENTS

[0004] Embodiments disclosed herein are directed to prodrug compounds
5 having the following formula: PEG-D(X)-(L-BA)_m-d_n, wherein PEG is a polyethylene glycol moiety, and has an average molecular weight of about 44 to about 40,000 Dalton; D(X) is a dendritic polymer moiety having one or more branched monomer units (X); L is an optional linker group; BA is at least one boronic acid moiety; d is at least one 1, 2- or 1, 3- cis-diol or catechol containing therapeutic compound, or an α-
10 or β- hydroxyl ketone containing therapeutic compound; and subscript m is an integer from 2 to 16 and subscript n is any number that greater than 0 and less than m. Other embodiments are directed to prodrug compounds, wherein the polyethylene glycol moiety has an average molecular weight of about 5000 Da. According to other embodiments disclosed herein, the prodrug compounds have branched monomer unit
15 X that is a natural or synthetic amino acids with more than one amine group. According to a particular embodiment the prodrug compound each branched monomer unit X is a lysine moiety.

[0005] According to embodiments disclosed herein, the prodrug compounds comprise a boric acid that can be an aryl boronic acid or an aliphatic boronic acid and
20 according to embodiments that boric acid component is a phenylboronic acid or a phenylboronic acid comprising a substitute group on the phenyl ring. Moreover, embodiments include a prodrug compound having a nonlinear dendritic structure, and according to an embodiment the X unit is a lysine moiety. According to an embodiment, the therapeutic compound of the prodrug is covalently bonded to the boric acid BA via
25 a reversible boronate ester bond.

[0006] According to embodiments disclosed herein, the prodrug compounds include at least one boronic acid moiety is selected from the group consisting of phenyl boronic acid, nitrophenyl boronic acid, methoxyl phenylboronic acid, benzyl boronic acid, methylene boronic acid, ethylene boronic acid, and proplene boronic acid.

30 [0007] According to embodiments the therapeutic drug molecule of the prodrug compound include curcumin, doxorubicin, daunorubicin, amphotericin B, nystatin, anidulafungin; tetracyclines, aminoglycosides, nicotinamide mononucleotide (NMN), and nicotinamide adenine dinucleotide (NAD/NADH). Further therapeutic compounds of the prodrug include tetracycline HCl, doxycycline, minocycline, tigecycline,

eravacycline, sarecycline, omadacycline, chlortetracycline, oxytetracycline, and chemically modified tetracycline 3 (CMT-3). Furthermore, therapeutic compounds of the prodrug include gentamycin, tobramycin, and kanamycine. And according to an embodiment the therapeutic compound is amphotericin B (AmB).

5 [0008] According to embodiments the linker (L) of the prodrug compound can be polyethylene glycol moiety, amino acid, polyamino acids, polyserine moiety, enzyme cleavable peptide moiety, disulfide bond moiety and acid labile moiety, polyglycine moiety, poly(serine-glycine) moiety, aliphatic amino acid moieties, 6-amino hexanoic acid moiety, 5-amino pentanoic acid moiety, 4-amino butanoic acid moiety, 10 and beta-alanine moiety. Other linker (L) groups include lysine, arginine, aspartic acid, glutamic acid, linear oligo-lysine, linear oligo-aspartic acid, linear oligo-arginine, and linear oligo-glutamic acid.

[0009] According to an embodiment the prodrug compound comprises self-assemble monodispersed micelles, and the micelles have a particle size of about 10- 15 20nm.

[0010] According to embodiments disclosed herein, the prodrug reduce toxic side effects, immunogenicity and improved efficacy of drug molecules when administered in therapeutically effective amounts to mammal in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

20 [0011] FIG. 1 presents a design of AmB conjugated telodendrimer: FIG. 1A presents model reaction of AmB and PBA conjugates by bivalent or trivalent boronate ester bond formation. FIG. 1B presents a schematic illustration for the architectures of PBA containing TDs: PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄, and PEG^{5k}BA₈. FIG. 1C presents a schematic illustration for AmB conjugation and release on PBA-containing 25 TDs via reversible boronate ester bond.

[0012] FIG. 2 presents the characterization of AmB-TDs prodrug formulations: FIG. 2A presents the particle size distribution of AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈. FIG. 2B presents TEM images of blank PEG^{5k}BA₄, PEG^{5k}BA₈, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈. FIG. 2C presents the 30 UV-Vis absorbance spectrum of AmB formulations including: Free AmB in DMSO, Fungizone, Ambisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈. FIG. 2D presents an in vitro drug release profile of Fungizone, Ambisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ in PBS at

37 °C. FIG. 2E presents an in vitro drug release profile of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ in neutral, acidic pH and with glucose at 37 °C.

[0013] FIG. 3 presents the antifungal activity, cytotoxicity and biocompatibility of AmB-TD formulations: FIG. 3A presents an MIC of AmB formulations for *C. albicans* inhibition. FIG. 3B presents candida albicans at invasion phase growth inhibition by Free AmB in DMSO, Fungizone, Ambisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at AmB concentration of 2.5 µg/mL. FIG. 3C presents cell viability of Free AmB in DMSO, Fungizone, Ambisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ in 293T, RAW 264.7, and CHO cells. FIG. 3D the hemolytic property of Free AmB in DMSO, Fungizone, Ambisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ for overnight incubation at 37 °C. FIG.3E presents the TNF-α production level of RAW 264.7 cells with Fungizone, Ambisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ for 2h incubation at 37 °C.

[0014] FIG. 4 presents the PK and biodistribution of AmB-TD formulations: FIG. 4A presents the PK of Fungizone, Ambisome, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at AmB dose of 1 mg/kg by i.v. injection. FIG. 4B presents the PK of Ambisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at AmB dose of 10 mg/kg by i.v. injection. FIG. 4C presents a summary of PK analysis of AmB formulations. FIG. 4D presents representative ex vivo images of major organs taken out at 24 h after i.v. injection of DiD encapsulated AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ in healthy and *C. albicans* infected mice. FIG. 4E presents an ex vivo semi-quantitative fluorescent intensity of major organs at 24 h post-injection.

[0015] FIG. 5 the MTD, blood chemistry and pathology profiles of mice treated by AmB formulations: FIG. 5A presents bodyweight changes of healthy mice administer intravenously with three consecutive Fungizone, Ambisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ and blank PEG^{5k}BA₄. FIGs. 5B-5F [present the BUN, CRE, K⁺, ALT and AST and TNF-α levels of 24 h after last dose of AmB formulations. FIG. 5G presents the histological examinations of the kidneys by H&E staining from animals treated with AmB formulations.

[0016] FIG. 6 presents the antifungal efficacy of AmB-PEG^{5k}BA₄ in *C. albicans* infected immunocompetent mice. FIG. 6A presents a schematic illustration of *C. albicans* infected immunocompetent mouse model and treatment. FIG. 6B presents the cumulative mice survival, FIG. 6C presents the bodyweight change, FIG. 6D

presents the body temperature changes of mice was monitored. FIG. 6E presents the colony count of *C. albicans* in major organ of mice treated by AmB formulations. FIG. 6F presents the TNF- α and IL-6 level in plasma of mice treated by AmB formulations. FIG. 6G presents GMS staining for *C. albicans* in the kidney of mice treated by AmB formulations. FIG. 6H presents the histological examinations of the kidneys by H&E staining of mice treated by AmB formulations.

[0017] FIG. 7 presents the antifungal efficacy of AmB-PEG5kBA₄ in *C. albicans* infected immunocompromised mice. FIG. 7A presents a schematic illustration of *C. albicans* infected immunocompromised mouse model and treatment. FIG. 7 presents FIG. 7B presents the cumulative mice survival, FIG. 7C presents the bodyweight change, of mice was monitored. FIG. 7D presents the colony count of *C. albicans* in major organ of mice treated by AmB formulations. FIG. 7E presents the TNF- α and IL-6 level in plasma of mice treated by AmB formulations. FIG. 7F presents the GMS staining for *C. albicans* in the kidney of mice treated by AmB formulations. FIG. 7G presents the histological examinations of the kidneys by H&E staining of mice treated by AmB formulations.

[0018] FIG. 8 presents a scheme of the chemical structures and synthesis route of PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈. FIG. 8A presents the chemical structures of PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈. FIG. 8B presents the synthesis route of PEG^{5k}BA₁. FIG. 8C presents the synthesis route of PEG^{5k}BA₂. FIG. 8D presents the synthesis route of PEG^{5k}BA₄. FIG. 8E presents the synthesis route of PEG^{5k}BA₈.

[0019] FIG. 9 Chemical structure of clinical medicine and compounds with 1,2- and 1,3 Cis-diol group that can be conjugated on BA containing TDs to form nanoprodug formulations.

[0020] FIG. 10 Chemical structure of clinical medicine and compounds with α -hydroxy ketone group that can be conjugated on BA containing TDs to form nanoprodug formulations.

[0021] FIG. 11 Chemical structure of clinical medicine and compounds with enolatable dione group that can be conjugated on BA containing TDs to form nanoprodug formulations.

[0022] FIG. 12 Chemical structure of PEG^{2k}BA₄, PEG^{2k}NBA₄, PEG^{5k}BA₄, PEG^{5k}NBA₄, PEG^{2k}Arg₄BA₄, PEG^{2k}Glu₄BA₄ and PEG^{2k}Glu₈BA₄. As shown in FIG. 12, we developed different types of boronic acid containing TDs. The TDs are prepared

with both PEG^{5k} and PEG^{2k}. The number of BA moieties in the TD are 1, 2, 4, and 8. PEG linker, C6 linker, Arginine, and glutamate are used as linker to increase flexibility of BA moieties on TD.

[0023] FIG. 13 BA-containing TD for TCL, DCL, and CMT-3 prodrug conjugation.

5 (A) TCL, DCL, and CMT-3 react with PBA small molecule to form boronate ester bond. (B) Schematic illustration for TCL, DCL, and CMT-3 conjugation and release on PBA-containing TDs via reversible boronate ester bond and acidic pH/ROS responsive for drug release. Tetracycline (TCL) and its derivatives doxycycline (DCL) and CMT-3 with 1,2- and 1,3-diol groups can be conjugated onto the boronic acid group containing
10 TDs to form prodrug nanoformulation via boronate ester bond, which is labile and responsive to acidic pH and ROS.

[0024] FIG.14 MALDI-TOF of CMT-3 react with (A) PBA and (B) NBA small molecules. MALDI-TOF result indicates CMT-3 can be reacted with PBA or NBA small molecules via boronate ester bonds in 1:1 or 1:2 molar ratio.

15 [0025] FIG. 15 Particle size of CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 with and without TEA during drug loading process. CMT-3 can be efficiently conjugated on both PEG2kBA4 and PEG2kNBA4 with TEA as catalyst and form homogeneous nanoparticles with particle size of 275 nm and 10 nm, respectively. CMT-3 can also efficiently be conjugated on PEG2kNBA4 without TEA at 1:2.5 mass
20 ratio. In comparison with PEG2kBA4, PEG2kNBA4 exhibits significantly higher efficiency for CMT-3 conjugation.

[0026] FIG. 16 MALDI-TOF of CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 formulations before and after CMT-3 conjugation. Free CMT-3 detection in CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 formulations. The MW of PEG2kBA4 and PEG2kNBA4 before and after CMT-3 conjugation were characterized by MALDI-TOF.
25 As shown in Figure 5, MW of TDs increased after CMT-3 conjugation. In addition, significant lower signal or non-signal of free CMT-3 were observed in the CMT-3-TD conjugated nanoprodug formulation. Based on the peak intensity ratio of free CMT-3 to CHCA matrix, the CMT-3 conjugation efficiency was calculated. The conjugation
30 efficiency of CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 are 84% and over 90%, respectively.

[0027] FIG. 17A UV spectrum of free CMT-3, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, and CMT-3 PEG5kCA4VE4 formulations. FIG. 17B Fluorescent intensity of free CMT-3 in DMSO, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, and CMT-

3 PEG5kCA4VE4 in PBS. FIG. 17 C Fluorescent intensity of CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 in PBS, pH 5.5 and pH 2.0 buffer. As shown in Fig.17A, significant red shift of CMT-3 peak were observed in CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, indicating efficient conjugation of CMT-3 on TDs, while CMT-3 encapsulated PEG5kCA4VE4 formulation exhibited similar UV spectrum with free CMT-3 without red-shift. CMT-3 showed significant fluorescent intensity increased after conjugation on PEG2kBA4 in comparison with free CMT-3 and CMT-3 encapsulated PEG5kCA4VE4 formulation, demonstrating successful conjugation of CMT-3 on TDs. Since the nitro group with strongly electron withdrawing property, the fluorescence of conjugated CMT-3 was significantly quenched by nitro group in CMT-3-PEG2kNBA4 nanoprodug formulation. The fluorescent intensity of CMT-3 was significantly reduced in acidic pH, indicating efficient cleavage of CMT-3 from TD at acidic pH.

[0028] FIG. 18 In vitro CMT-3 release profile CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, and CMT-3 PEG5kCA4VE4 at pH 7.4 and pH 5.5. CMT-3 release percentage at 8h. As shown in Fig.18, both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 exhibited significant prolonged drug release in comparison with CMT-3 encapsulated PEG5kCA4VE4 formulation at neutral pH. Acidic pH 5.5 accelerated the CMT-3 release in CMT-3-PEG2kBA4, while has slightly effect on release profile of CMT-3-PEG2kNBA4. In comparison with PEG2kNBA4, PEG2kBA4 exhibited higher sensitivity to acidic pH.

[0029] FIG. 19 MTS of CMT-3 and nanoformulations: (FIG. 19A) Cell viability of THP-1, HEK, and RAW 264.7 cells incubated with free CMT-3, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, and CMT-3 PEG5kCA4VE4 for 72 h. (FIG. 19B) Cell viability of THP-1, HEK, and RAW 264.7 cells incubated with blank polymer PEG2kBA4 and PEG2kNBA4 for 72 h. (FIG. 19 C) IC50 of free CMT-3, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, and CMT-3 PEG5kCA4VE4 in THP-1, HEK, and RAW 264.7 cells. As shown in FIG. 19, blank TDs exhibited non-cytotoxicity in THP-1 and RAW cells up to 1 mg/mL, while moderate cytotoxicity were shown in HEK cells at the highest concentration. Both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 showed more than 20-fold and over 3-fold IC50 than that of free CMT-3 in THP-1 and RAW cells, and HEK cells, respectively. Both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 significantly reduced the cytotoxicity of CMT-3.

[0030] FIG. 20 Cellular uptake of CMT-3: (FIG. 20A) Fluorescence intensity of RAW 264.7 and THP-1 cells incubated with free CMT-3, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4, and CMT-3 PEG5kCA4VE4 at 4h and 8h. (FIG. 20B) Fluorescent images of RAW 264.7 cells incubated with CMT-3, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4 at 4h. As shown in FIG 20, both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 exhibited significantly lower cellular uptake in comparison with random diffusion of free CMT-3 in THP-1 and RAW cells at 4 and 8 h incubation.

[0031] FIG. 21 Hemolytic toxicity of (FIG. 21A) CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4, and (FIG. 21B) blank TD PEG2kBA4 and PEG2kNBA4 at 30 min, 4h, and overnight incubation. Nonhemolytic toxicity has been found in both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4, and blank TDs at 30 min, 4 h, and overnight incubation.

[0032] FIG. 22 A Particle size of CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4. FIG. 22B Fluorescent spectrum and intensity of free CMT-3, CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4. FIG. 22C UV spectrum of free CMT-3, CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4. CMT-3 can be effectively conjugated on both PEG5kBA4 and PEG5kNBA4 with homogeneous particle size of 315 nm and 11 nm, respectively. In comparison with free CMT-3, fluorescent intensity of CMT-3 significantly increased after conjugation in PEG5kBA4 and PEG5kNBA4. Red shift of UV spectrum was observed in both CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4, indicating efficient conjugation of CMT-3 on BA containing TDs.

[0033] FIG. 23 MTS of CMT-3 and nanoformulations: FIG. 23A Cell viability of THP-1, HEK, and RAW 264.7 cells incubated with free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for 72 h. (FIG. 23B) Cell viability of THP-1, HEK, and RAW 264.7 cells incubated with blank polymer PEG2kBA4 and PEG2kNBA4 for 72 h. (FIG. 23C) IC50 of free CMT-3, CMT-3-PEG2kBA4, CMT-3-PEG2kNBA4 in THP-1, HEK, and RAW 264.7 cells. As shown in FIG. 23, blank TDs exhibited non-cytotoxicity in THP-1, RAW, and HEK cells up to 1 mg/mL. Both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 showed more than 50-fold and over 3-fold IC50 than that of free CMT-3 in HEK and RAW cells, and THP-1 cells, respectively. Both CMT-3-PEG2kBA4 and CMT-3-PEG2kNBA4 significantly reduced the cytotoxicity of CMT-3.

[0034] FIG. 24 A Cell viability of RAW 264.7 cells incubated with free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for overnight with or without LPS. FIG. 24B LPS induced proinflammatory cytokine IL-6 and TNF- α production in RAW 264.7 cells

treated by free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for overnight incubation. RAW cells incubated with CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 exhibited significant higher cell viability than that of free CMT-3. Free CMT-3 significantly reduced TNF- α and IL-6 production induced by LPS at three highest concentrations due to its high cytotoxicity, while CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 with much higher cell viability showed no inhibition effect on proinflammatory cytokine production.

[0035] FIG 25A LTA or LPS induced TLR activation in HEK TLR-2, HEK TLR-4 and THP-1 Blue cells treated by free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for overnight incubation. FIG 25B Cell viability of HEK TLR-4 and THP-1 Blue cells incubated with free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for overnight with LPS. Free CMT-3 exhibited dose-dependent inhibition of TLR signaling pathway activation by LTA/LPS in HEK TLR2, HEK TLR4, and THP-1 blue cells due to its cytotoxicity. CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 showed dose-dependent inhibition of TLR signaling pathway activation by LPS in HEK TLR4, and THP-1 blue cells, rather than HEK TLR2 cells. CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 significantly reduced cytotoxicity of CMT-3 in HEK TLR and THP-1 blue cells during overnight incubation.

[0036] FIG. 26A Growth curve of gram-positive and gram-negative bacteria treated with different concentration of free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for overnight incubation. FIG. 26B MIC of free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 in gram-positive and gram-negative bacteria. In comparison with free CMT-3, CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 kept similar potency of antibacterial activity or slightly increased MIC in E.Coli. (K12), E.Coli (DH5 α), Staph. aureus, Strep. Pneumoniae and Listeria, indicating efficient drug release of CMT-3 in nanoprodrug formulations, while antibacterial activity of CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 reduced in Pseudo. Aeruginosa.

[0037] FIG. 27A CMT-3 adsorption on hydroxyapatite. FIG. 27B CMT-3 degradation by UV. FIG. 27C Cell viability of RAW 264.7 cells incubated with UV degraded of free CMT-3, CMT-3-PEG5kBA4, CMT-3-PEG5kNBA4 for 72 h. As shown in FIG. 27A, CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 exhibited less hydroxyapatite adsorption in comparison with free CMT-3, demonstrating CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 may reduce the damage of bone tissue. Less UV degradation of CMT-3-PEG5kNBA4 has been found. Higher cell viability of RAW

cells incubated with UV treated CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 in comparison with RAW cells incubated with UV treated free CMT-3, indicating CMT-3-PEG5kBA4 and CMT-3-PEG5kNBA4 reduce the phototoxicity of CMT-3.

[0038] FIG. 28 PK and biodistribution of CMT-3-PEG5kBA4 via i.v. injection.

5 FIG. 28A Body weight change of the mice treated by 10 mg/kg CMT-3-PEG5kBA4. (. FIG. 28B) Pharmacokinetics of CMT-3 in mice treated by CMT-3-PEG5kBA4 via i.v. injection. (. FIG. 28C) Biodistribution of CMT-3 in mice treated by CMT-3-PEG5kBA4 via i.v. injection after 24h. Less than 2% body weight loss shown in the mice treated by 10 mg/kg CMT-3-PEG5kBA4 via i.v. injection, indicating minimized toxicity of
10 nanoprodug formulation. CMT-3-PEG5kBA4 significantly prolong the blood circulation time of CMT-3 with over 57-fold AUC in comparison with CMT-3 encapsulated PEG5kCA4VE4 formulation. After 24 h of the injection, majority of CMT-3 were distributed in the liver and spleen.

[0039] FIG. 29 Particle size of TCL-PEG2kBA4, TCL-PEG2kNBA4 and TCL-

15 PEG5kBA4, TCL-PEG5kNBA4. TCL can be effectively conjugated on PEG2kBA4, PEG2kNBA4, PEG5kBA4 and PEG5kNBA4 with homogeneous particle size of 269 nm, 9 nm, 318 nm and 11 nm, respectively.

[0040] FIG.30A UV spectrum (FIG.30B) Fluorescent spectrum and intensity of

free TCL, TCL-PEG2kBA4, TCL-PEG2kNBA4 and TCL-PEG5kBA4, TCL-
20 PEG5kNBA4. (FIG.30C) In vitro TCL release profile of free TCL, TCL-PEG2kBA4, TCL-PEG2kNBA4 and TCL-PEG5kBA4, TCL-PEG5kNBA4 at pH 7.4 and pH 5.5. Red shift of UV spectrum was observed in TCL-PEG2kBA4, TCL-PEG2kNBA4 and TCL-PEG5kBA4, TCL-PEG5kNBA4, indicating efficient conjugation of TCL on BA containing TDs. In comparison with free TCL, fluorescent intensity of TCL significantly
25 increased after conjugation in nanoprodug formulations. TCL-PEG2kBA4, TCL-PEG2kNBA4 and TCL-PEG5kBA4, TCL-PEG5kNBA4 significantly prolonged drug release profile in comparison with free TCL. Acidic pH 5.5 accelerated the TCL release in TCL-PEG2kBA4, TCL-PEG5kBA4, and TCL-PEG2kNBA4 while has slightly effect on release profile of TCL-PEG5kNBA4. In comparison with PEG5kNBA4, PEG5kBA4
30 exhibited higher sensitivity to acidic pH.

[0041] FIG. 31A Fluorescent intensity of TCL in free TCL, TCL-PEG2kBA4,

TCL-PEG2kNBA4 mixed with Ca^{2+} at different concentration for 10 min and FIG. 31B 5 h. Fluorescent intensity of free TCL significantly increased by interaction with Ca^{2+} in a dose-dependent manner. Only slightly fluorescent intensity increase has been

shown in both TCL-PEG2kBA4 and TCL-PEG2kNBA4 at highest Ca²⁺ concentration, indicating nanoprodru drug nanoformulation TCL-PEG2kBA4 and TCL-PEG2kNBA4 efficiently prevent TCL interacting with Ca²⁺, which may further reduce the damage of bone tissue.

5 [0042] FIG. 32 Cytotoxicity of free TCL, TCL-PEG2kBA4, TCL-PEG2kNBA4. (FIG. 32 A) Cell viability of RAW 264.7, THP-1 and HEK cells incubated with free TCL, TCL-PEG2kBA4, TCL-PEG2kNBA4 for 72 h. (FIG. 32 B) Hemolytic toxicity of free TCL, TCL-PEG2kBA4, TCL-PEG2kNBA4 for 0.5 h, 4 h and overnight incubation. In comparison with free TCL, both TCL-PEG2kBA4 and TCL-PEG2kNBA4 show nearly
10 100% cell viability up to 100 µg/mL, demonstrating reduced cytotoxicity of TCL in nanoprodru drug formulation. Nonhemolytic toxicity has been found in both TCL-PEG2kBA4, TCL-PEG2kNBA4, TCL-PEG5kBA4 and TCL-PEG5kNBA4 at 30 min, 4 h, and overnight incubation.

[0043] FIG. 33 Particle size of DCL-PEG2kBA4, DCL-PEG2kNBA4 and DCL-PEG5kBA4, DCL-PEG5kNBA4. TCL can be effectively conjugated on PEG2kBA4, PEG2kNBA4, PEG5kBA4 and PEG5kNBA4 with homogeneous particle size of 181
15 nm, 192 nm, 261 nm and 11 nm, respectively.

[0044] FIG. 34A UV spectrum (FIG. 34B) Fluorescent spectrum and intensity of free DCL, DCL-PEG2kBA4, DCL-PEG2kNBA4 and DCL-PEG5kBA4, DCL-PEG5kNBA4. (FIG. 34C) In vitro DCL release profile of free DCL, DCL-PEG2kBA4, DCL-PEG2kNBA4 and DCL-PEG5kBA4, DCL-PEG5kNBA4 at pH 7.4 and pH 5.5. Red
20 shift of UV spectrum was observed in DCL-PEG2kBA4, DCL-PEG2kNBA4 and DCL-PEG5kBA4, DCL-PEG5kNBA4, indicating efficient conjugation of DCL on BA containing TDs. In comparison with free DCL, fluorescent intensity of DCL significantly increased after conjugation in nanoprodru drug formulations. DCL-PEG2kBA4, DCL-PEG2kNBA4 and DCL-PEG5kBA4, DCL-PEG5kNBA4 significantly prolonged drug
25 release profile in comparison with free TCL. Acidic pH 5.5 accelerated the DCL release in DCL-PEG2kBA4 and DCL-PEG5kBA4, while has slightly effect on release profile of DCL-PEG5kNBA4 and DCL-PEG2kNBA4. In comparison with PEGnNBA4, PEGnBA4 exhibited higher sensitivity to acidic pH.
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[0045] FIG. 35 Cytotoxicity of free DCL, DCL-PEG2kBA4, DCL-PEG2kNBA4. (FIG. 35 A) Cell viability of RAW 264.7, THP-1 and HEK cells incubated with free DCL, TCL-PEG2kBA4, DCL-PEG2kNBA4 for 72 h. (FIG. 35 B) Hemolytic toxicity of free DCL, DCL-PEG2kBA4, DCL-PEG2kNBA4 for 0.5 h, 4 h and overnight incubation. (FIG.

35 C) Fluorescence intensity of RAW 264.7 cells incubated with free DCL, TCL-PEG2kBA4, DCL-PEG2kNBA4 for 4 and 8h. In comparison with free DCL, both DCL-PEG2kBA4 and DCL-PEG2kNBA4 show nearly 100% cell viability up to 100 µg/mL, demonstrating reduced cytotoxicity of DCL in nanoprodug formulation. Nonhemolytic toxicity has been found in both DCL-PEG2kBA4, DCL-PEG2kNBA4, DCL-PEG5kBA4 and DCL-PEG5kNBA4 at 30 min, 4 h, and overnight incubation. Both DCL-PEG2kBA4 and DCL-PEG2kNBA4 exhibited significantly lower cellular uptake in comparison with random diffusion of free DCL in RAW cells at 4 and 8 h incubation.

[0046] FIG. 36 Anti-bacterial activity of DCL and its nanoprodug formulations.

(FIG. 36A) Growth curve of gram-positive and gram-negative bacteria treated with different concentration of free DCL, DCL-PEG2kBA4, DCL-PEG2kNBA4 for overnight incubation. (FIG. 36B) MIC of free DCL, DCL-PEG2kBA4, DCL-PEG2kNBA4 in gram-positive and gram-negative bacteria. In comparison with free DCL, DCL-PEG2kBA4 and DCL-PEG2kNBA4 kept similar potency of antibacterial activity or slightly increased MIC in E.Coli. (K12), Pseudo. Aeruginosa, Staph. aureus, Strep. Pneumoniae and Listeria, indicating efficient drug release of CMT-3 in nanoprodug formulations.

[0047] FIG. 37 BA-containing TD for CUR produg conjugation. (FIG. 37A) CUR react with PBA small molecule to form boronate ester bond. (FIG. 37B) Schematic illustration for CUR conjugation and release on PBA-containing TDs via reversible boronate ester bond and acidic pH/ROS responsive for drug release. Curcumin (CUR) with 1,3-diol groups can be conjugated onto the boronic acid group containing TDs to form produg nanoformulation via boronate ester bond, which is labile and responsive to acidic pH and ROS.

[0048] FIG. 38 Particle size of CUR-PEG2kBA4, CUR-PEG2kNBA4. CUR can be effectively conjugated on PEG2kBA4, PEG2kNBA4, with homogeneous particle size of 13 nm and 11 nm, respectively.

[0049] FIG. 39A Free CUR detection in CUR-PEG2kBA4 and CUR-PEG2kNBA4 formulations. (FIG. 39B) MALDI-TOF of CUR-3-PEG2kBA4 and CUR-3-PEG2kNBA4 formulations before and after CMT-3 conjugation. The MW of PEG2kBA4 and PEG2kNBA4 before and after CUR conjugation were characterized by MALDI-TOF. As shown in Figure 28, MW of TDs increased after CUR conjugation. In addition, significant lower signal or non-signal of free CUR were observed in the CUR-TD conjugated nanoprodug formulation. Based on the peak intensity ratio of

free CUR to CHCA matrix, the CUR conjugation efficiency was calculated. The conjugation efficiency of CUR-PEG2kBA4 and CUR-PEG2kNBA4 are over 95%.

[0050] FIG. 40A UV spectrum (FIG. 40B) Fluorescent spectrum and intensity of free CUR, CUR-PEG2kBA4, CUR-PEG2kNBA4 and CUR PEG5kBA4, CUR -
5 PEG5kNBA4. (FIG. 40C) In vitro CUR release profile of free CUR, CUR-PEG2kBA4, CUR-PEG2kNBA4 and CUR-PEG5kBA4, CUR-PEG5kNBA4 at pH 7.4, pH 8.5, pH 5.5 and pH 1.6. Red shift of UV spectrum was observed in CUR-PEG2kBA4 and CUR-PEG2kNBA4, indicating efficient conjugation of CUR on BA containing TDs. In comparison with free CUR, fluorescent intensity of CUR significantly reduced after
10 conjugation in nanoprodug formulations. CUR-PEG2kBA4 and CUR-PEG2kNBA4 significantly prolonged drug release to 96 h. Alkaline pH 8.5 accelerated the CUR release in CUR-PEG2kBA4 and CUR-PEG2kNBA4, while acidic pH further slowdown the release profile of CUR-PEG5kNBA4 and CUR-PEG2kNBA4.

[0051] FIG. 41 MTS of CUR and nanoformulations. (FIG. 41A) Cell viability of
15 THP-1, HEK, and RAW 264.7 cells incubated with free CUR, CUR-PEG5kBA4, CUR-PEG5kNBA4 for 72 h. (FIG. 41B) Cell viability of THP-1, HEK, and RAW 264.7 cells incubated with blank polymer PEG2kBA4 and PEG2kNBA4 for 72 h. (FIG. 41C) IC₅₀ of free CUR, CUR-PEG2kBA4, CUR-PEG2kNBA4 in THP-1, HEK, and RAW 264.7 cells. As shown in FIG. 41, blank TDs exhibited non-cytotoxicity in THP-1 and RAW
20 cells up to 1 mg/mL, while moderate cytotoxicity were shown in HEK cells at the highest concentration. Both CUR-PEG2kBA4 and CUR-PEG2kNBA4 showed over 2-fold IC₅₀ than that of free CMT-3 (EtOH) in THP-1 and RAW cells, respectively. Both CUR PEG2kBA4 and CUR-PEG2kNBA4 significantly reduced the cytotoxicity of CUR.

[0052] FIG. 42A LTA or LPS induced TLR activation in HEK TLR-2, HEK TLR-
25 4 and THP-1 Blue cells treated by free CUR, CUR-PEG5kBA4, CUR-PEG5kNBA4 for overnight incubation. (FIG. 42B) Cell viability of HEK TLR-4 and THP-1 Blue cells incubated with free CUR, CUR-PEG5kBA4, CUR-PEG5kNBA4 for overnight with LPS. Free CUR, CUR-PEG5kBA4 and CUR-PEG5kNBA4 exhibited dose-dependent inhibition of TLR signaling pathway activation by LTA/LPS in HEK TLR2, HEK TLR4, and THP-1 blue cells due to their cytotoxicity. Free CUR, CUR-PEG5kBA4 and CUR-PEG5kNBA4 showed dose-dependent inhibition cell viability in HEK TLR and THP-1
30 blue cells. CUR-PEG5kBA4 and CUR-PEG5kNBA4 significantly reduced cytotoxicity of CUR in HEK TLR and THP-1 blue cells up to 10 µg/mL during overnight incubation.

[0053] FIG. 43A Cell viability of RAW 264.7 cells incubated with free CUR, CUR-PEG5kBA4, CUR-PEG5kNBA4 for overnight with or without LPS. (FIG. 43B) LPS induced proinflammatory cytokine IL-6 and TNF- α production in RAW 264.7 cells treated by free CUR, CUR-PEG5kBA4, CUR-PEG5kNBA4 for overnight incubation. (FIG. 43C) Anti-bacterial activity of free CUR, CUR-PEG5kBA4, CUR-PEG5kNBA4. RAW cells incubated with CUR-PEG5kBA4 and CUR-PEG5kNBA4 exhibited significant higher cell viability than that of free CUR. Free CUR, CUR-PEG5kBA4 and CUR-PEG5kNBA4 significantly reduced TNF- α and IL-6 production induced by LPS in a dose-dependent manner due to their cytotoxicity. Free CUR exhibited moderate antibacterial activity at 100 μ g/mL in both gram-positive and gram-negative bacteria. CUR-PEG5kBA4 and CUR-PEG5kNBA4 exhibited less antibacterial activity, indicating the effective conjugation of CUR on the BA containing TDs.

[0054] FIG. 44 Treatment of LPS induced sepsis mouse model. (FIG. 44A) Body temperature and body weight change of LPS-induced sepsis mice without treatment and treated by CUR-PEG5kBA4 and CMT-3-PEG5kBA4. (FIG. 44B) Proinflammatory cytokine TNF- α and IL-6 in serum of LPS-induced sepsis mice without treatment and treated by CUR-PEG5kBA4 and CMT-3-PEG5kBA4. CUR-PEG5kBA4 and CMT-3-PEG5kBA4 significantly improve the body temperature of the LPS-induced sepsis mice at 24 h. The body weight change showed no significant difference between treated and untreated group. In comparison with untreated group, CMT-3-PEG5kBA4 significantly reduced TNF- α production at 1 h, while CUR-PEG5kBA4 significantly reduced IL-6 production at 24 h.

[0055] FIG. 45 Particle size of PEG2kArg4BA4, PEG2kGlu4BA4, PEG2kGlu8BA4, CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4, and CUR-PEG2kGlu8BA4. CUR can be effectively conjugated on PEG2kArg4BA4 and PEG2kGlu4BA4 with homogeneous particle size of 19 nm and 13 nm, respectively. Multiple peaks were observed in CUR-PEG2kGlu8BA4.

[0056] FIG. 46A Free CUR detection in CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4 formulations. (FIG. 46B) MALDI-TOF of CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4 formulations before and after CUR conjugation. The MW of PEG2kArg4BA4 and PEG2kGlu4BA4 before and after CUR conjugation were characterized by MALDI-TOF. As shown in Figure 35, MW of TDs increased after CUR conjugation. In addition, significant lower signal or non-signal of free CUR were observed in the CUR-TD conjugated

nanoprodru formulation. Based on the peak intensity ratio of free CUR to CHCA matrix, the CUR conjugation efficiency was calculated. The conjugation efficiency of CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 are over 80%.

[0057] FIG. 47A UV spectrum FIG. 47B Fluorescent spectrum and intensity of CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4. Red shift of UV spectrum was observed in CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4, indicating efficient conjugation of CUR on BA containing TDs. In comparison with free CUR, fluorescent intensity of CUR significantly reduced after conjugation in nanoprodru formulations.

[0058] FIG. 48 Cell viability of RAW 264.7 incubated with CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4, PEG2kArg4BA4, PEG2kGlu4BA4 and PEG2kGlu8BA4 for 72 h. As shown in FIG. 48, blank TDs exhibited non-cytotoxicity in RAW cells up to 1 mg/mL. CUR-PEG2kArg4BA4 exhibited 4-5-fold higher IC₅₀ than PEG2kGlu4BA4 and PEG2kGlu8BA4 in RAW cells. All the CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4 significantly reduced the cytotoxicity of CUR.

[0059] FIG. 49A Cell viability of RAW 264.7 and THP-1 cells incubated with CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4, PEG2kArg4BA4, PEG2kGlu4BA4 and PEG2kGlu8BA4 for overnight with LPS. (FIG. 49B) LPS-induced proinflammatory cytokine TNF- α production in RAW 264.7 and THP-1 cells incubated with CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4, PEG2kArg4BA4, PEG2kGlu4BA4 and PEG2kGlu8BA4. Blank TDs exhibited non-cytotoxicity in RAW and THP-1 cells up to 1 mg/mL. CUR-PEG2kArg4BA4 exhibited less cytotoxicity than PEG2kGlu4BA4 and PEG2kGlu8BA4. CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 and CUR-PEG2kGlu8BA4 significantly reduced TNF- α and IL-6 production induced by LPS at the highest concentration due to their cytotoxicity. Blank TDs exhibited no inhibition of TNF- α and IL-6 production induced by LPS.

[0060] FIG. 50 Particle size of CMT-3-PEG2kArg4BA4, CMT-3-PEG2kGlu4BA4, and CMT-3-PEG2kGlu8BA4. CMT-3 can be effectively conjugated on PEG2kArg4BA4 and PEG2kGlu4BA4 with homogeneous particle size of 17 nm and 107 nm, respectively. Precipitation was observed in CUR-PEG2kGlu8BA4 on the second day.

[0061] FIG. 51A Free CMT-3 detection in CMT-3-PEG2kArg4BA4, CMT-3-PEG2kGlu4BA4 and CMT-3-PEG2kGlu8BA4 formulations. (FIG. 51B) MALDI-TOF of CMT-3-PEG2kArg4BA4, CMT-3-PEG2kGlu4BA4 and CMT-3-PEG2kGlu8BA4 formulations before and after CUR conjugation. The MW of PEG2kArg4BA4 and PEG2kGlu4BA4, PEG2kGlu8BA4 before and after CMT-3 conjugation were characterized by MALDI-TOF. As shown in Figure 40, MW of TDs increased after CMT-3 conjugation. In addition, significant lower signal or non-signal of free CMT-3 were observed in the CMT-3-TD conjugated nanoprodug formulation. Based on the peak intensity ratio of free CMT-3 to CHCA matrix, the CUR conjugation efficiency was calculated. The conjugation efficiency of CUR-PEG2kArg4BA4, CUR-PEG2kGlu4BA4 are nearly 100%.

[0062] FIG. 52 BA-containing TD for NMN produg conjugation. NMN react with PBA small molecule to form boronate ester bond. Nicotinamide mononucleotide (NMN) with 1,2-diol groups can be conjugated onto the boronic acid group containing TDs to form produg nanoformulation via boronate ester bond, which is labile and responsive to acidic pH and ROS.

[0063] FIG. 53 Particle size of NMN-PEG2kBA4, NMN-PEG2kArg4BA4, NMN-PEG2kGlu4BA4, and NMN-PEG2kGlu8BA4. NMN can be effectively conjugated on PEG2kBA4, PEG2kArg4BA4 and PEG2kGlu4BA4 with homogeneous particle size of 223 nm, 14 nm, and 253 nm, respectively. Multiple peaks were observed in NMN-PEG2kGlu8BA4 on the second day.

[0064] FIG. 54A Free NMN detection in NMN-PEG2kBA4, NMN-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4 formulations. (FIG. 54B) MALDI-TOF of NMN-3-PEG2kBA4, NMN-3-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4 formulations before and after CUR conjugation. The MW of PEG2kBA4, PEG2kArg4BA4 and PEG2kGlu4BA4, PEG2kGlu8BA4 before and after CMT-3 conjugation were characterized by MALDI-TOF. As shown in Figure 43, MW of TDs increased after NMN conjugation. In addition, significant lower signal or none signal of NMN were observed in the NMN-TD conjugated nanoprodug formulation. Based on the peak intensity ratio of free NMN to CHCA matrix, the CUR conjugation efficiency was calculated. The conjugation efficiency of NMN-PEG2kBA4, NMN-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4 are nearly 100%.

[0065] FIG. 55A Cell viability of RAW 264.7 and THP-1 cells incubated with NMN-PEG2kBA4, NMN-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4 for overnight with LPS. (FIG. 55B) LPS-induced proinflammatory cytokine TNF- α production in RAW 264.7 and THP-1 cells incubated with NMN-3-PEG2kBA4, NMN-3-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4. (FIG. 55C) Cell viability of RAW 264.7 cells incubated with NMN-3-PEG2kBA4, NMN-3-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4 for 72 h. NMN-PEG2kBA4, NMN-PEG2kArg4BA4, NMN-PEG2kGlu4BA4 and NMN-PEG2kGlu8BA4 exhibited no cytotoxicity in RAW cells for overnight incubation. None of the NMN nanoprodug formulations can reduce TNF- α and IL-6 production induced by LPS. In comparison with other NMN nanoprodug formulations, NMN-PEG2kArg4BA4 can significantly improve the cell viability in a dose dependent manner.

[0066] FIG. S1 presents MALDI-TOF MS characterization of TD intermediates and TDs. FS1A presents an MS spectrum detected by MALDI-TOF MS of TD intermediates: PEG^{5k}NH₂, PEG^{5k}Lys₁(NH₂)₂, PEG^{5k}Lys₃(NH₂)₄, and PEG^{5k}Lys₇(NH₂)₈. FIG. S1B presents an MS spectrum detected by MALDI-TOF MS of TDs: PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈.

[0067] FIG. S2 present a scheme and MS spectrum of AmB-PBA conjugation in AmB and PBA model reaction.

[0068] FIG. S3 presents ¹H NMR characterization of PBA-containing TDs: FIG. S3A presents ¹H NMR spectra of PEG^{5k}BA₁. S3B presents ¹H NMR spectra of PEG^{5k}BA₂. S3C presents ¹H NMR spectra of PEG^{5k}BA₄. S3D presents ¹H NMR spectra of PEG^{5k}BA₈.

[0069] FIG. S4 presents the characterization of AmB and PBA-containing TDs conjugation. FIG. S4A presents characterization of conjugation between AmB and TDs-PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈ by TLC. FIG. S4B presents fluorescent intensity of Fungizone, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ at neutral and acidic pH.

[0070] FIG. S5 presents the characterization of AmB conjugation on TDs by MALDI-TOF MS. FIG. S5A presents the MS spectra of AmB-PEG^{5k}BA₁ at neutral and acidic pH. FIG. S5B presents the MS spectra of AmB-PEG^{5k}BA₂ at neutral and acidic pH. FIG. S5C presents the MS spectra of AmB-PEG^{5k}BA₄ at neutral and acidic pH. FIG. S5D presents the MS spectra of AmB-PEG^{5k}BA₈ at neutral and acidic pH.

[0071] FIG. S6 presents particle size distributions and zeta potentials measured by DLS of blank TDs and AmB-TDs. FIG. S6A presents particle size distributions of PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈. FIG. S6B presents the zeta potentials of PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈. S6C presents the zeta potentials of AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈. S6D presents the particle size distributions of AmB-PEG^{5k}BA₁ storage overnight, AmB-PEG^{5k}BA₂ storage 4-day, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ storage over 1 month.

[0072] FIG. S7 presents the particle size distribution by TEM and CMC of blank TDs and AmB-TDs. FIG. S7A presents the particle size distributions of PEG^{5k}BA₄, PEG^{5k}BA₈, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ from TEM images. FIG. S7B presents the CMC of PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈. FIG. S7C presents the CMC of AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈.

[0073] FIG. S8 presents *in vitro* cytotoxicity of blank TDs-PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈ in 293T, RAW 264.7, and CHO cells by MTS assay.

[0074] FIG. S9 presents hemolytic toxicity in vitro of AmB formulations and blank TDs. FIG. S9A presents hemolysis of Free AmB, Fungizone, AmBisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ for 30 min and 4h at 37 °C. FIG. S9B presents hemolysis of blank TDs-PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈ for 30 min, 4h, and overnight incubation at 37 °C.

[0075] FIG. S10 presents the fluorescent intensity of Doxil incubated with Fungizone, AmBisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ at 1:1 mass ratio for 30 min and overnight.

[0076] FIG. S11 presents the TNF-α production level in RAW 264.7 cells induced by Fungizone, AmBisome, AmB-PEG^{5k}BA₁, AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ with concentration of AmB at 5μ g/mL for 1 h incubation.

[0077] FIG. S12 present the MTD, blood chemistry of mice treated by AmB formulations. FIG. S12A present the bodyweight changes of healthy mice administer intravenously with seven consecutive AmBisome, and AmB-PEG^{5k}BA₄ at 15 mg/kg dose daily. FIGs. S12B – S12E present BUN, CRE, ALT and AST levels of 24 h after last dose of AmB formulations (n=5).

[0078] FIG. S13 presents the histological examinations of the damaged livers by H&E staining from animals treated with Fungizone (1.5 mg/kg×3), AmB-PEG^{5k}BA₄ (15 mg/kg×7), and AmBisome (15 mg/kg×7).

[0079] FIG S14 presents the histological examinations of the damaged hearts by H&E staining from animals treated with Fungizone (1.5 mg/kg×3), AmB-PEG^{5k}BA₄ (15 mg/kg×7), and AmBisome (15 mg/kg×7).

[0080] FIG. S15 presents the antifungal efficacy of AmB-PEG^{5k}BA₄ in *C. albicans* infected immunocompetent mice. FIG. S15A presents a schematic illustration of *C. albicans* infected immunocompetent mouse model and treatment extension. FIG. S15B presents the cumulative mice survival. FIG. S15C presents the bodyweight change. FIG. S15D presents the monitored body temperature changes of the infected mice. FIG. S15E presents the colony count of *C. albicans* in major organ and blood of mice treated by AmB formulations. FIG. S15F presents GMS staining for *C. albicans* in the heart of mice treated by AmB formulations. FIG. S15G presents the histological examinations of the hearts by H&E staining of mice treated by AmB formulations.

[0081] FIG. S16 presents the characterization of immunocompromised mice induced by CY. FIG. S16A presents the WBC counting change of mice treated by 200 mg/kg CY via i.p. injection. FIG. S16B presents the bodyweight change of mice treated by CY. FIG. S16C presents the body temperature change of mice treated by CY.

[0082] FIG. S17 presents the antifungal efficacy of AmB-PEG^{5k}BA₄ in *C. albicans* infected immunocompromised mice. FIG. S17A presents the body temperature change of mice was monitored. FIG. S17B presents the colony count of *C. albicans* in major organs and blood of mice treated by AmB formulations. FIG. S17C presents the GMS staining for *C. albicans* in the heart of mice treated by AmB formulations. FIG. S17D presents the histological examinations of the hearts by H&E staining of mice treated by AmB formulations.

[0083] FIG. S18 presents the antifungal efficacy of AmB-PEG^{5k}BA₄ in *C. albicans* infected immunocompromised mice for kept 1-2 months. FIG. S18A presents the body weight change of mice was monitored. FIG. S18B presents the GMS staining for *C. albicans* in the kidney of mice treated by AmB-PEG^{5k}BA₄. FIG. S18C presents the histological examinations of the hearts by H&E staining of mice treated by AmB-PEG^{5k}BA₄. FIG. S18D presents the histological examinations of the kidney by H&E

staining of mice treated by AmB-PEG^{5k}BA₄. FIG. S18E presents the histological examinations of the livers by H&E staining of mice treated by AmB-PEG^{5k}BA₄.

[0084] FIG. S19 presents an illustrative graphic for the prodrug design of AmMB for in vivo fungal infection control with preferred release in acidic extracellular and intracellular environments.

[0085] Before embodiments are further described, it is to be understood that this disclosure is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0086] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0087] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

DETAILED DESCRIPTION OF THE INVENTION

[0088] Embodiments disclosed herein are directed to biocompatible well-defined AmB-conjugated TD prodrug nanoformulations that have been rationally designed and prepared efficiently via reversible boronate ester chemistry for AmB systemic delivery. The AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ prodrug nanoformulations have stable and small particle sizes with zeta potentials close to neutral, which are desired for improving macrophage targeting for systemic drug

delivery. The burst release was significantly minimized by increasing density of phenylboronic acid (PBA) moieties of TDs. Embodiments herein utilize the sustained drug release profiles of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ that can reduce systemic toxic effects of Fungizone[®]. In comparison with AmB-PEG^{5k}BA₈, efficient drug release and higher efficacy for invasive phase C. albicans inhibition were shown in AmB-PEG^{5k}BA₄. Therefore, AmB-PEG^{5k}BA₄ is promising to further development in systemic delivery of AmB in severe fungal infections. Embodiment herein provide AmB-PEG^{5k}BA₄ having exhibited prolonged blood circulation time, comparable MTD with AmBisome[®], reduced nephrotoxicity, and significantly improved antifungal treatment efficacy and survival rate than Fungizone[®] and AmBisome[®] in immunocompromised mouse model. Embodiments herein provide stable and efficient reversible AmB conjugation in the small-sized TD nanocarriers with optimized release profile is crucial for optimizing systemic drug delivery, minimizing nephrotoxicity, administration-related toxicity and improving treatment efficacy in vivo. Therefore, cost-effective AmB-PEG^{5k}BA₄ prodrug formulation significantly enhanced systemic antifungal treatment efficacy than Fungizone[®] and AmBisome[®], which holds a great potential for clinical translation.

[0089] Embodiments disclosed herein are directed to a novel pH sensitive prodrug strategy to reversibly conjugate 1, 2 or 1, 3 cis-diol containing drug molecules in a telodendrimer via boronate chemistry to improve the drug solubility, targeted drug delivery and controlled release for improved drug availability, reduced toxicity, and enhance drug efficacy. Embodiments can be applied broadly to different kinds of drugs, e.g. antibiotics, antifungal drug, anticancer drugs etc.

[0090] Polymeric micelles and nanoparticles have been widely applied for hydrophobic drug delivery, including AmB, via non-covalent physical interactions. For example, chitosan, gelatin, poly glycolic acid (PGA), and poly lactide-co-glycolide acid (PLGA) have been studied for AmB encapsulation. The AmB encapsulated PLGA formulation with reduced renal toxicity and high oral bioavailability has potential for AmB oral delivery. We have systemically engineered the core structure of telodendrimer micelles for monomeric AmB delivery, which reduces AmB toxicity to mammalian cells. Prodrug conjugation of AmB to protein or polymers via amide or imine Schiff base linkages significantly reduce the hemolysis and cytotoxicity, however, also significantly compromise the antifungal potency of AmB.

[0091] It is known that the 1,3-cis-diols has lower reactivity than the 1,2-cis-diol for boronic acid coupling, thus the increased valency of both cis-diols and boronic acids in the coupling pairs may increase the chance for efficient coupling. AmB has three pairs of 1,3-cis-diols at the polar side of molecule for pore formation in fungal plasma membrane. However, the polyenes in AmB confine a very rigid molecular conformation, which limits the multivalent effects for reaction. On the contrary, a multivalent and flexible boronic acid scaffold can maximize the multivalence for AmB conjugation via boronate ester. The reversible boronate chemistry enables the drug release at slightly acidic environment, or upon the competition by the diol-containing glucose. The well-defined linear-dendritic telodendrimer nanoplatfrom can be decorated precisely with functional moiety for efficient drug interactions in nanocarrier. In our previous study, we developed a cis-diol-containing telodendrimer nanoplatfrom for boronic acid anti-cancer drug bortezomib delivery via reversible boronate chemistry for ovarian cancer treatment. In this invention, we introduced controlled number of boronic acid moieties onto the periphery of the flexible dendron in telodendrimer to optimize cis-diols containing AmB conjugation via the reversible boronate bond formation and fine-tune the release profile of AmB. The optimal constructs of boronate moieties in TD enable effective AmB prodrug conjugation and encapsulation in a solvent-free and catalyst-free manner, which ease the formulation process for clinical translation. As expected, the optimized AmB-TD nanoformulations improve the bioavailability, reduce its nephrotoxicity and administration related side effects, while sustaining the antifungal activity of AmB both in vitro and in vivo. We have systematically characterized the pharmacokinetics, toxicity, cytokine production and antifungal efficacy in comparison with the commercial Fungizone and Ambisome in vivo in both immunocompetent and immunocompromised mouse models.

[0092] Amphotericin B (AmB) is the gold standard treatment of life-threatening systemic fungal infections. The severe nephrotoxicity and the infusion side effects hinder its clinical application. To develop a cost-effective and safe AmB formulation, we rationally introduce numbers of phenylboronic acid (PBA) moieties on a linear dendritic telodendrimer (TD) scaffold for reversible AmB conjugation via boronate ester bonds with the cis-diol in AmB. Optimized AmB-TDs prodrug self-assemble into monodispersed micelles with small particle size from 14-16 nm with neutral surface zeta potential. Similar to the commercial liposome AmBisome, the optimized AmB prodrugs sustain the drug release with approximately 30% drug released after 8 h

dialysis in a sink condition, whereas burst release was observed in the conventional Fungizone and suboptimal AmB prodrug formulations. In addition, AmB-TD prodrugs respond to acidic pH for accelerated drug release, but not sensitive to glucose competition. UV-vis spectrum indicates that AmB prodrugs reduce the aggregation status of AmB, indicating the reduced cytotoxicity and hemolytic activity, in comparison to both Fungizone and AmBisome. All AmB-TDs prodrugs exhibit comparable antifungal activity to Fungizone and more effective than Ambisome. The optimal AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ remarkably prolonged AmB circulation time in the blood without the accelerated clearance observed after repeated injections and reduced TNF- α production for innate immune cell stimulation. AmB-TD prodrug demonstrates a comparable maximum tolerate dose (MTD) with AmBisome and an over-20 fold MTD enhancement than Fungizone. Finally, a high single dose of AmB-PEG^{5k}BA₄ were effective in both immunocompetent and immunocompromised mice for treatment of systemic fungal infections caused by *Candida albicans*. Therefore, AmB-PEG^{5k}BA₄ prodrug nanoformulation seems to be very promising as an alternative for the treatment of systemic fungal infections.

[0093] Material and methods: Amphotericin B was purchased from Gold Biotechnology, Inc. (St Louis, MO). Fungizone (Amphotericin B solubilized) was purchased from Sigma-Aldrich (St. Louis, MO). Ambisome was received from Pharmacy of SUNY Upstate Medical University. Monomethyl-terminated poly(ethylene glycol) monoamine hydrochloride (MeO-PEG-NH₂·HCl, *M_w* 5 kDa) was purchased from Biopharma PEG Scientific Inc. (Watertown, MA). (Fmoc)Lys(Fmoc)-OH were purchased from Chem-Impex International Inc. (Wood Dale, IL). 4-carboxyphenylboronic acid was obtained from Combi-Blocks Ins (San Diego, CA). N,N'-diisopropylcarbodiimide (DIC), N-hydroxybenzotriazole (HOBt), 4-methylpiperidine, dimethyl-sulfoxide (DMSO), dimethyl-sulfoxide-d₆ (DMSO-d₆), α -cyano-4-hydroxycinnamic acid (α -CHCA), dimethylformamide (DMF), methyl ether, dichloromethane (DCM), methanol, UranyLess, cyclophosphamide (CY) and all other chemicals were purchased from Sigma-Aldrich and Acros organics and used without further purification. CellTiter 96® AQueous MTS reagent powder was purchased from Promega (Madison, WI, USA). TNF- α and IL-6 mouse uncoated ELISA kits were obtained from ThermoFisher Scientific. VetScan Critical care plus rotors were purchased from Zoetis (United States). Pointe Liquid ALT (SGPT), AST (SGPT),

Creatinine, and Urea Nitrogen (BUN) Reagent Set were obtained from MedTextDX (Canton MI).

[0094] The nomenclatures of the TDs follow the system used in our previous publications ("Well-defined, size-tunable, multifunctional micelles for efficient paclitaxel delivery for cancer treatment," 2010 Jul 21;21(7):1216-24. doi: 10.1021/bc1000033; incorporated herein by reference), as shown in FIG. 8A, TD named PEG^{5k}BA₄ means that the molecular weight of PEG is 5 kDa with four phenylboronic acid (PBA) moieties conjugated on the periphery amino groups at both α and ϵ position of lysine in the TD.

[0095] TD Synthesis: General preparation for TD synthesis has been reported in our previous publications (*Id*), and is described mainly as following: the TDs were synthesized by solution-phase peptide chemistry beginning from MeO-PEG^{5k}-NH₂·HCl. *N*-terminal-protected lysine was used to synthesize the branched scaffold of TD. First, triethylamine (TEA, 1 equiv) was added to neutralize the hydrochloride on PEG together with *N,N'*-Diisopropylcarbodiimide (DIC, 3 equiv) and *N*-hydroxybenzotriazole (HOBt, 3 equiv) added as coupling reagents in dimethylformamide (DMF) to catalyze amide bond formation for 4-carboxyphenylboronic acid coupling to generate PEG^{5k}BA₁ (FIG. 8B) or Fmoc-Lys(Fmoc)-OH coupling. The reaction completion was confirmed by the negative Kaiser test result. The ice-chilled ether was added to the reaction solution to precipitate PEGylated intermediates, and then washed by chilled ether for three times. Then Fmoc-protection groups were removed by the treatment with 20% 4-methylpiperidine in DMF for 30 min. PEGylated intermediate was precipitated and washed with chilled ether for three times. Then 4-carboxyphenylboronic acid was conjugated was conjugated to generate PEG^{5k}BA₂ (FIG. 8C). On the other hand, the branched scaffold was further branched by another step of Fmoc-Lys (Fmoc)-OH coupling and then 4-carboxyphenylboronic acid was coupled to generate PEG^{5k}BA₄ (FIG. 8D) or Fmoc-Lys(Fmoc)-OH were conjugated on the peripheries of dendritic oligolysine. After removal of Fmoc groups, the intermediate was coupled with 4-carboxyphenylboronic acid to generate PEG^{5k}BA₈ (FIG. 8E). The molecular weight of intermediates and final products were characterized by MALDI-TOF mass (Bruker Microflex). The structure of final products was detected by ¹H NMR (Bruker ABANCE III 600 MHz) by dissolving TDs in DMSO-*d*₆ at 5 mg/mL.

[0096] AmB Conjugation and Characterization: AmB conjugation on telodendrimer micelles were carried out through thin-film dispersion method. In thin-film dispersion method, AmB was dissolved in DMSO and added into TD solution in DCM/MeOH (3:1 v/v) and 10 μ L TEA to form homogenous drug solution at 1:10 (w/w) ratio of TD/AmB. Then, solvents were evaporated to dryness and homogenous thin film of AmB-TD conjugates was coated on the flask wall. After further drying of film by oil pump, PBS was added into flask to disperse thin film into solution via vortex and sonication. The efficiency of AmB conjugation to TDs was confirmed by TLC, MALDI-TOF mass, and fluorescent intensity with ex/em 350/470 nm. The particle size distributions and zeta potentials of NPs and AmB-NPs were measured by Malvern Nano-Zetasizer (Malvern Panalytical Ltd, UK) at room temperature in PBS and H₂O, respectively. The morphology and particle size were further measured by transmission electron microscopy (TEM) (JEOL JEM-1400 instrument at 80 kV) with negative staining by UranylLess.

[0097] Critical Micelle Concentration (CMC) of TDs and AmB-TDs: The CMC of TD micelles was measured by fluorescence spectrometry via Nile Red as a hydrophobic fluorescent probe. The micelles were serially diluted in PBS to provide the concentrations ranging from 3.125 to 5000 μ g/mL. 5 μ L of 0.5 mg/mL Nile Red methanol solution was added to a series of vials in 96-well plate. After methanol was evaporated under the vacuum, 200 μ L of TD solutions at different concentrations was added to each vial, and the 96-well plate was left to shake overnight at room temperature with foil preventing from the light. Fluorescent emission intensity was measured by microplate reader (BioTek Synergy H1) at the wavelength of 620 nm with the excitation at 543 nm. CMC of TDs were determined at the intersection of the tangents to the two linear fitting of the curve of fluorescence intensity as a function of the log concentrations of the TDs and AmB-TDs.

[0098] In vitro Drug Release: The drug release profiles of AmB formulations were measured by a dialysis method. 300 μ L of Fungizone, Ambisome, AmB-TDs nanoformulations at 1 mg/mL AmB concentration were loaded into dialysis cartridges with 3.5 kDa MWCO dialysis membranes (Thermo Scientific, Rochford, IL). The cartridge was dialyzed against 40 mL PBS, pH 5.5 buffer, or PBS with 850 mg/mL glucose and gently shaken at 37 °C water bath. The PBS solution was changed every 4 h. 2 μ L of AmB solution within dialysis cartridge was withdrawn at different time

points and UV-Vis absorbance was measured by NanoDrop. Results were reported as the average percentage of AmB release for triplicate samples.

[0099] Cell and Fungi Culture and Animals: Murine macrophage cell line Raw 264.7, Chinese hamster ovary cells CHO, and human embryonic kidney cell line HEK293 were purchased from American Type Culture Collections (Manassas, VA, USA). Raw 264.7 and HEK293 cells were cultured in DMEM medium, while THP-1 was cultured in RPMI 1640 medium, supplemented with 10% FBS, 100 U/mL penicillin G and 100 µg/mL streptomycin at 37 °C using a humidified 5% CO₂ incubator. *Candida albicans* (*C. albicans*) was obtained from ATCC (Manassas, VA, USA). It was cultured in Sabouraud's broth or agar at 28 °C and room temperature, respectively. C57BL/6J mice aged 6-8 weeks were purchased from The Jackson Laboratory (Bar Harbor, Maine). All the animals were kept under pathogen-free conditions according to the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) guidelines and were allowed to acclimatize for at least 7 days before any experiments. All the animal experiments were performed in the compliance with the institutional guidelines and according to the protocol approved by the committee for the Humane Use of Animals of State University of New York (SUNY) Upstate Medical University.

[00100] Antifungal Susceptibility Testing: Antifungal susceptibility tests for AmB formulations were performed using the broth microdilution method according to study. The AmB formulations were prepared as stock solutions at 1 mg/mL concentration of AmB in water or 100% DMSO for free AmB. The final concentrations of AmB were 0.0125 to 0.2 µg/mL. Total volume of 200 µL of *Candida albicans* in culture medium with different AmB concentration of different AmB formulations were conducted in a 96-well plate at 28 °C. The fungi growth was monitored at OD of 530 nm using a microplate reader (BioTek Synergy H1). Minimum inhibitory concentration (MIC) of AmB was defined as the lowest concentration resulting in at least 90% growth inhibition after overnight incubation. To further evaluate the antifungal activity of AmB formulations of *Candida albicans* at invasive phase. Total volume of 200 µL of *Candida albicans* in culture medium without AmB were conducted in a 96-well plate at 28 °C for 12 h. Then AmB of different formulations were added into 96-well plate with *Candida albicans* growth for 12 h with the final concentration of AmB at 2.5 µg/mL. The fungi growth was monitored at OD of 530 nm using a microplate reader (BioTek Synergy H1) after overnight incubation.

[00101] Cell Viability Study: Raw 264.7, CHO and HEK293 cells were seeded in a 96-well plate with the cell density of 4×10^3 cells per well for Raw 264.7 and HEK 293 cells, and 6×10^3 cells per well for THP-1 cell. After an overnight incubation, the cells were treated with different concentrations of AmB formulations, as well as blank TDs. After 72 h incubation, Cell Titer 96 Aqueous Cell proliferation Reagent, which is composed of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) and an electron-coupling reagent phenazine methosulphate (PMS), was added to each well according to the manufacturer's instructions and further incubated for 1-4 h at 37 °C. The cell viability was determined by measuring the absorbance at 490 nm using a microplate reader (BioTek Synergy H1). Untreated cells served as a control. Results were shown as the average cell viability of triplicate wells via a equation: Cell viability %=[(OD_{treat}-OD_{blank})/(OD_{control}-OD_{blank})×100%].

[00102] Hemolytic Assays: Fresh blood from a healthy human volunteer was collected into PBS solution with 20 mM EDTA. Red blood cells (RBCs) were separated by centrifugation at 1000 rpm for 10 min. Then the RBCs were washed by PBS for three times and were re-suspended in PBS. AmB formulations or blank TDs were added into 200 µL RBC solutions with the concentration range of AmB at 10, 50 and 100 µg/mL, and 100, 500 and 1000 µg/mL of blank TDs followed by gentle mix and incubation at 37 °C for 0.5 h, 4 h and overnight, respectively. The samples were centrifuged at 1000 rpm for 5 min. The hemoglobin in supernatant was measured by the UV absorbance at 540 nm using a NanoDrop spectrophotometer. PBS and Triton X-100 (2%) were also incubated with RBCs for a negative and positive control, respectively. The hemolytic toxicity was calculated by the following equation: Hemolysis%=[(OD_{sample}-OD_{PBS})/(OD_{triton}-OD_{PBS})]×100%.

[00103] In vitro Cytokine Analysis: Raw 264.7 were seeded into a well of 96-well plate with the cell density of 1.5×10^4 per well. After an overnight incubation, the cells were treated with different concentrations of AmB formulations, as well as blank TDs incubated for 1h and 2h. Then the supernatant of cell culture medium was collected and stored at -80 °C for cytokine analysis by mouse TNF-α and IL-6 ELISA assays.

[00104] Maximum Tolerated Dose (MTD) Studies: 6-week-old healthy C57BL/6J mice were administrated intravenously with AmB formulations at the AmB doses of 1, 10, 15 mg/kg for single, three or seven consecutive doses (n=3-5). The

blank PEG5kBA4 was injected intravenously at 500 mg/kg by three doses. After 24h of the last dose, the mice were euthanized to collect blood plasma and critical organs e.g. the heart and kidney, and liver for blood chemistry and histology studies. The blood chemistry was conducted by VetScan Critical care plus rotors via Vetscan VS2 (Zoetis) or by reagent set (MedTextDX). Mice survival or physical conditions e.g. body weight changes were monitored daily until the mice euthanized. The mouse serum was collected and stored at -80 °C for cytokine analysis by mouse TNF- α and IL-6 ELISA assays. The MTD was defined as the allowance of 15% loss of median body weight and causing neither death due to toxic effects nor significant changes in the general signs within 24 h after the last dose.

[00105] Pharmacokinetic Studies: Healthy 6-week-old C57BL/6J mice were administrated intravenously with Fungizone, Ambisome, and AmB TD formulations at an AmB single dose of 1 or 10 mg/kg body weight, respectively (n=3). Blood was collected from mice tail vein at different time points in a heparinized tube. Plasma was isolated and collected by centrifugation and diluted with DMSO for UV-Vis absorbance measurement via NanoDrop. The standard curve of AmB content in the plasma was set up by adding different concentration of AmB in the plasma and then diluted by DMSO, and measured UV absorbance by NanoDrop. The pharmacokinetic parameters were calculated by an add-in program PKsolver in Microsoft Excel. AUC (area under the curve), Cmax (maximum drug concentration), t_{1/2} (terminal half-life), and Cl (total body clearance) were evaluated.

[00106] In vivo Imaging System (IVIS) Animal Imaging: The fluorescent nanoformulations for in vivo near infrared fluorescence (NIRF) optical imaging were prepared by loading of a hydrophobic near-infrared dye DiD into AmB TD formulations with a ratio of 0.2:1:10 (w/w/w, DiD/AmB/TD). Then the fluorescent nanoformulations were filtered with a 0.22 μ m sterile filter. Healthy 6-week-old C57BL/6J mice with or without *Candida albicans* inoculation via tail vein injection were administered with DiD-encapsulated AmB-TDs formulations. The mice were anaesthetized and imaged by IVIS 50 at different time points. Finally, after 24h of the nanoformulations injection, the mice were euthanized, all the major organs were excised for ex vivo imaging. The associated fluorescent intensities were analyzed by Living Image software (Caliper Life Sciences) using operator-defined regions of interest (ROI) measurements.

[00107] In vivo Efficacy for *Candida albicans* Infection Treatment and *Candida albicans* Infected Immunocompetent Mouse Model: 4×10^7 /kg cell suspensions of

Candida albicans were injected into the tail veins of healthy 6-week-old C57BL/6J mice. The size of the inoculum was confirmed by fungi cell counting in a hemocytometer. 24 h after the inoculation with Candida albicans, animals were assigned at random to receive an intravenous injection of Fungizone at a single dose of 1 mg/kg AmB, or Ambisome, AmB-PEG^{5k}BA₄ at a single dose of 10 mg/kg AmB or non-treatment (n=3-5). Survival rates, body weight change and body temperature were observed until Day 12. To further explore the severe infection, 1×10⁶/mice and 5×10⁶/mice were injected intravenously to the mice on Day 12 and 14, respectively. Another single dose of Fungizone, Ambisome and AmB-PEG^{5k}BA₄ were administered to the mice intravenously. Survival rates, body weight change and body temperature were observed until Day 21. All the mice were euthanized at Day 21. The liver, spleen, heart, lungs and kidneys were removed aseptically, weighed, and ground with sterile Sabouraud's broth to obtain a fairly homogeneous solution. The tissue and blood samples were made in sterile Sabouraud's broth and plated on Sabouraud-dextrose agar. Gentamicin (40 µg/mL) and chloramphenicol (200 µg/mL) were added into Sabouraud-dextrose agar for fungi selective growth. Colonies were counted after incubation at room temperature for 48 h. The plasma was collected and stored at -80 °C for cytokine analysis by mouse TNF-α and IL-6 ELISA assays.

[00108] Candida albicans Infected Immunocompromised Mouse Model: Healthy 6-week-old C57BL/6J mice were treated with immunosuppressive agent cyclophosphamide (CY) at a dosage of 200 mg/kg by I.P. injection. Mice were monitored daily post-treatment to evaluate blood leucocyte count and by body weight. Three days after the CY treatment, the candida albicans with cell concentration at 7×10⁶ was injected intravenously through tail veins of the mice. 24 h after the inoculation with Candida albicans, animals were assigned at random to receive an intravenous injection of Fungizone at twice dose of 0.5mg/kg AmB, or Ambisome, AmB-PEG^{5k}BA₄ at a single dose of 10 mg/kg AmB or non-treatment (n=5-8). Survival rates, body weight change and body temperature were observed until Day 6-8. The survived the mice were euthanized at Day 6-8. The liver, spleen, heart, lungs and kidneys were collected aseptically, weighed, and ground with sterile Sabouraud's broth to obtain a fairly homogeneous solution. The tissue and blood samples were made in sterile Sabouraud's broth and plated on Sabouraaud-dextrose agar. Gentamicin (40 µg/mL) and chloramphenicol (200 µg/mL) were added into Sabouraud-dextrose agar for fungi selective growth³². Colonies were counted after incubation at

room temperature for 48 h. The plasma was collected and stored at -80 °C for cytokine analysis by mouse TNF- α and IL-6 ELISA assays.

[00109] Histology and Fungi Staining of Tissues: The heart, kidney and liver harvested from the mice that used for MTD and treatment efficacy studies were embedded in O.C.T (Optimal Cutting Temperature Compound, Sakura Finetek USA, Inc) and store at -80 °C. Tissue were cut into serial 5 μ m sections using a Cryostat and fixed by 95% ethanol for 15-20 min, and subjected to the pathological analysis by hematoxylin and eosin (H&E) staining for histology evaluation or Grocott's methenamine silver (GMS) staining for identification of *Candida albicans* on tissue sections. Cover slips were mounted with the slides and imaged by an ImageXpress Pico Automated Cell imaging system.

[00110] Statistical analysis: Data are presented as means $\pm$ standard deviation (SD). Statistical analysis will be performed by Students t-test for the comparison of two group, and one-way analysis of variance (ANOVA) for multiple groups. The significance level with $P < 0.05$ is considered statistically significant. Linear regression model was fitted by ordinary least square in the correlation studies. The values of IC₅₀ were calculated from the curves of cell viability via a dose-response model of sigmoidal function with variable Hill slope fitting. A two-compartment model was used to fit the pharmacokinetic data. The sample size of in vivo efficacy study was based on our previous studies^{27, 29, 31}. In the treatment group, an 80% power will be obtained from a sample size of 5 in each group with a significance level of 0.05. Kaplan Meier method was used to analyze animal survival data, and the differences of mean survival times between two groups were assessed by Student's t-test.

EXAMPLES

[00111] Telodendrimer synthesis: In order to explore the reactivity of 1,3-cis diols in AmB for boronate formation, we first applied small molecule phenylboronic acid (PBA) to react with AmB as shown in FIG. 1A. As expected, the conjugates of AmB with PBA were detected in MALDI-TOF MS as shown in FIG. S3 and Table S2 (below), with the detection of the mono-valent AmB-PBA, bivalent AmB-PBA₂ (m/z 1206.559), and the tri-valent AmB-PBA₃ conjugates (m/z 1313.529). The relative strong peak intensity of both bivalent and trivalent conjugates indicate the feasibility of AmB prodrug design via multi-valent boronate conjugation. Thus, PBA was selected to functionalize TD platform with defined numbers to fine-tune AmB conjugation and release in TD nanocarriers.

[00112] Table S2: MW of AmB-PBA conjugation in AmB and PBA model reaction detected by MALDI-TOF MS.

Molecule	MW (Calc.)	MW (Detected)
AmB (DMSO+H ₂ O)+H ⁺	1020.556	1020.868
PBA-AmB (DMSO+H ₂ O)+H ⁺	1150.579	1150.789
2PBA-AmB+H ⁺	1206.559	1206.160
3PBA-AmB+H ⁺	1313.592	1312.928
3PBA-AmB+Na ⁺	1336.581	1336.281

5 [00113] The TDs were synthesized using solution peptide chemistry with the structural scaffold shown in FIG. 1B, TDs were synthesized from PEG^{5k}-NH₂, and the stepwise synthetic routes are illustrated in detail in FIG. 8B-8E. The dendritic oligolysine scaffold was synthesized by the coupling of the lysine protected with Fmoc groups. The efficient amide bond formation allows the precise and controlled construction of TD scaffold with precise number of primary amino groups for PBA conjugation. The molecular weight of all the intermediates in TD synthesis have been confirmed by MALDI-TOF MS (FIG. S1), revealing the accurate mass increase accordingly throughout the TD synthesis step by step. At the final step, one, two, four and eight PBAs were conjugated on TD through amide bond formation. As shown in
10 FIG. 1B, the TDs bearing different number of PBA are denoted as PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈ (chemical structures are shown in FIG. 8A).

[00114] The successful prodrug conjugation and the molecular weight distribution of the final PBA-containing TDs were characterized by MALDI-TOF MS. Like the starting material PEG^{5k}-NH₂, monomodal and narrowly dispersed molecular weight distributions were detected with molecular peaks centered at m/z of 4976, 5362, 5945 and 7263 for PEG^{5k}BA₁, PEG^{5k}BA₂, PEG^{5k}BA₄ and PEG^{5k}BA₈ TDs, respectively
20 (Table S1 below, and FIG. S2). The observed molecular weights are close to the calculated values based on the PEG^{5k} starting material. The chemical compositions of TDs were further confirmed using ¹H NMR spectroscopy. The representative spectrum for PEG^{5k}BA₁ is shown in FIG. S3A. Well resolved resonances of the aromatic protons at 7.79 and 7.80 ppm, 7.84 and 7.85 ppm were observed. The integration of these signature peaks match closely to the predicted values relative to the characteristic methoxy proton of MeO-PEG at 3.25 ppm, indicating well-defined
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structure of the TD. Therefore, based on the ^1H NMR integration (FIG. S3), the chemical formulas of TDs were $\text{PEG}^{5k}\text{BA}_{1.0}$, $\text{PEG}^{5k}\text{BA}_{1.9}$, $\text{PEG}^{5k}\text{BA}_{3.9}$ and $\text{PEG}^{5k}\text{BA}_{8.0}$, which are very close to the designed structures.

[00115] Table S1: MW of TDs detected by MALDI-TOF MS

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TD Polymer	MW (Calculated)	MW (Detected)
$\text{PEG}^{5k}\text{BA}_1$	5149.041	4976.441
$\text{PEG}^{5k}\text{BA}_2$	5425.169	5362.021
$\text{PEG}^{5k}\text{BA}_4$	5977.425	5945.447
$\text{PEG}^{5k}\text{BA}_8$	7081.938	7262.886

[00116] AmB-TD prodrug conjugation: The presence of boronic acid (BA) groups on the designed TDs provide efficient anchors for AmB conjugation via reversible boronate ester bond. Here we disclose the drug loading contents of AmB were set at 10% in TD-prodrug nanoformulations production. To form AmB-TD prodrug, 10% weight ratio of AmB were fed to TD in organic solvent (e.g., chloroform with some methanol) with TEA base added. The progress of the coupling reaction was monitored by thin layer chromatography (TLC) and fluorescence intensity. The molecular weight of final prodrug products after AmB conjugation were confirmed by MALDI-TOF MS. The TLC results (FIG. S4A) revealed that AmB coupling reaction completed very efficiently after thin film formation. In comparison with free AmB that migrating on the TLC, none-free AmB was observed from TLC of AmB-TD prodrug formulations after PBS dispersion, indicating nearly 100% conjugation efficiency of each AmB-TDs. In addition, the fluorescence intensity of AmB were significantly decreased due to AmB conjugation onto TDs. AmB- $\text{PEG}^{5k}\text{BA}_1$ and AmB- $\text{PEG}^{5k}\text{BA}_2$ at the same concentration exhibited nearly 50% fluorescent intensity of Fungizone at the same concentration, while over 90% and 80% fluorescent reduction for AmB- $\text{PEG}^{5k}\text{BA}_4$ and AmB- $\text{PEG}^{5k}\text{BA}_8$ at neutral pH (FIG. S4B), indicating more effective conjugation and molecular aggregation with the increased PBA valency in TD. MALDI-TOF MS was applied to detect change of TD molecular weight after AmB conjugation to evaluate the ratio of AmB and TD in the prodrug. As shown in Table 1, significant increase of molecular weight was observed in each AmB-TD formulation as compared with blank TDs, conforming AmB successfully conjugated on the PBA groups of TDs. According to the change of molecular weight (ΔMW) shown in Table 1, the molar ratios

for AmB conjugated to TD prodrug formulations were obtained. The detected molar ratios AmB/TD are around 1:1.5 for PEG^{5k}BA₁, PEG^{5k}BA₂, and PEG^{5k}BA₄ TDs, and 1:1.04 was determined for AmB-PEG^{5k}BA₈ TD conjugate, indicating excessive PBA in TDs for AmB conjugation via the formation of multivalent boronate linkages. In comparison with the theoretically molar ratio, the detected molar ratio of AmB to TD showed less molar of TDs are required to conjugate with AmB, indicating partial of the TDs in the polymeric micelles are without AmB conjugation, which may explain two populations of TD molecular weight in AmB-PEG^{5k}BA₁ MALDI-TOF MS spectra shown in FIG. S5A. After characterization of AmB conjugation on the TDs, we continuously incubate the AmB-TD prodrug formulations with pH 5.5 buffer to further confirm that AmB can cleavage from the TDs reversibly at slight acidic condition. As shown in FIG. S4B, fluorescent intensity of AmB-TDs formulations was significantly increased at pH 5.5 as compared that in neutral pH, indicating efficient AmB cleavage from PBA moieties of TDs. The molecular weight of pH 5.5 buffer treated AmB-TD nanoformulations were detected by MALDI-TOF MS. As shown in Table 1 and FIG. S5, the molecular weight of each AmB-TD formulations was significantly reduced and quite closed to the molecular weight of blank TD, demonstrating slight acidic environment trigger the efficient reversible cleavage between AmB and TD.

[00117] Table 1: MW characterization of blank TDs, AmB-TDs prodrug formulations.

Formulation (1:10 w/w)	AmB:TD:BA (Theor. MR)	Blank TD MW	AmB-TD MW	A MW	AmB:TD Conj. (Detected MR)	MW after cleav. pH 5.5
AmB-PEG ^{5k} BA ₁	1:1.06:2.0	4976.441	5644.657	668.216	1:1.32	4905.384
AmB-PEG ^{5k} BA ₂	1:1.85:3.7	5362.021	5982.328	620.307	1:1.53	5218.356
AmB-PEG ^{5k} BA ₄	1:1.68:3.7	5945.447	6583.439	637.992	1:1.32	5973.640
AmB-PEG ^{5k} BA ₈	1:1.41:11.2	7262.886	8161.902	899.016	1:1.04	7314.074

[00118] AmB-TD prodrug nanocarrier Characterization: The self-assembly behavior of TDs was characterized by dynamic light scattering (DLS) and critical micelle concentration (CMC) measurements. As shown in Table 2, the particle size of AmB-TDs was significantly increased after AmB conjugation as compared with blank TDs. As expected, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ self-assembled into monodispersed micelles with small particle size and homogenous distribution ranging from 14-16 nm and exhibit great stability with particle size of 13 nm of both formulations for storage over a month with at 4 °C, as evident by both DLS and TEM studies (FIG. 2A, FIG. 2B and FIG. S6D). While large particles aggregation and multiple peaks of

particle distribution were observed in AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ as well as the precipitation of AmB free drug molecules after 1-4 days storage at 4 °C (FIG. S6D), indicating less stable micellar formation due to less PBA functional moieties of PEG^{5k}BA₁ and PEG^{5k}BA₂ for efficient AmB-conjugation. Representative TEM images (FIG. 2B) showed that the blank PEG^{5k}BA₈, AmB conjugated PEG^{5k}BA₄ and PEG^{5k}BA₈ are spherical micelles in shape. The relatively aggregation particles observed from blank PEG^{5k}BA₄ and PEG^{5k}BA₈ of TEM images may be due to their high CMCs and strong hydrogen bond formation between high density of PBA moieties on TDs during the TEM grid dryness process (FIG. S7A). After hydrophobic AmB conjugation, the well dispersed spherical structure was observed in both AmB-TDs, suggesting lower CMC and disruption of strong hydrogen bond among PBA moieties. Since PBA with relatively high-water solubility were hardly able to form hydrophobic core of micelles, as shown in Table 2 and Fig. S7B, neither blank PEG^{5k}BA₁ nor PEG^{5k}BA₂ were able to form micelles up to 5 mg/mL. With increased density of PBA moieties conjugated on TDs, blank PEG^{5k}BA₄ and PEG^{5k}BA₈ show decreased values of CMC with 784.2 and 277.2 µg/mL, respectively. Despite both AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ exhibit lower CMC after hydrophobic AmB conjugation, similar CMC value of 152 and 167 µg/mL were detected in AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ (FIG. S7C). The comparable CMC after AmB conjugation may be caused by their similar AmB-TD conjugation molar ratio of 1:1.47 and 1:1.04 for AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈, respectively. CMC of AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ were not detectable due to less stable micellar formation, which is supported by their particle size measurement in DLS. Blank PEG^{5k}BA₁, PEG^{5k}BA₂, and PEG^{5k}BA₄ showed relatively negative zeta potential ranging from -8~-17 mv, while zeta potential of PEG^{5k}BA₈ was close to neutral. Blank PEG^{5k}BA₁, PEG^{5k}BA₂ and their AmB conjugated formulations were not able to form stable micelles, therefore, the negatively charged PBA moieties significantly affect their zeta potential measurement. Negatively charged PBA moieties in blank PEG^{5k}BA₄ with relatively high CMC may form micelle with loosed core and reduce its zeta potential. After AmB-conjugation, zeta potentials of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ are close to neutral due to stable micelles formation.

[00119] Aggregation state of AmB-TD conjugates: Since UV absorption spectrum of AmB is highly sensitive to its local environment, the aggregation state of AmB was determined by UV-Vis absorption spectrum. The spectra of AmB in water or similar polar environment reveals a typical UV spectrum of aggregation with a main

peak in low to mid 300 nm, while the main peak of monomer is in the low 400 nm region. FIG. 2C shows the spectra of free AmB in DMSO, and Fungizon®, AmBisome®, and AmB-TD prodrug formulations in aqueous solution. The main absorbance of monodispersed free AmB in DMSO are at wavelength of 372, 392, and 416 nm. In contrast, Fungizone® show highly aggregated state of AmB in aqueous solution with significantly increased absorbance at 326 nm and reduced monomer signal at 420 nm. AmB also dimerizes and aggregates in the lipid environments due to its physicochemical properties. Therefore, strong absorbance of oligomer at 324 nm and quite weak signal at 416 were observed in AmBisome UV-Vis spectra. Similar spectra pattern was shown in AmB-PEG^{5k}BA₁ prodrug, demonstrating PEG^{5k}BA₁ fail to reduce the aggregation state of AmB after conjugation due to low density of PBA moieties and less stable micellar formation, as evident by both DLS and CMC studies. Unlike AmB-PEG^{5k}BA₁, spectra of AmB-PEG^{5k}BA₂, AmB conjugated PEG^{5k}BA₄ and PEG^{5k}BA₈ show red-shift of oligomer aggregation absorbance from 324-326 nm towards 354-368 nm region and merged partially with monodispersed absorbance at 372 nm as well as increased intensity of monodispersed absorbance at 422 nm, confirming reduced AmB oligo-aggregation and formation of monomer or partial dimer-aggregation. The ratio of oligomer and monomer absorbance intensity was used to evaluate the degree of AmB aggregation. As shown in Table 3, the ratios of Fungizone® and AmB-PEG^{5k}BA₁ prodrug were reach to 9.79 and 7.87, respectively, while the ratios of AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ remarkably decreased to 0.88, 0.69 and 0.54. The dramatically reduced ratios indicate AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ nanoformulations significantly reduced the aggregation status of AmB, with the density of PBA increased, less aggregation status of AmB formed, while PEG^{5k}BA₁ showed no effect. Main peak ratio (MPR, OD_{dimer}/OD_{mono}) can estimate the ratio between the population size of the loose aggregate and monomers³³. As shown in Table 3, the mpr result indicates dimer aggregation was mainly observed in Fungizone, Ambisome and AmB-PEG^{5k}BA₁. AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ nanoformulations exhibit comparable dimeric aggregation other than oligomer. The UV-Vis spectra of AmB formulations suggest the aggregation state of is highly dependent on the density of PBA moieties of TDs, which serve as anchor to conjugate AmB in its monomeric or dimeric state by boronate ester bond in the micelle nanoformulations. The remarkable reduction in aggregation state of AmB in the AmB-TDs prodrug micelles is

potentially beneficial for reducing the toxicity of AmB. Although some studies mentioned that dimeric/loose aggregates show increase the toxicity than that of the tight/oligomer aggregates due to the faster release, these formulations were limited to non-covalent lipid-based AmB-encapsulated formulations. In the AmB-TDs prodrug formulations, the AmB were covalently bind to the nanocarriers, therefore, AmB can be released in monomeric forms other than aggregated forms to lowered toxicity for systemic application.

[00120] Table 2: Characterization of blank TDs, AmB-TDs prodrug formulations.

Formulation (1:10 w/w)	Blank TD (nm)	AmB-TD (nm)	Blank TD ζ (mv)	AmB-TD ζ (mv)	Blank TD (CMC $\mu\text{g/mL}$)	AmB-TD (CMC $\mu\text{g/mL}$)
AmB-PEG ^{5k} BA ₁	4	1398±122	-8.35	-11.06	ND	ND
AmB-PEG ^{5k} BA ₂	4	Multiple peaks	-16.95	-14.82	ND	ND
AmB-PEG ^{5k} BA ₃	5	16±2	-16.69	-6.32	784.2	151.9
AmB-PEG ^{5k} BA ₈	7	14±1	-4.28	-6.70	277.2	166.8

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[00121] Table 3: UV-Vis Peak intensity of AmB formulations

Formulation (1:10 w/w)	324-328 nm (oligo)	354-368 nm (dimer)	416-422 nm (mono)	OD _{oligo} /OD _{mono}	OD _{dimer} /OD _{mono} (MPR)
AmB (DMSO)	0.089	0.369	0.927	0.096	0.398
Fungizone	1.400	0.311	0.143	9.790	2.175
AmBisome	1.400	0.414	0.178	7.875	2.326
AmB-PEG ^{5k} BA ₁	1.367	0.346	0.153	8.935	2.261
AmB-PEG ^{5k} BA ₂	0.296	1.253	0.336	0.881	3.729
AmB-PEG ^{5k} BA ₃	0.255	1.093	0.370	0.689	2.954
AmB-PEG ^{5k} BA ₈	0.255	1.249	0.469	0.544	2.663

[00122] In vitro drug release: The *in vitro* drug release profiles of AmB formulations were evaluated in PBS at 37 °C under a sink condition, i.e. frequently refreshed PBS. After 8 h dialysis (FIG. 2D), less than 30% of AmB wase release from AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ prodrug conjugates, which were significantly slower than the nearly 50% AmB release of Fungizone, AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂. With the burst release of AmB in the first 8 h, Fungizone, AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ may induce high drug concentration accumulation causing acute toxicity for systemic application. Slow AmB release was observed in AmBisome and AmB-PEG^{5k}BA₈ formulations, revealing only less than 5% of AmB release during 8-24 h, 50-60% AmB-release in 144 h and nearly 80% in 216 h, which may limit AmB availability in fungi infection treatment when systemic administration

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especially with a low dose therapy. In comparison with other AmB-formulations, AmB-PEG^{5k}BA₄ exhibited a relatively slow release of less than 30% in first 8 h and an effective AmB release of nearly 20% during 8-24 h, which may reduce the toxicity of AmB as AmBisome during the systemic administration as well as retain the potency and availability of the drug even with a low dose application. The differences in release profiles from four AmB-TDs formulations is attributed to the density of PBA functionalities for AmB conjugation via boronate ester bond. Increased number of PBA moieties on TDs exhibits higher capability AmB conjugation and superior stability of the micelles, leading to reduced burst release and slower sustained release. In addition, the higher branched structure in PEG^{5k}BA₄ and PEG^{5k}BA₈ create additional barrier to prevent cleaved AmB diffusion.

[00123] The boronate ester bond is sensitive to both acidic pH and cis-diol containing sugar, e.g. glucose. As shown in FIG. 2E, the AmB release from PEG^{5k}BA₄ and PEG^{5k}BA₈ conjugates were found to be efficient responsive to acidic pH at 5.5. Over 60% and 42% AmB was released from PEG^{5k}BA₄ and PEG^{5k}BA₈ in 24 h at pH 5.5, which was faster than AmB release of two formulations at neutral pH with 50% and 33%, respectively. The moderate pH responsive AmB release may be because the primary amino moiety of AmB can be protonated at acidic condition and further neutralize the pH locally in the micelle. The existing of glucose slightly accelerated AmB release in PEG^{5k}BA₄ formulation after 24 h dialysis other than PEG^{5k}BA₈, indicating minimized burst drug release of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ during the blood circulation after systemic administration. This highly contributes to the low associate constant of glucose to PBA and certain number of AmB-unconjugated PBA moieties on TDs for alleviating the competition by glucose, which is potentially beneficial for minimizing the toxicity induced by AmB burst release.

[00124] In vitro antifungal activity: To evaluate the in vitro antifungal activity of AmB-TDs prodrug formulations, MIC was determined against *C. albicans*, which is commonly susceptible to AmB. As shown in FIG. 3A, free AmB exhibits lowest MIC values of 0.05 µg/mL, all the AmB-TDs show similar MIC value of 0.1 µg/mL with that of Fungizone, while AmBisome shows slightly higher MIC of 0.2 µg/mL in comparison with other formulations. The possible explanation for the different in vitro antifungal activity of the AmB formulations could be associated with their in vitro drug release profiles. Although AmBisome and AmB-PEG^{5k}BA₈ exhibited similar drug release profiles at first 24 h, the slightly acidic pH 5.6 of Sabouraud's broth for *C. albicans*

culture promote faster release of AmB in AmB-PEG^{5k}BA₈ due to the pH responsive boronate ester linkage. No anti-fungal activities were observed in all the blank TDs (Table S3). *C. albicans* are reported to actively neutralize the environment from either acidic or alkaline pHs. These fungi can raise pH from 4 to over 8 in less than 12 h by release of ammonia and introduce the yeast-hyphal transition. Then we further evaluated in vitro anti-fungal activity of AmB formulations in the *C. albicans* that pre-growth in the Sabouraud's broth for 12 h with abundant hyphae growth. Different AmB formulations with AmB were added into *C. albicans* in the invasion phase after 12 h growth at final concentration at 2.5 µg/mL. The fungi growth was further monitored by absorbance at 530 nm in the next 24 h. As shown in FIG. 3B, most of the AmB formulations besides AmB-PEG^{5k}BA₈ successfully inhibited *C. albicans* growth in the invasion phase. AmB-PEG^{5k}BA₈ reduced nearly 50% of the fungi growth after 24 h incubation, which may be due to the less effective drug release at slightly alkaline pH triggered by *C. albicans*. Therefore, AmB-PEG^{5k}BA₄ showed most optimized features of micellar stability and anti-fungal activity in vitro among all the AmB-TDs prodrug formulations.

[00125] Table S3 MIC of TDs for *Candida albicans*:

Blank TD	MIC (µg/mL)
PEG ^{5k} BA ₁	ND
PEG ^{5k} BA ₂	ND
PEG ^{5k} BA ₄	ND
PEG ^{5k} BA ₈	ND

[00126] In vitro cytotoxicity of AmB-TDs formulations: The *in vitro* toxicity of the designed AmB-TDs prodrug nanoformulations were evaluated in terms of cytotoxicity via mammalian cells and hemolytic property in human red blood cells (RBCs). Free AmB and Fungizone exhibited significant cytotoxicity in all three mammalian cells. In comparison with free AmB, AmBisome and AmB-TDs conjugates significantly reduced the drug cytotoxicity in MTS studies (FIG. 3C). All the TDs with concentrations ranging from 100-5000 µg/mL exhibited non-cytotoxicity as determined by MTS assay. AmB-PEG^{5k}BA₁ showed comparable cell viability profile with Fungizone in both RAW and CHO cells. AmB-PEG^{5k}BA₂ significantly reduced cytotoxicity in RAW 264.7 cells and slightly increased IC₅₀ in CHO cells. AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ exhibited comparable or even superior biocompatibility as compared with AmBisome. In vitro

MTS studies in human kidney 293T cells revealed AmBisome, AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ conjugates significantly enhanced the drug IC₅₀ to 428, 471 and 508 µg/mL as well as AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ increased IC₅₀ to 250 and 333 µg/mL, in comparison with the IC₅₀ of free AmB and Fungizone, which is 1.4 and 79 µg/mL, respectively (Table S4). This result demonstrates cytotoxicity of AmB were significantly reduced by conjugation with PBA of TDs, which may benefit to minimize the nephrotoxicity of AmB in vivo. In addition, more than 90% cell viability (FIG. 3C) was observed in Raw 264.7 and CHO cells treated with AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈ prodrug formulations at AmB concentration of 500 µg/mL for 72 h, while only 30% and 70% cell viability were shown in AmBisome at the same condition, demonstrating lower cytotoxicity and improved biocompatibility of AmB-PEG^{5k}BA₄, and AmB-PEG^{5k}BA₈. Up to 5 mg/mL, nonsignificant cytotoxicity of all the blank TDs with over 90% cell viability was detected in all of the three mammalian cells (FIG. S8).

[00127] Table S4 IC₅₀ of AmB formulations in 293T, RAW 264.7, and CHO cells by MTS assay:

IC ₅₀ (µg/mL)	AmB	Fungizone	Ambisome	AmB-PEG ^{5k} BA ₁	AmB-PEG ^{5k} BA ₂	AmB-PEG ^{5k} BA ₄	AmB-PEG ^{5k} BA ₈
293T	1.400	78.89	427.8	249.8	332.6	471.0	508.0
RAW 264.7	1.513	44.92	405.1	57.54	215.3	ND	ND
CHO	1.619	42.57	ND	23.18	6.054	ND	ND

[00128] Hemolysis is another general parameter for determining the biocompatibility of AmB formulations. Free AmB with AmB concentration ranging from 10 -100 µg/mL triggered over 100% of hemolysis within 30 min, as considered hemolysis of 2% triton X-100 is 100%. In comparison with free AmB, Fungizone, AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ delayed the onset of hemolysis to 4h and exhibited dose-dependent hemolysis property. After incubation for overnight, as shown in FIG. 3D, Fungizone with AmB concentration ranging from 10-100 µg/mL reached to over 100% of hemolysis. AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ resulted in nearly 90% of hemolysis at 50 and 100 µg/mL and over 50% hemolysis at 10 µg/mL of AmB after overnight incubation. In contrast, non-hemolysis was observed in AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ treated RBCs at all the

concentration of AmB during the different incubation time of 30 min, 4h and overnight (FIG. 3D and FIG. S9). Non-hemolytic toxicity was found in all the blank TDs with their concentration up to 1000 µg/mL during the incubation. To further confirm the interaction between lipid and AmB of each AmB formulation, Doxil, a liposome formulation of doxorubicin were incubated with AmB formulations for 0.5 h and overnight. As shown in FIG. S10, fluorescence intensity of Doxil significantly increased after incubation with Fungizone, AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂, indicating membrane damage of the liposome, resulting in doxorubicin released from liposome. Whereas the fluorescent intensity of Doxil that incubated with AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ were kept similar with Doxil only, indicating intact lipid membrane of Doxil.

[00129] Among these AmB-formulations, free AmB and Fungizone exhibited most severe cytotoxicity and hemolytic toxicity due to their rapid drug release and high aggregated AmB state. AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ with less effective micellar formation (none-detectable CMC), relatively fast drug release and high aggregated AmB in PEG^{5k}BA₁ slightly reduced the cytotoxicity and hemolysis. AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ exhibited non-hemolytic toxicity, comparable or even less cytotoxicity than AmBisome. The favorable biocompatibility of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ mainly attribute to their high stability micelles, significantly reduced aggregation degree and optimized drug release profiles.

[00130] Innate immune cell stimulation by AmB and TNF-α production: A “sepsis-like syndrome” infusion-related toxicity of AmB distinguished by inflammatory cytokine release has been reported up to 70% of patients at 1-3 h starting to receive AmB. AmB as a microbial product can stimulate immune cells through Toll-like receptors (TLR)2 and TLR4 with CD14. AmB initiates production of inflammatory cytokines e.g. TNF-α, IL-6, and IL-1β, chemokines, e.g. IL-8 and MCP-1, and nitric oxide (NO) in murine and human innate immune cells in vitro. We evaluated the proinflammatory cytokine TNF-α production in RAW 264.7 cell treated by various AmB formulations. Non-noticeable amount of TNF-α production induced by any AmB formulations after 1 h incubation with concentration of AmB at 5 µg/mL (FIG. S11), while after 2 h, Fungizone, AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ treated Raw cells were stimulated to produce remarkably high level of TNF-α. With PBA density increased, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ only slightly increase the TNF-α level in the RAW cells after 2 h incubation as compared with control and AmBisome groups

(FIG. 3E). The stimulation of RAW cells by AmB-TDs formulations may attribute to the concentration applied to the Raw cells was much lower than the CMCs of AmB-TD conjugates prodrugs, inducing less stability of micelles and rapid release of AmB. Still with higher density of PBA, the AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ significantly reduced TNF- α production from innate immune cells.

[00131] Pharmacokinetics (PK) and biodistribution of AmB-TDs: The PK profiles of AmB-PEG^{5k}BA₂, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ were investigated in C57BL/6J mice after intravenous administration in comparison with Fungizone and AmBisome at the dose of AmB of 1 mg/kg, respectively. As shown in FIG. 4A and Table 5, Fungizone and AmB-PEG^{5k}BA₂ administration at 1 mg/kg has a rapid clearance from blood circulation with area under the curve (AUC) of 15.1 and 9.3 $\mu\text{g/mL/h}$ and half-life ($t_{1/2\alpha}$) of 0.004 h and 0.003 h, respectively, analyzed by UV-Vis of AmB in plasma at different time points. AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at 1 mg/kg exhibit significantly prolonged blood circulation time as compared with Fungizone. AmB-PEG^{5k}BA₄ exhibited comparable AUC of PK profile with AmBisome, which contributes to over 3-fold increase of AUC relative to Fungizone. In comparison with AmBisome with $t_{1/2\alpha}$ of 0.022 h, slightly longer $t_{1/2\alpha}$ of 0.099 h were observed in AmB-PEG^{5k}BA₄. AmB-PEG^{5k}BA₈ performed the longest blood circulation time among various AmB formulations with $t_{1/2\alpha}$ of 1.5 h and over 12-fold higher AUC than that of Fungizone. The prolonged circulation time of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at 1 mg/kg can be attributed to high density of PBA in TD for AmB conjugation to minimize initial burst drug release and the existing of AmB-unconjugated PBA moieties for alleviating the competition by glucose as demonstrated in FIG. 2E.

[00132] Some studies reported anti-PEG IgM might be induced after repeated administration of PEGylated NPs, resulting in the fast in vivo clearance of nanoformulations in the repeated administrations. To address this concern, after a week, we continuously evaluated PK profile of AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at 10 mg/kg by using the identical C57BL/6J mice treated with same AmB formulation at 1 mg/kg. As shown in FIG. 4B and FIG. 4C, AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ exhibited comparable $t_{1/2\alpha}$ and $t_{1/2\beta}$ to the previous injection at 1 mg/kg. No accelerated clearance of three AmB formulations was observed after repeated administration, indicating the low immunogenicity of AmB-TDs prodrug formulations. It has been also reported suppression of the contacted immune cells by

the payload drug and further inhibits anti-PEG IgM production. In comparison with AmBisome, 2.7 and 3.6-fold of AUC and prolonged blood circulation time of both $t_{1/2\alpha}$ and $t_{1/2\beta}$ were observed in AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at 10 mg/kg dose. The further improved PK profiles at 10 mg/kg dose of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ are largely due to the superior stability of micelles at high dose injection. After I.V. injection, the concentration of AmB-TDs was still above their CMCs through rapid dilution by the blood at 10 mg/kg dose, forming stable micelles during the blood circulation, whereas AmB-TDs concentrations were close to their CMCs after I.V. injection at 1 mg/kg resulting in less stability of micelles.

10 [00133] IVIS was utilized as a non-invasive method to monitor real time tissue distribution of AmB-TD formulations at healthy and *C. albicans* infected mice in vivo. DiD, a near-infrared fluorescent (NIRF) dye, was co-loaded into AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ to probe in vivo and ex vivo biodistribution of micelles. After 24 h post-injection, major organs were harvested for ex vivo NIRF imaging to compare the tissue distribution of formulations. As shown in FIG. 4D and FIG. 4E, the biodistribution of both AmB-TDs formulations exhibited a similar pattern in uninfected mice as well as the mice infected with *C. albicans*, which the immune status of the mice had little influence on the disposition of the drug. The highest fluorescent signal was observed in the liver. AmB-PEG^{5k}BA₈ showed higher accumulation in the spleen and the lung than that from AmB-PEG^{5k}BA₄. The higher accumulation of AmB-PEG^{5k}BA₈ in the spleen was probably due to its relatively longer circulation time than AmB-PEG^{5k}BA₄. Both liver and spleen are considered as reservoir organs for AmB due to high accumulation in the reticuloendothelial system (RES). The high fluorescent signal of AmB-TDs conjugates in the organs with RES was probably related to the appropriate size of micellar formulations of AmB which is enough to be less opsonized by the contents of the plasma. The optimal particle sizes and prolonged blood circulation time allow the AmB-TDs conjugates accumulate and penetrate to the targeted tissues. The signal of DiD in the lung was less compared with that in the liver but was still higher than the fluorescent signals from other critical organs, e.g. the heart, brain, kidney, colony, and intestine, indicating possibility of lung fungal infection treatment. Both AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ showed dramatically lower levels of fluorescent signals to 25% and 40% of those from the liver. The fluorescent signal from AmB-PEG^{5k}BA₄ in the kidney further reduced kidney

accumulation than that from AmB-PEG^{5k}BA₈, demonstrating minimized nephrotoxicity during the systemic administration.

[00134] Maximum tolerance dose (MTD) and histology studies: Conventional AmB has been reported to produce significant cardiac toxicity with ventricular
5 arrhythmias and nephrotoxicity with acute renal failure in the patients overdoses or even administered in conventional dosages and infusion rates. Therefore, we evaluated MTD of AmB-TDs conjugates by dose escalation in C57BL/6J mice using a single dose intravenous injection or a schedule of multiple doses with consecutive I.V. injection for three or seven days. According to the treatment dose of AmBisome
10 applied in the clinic, we tested the MTD of various AmB formulations up to 10 mg/kg at single dose and triple-dose treatment studies. In single dose injection, AmB-PEG^{5k}BA₁ and AmB-PEG^{5k}BA₂ were significantly increased MTD to 5 mg/kg as compared with MTD of Fungizone at 1 mg/kg. All the mice treated by AmBisome, AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ at 10 mg/kg survived and barely show any
15 noticeable changes of behavior (Table S5). In the three consecutive doses treatment studies, mice lost over 5% body weight in the groups treated with 1.5 mg/kg of Fungizone. The mice treated with AmBisome and AmB-PEG^{5k}BA₄ and PEG^{5k}BA₄ barely showed weight loss or significant changes in behavior up to three consecutive dose of 10 mg/kg (FIG. 5A). To further investigate nephrotoxicity and other infusion
20 related toxicities, we test the bold enzyme levels of mice in the triple-dose treatments. As shown in FIG. 5B, one out of three mice in three doses Fungizone of 1 mg/kg and 1.5 mg/kg groups showed 1.8 and 3.2-fold increased level of BUN as compared with that of control group, indicating kidney dysfunction. In addition, 1.7-fold of creatinine level (FIG. 5C) higher than control group was observed the serum from the identical
25 mice with high BUN in 1 mg/kg Fungizone group, which further confirmed the incidence of damage of kidney by nephrotoxicity. The serum potassium level of 8.0 ± 0.85 mmol/L was noted as the upper limit of a normal reference range. The serum from the mouse with high BUN level in triple doses of Fungizone at 1.5 mg/kg also exhibited high serum potassium level of 10.3 mmol/L. The serum potassium of one
30 mouse in 3 mg/kg Fungizone treated group increase to 9.7 mmol/L as well shown in FIG. 5D. The hyperkalemia-like symptom may reveal acute kidney failure and the high potassium level in the serum could lead a high risk to develop heart attack. In strong contrast, the mice treated by triple doses of AmBisome, AmB-PEG^{5k}BA₄ and blank PEG^{5k}BA₄ at 1 or 10 mg/kg and 500 mg/kg, respectively, exhibited comparable level

of BUN, creatinine, and serum potassium located within the normal range as compared with mice in control group, demonstrating normal function of kidney due to significantly minimized nephrotoxicity of AmBisome and AmB-PEG^{5k}BA₄ prodrug formulation. The liver function evaluation typically includes alanine transaminase (ALT) and aspartate transaminase (AST). As shown in FIG. 5E, all the mice treated by triple doses of AmB-PEG^{5k}BA₄ at 1 mg/kg and PEG^{5k}BA₄ at 500 mg/kg showed similar levels of ALT and AST with those in control group. Slightly higher serum level of ALT (17-77 U/L) and AST (54-298 U/L) than their normal ranges were observed in one mouse of each Fungizone treated group, one mouse treated by AmB-PEG^{5k}BA₄ at 10 mg/kg, and two mice injected by AmBisome at 10 mg/kg, which may indicate possible liver toxicity due to the higher accumulation of nanoformulations in the liver observed from biodistribution studies. Necrosis in the liver was only found in the mice treated by triple doses of Fungizone at 1.5 mg/kg from pathology studies (FIG. S13). Seven consecutive doses of 15 mg/kg AmBisome and AmB-PEG^{5k}BA₄ were further evaluated in C57BL/6J mice. As shown in FIG. S12A, the bodyweight changes of both groups were less than 10%. In addition, seven doses i.v. injections at 15 mg/kg of AmBisome and AmB-PEG^{5k}BA₄ were well tolerated without noticeable changes in their blood chemistry evaluation including BUN, CRE, ALT and AST shown in FIG. S12B – FIG. S12E. The infusion-related toxicity was further evaluated by determining the proinflammatory cytokine TNF- α production level in the serum. High level of TNF- α production of 210 pg/mL was only observed in one mouse treated by 3 mg/kg Fungizone rather than other mice in different AmB treatment groups (FIG. 5F).

[00135] Table S5. MTD of single dose and multiple doses of AmB formulations:

Formulation	Single dose MTD (i.v. mg/kg)	Multiple doses MTD (i.v. mg/kg)
Fungizone	1	1.5×3
Ambisome	10	15×7
AmB-PEG ^{5k} BA ₁	5	NA
AmB-PEG ^{5k} BA ₂	5	NA
AmB-PEG ^{5k} BA ₄	10	15×7
AmB-PEG ^{5k} BA ₈	10	NA

[00136] To further characterize the potential organ damage in mice after MTD studies, mice were sacrificed, and the heart, liver, kidney were harvested for histology analysis on 24 h after the last dose. We carefully examined pathologic structures of kidney in the mice treated by various AmB formulations. In comparison with normal

kidney organ, 90% necrotic proximal tubules were observed in the mice treated by triple doses of Fungizone at 1.5 mg/kg (FIG. 5G). The mice treated by triple doses of Fungizone at 1 mg/kg, AmBisome at 10 mg/kg, AmB-PEG^{5k}BA₄ at 1 and 10 mg/kg exhibited less than 5% necrotic proximal tubules, while with increased treatment doses of AmB, mice treated by seven doses of AmBisome and AmB-PEG^{5k}BA₄ at 15 mg/kg showed necrotic proximal tubules of 5%. Non-pathologic changes of the kidney in the blank PEG^{5k}BA₄ group. The pathology results of the kidney treated by various AmB-formulations demonstrated the remarkably superior biocompatibility and less nephrotoxicity of AmB-PEG^{5k}BA₄ than Fungizone and comparable safety with AmBisome for i.v. injection in vivo. As shown in FIG. S13 and FIG. S14, marginally necrosis in the liver and heart was found in the mice treated by triple doses of Fungizone at 1.5 mg/kg and seven doses of AmBisome and AmB-PEG^{5k}BA₄ at 15 mg/kg. The slightly increased ALT and AST might only indicate the transient alteration in the liver function since magnitude of change in both parameters has no relationship to the prognosis or severity of liver damage. As a result, AmB-PEG^{5k}BA₄ showed comparable MTD level (15 mg/kg×7) of AmBisome and reached to over 20-fold higher than the MTD (1.5 mg/kg×3) of Fungizone.

[00137] Treatment efficacy of AmB-PEG^{5k}BA₄ in immunocompetent mouse model: To evaluate treatment efficacy of AmB-PEG^{5k}BA₄ nanoformulation in comparison with commercialized AmB formulations i.e., Fungizone and AmBisome, we used experimental systemic candidiasis induced in immunocompetent mice, the most widely used experimental animal model for investigating systemic mycosis. The evaluation was based on mortality and morbidity, the latter mainly being assessed by quantitative determination of fungal infection of the major organs e.g. blood, the kidney, liver, heart, lung, and spleen. Treatment of Fungizone at 0.5 mg/kg×2 in one day, AmBisome and AmB-PEG^{5k}BA₄ at 10 mg/kg single dose was administered intravenously after 24 h of *C. albicans* inoculation (FIG. 6A). The results shown in FIG. 6B reveal that all the formulations of AmB with survival of 100% significantly increased survival of mice in comparison with untreated controls of 60% survival, which attributes to the functional immune system for effective clearance of fungal pathogens. The bodyweight changes (FIG. 6C) of AmB-PEG^{5k}BA₄ group exhibited the most rapid recovery in comparison with both Fungizone and AmBisome treated groups, indicating efficient and timely fungal elimination and less toxicity in vivo. In comparison with AmB

formulation treated groups shown in FIG. 6D, the body temperature of untreated group was significantly reduced on Day 1 due to the low temperature of dying mice.

[00138] To challenge the severe fungal infection, two batches of *C. albicans* were inoculated intravenously in the survived mice on Day 12 and 14. The same dose treatment of AmB was administered on Day 15 as shown in FIG. S15A. No death of mouse was observed in all the groups including the untreated control (FIG. S15B – FIG. S15D), indicating effective elimination or inhibition of *C. albicans* via immune system. All the mice were euthanized on Day 21 to further evaluate the morbidity of each group. As shown in FIG. 6E, remarkably high number of colonies in the heart, lung and kidney were observed in both dead and survived mice of untreated group. All the infected mice treated by AmBisome and AmB-PEG^{5k}BA₄ at 10 mg/kg showed non-colony in their major organs, while small number of colonies were observed in the kidney, lung, liver and spleen of one or two mice treated by Fungizone (FIG. 6E and FIG. S15E). The dead mouse in untreated group exhibited extremely high level of TNF- α and IL-6 in serum, revealing the fungal infection leaded to cytokine storm and caused mice death. The AmB formulation treatment and functional immune system efficiently eliminated the fungal pathogens, therefore, prevented the development of cytokine storm and saved the mice.

[00139] To further evaluate anti-fungal treatment efficacy and organ damage by fungal infection and AmB treatment, GMS and H&E staining were applied for distinguishing presence of *Candida* organisms and pathology evaluation. The GMS results show 1% presence of hyphae candida organisms in the kidney of untreated survived mouse, while budding yeast-like cells were observed in the kidney of Fungizone treated mouse (FIG. 6G), indicating the environmental stress cause by Fungizone treatment prevented the transformation of *C. albicans* from budding yeast to hypha. No *Candida* organisms were detected in the kidney of mice in both AmBisome and AmB-PEG^{5k}BA₄ treatment groups, as supported by the colony counting results. As shown in FIG. 6H, the kidney histology of untreated groups demonstrated over 50% necrotic proximal tubules and inflammation. 10% of necrotic proximal tubules and inflammation were detected in the kidney in Fungizone treated group. Both AmBisome and AmB-PEG^{5k}BA₄ treatment groups showed the lowest level of the damage in the kidney, further verifying their efficacy of efficient and timely elimination of fungal pathogens and minimized nephrotoxicity. Heart GMS images showed non-*Candida* organisms in both untreated and AmB-treated groups (FIG.

S15F). Focal necrosis was observed in the heart of untreated survived mouse (FIG. S15G). The heart of AmB-treated group showed less damage and inflammation than the untreated control.

[00140] Table S6 provides a summary of candida organisms presence, percentage of necrotic proximal tubules, and inflammation in the kidneys from the immunocompromised mice treated by AmB formulations:

Kidney	Presence of candida organisms	% necrotic proximal tubules	Inflammation %
None-treatment	>10%	5	minimal
Fungizone dead	5%	<5	minimal
Fungizone survived	1%	20	minimal
Ambisome dead	2%	50	15
Ambisome survived	0	5	minimal
AmB-PEG ^{5k} BA ₄ dead	2%	50	10
AmB-PEG ^{5k} BA ₄ survived	0	10	minimal

[00141] Table S7 provides a summary of candida organisms presence and inflammation percentage with tissue injury in the hearts from the immunocompromised mice treated by AmB formulations:

[00142] Heart	Presence of candida organisms	Inflammation % with tissue injury
None-treatment	>10%	5
Fungizone dead	10%	5
Fungizone survived	5%	15
Ambisome dead	2%	25
Ambisome survived	0	10
AmB-PEG ^{5k} BA ₄ dead	2%	10
AmB-PEG ^{5k} BA ₄ survived	0	<5

[00143] Treatment efficacy of AmB-PEG^{5k}BA₄ in immunocompromised mouse model: Immunocompromised patients are highly susceptible to Candida species infections. Preclinical studies in animal models can provide valuable information for optimizing treatment for immunocompromised patients. In this study, cyclophosphamide (CY) was used to achieve immunocompromised model in mice shown in FIG. 7A. CY is an alkylating agent with myelosuppressive activity can interfere DNA replication causing cell apoptosis. It is used to treat aplastic anemia

and to prevent rejection of organ transplants. As shown in FIG. S16A, mice treated by CY showed a decrease in the number of WBCs. The lowest level was noted on the day 3 after CY treatment with a significant reduction of WBCs from 15 to 2.3 k/ μ L. The bodyweight reveals nearly 5% reduce after 24 h of CY injection (FIG. S16B), and then became stable. Therefore, day 3 post-CY treatment was selected for *C. albicans* inoculation in an immunocompromised status.

[00144] As presented in FIG. 7B, CY induced immunosuppression in mice increased the mortality rate elicited by *C. albicans*. The untreated control of immunocompromised mice exhibited 100% mortality within a 5-day period. The treatment of Fungizone at 0.5 mg/kg $\times$ 2 in one day, AmBisome and AmB-PEG^{5k}BA₄ at 10 mg/kg of one dose was administered to immunocompromised mice 24 h after *C. albicans* infection. In comparison with untreated group, the AmB formulation treatment postponed the incidence of death from 29 h to over 40 h. The survival percentage was significantly increase to 20%, 37.5% and 62.5% by the treatment of Fungizone, AmBisome, and AmB-PEG^{5k}BA₄, respectively, as compared with untreated group. Bodyweight of mice shown in FIG. 7C started recovery on Day 4 in AmBisome and AmB-PEG^{5k}BA₄ treatment groups, while bodyweight continuously lost over 20% was observed in untreated and Fungizone treated group, which may be due to toxicity of Fungizone despite the low dose. All the survived mice were euthanized on Day 6 or 8 to collect the major organ as well as organs of dead mice for evaluating antifungal treatment efficacy via colony counting. As shown in FIG. 7D, the survived mice in AmBisome and AmB-PEG^{5k}BA₄ treatment groups exhibited no colony growth in all the major organs including the heart, lung, kidney, liver, spleen as well as blood, while a few of colonies were observed in the major organs of the survived mouse treated by Fungizone. The result reveals higher single dose treatment of AmB achieved by AmBisome and AmB-PEG^{5k}BA₄ can efficiently eliminated all the cells of *C. albicans* showing higher antifungal efficacy than the low dose treatment of Fungizone. A significant large number of colonies were found in major organs of dead mice in both untreated and Fungizone treatment group, also indicating the relatively low anti-fungal treatment efficacy of Fungizone. In contrast, few colonies shown in the dead mice that treated by either AmBisome or AmB-PEG^{5k}BA₄ further demonstrated their efficient antifungal activity. The level of proinflammatory cytokines i.e., TNF- α and IL-6 in the serum were determined in both survived and dead mice. As shown in FIG. 7E, survived mice treated by AmBisome and AmB-PEG^{5k}BA₄ exhibited little TNF- α and IL-

6. Low level of TNF- α and IL-6 were detected in the survived mouse receiving Fungizone. All the dead mice showed remarkably high level of TNF- α and IL-6, indicating the mice treated by AmBisome and AmB-PEG^{5k}BA₄ may have died of cytokine storm caused tissue damage, despite the infection of *C. albicans* has already
5 been inhibited effectively. To validate this hypothesis, we further examined the organs from both survived and dead mice in GMS and H&E staining to evaluate their presence of candida organisms and tissue damage via pathology analysis.

[00145] Macroscopic observation of the kidneys from dead mice infected with *C. albicans* in untreated and Fungizone treated groups shown in FIG. 7F revealed
10 abundant microabscesses with over 10% and 5% presence of candida organisms, respectively, which upon microscopic observation demonstrated hyphae and yeasts. The kidneys from dead mice treated by both AmBisome and AmB-PEG^{5k}BA₄ showed 2% candida organisms presence with most budding-yeasts. No candida organisms were found in the kidney from survived mice treated by AmBisome and AmB-
15 PEG^{5k}BA₄, while 1% budding-yeasting candida organisms were detected in survived mouse receiving Fungizone. Similar results were also shown in the hearts from dead and survived mice in each group (FIG. S17C and FIG. S17D). This GMS staining results are well correlated with the results of colony culture and counting from major organs shown in FIG. 7E. The pathology analysis of kidney and heart were also
20 conducted in survived and dead mice of each group. As we expected, 50% necrotic proximal tubules and 10-15% inflammation were observed in the kidneys (FIG. 7F) from dead mice treated by AmBisome and AmB-PEG^{5k}BA₄, indicating the cytokine storm with high level of proinflammatory cytokines in serum induced severe damage of kidney and heart and finally caused mice death. In contrast, only 5% necrotic
25 proximal tubules and minimal inflammation shown in the kidneys from untreated and Fungizone groups, therefore, mice dead in untreated and Fungizone group may be due to severe infection status of *C. albicans* other than the tissue damage by the proinflammatory cytokines. The survived mice receiving Fungizone exhibited the most severe tissue damage level with 20% proximal tubules necrosis in the kidney and 15%
30 inflammation in the heart among the survived mice treated by AmBisome and AmB-PEG^{5k}BA₄. 5% proximal tubules necrosis in the kidney and 10% inflammation in the heart as well as 10% proximal tubules necrosis in the kidney and less than 5% inflammation in the heart were observed in survived mice of AmBisome and AmB-PEG^{5k}BA₄ groups. Two survived mice from AmB-PEG^{5k}BA₄ treatment group were

continuously monitored for over two months to confirm the completely elimination of *C. albicans* infection by single dose treatment and the animal fully recovery from the tissue damage. The bodyweight of two mice were gradually recovered, and non-candida organisms were observed from their major organs. Less than 5% necrosis in heart, less than 1% necrosis in the kidney and liver (Table S8) were evaluated in the pathological H&E staining images shown in FIG. S18.

[00146] Table S8 provides a summary of tissue damage and candida organism presence of the survived immunocompromised mice treated by AmB-PEG^{5k}BA₄:

Heart AmB-PEG ^{5k} BA ₄	Necrosis	Candida organism
980	1%	no
null	5%	no
Kidney AmB-PEG ^{5k} BA ₄	Necrosis	Candida organism
980	10%	No
null	Less than 1%	No
Liver AmB-PEG ^{5k} BA ₄	Necrosis	Candida organism
980	1%	No
null	Less than 1%	No

[00147] The treatment efficacy results indicate that AmB-PEG^{5k}BA₄ were effective in immunocompromised mice for treatment of systemic infection caused by *C. albicans*, especially when high single dose was used. This result is relevance as the immunocompromised status that similar to clinical situations with a high risk for candidiasis development. Therefore, it may be demonstrated that AmB-PEG^{5k}BA₄ conjugates seem to be very promising as alternatives for the treatment of systemic fungal infections.

[00148] As disclosed herein, biocompatible well-defined AmB-conjugated TD prodrug nanoformulations have been rationally designed and prepared efficiently via reversible boronate ester chemistry for AmB systemic delivery. The AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ prodrug nanoformulations have stable and small particle sizes with zeta potentials close to neutral, which are desired for improving macrophage targeting for systemic drug delivery. The burst release was significantly minimized by increasing density of PBA moieties of TDs. The sustained drug release profiles of AmB-PEG^{5k}BA₄ and AmB-PEG^{5k}BA₈ can reduce systemic toxic effects of Fungizone®.

In comparison with AmB-PEG^{5k}BA₈, efficient drug release and higher efficacy for invasive phase C. albicans inhibition were shown in AmB-PEG^{5k}BA₄. Therefore AmB-PEG^{5k}BA₄ is promising to further development in systemic delivery of AmB in severe, fungal infections. As a result, AmB-PEG^{5k}BA₄ exhibited prolonged blood circulation
 5 time, comparable MTD with AmBisome[®], reduced nephrotoxicity, and significantly improved antifungal treatment efficacy and survival rate than Fungizone[®] and AmBisome[®] in immunocompromised mouse model. Stable and efficient reversible AmB conjugation in the small-sized TD nanocarriers with optimized release profile is crucial for optimizing systemic drug delivery, minimizing nephrotoxicity, administration-
 10 related toxicity and improving treatment efficacy in vivo. Therefore, cost-effective AmB-PEG^{5k}BA₄ prodrug formulation significantly enhanced systemic antifungal treatment efficacy than Fungizone[®] and AmBisome[®], which holds a great potential for clinical translation.

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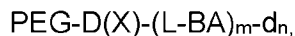
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CLAIMS:

Claim 1. A prodrug compound having the following formula:



wherein PEG is a polyethylene glycol moiety, and has an average molecular weight
5 of about 44 to about 40,000 Dalton; D(X) is a dendritic polymer moiety having one or
more branched monomer units (X); L is an optional linker group; BA is at least one
boronic acid moiety; d is at least one 1, 2- or 1, 3- cis-diol or catechol containing
therapeutic compound, or an α - or β - hydroxyl ketone containing therapeutic
compound; and subscript m is an integer from 2 to 16 and subscript n is any number
10 that greater than 0 and less than m.

Claim 2. The prodrug compound of claim 1, wherein the polyethylene glycol
moiety, and has an average molecular weight of about 5000 Da.

Claim 3. The prodrug compound of claim 1, wherein each branched monomer
unit X is a natural or synthetic amino acids with more than one amine groups.

15 Claim 4. The prodrug compound of claim 1, wherein each branched monomer
unit X is a lysine moiety.

Claim 5. The prodrug compound of claim 1, wherein BA is at least one selected
from the group consisting of aryl boronic acid and aliphatic boronic acid.

20 Claim 6. The prodrug compound of claim 5, wherein BA is phenylboronic acid or
phenylboronic acid comprising a substitute group on the phenyl ring.

Claim 7. The prodrug compound of claim 1, wherein D is a nonlinear dendritic
structure and X is a lysine moiety.

Claim 8. The prodrug compound of claim 1, wherein d is covalently bonded to
BA via a reversible boronate ester bond.

25 Claim 9. The prodrug compound of claim 8, wherein the boronate bond is
cleavable in an aqueous solution.

Claim 10. The prodrug compound of claim 8, wherein the boronate bond can be
cleaved upon a change to the pH in an aqueous solution.

Claim 11. The prodrug compound of claim 1, wherein the at least one boronic acid moiety is selected from the group consisting of phenyl boronic acid, nitrophenyl boronic acid, methoxyl phenylboronic acid, benzyl boronic acid, methylene boronic acid, ethylene boronic acid, and proplene boronic acid.

5 Claim 12. The prodrug compound of claim 1, wherein d is at least one a therapeutic drug molecule selected from the group consisting of curcumin, doxorubicin, daunorubicin, amphotericin B, nystatin, anidulafungin; tetracyclines, aminoglycosides, nicotinamide mononucleotide (NMN), and nicotinamide adenine dinucleotide (NAD/NADH),

10 Claim 13. The prodrug compound of claim 12, wherein said tetracycline is at least one selected from the group consisting of tetracycline HCl, doxycycline, minocycline, tigecycline, eravacycline, sarecycline, omadacycline, chlortetracycline, oxytetracycline, and chemically modified tetracycline 3 (CMT-3).

Claim 14. The prodrug compound of claim 12, wherein said aminoglycoside is at
15 least one selected from the group consisting of gentamycin, tobramycin, and kanamycine.

Claim 15. The prodrug compound of claim 1, wherein the at least one therapeutic compound is amphotericin B (AmB).

Claim 16. The prodrug compound of claim 1, wherein the L is at least one
20 selected from the group consisting of linker group or a bond selected from the group consisting of a polyethylene glycol moiety, amino acid, polyamino acids, polyserine moiety, enzyme cleavable peptide moiety, disulfide bond moiety and acid labile moiety, polyglycine moiety, poly(serine-glycine) moiety, aliphatic amino acid moieties, 6-amino hexanoic acid moiety, 5-amino pentanoic acid moiety, 4-amino
25 butanoic acid moiety, and beta-alanine moiety.

Claim 17. The prodrug compound of claim 16, wherein the at least one L is selected from the group consisting of lysine, arginine, aspartic acid, glutamic acid, linear oligo-lysine, linear oligo-aspartic acid, linear oligo-arginine, and linear oligo-glutamic acid.

30 Claim 18. The prodrug compound of claim 1, wherein the prodrug compound is a linear dendritic telodendrimer (TD) scaffold.

Claim 19. The prodrug compound of claim 18, wherein the prodrug compound comprises self-assemble monodispersed micelles.

Claim 20. The prodrug compound of claim 19, wherein the micelles have a particle size of about 10-20nm.

- 5 Claim 21. The prodrug compound of claim 1, wherein the compound reduces toxic side effects, immunogenicity and improved efficacy of drug molecules when administered in therapeutically effective amounts to mammal in need thereof.

FIG. 1A

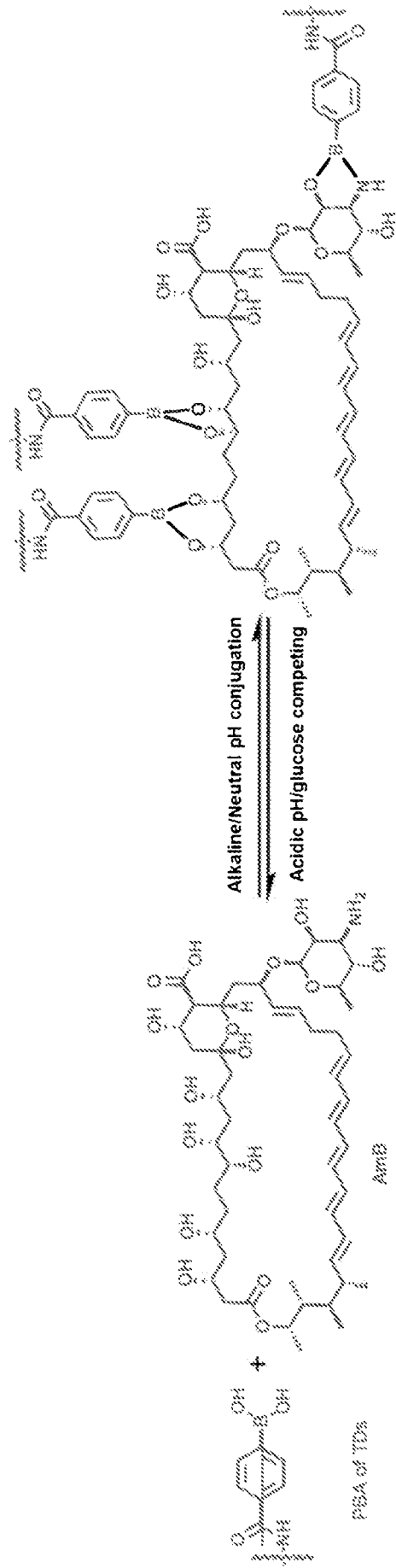


FIG. 1B

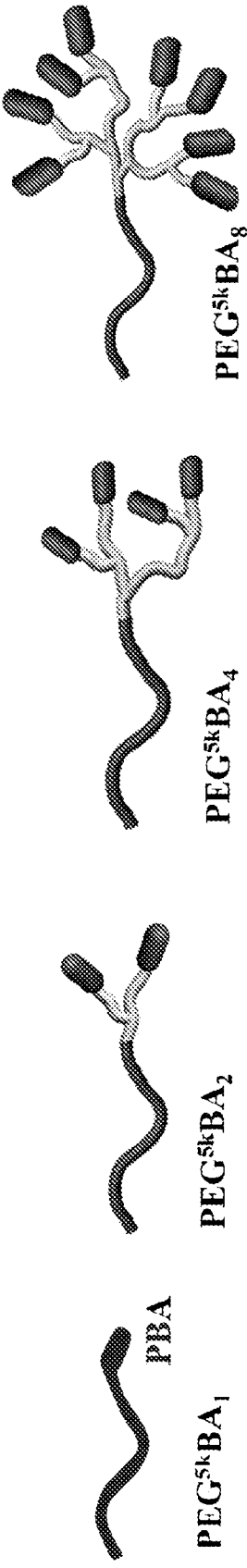


FIG. 1C

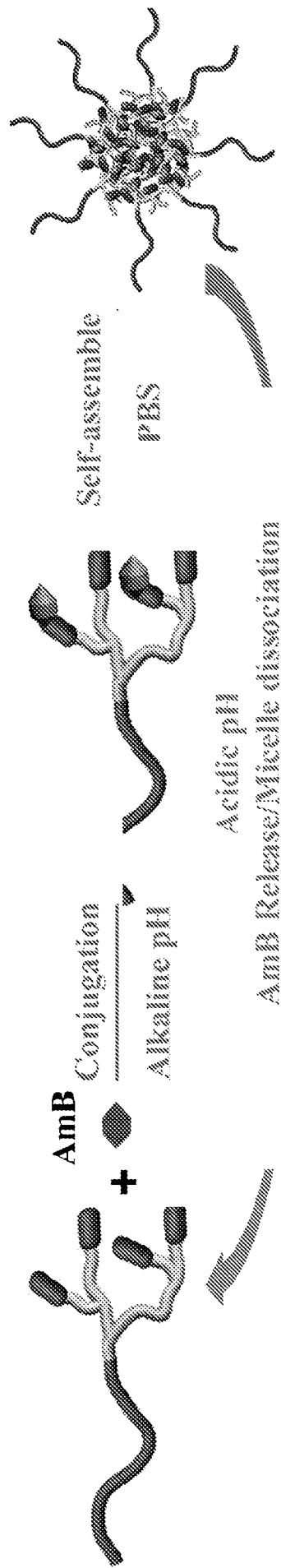
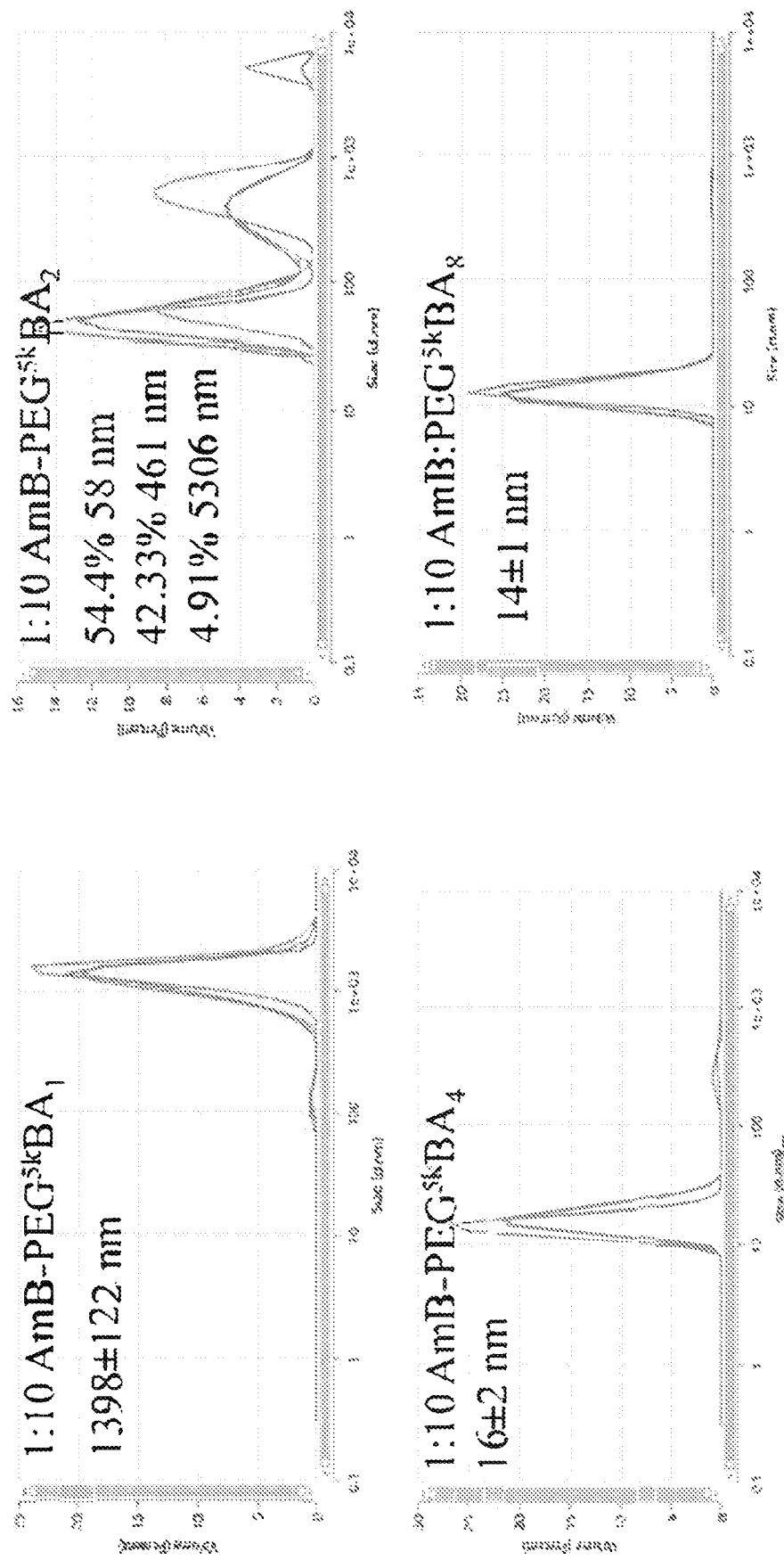


FIG. 2A



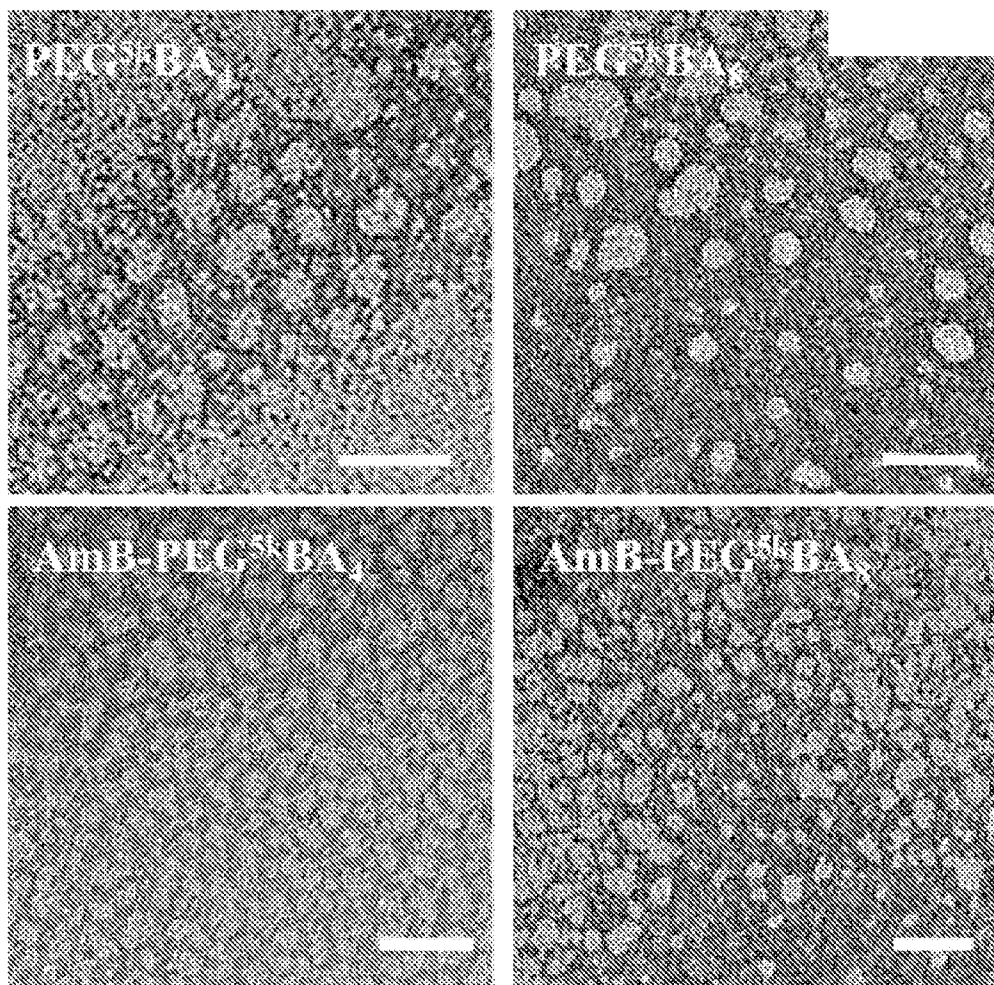


FIG. 2B

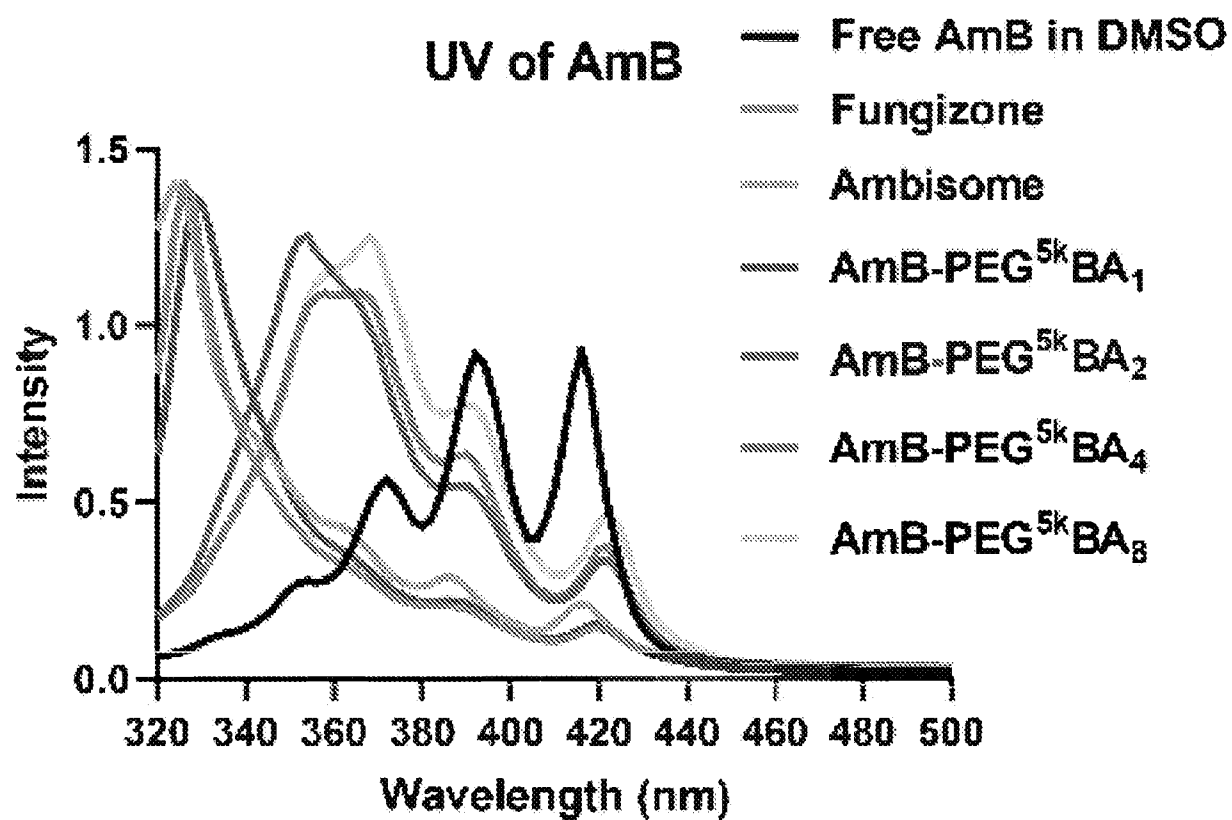


FIG. 2C

Amb release in PBS

FIG. 2D

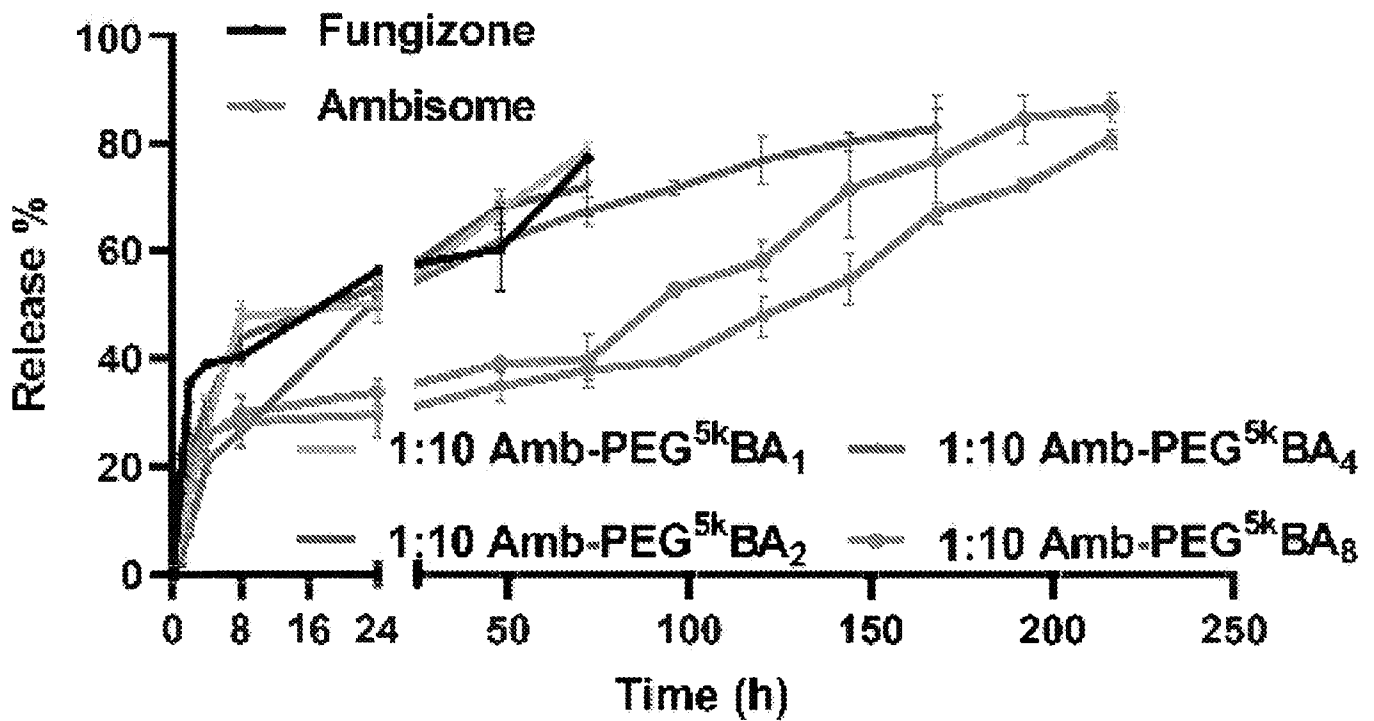
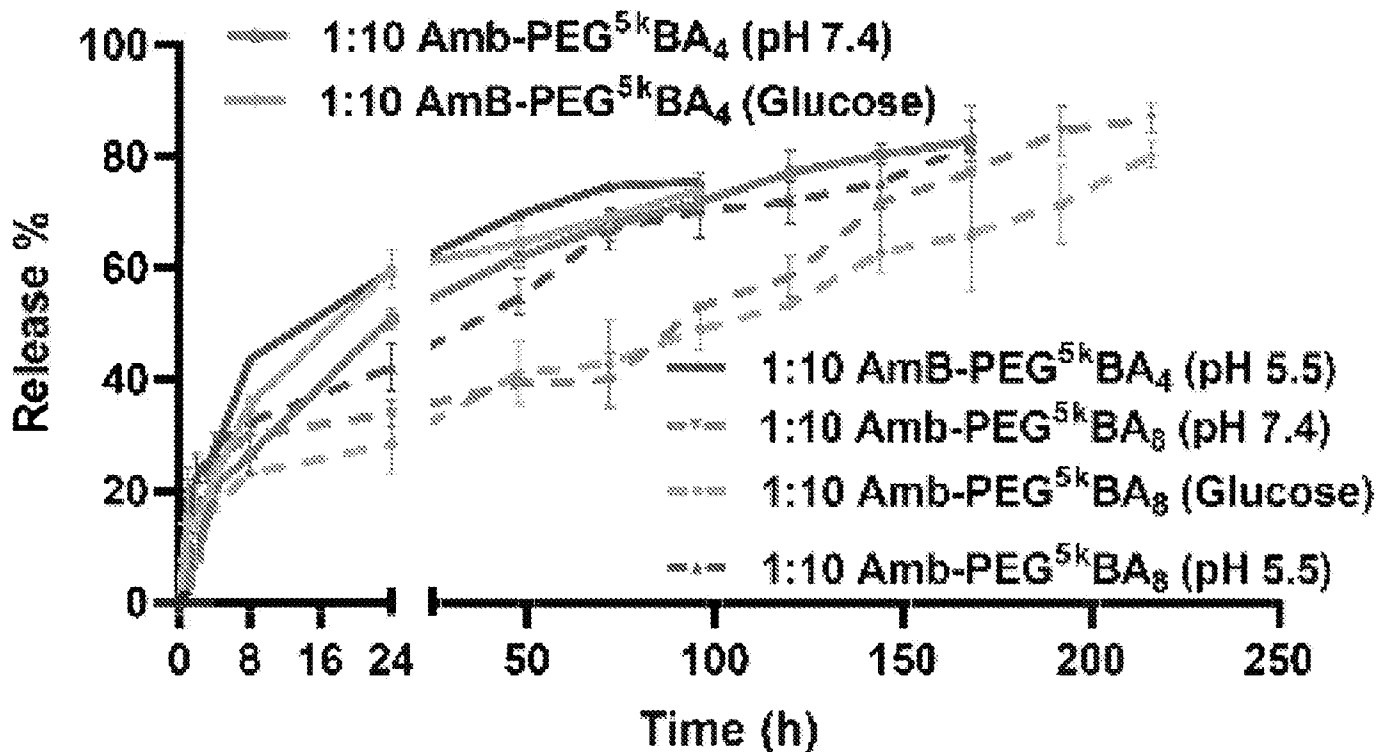
**AmB Release**

FIG. 2E



MIC of AmB formulations for *C. albicans*

Formulation	MIC ($\mu\text{g/mL}$)
AmB	0.05
Fungizone®	0.1
AmBisome®	0.2
AmB-PEG ^{5k} BA ₁	0.1
AmB-PEG ^{5k} BA ₂	0.1
AmB-PEG ^{5k} BA ₄	0.1
AmB-PEG ^{5k} BA ₈	0.1

FIG. 3A

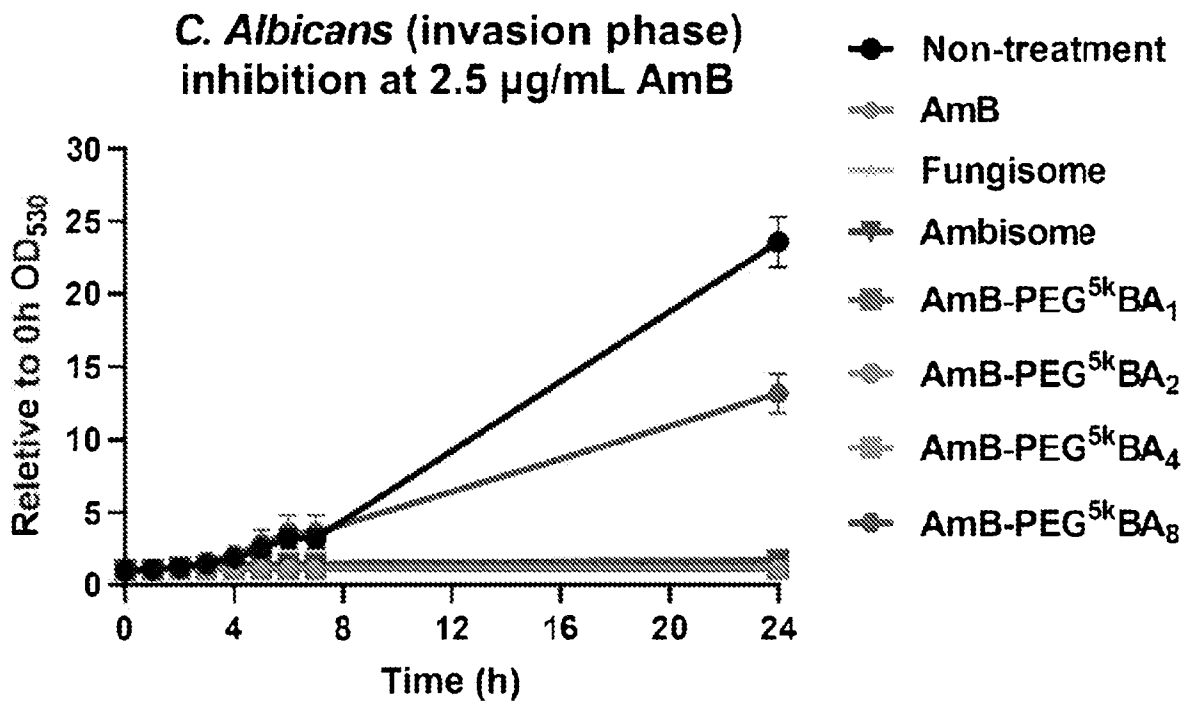
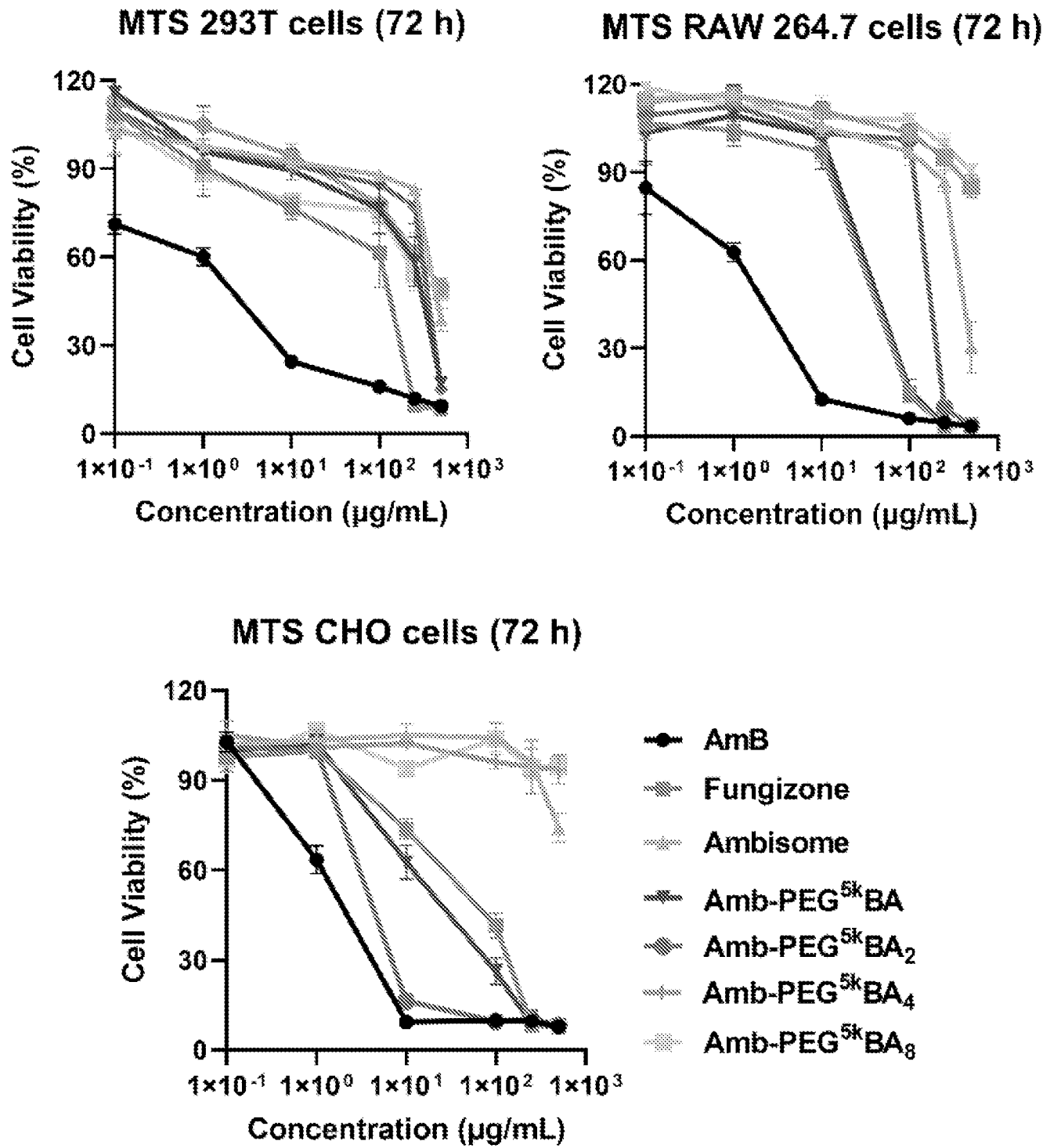


FIG. 3B

FIG. 3C



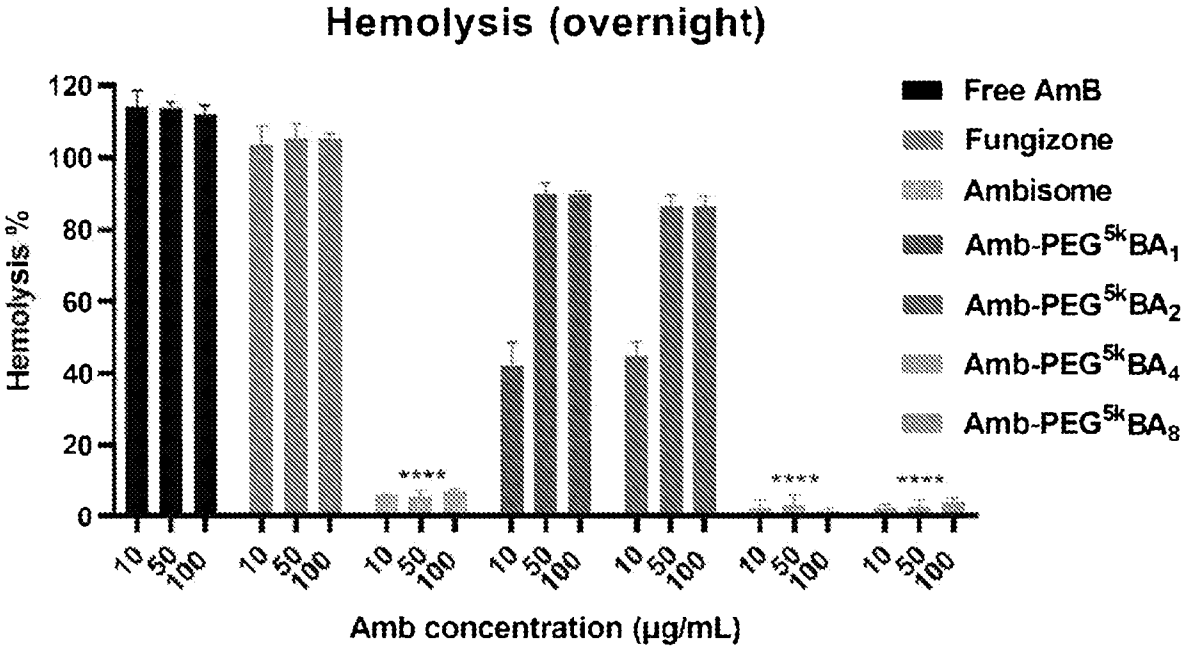


FIG. 3D

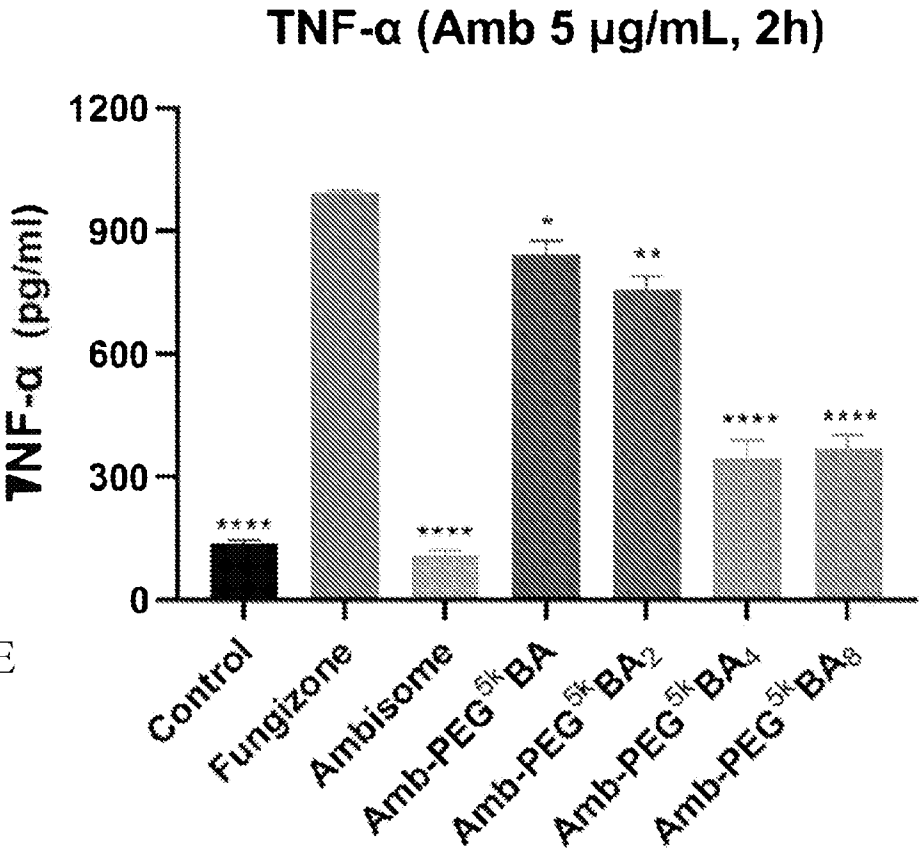


FIG. 3E

FIG. 4A

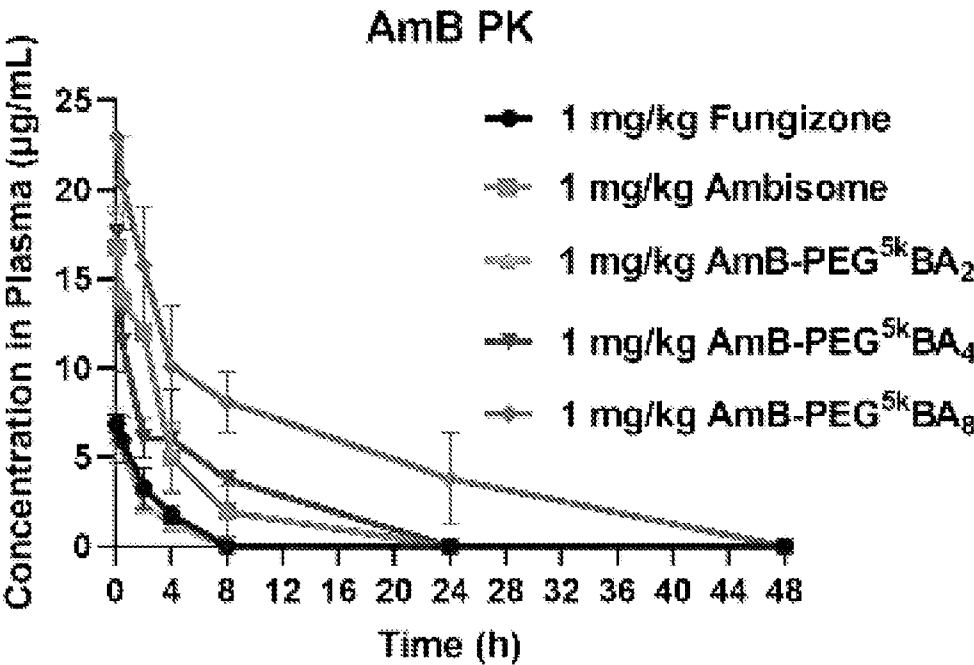


FIG. 4B

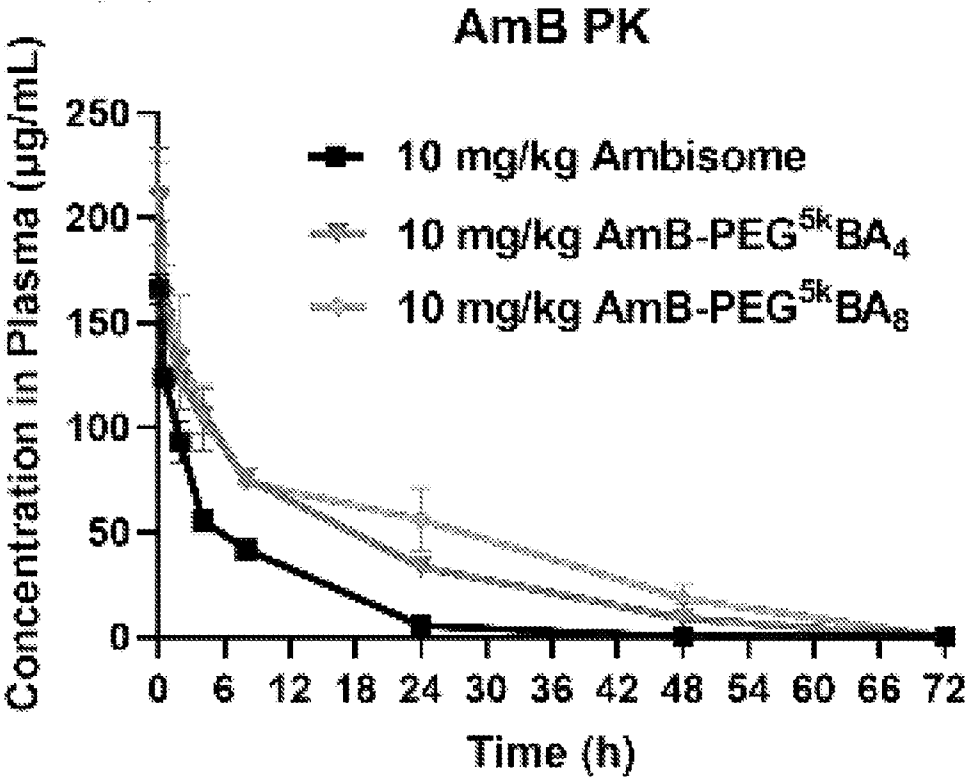


FIG. 4C

PK analysis of AmB formulations

Formulations	AUC ($\mu\text{g/mL/h}$)	$t_{1/2\alpha}(\text{h})$	$t_{1/2\beta}(\text{h})$
1 mg/kg Fungizone	15.103	0.004	1.925
1 mg/kg Ambisome	57.625	0.022	2.792
1 mg/kg AmB-PEG ^{5k} BA ₂	9.503	0.003	3.877
1 mg/kg AmB-PEG ^{5k} BA ₄	50.985	0.099	4.604
1 mg/kg AmB-PEG ^{5k} BA ₈	187.068	1.513	17.030
10 mg/kg Ambisome	756.690	0.051	4.002
10 mg/kg AmB-PEG ^{5k} BA ₄	2063.899	0.190	10.876
10 mg/kg AmB-PEG ^{5k} BA ₈	2712.941	1.326	16.121

FIG. 4D

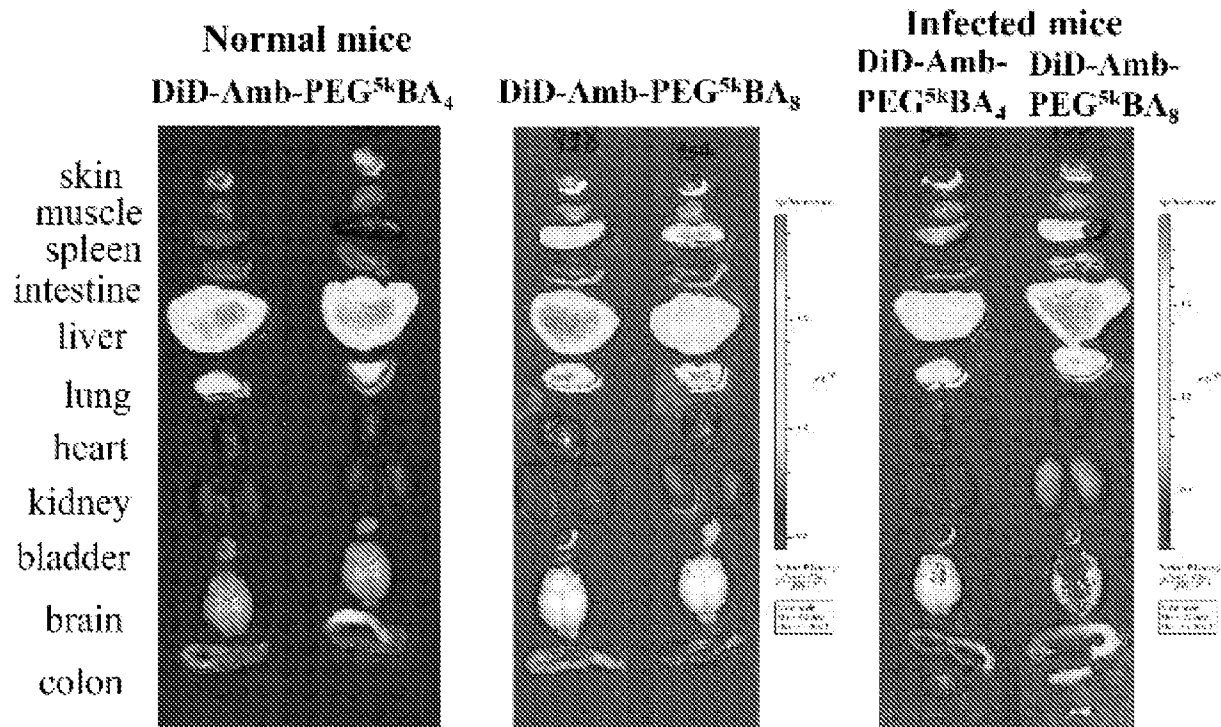


FIG. 4E

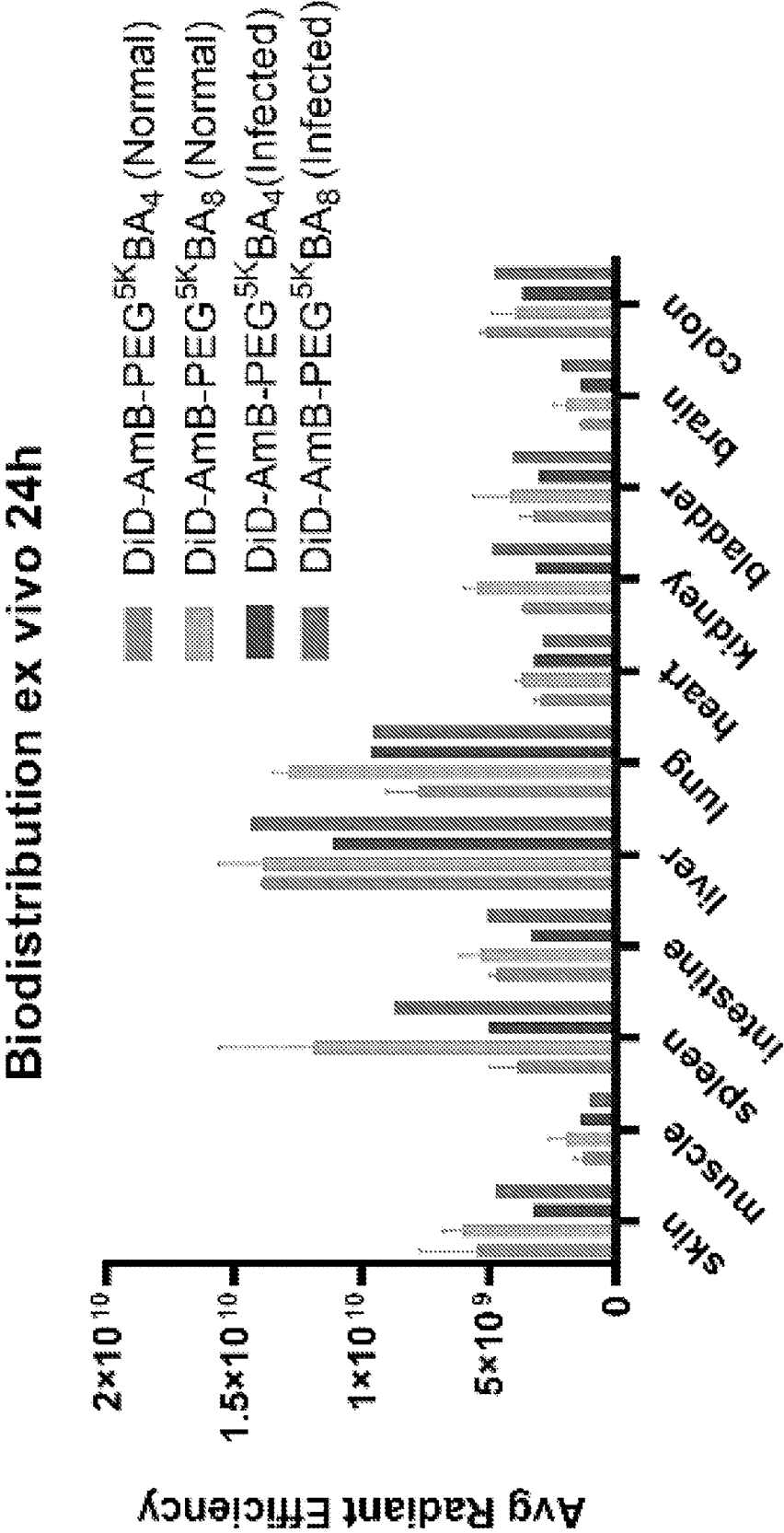


FIG. 5A

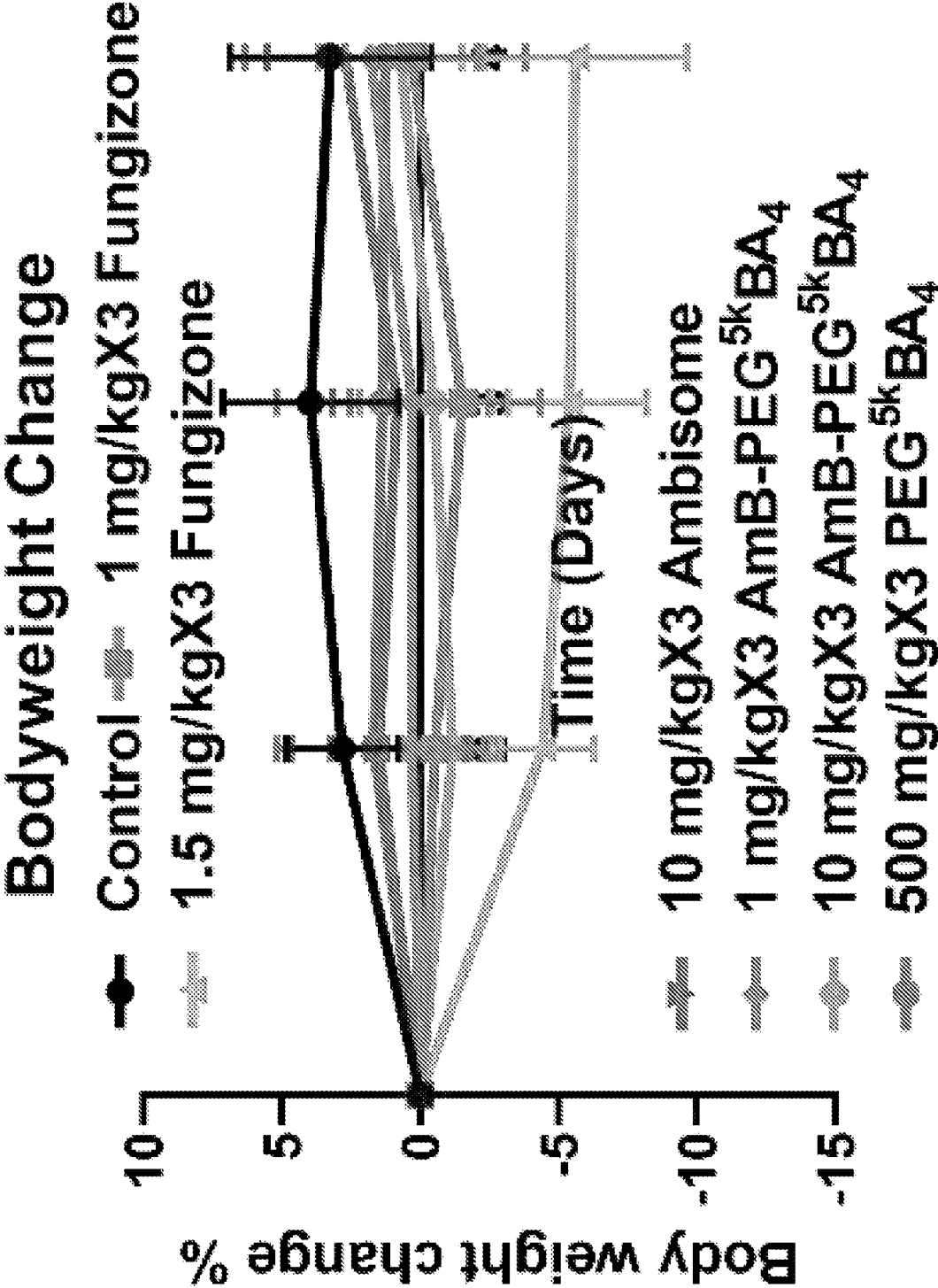


FIG. 5C

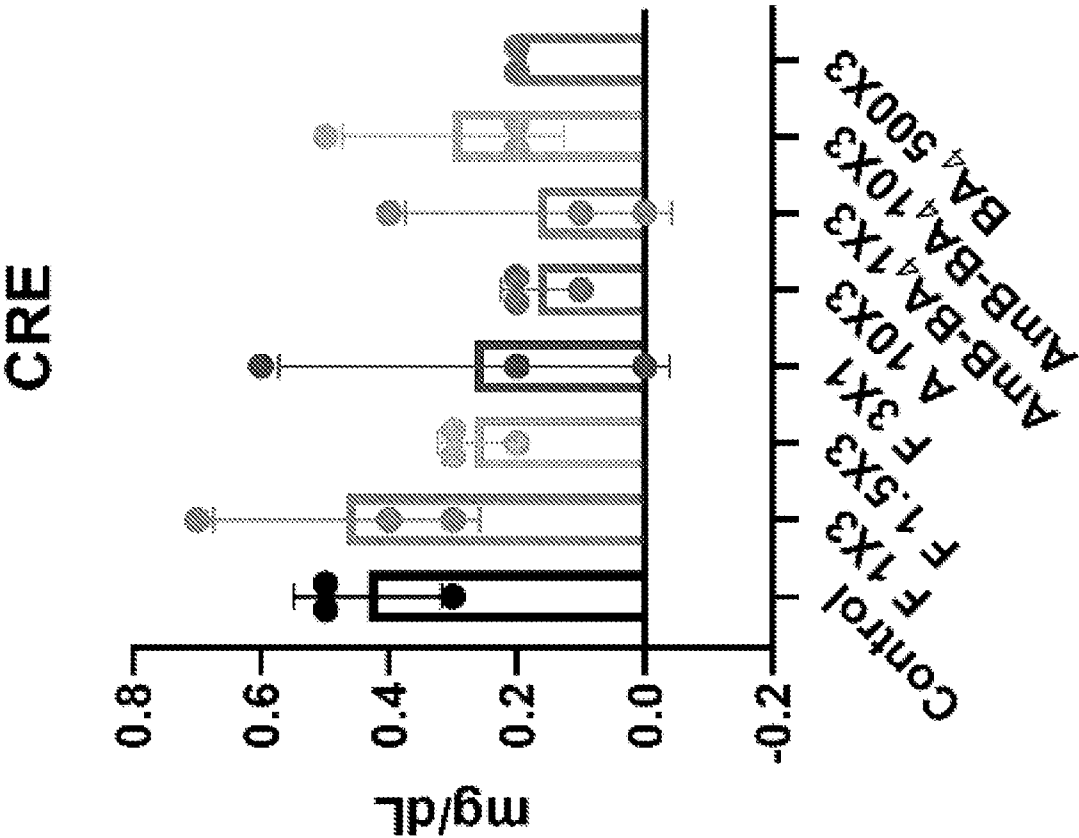


FIG. 5B

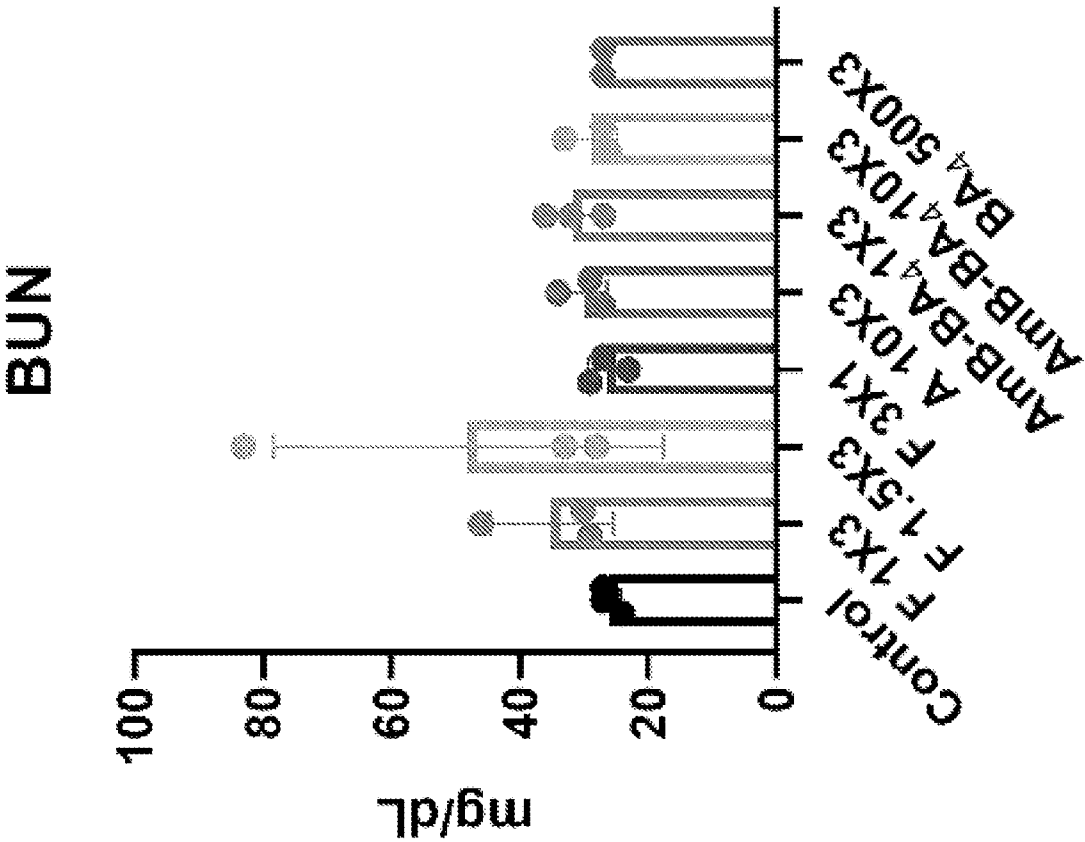


FIG. 5E

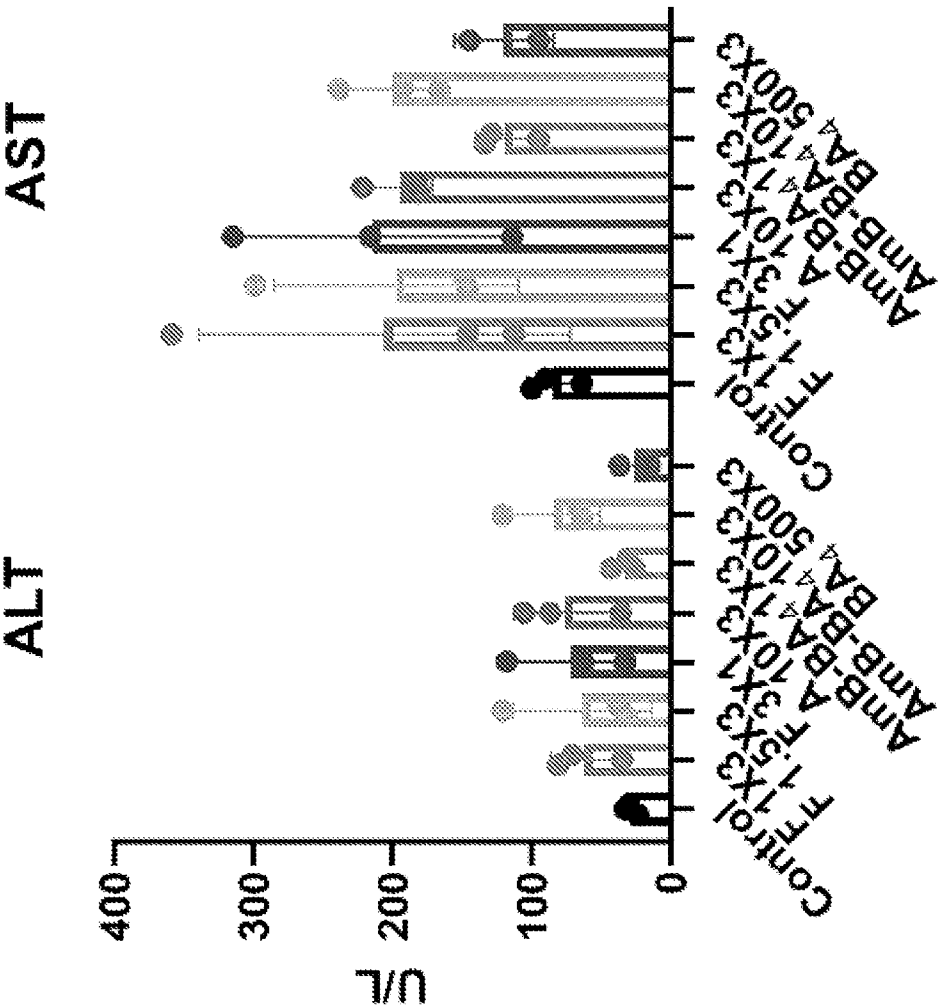


FIG. 5D

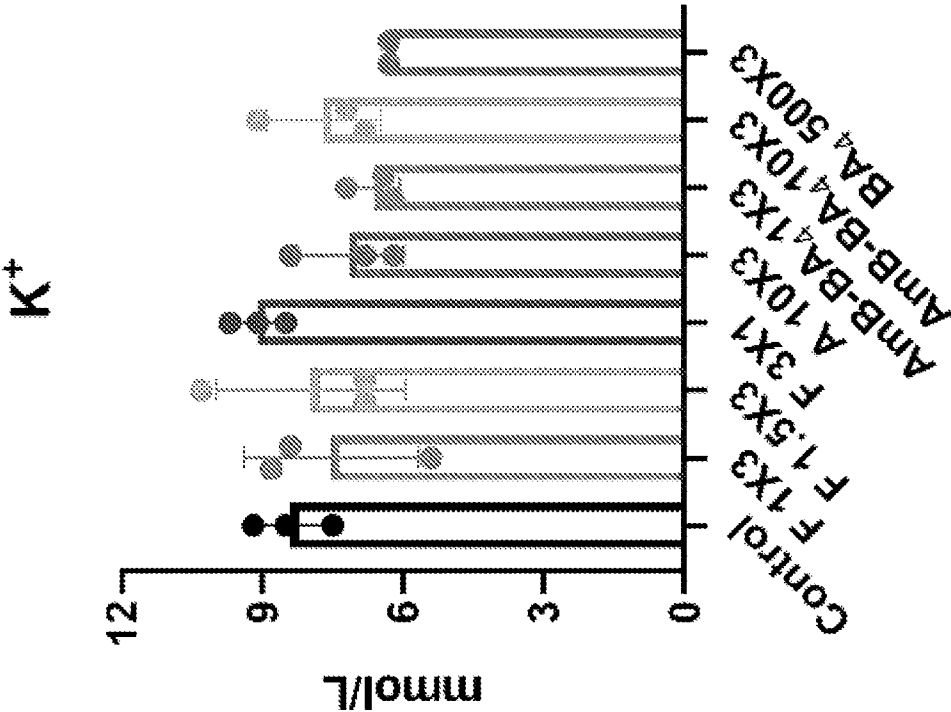


FIG. 5F

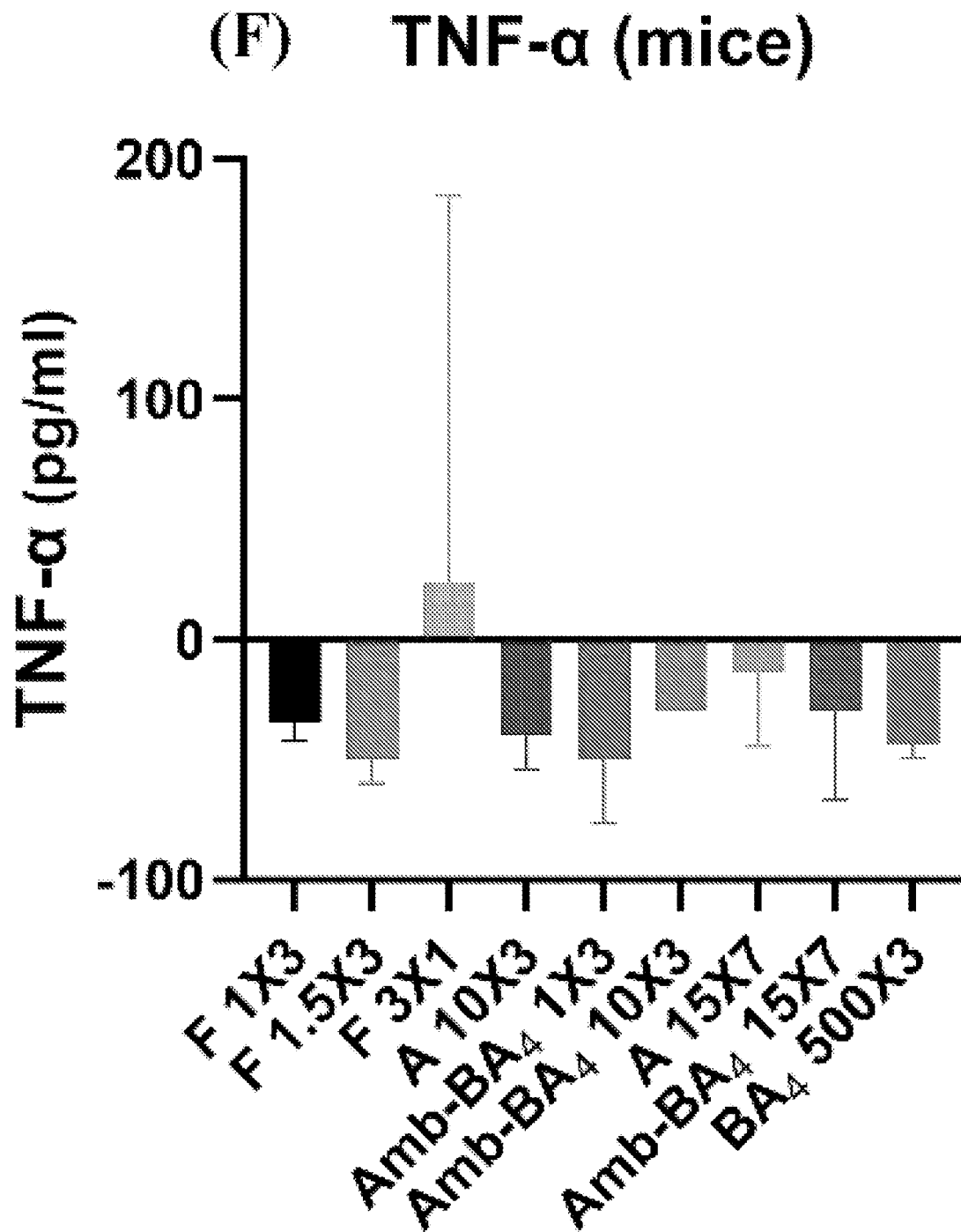


FIG. 5G

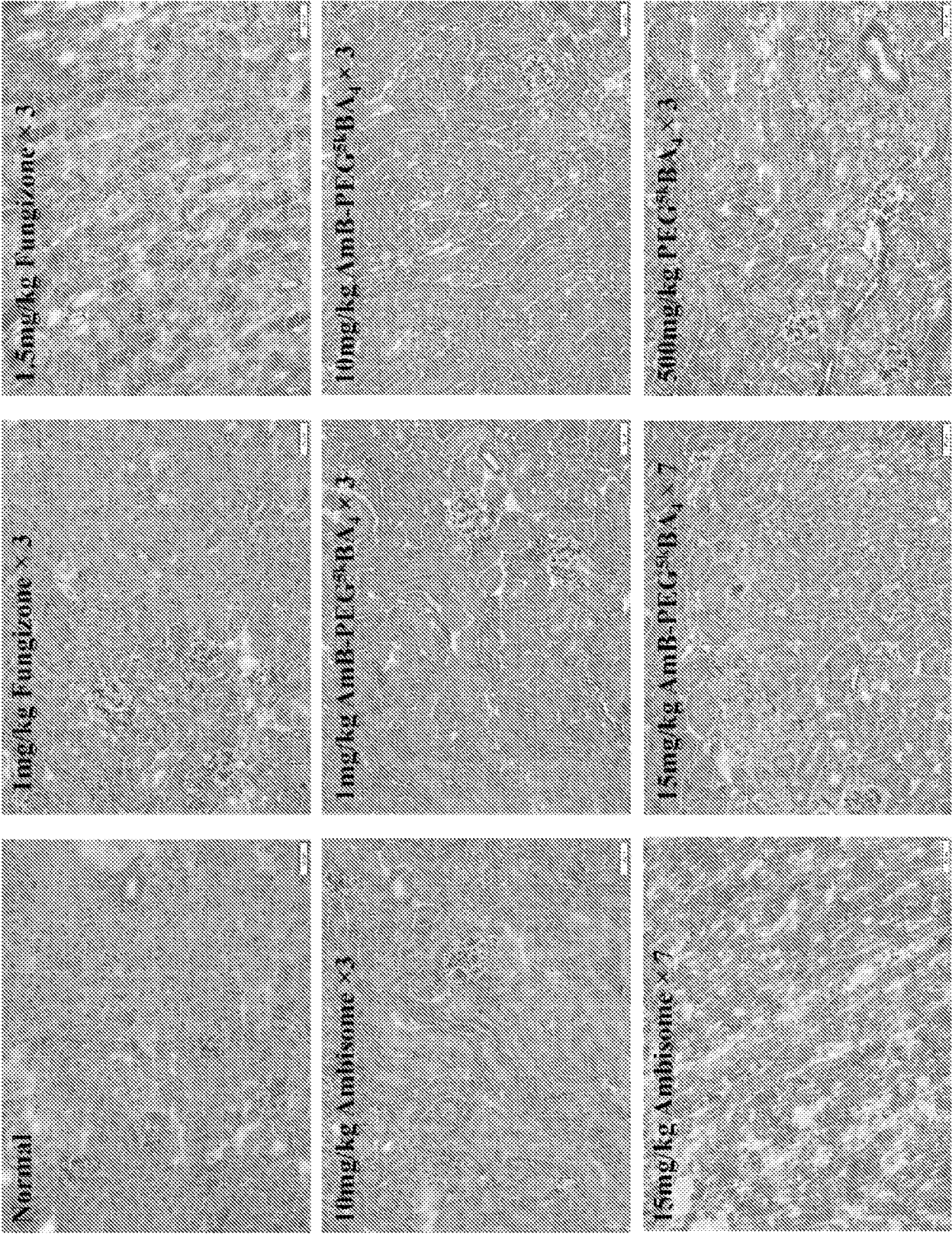


FIG. 6A

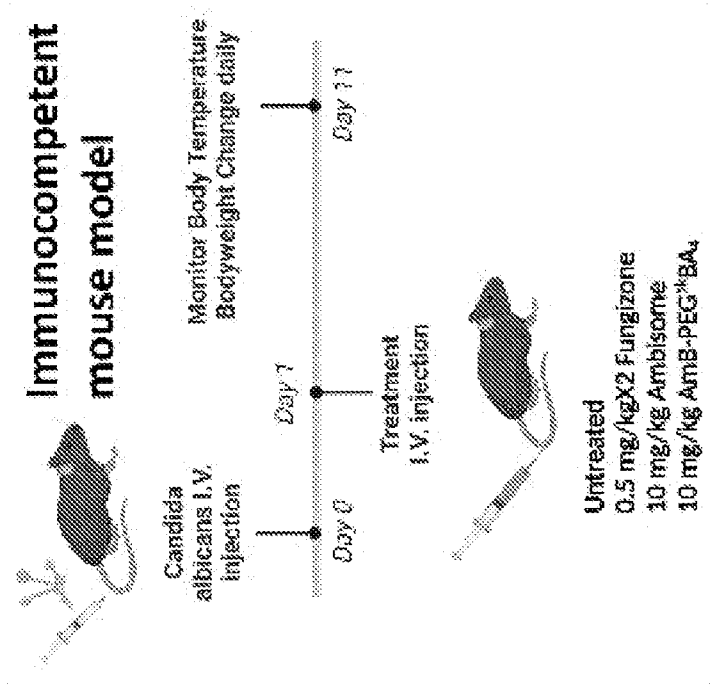


FIG. 6B

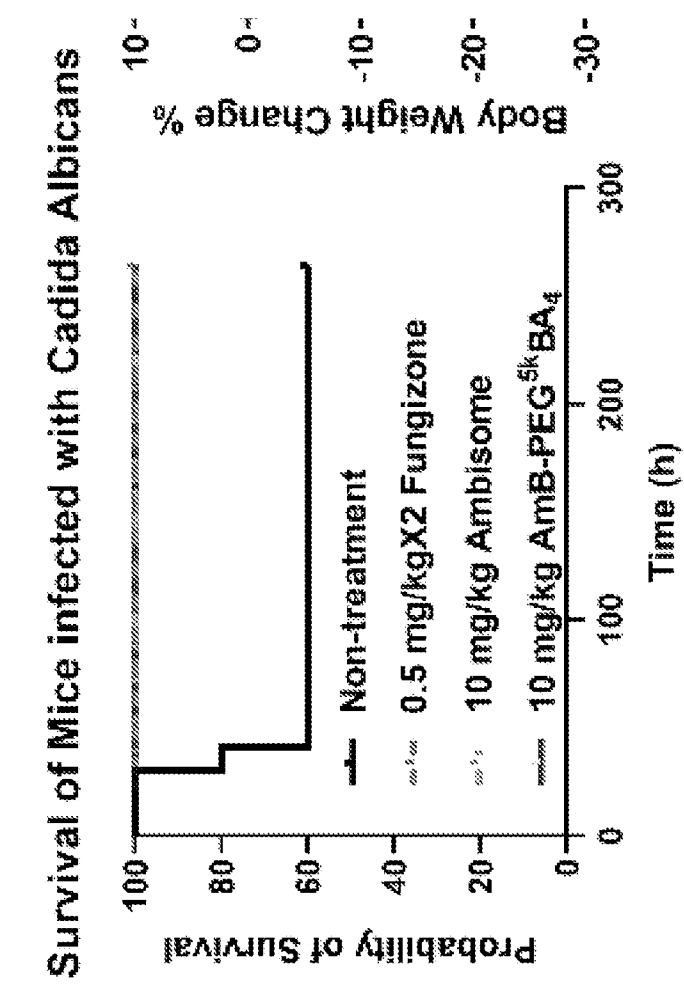


FIG. 6D

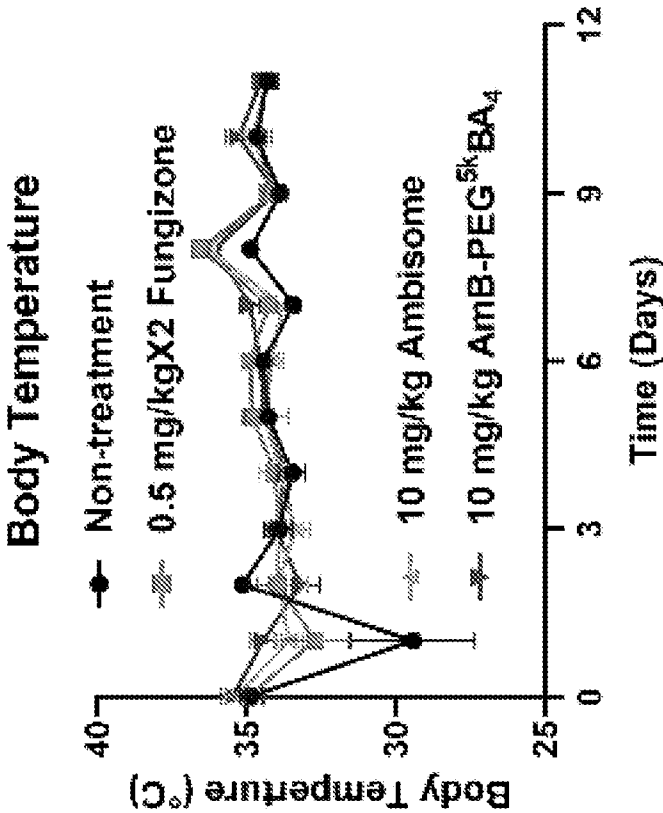


FIG. 6C

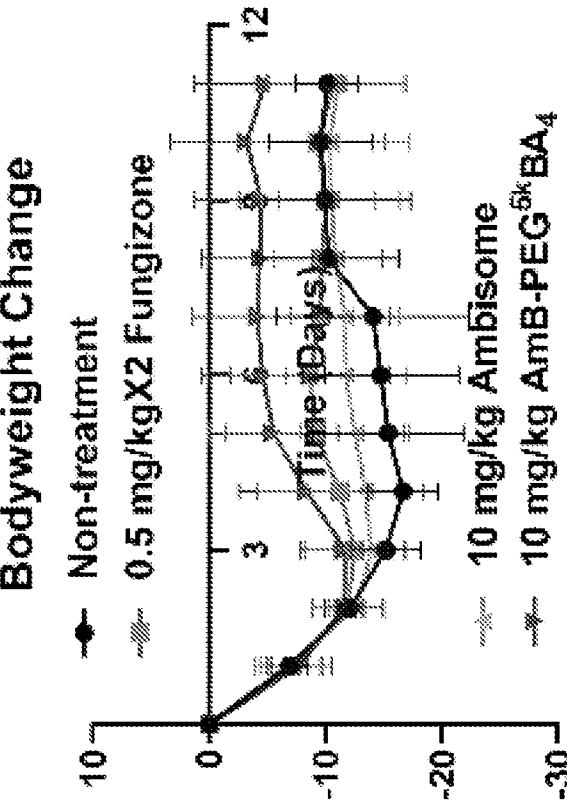


FIG. 6E

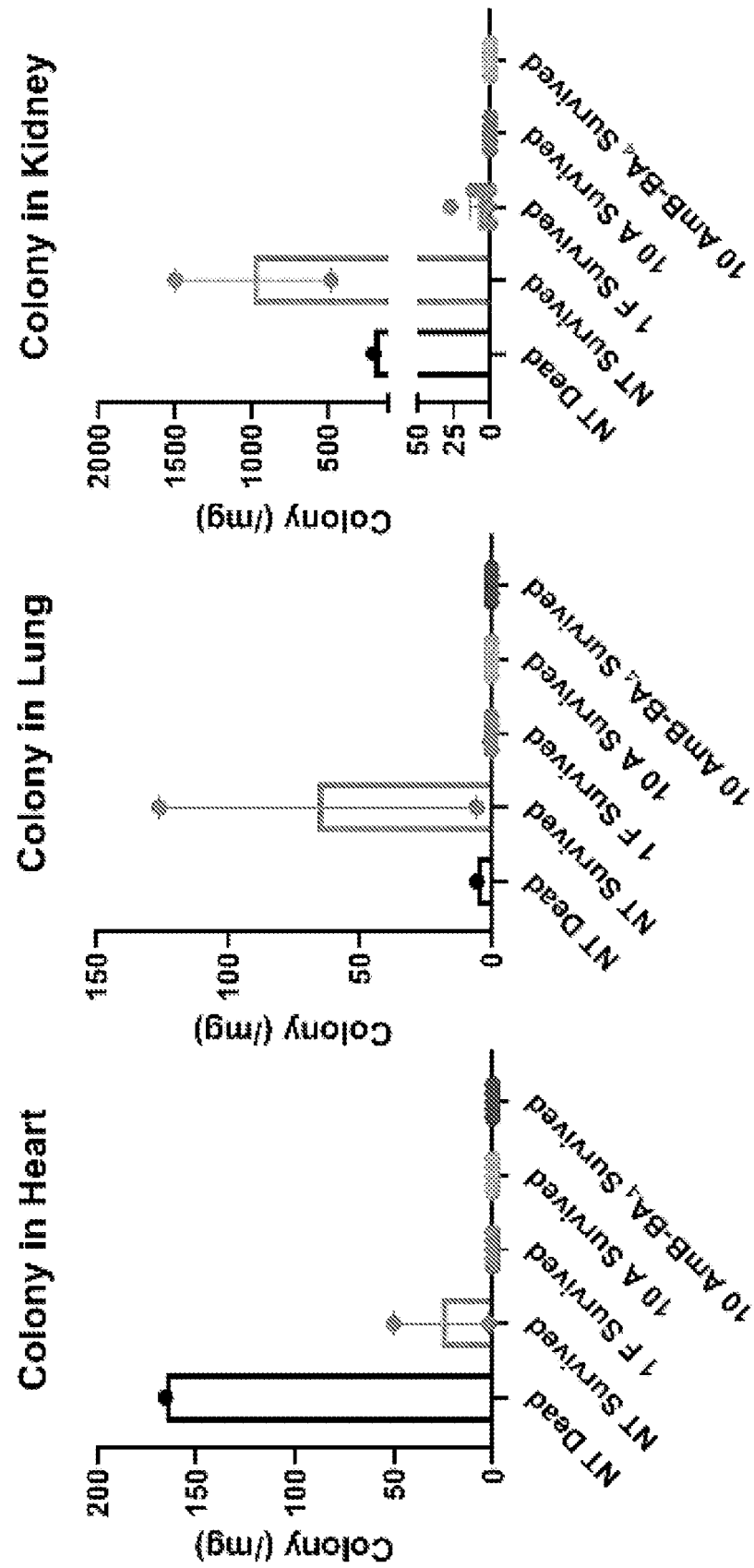


FIG. 6F

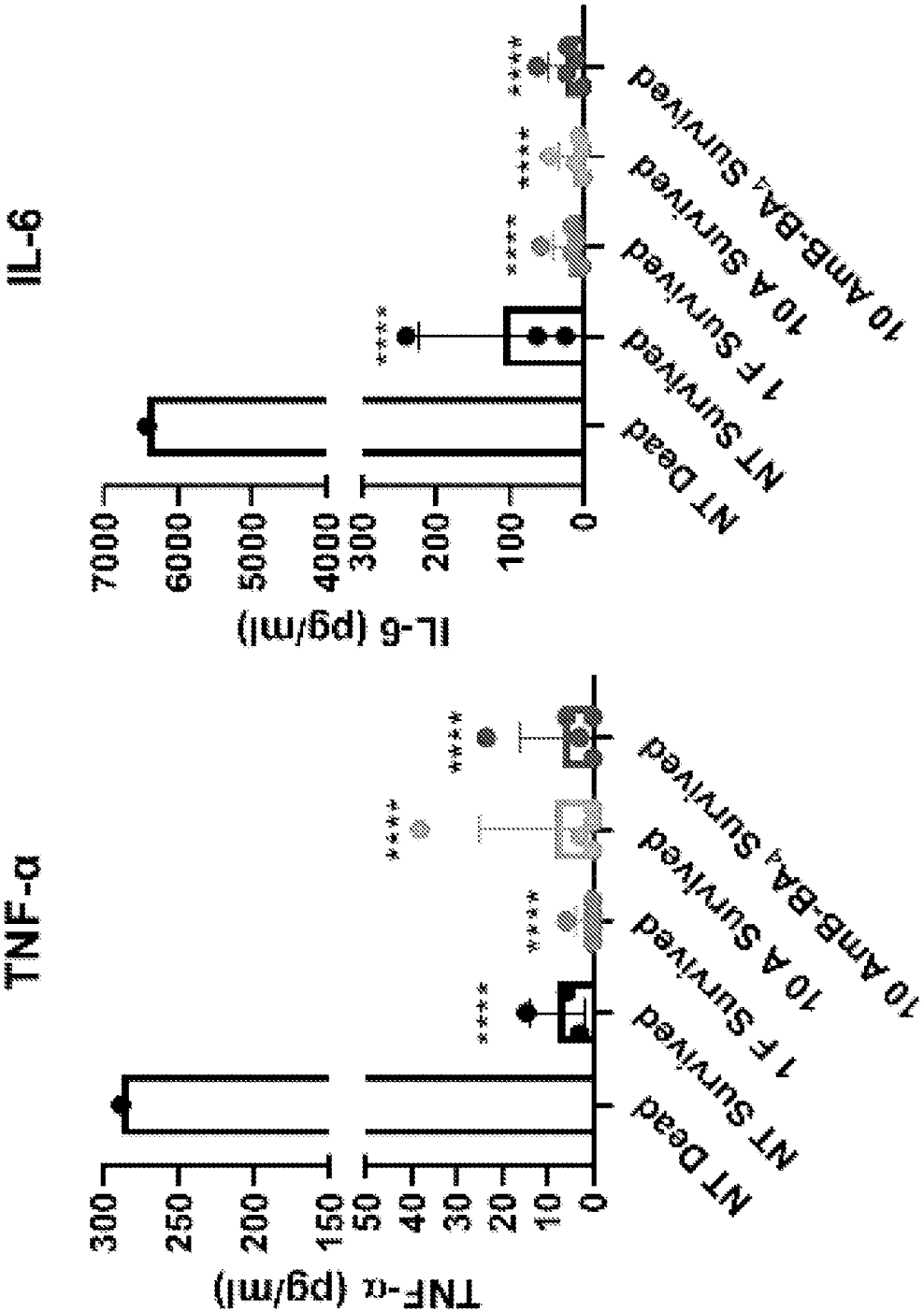


FIG. 6G

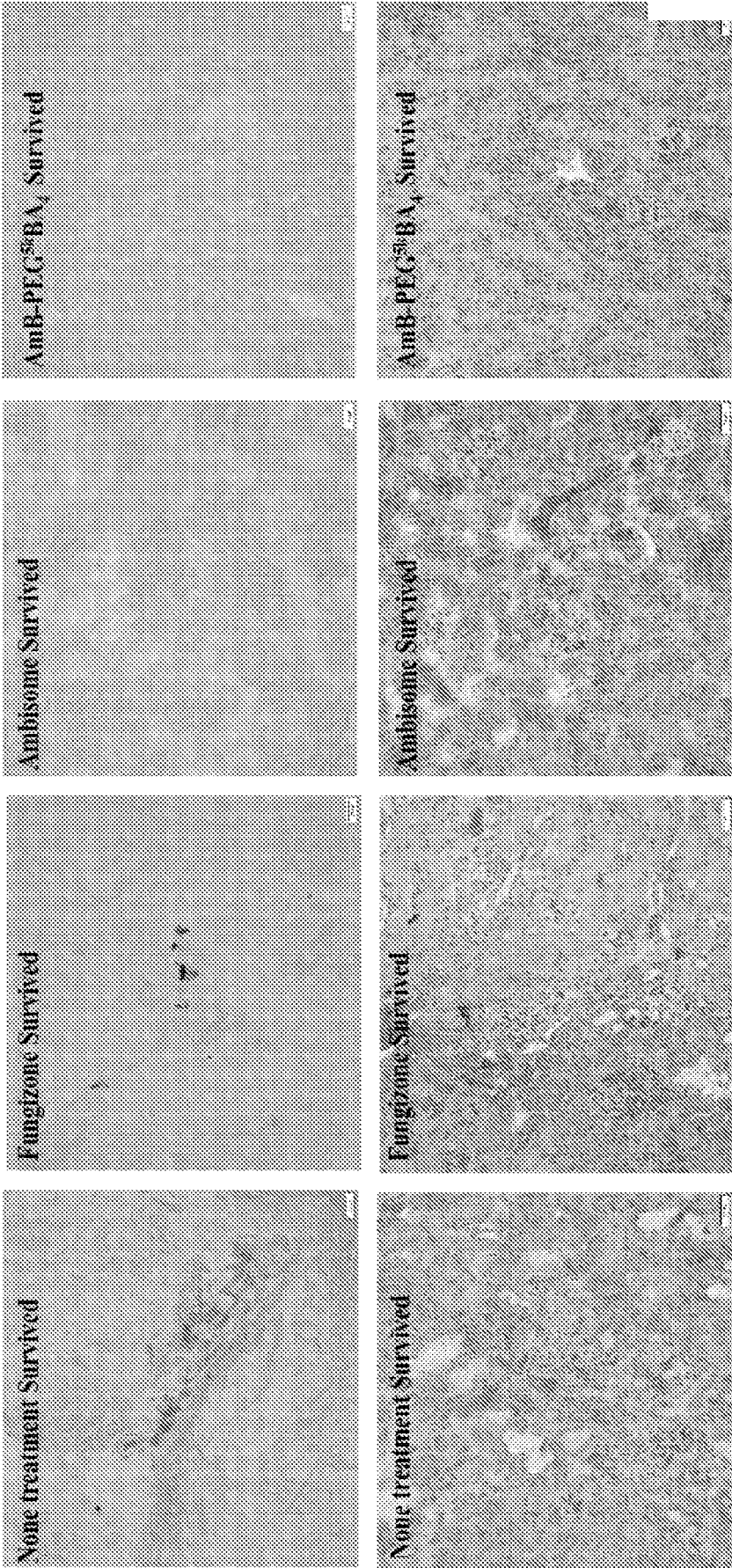


FIG. 6H

FIG. 7A

Immunocompromised mouse model

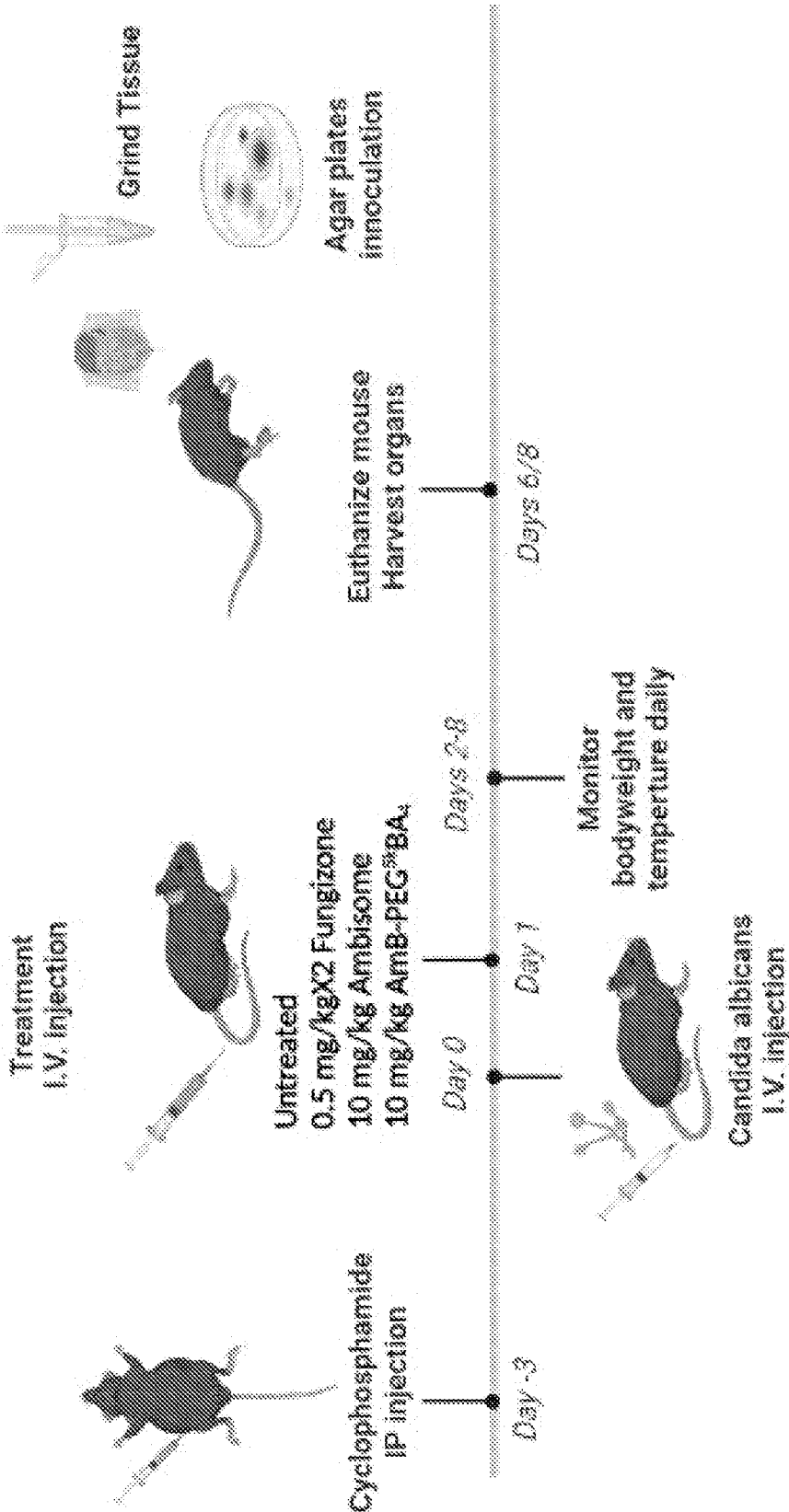


FIG. 7B

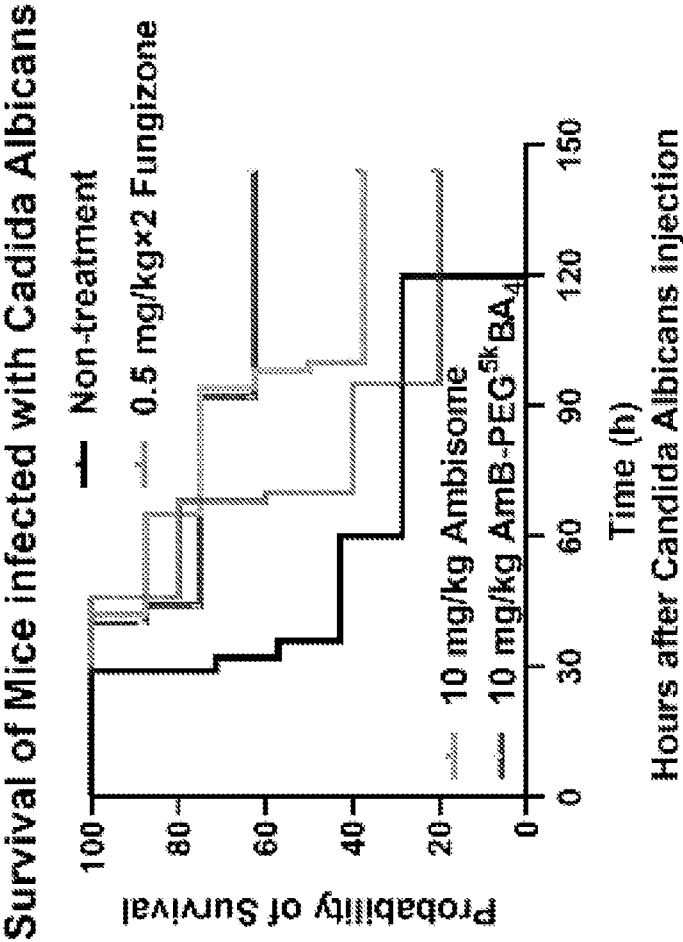


FIG. 7C

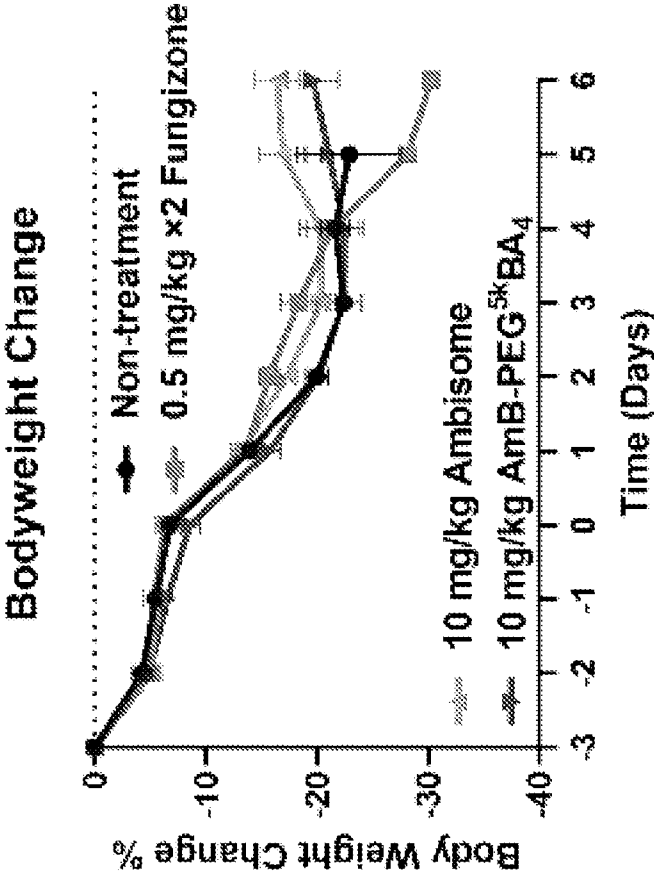


FIG. 7D

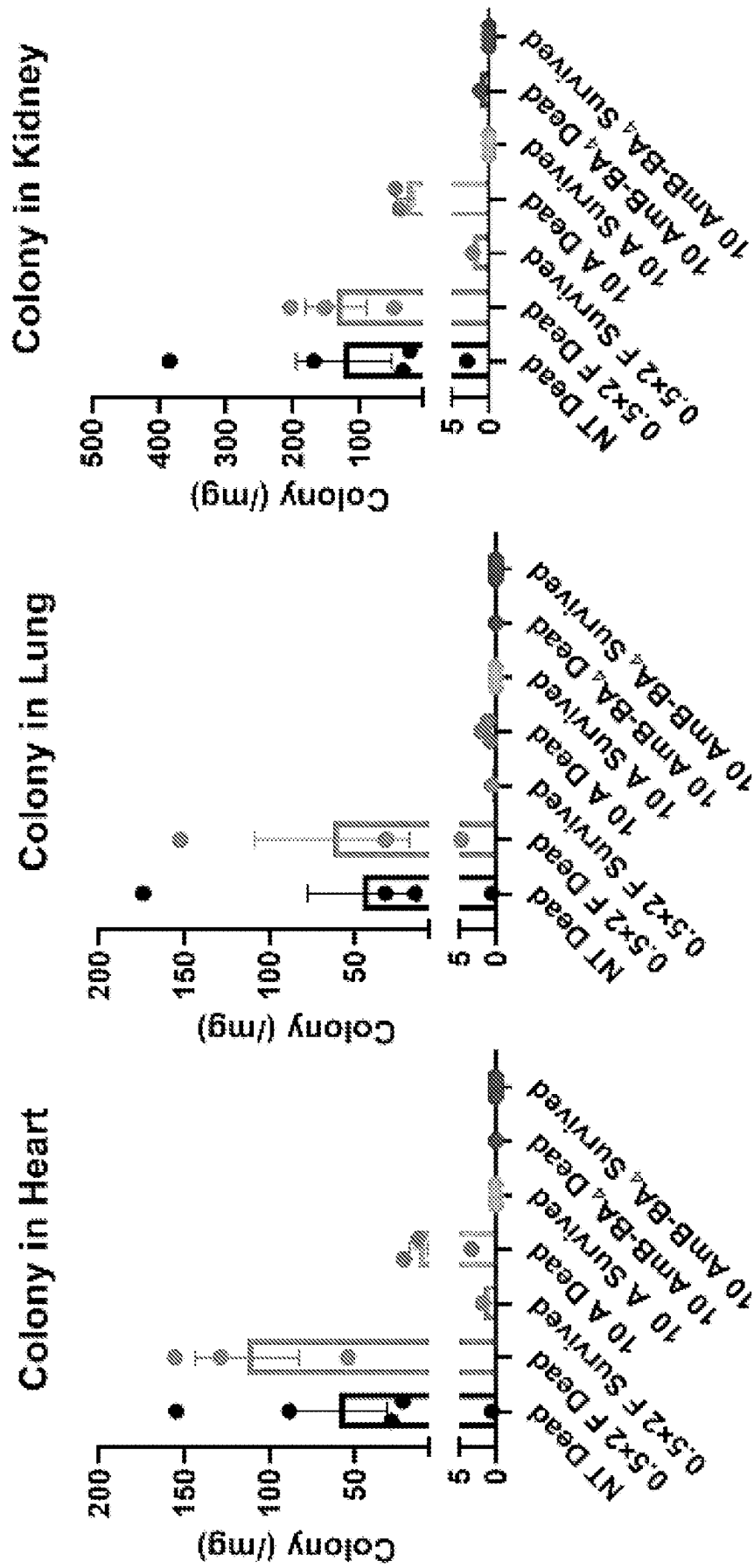


FIG. 7E

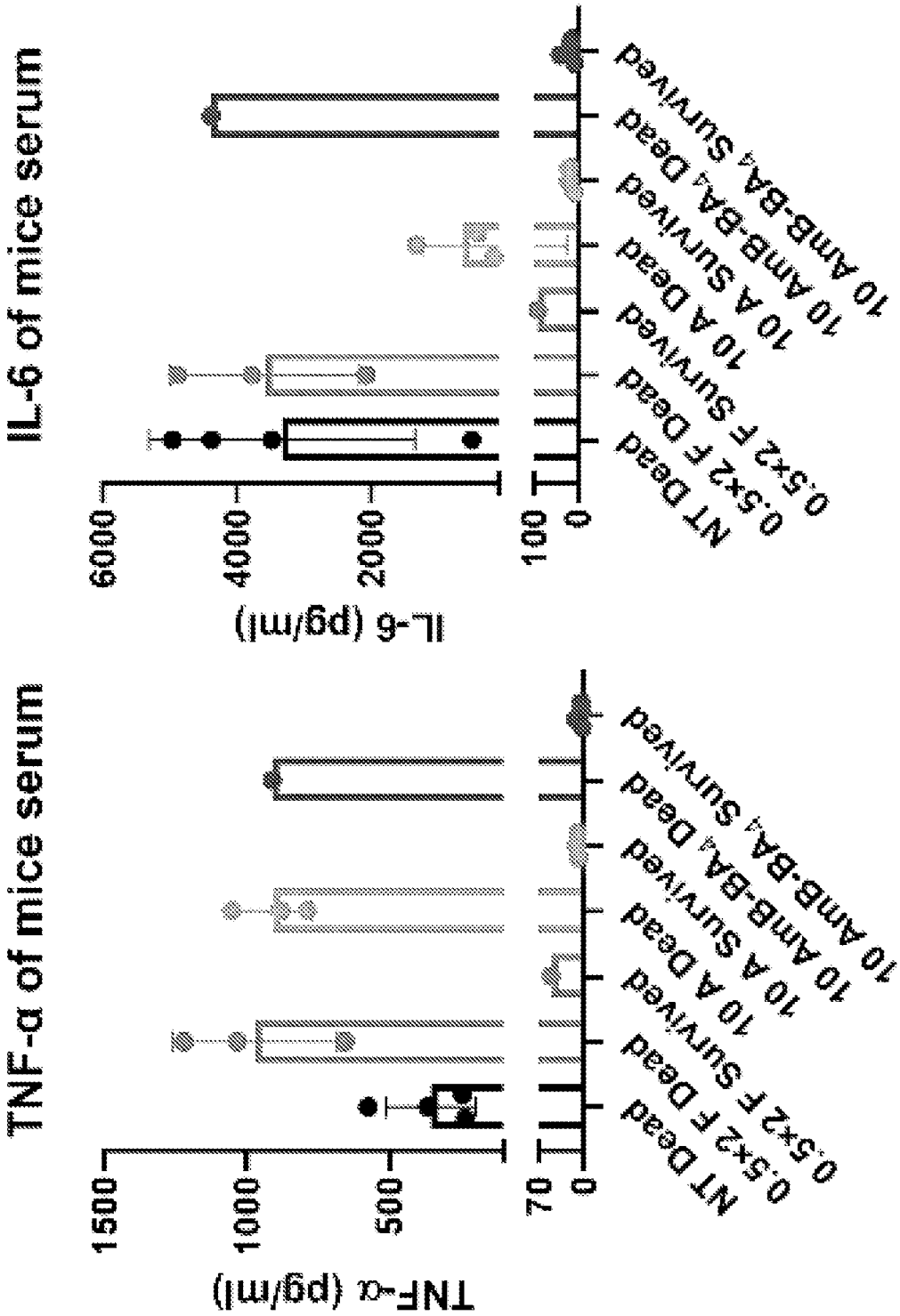


FIG. 7F

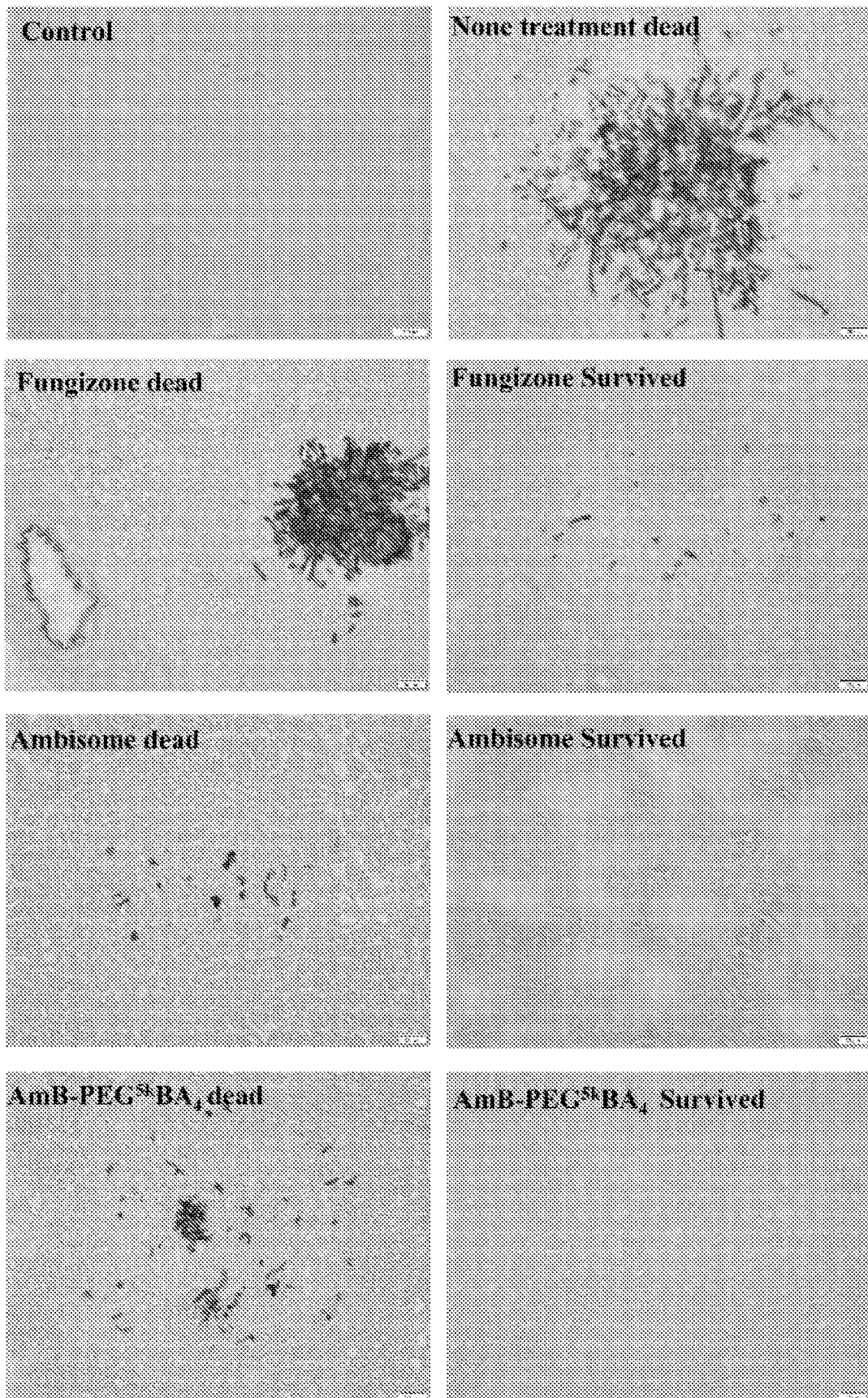


FIG. 7G

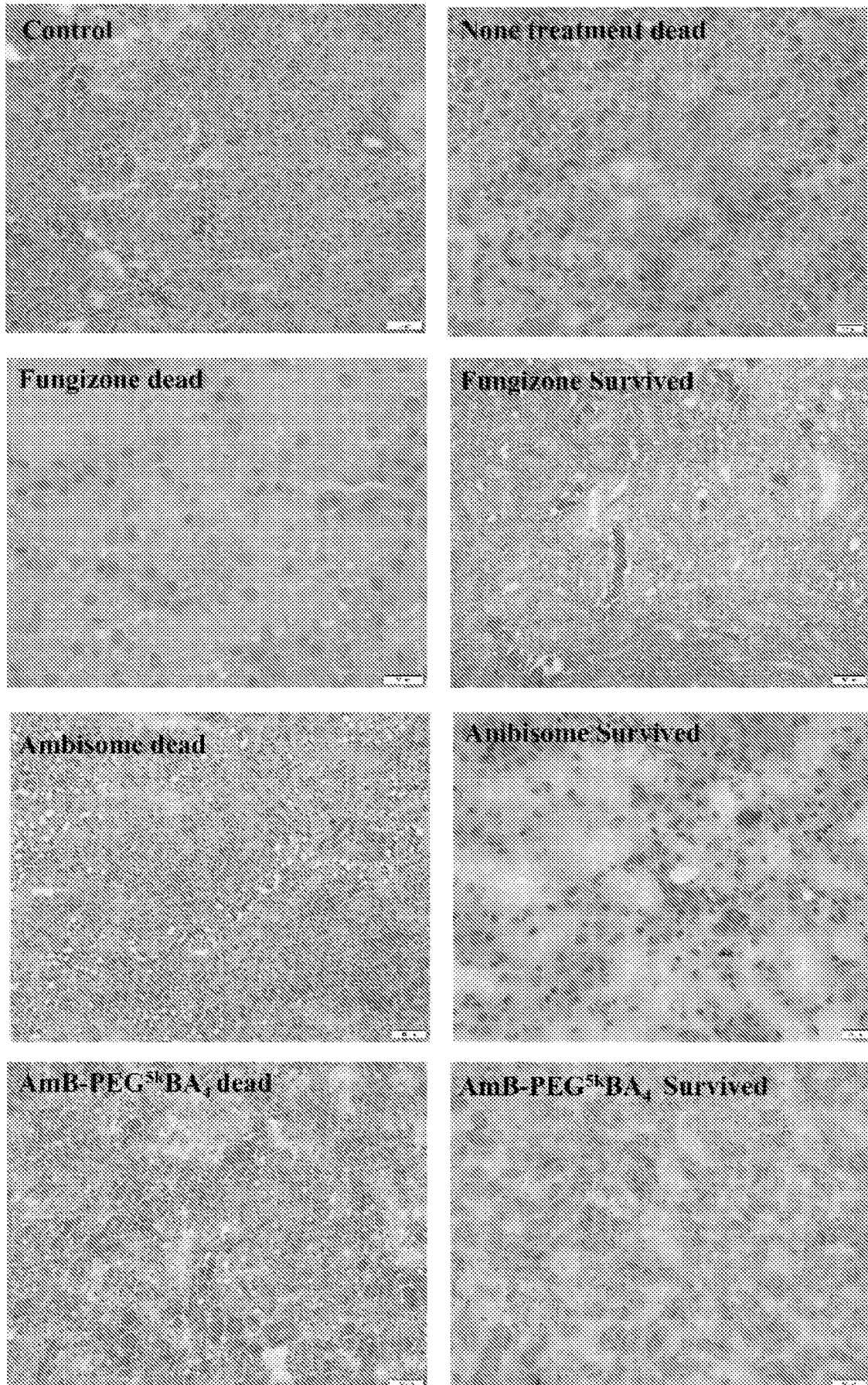


FIG. 8A

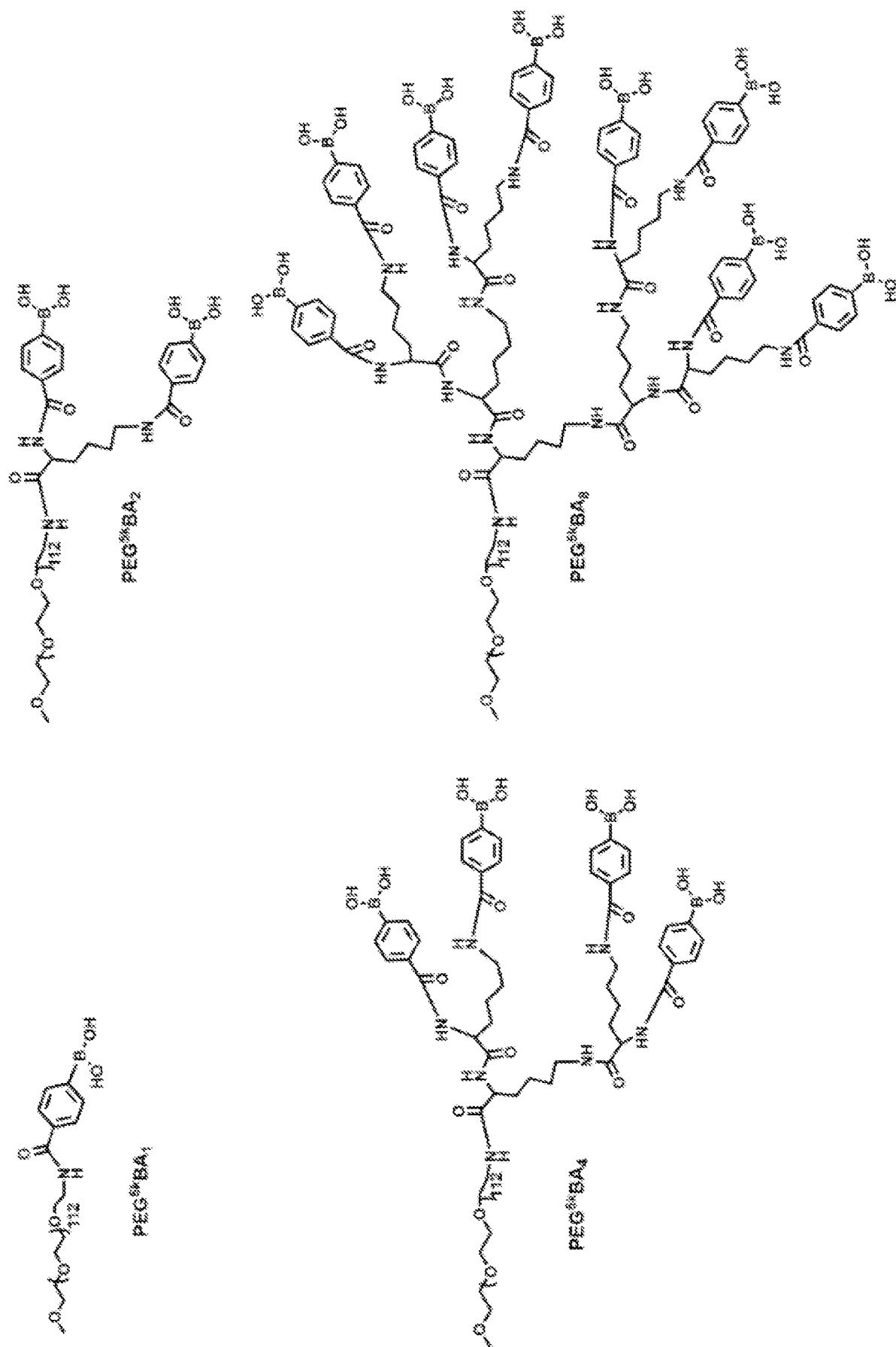


FIG. 8B

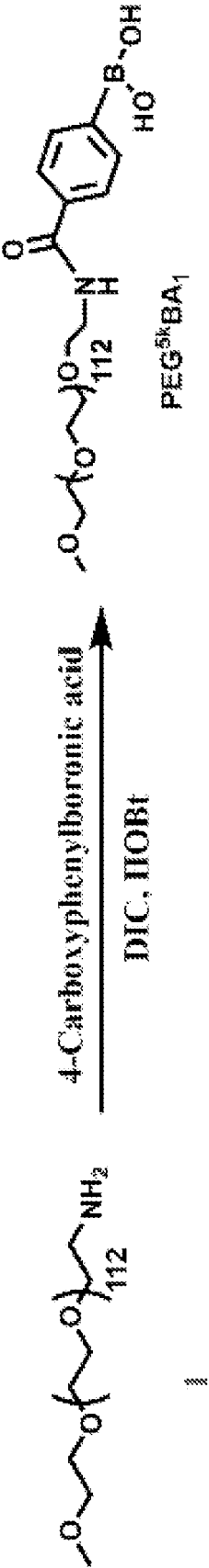


FIG. 8C

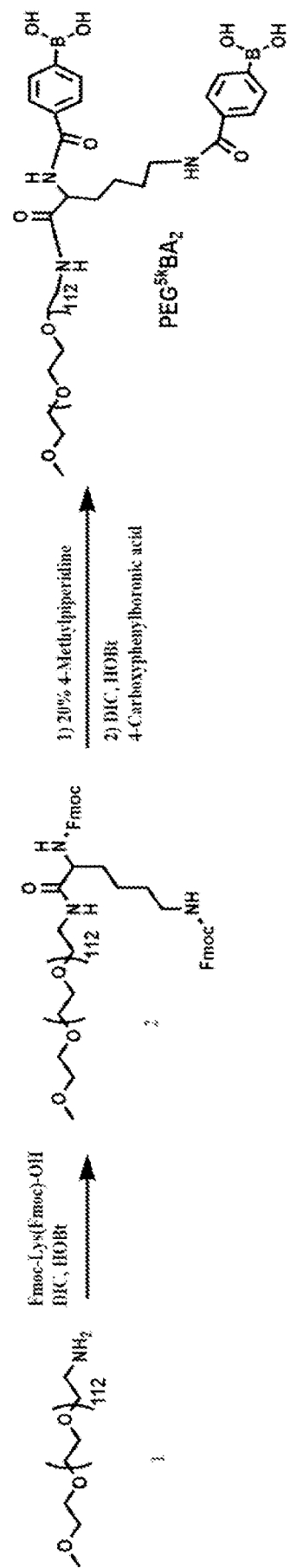


FIG. 8D

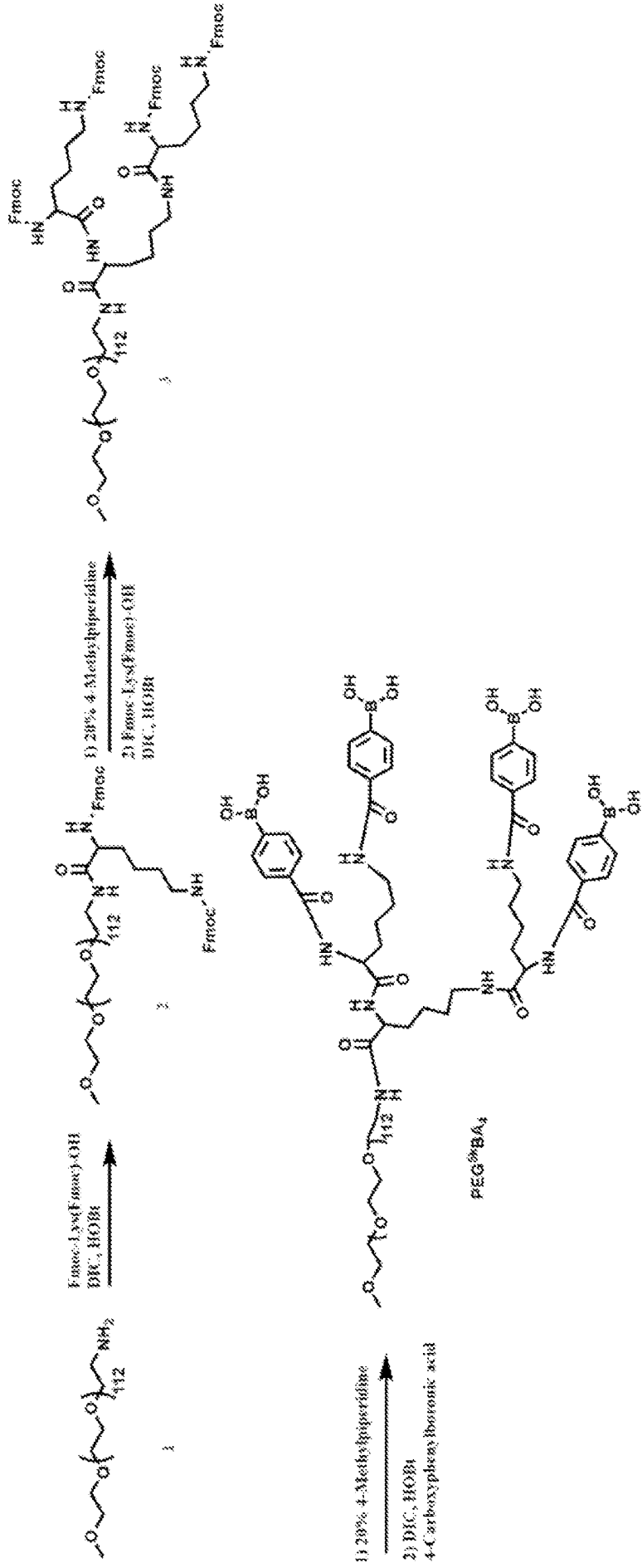


FIG. 8E

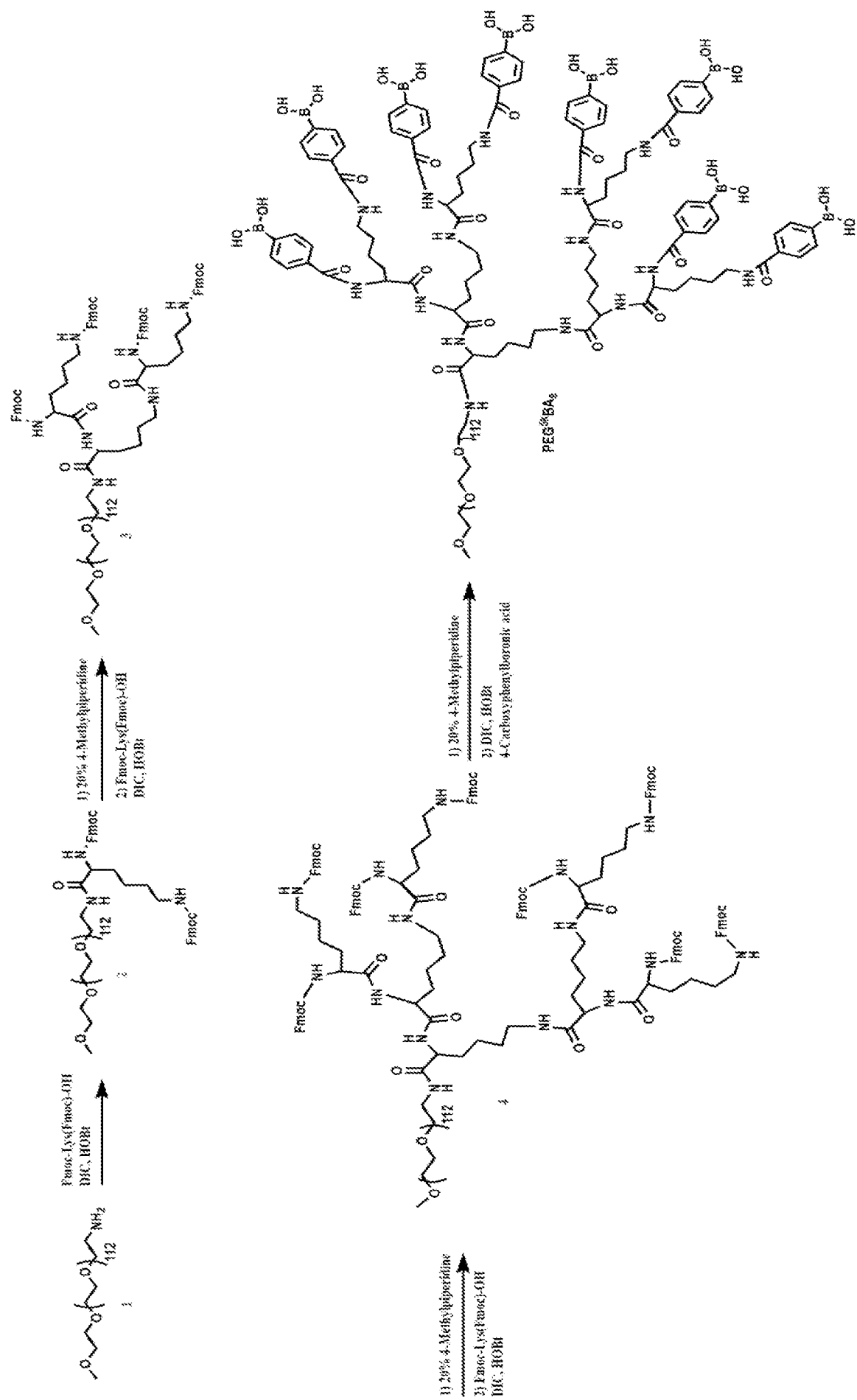


FIG. 9

1,2- and 1,3- Cis-diol containing drugs and
bioactive molecules

- AmB
- NMN, NAD/NADH
- Tetrodotoxin
- Aminoglycoside antibiotics, e.g. Tobramycin

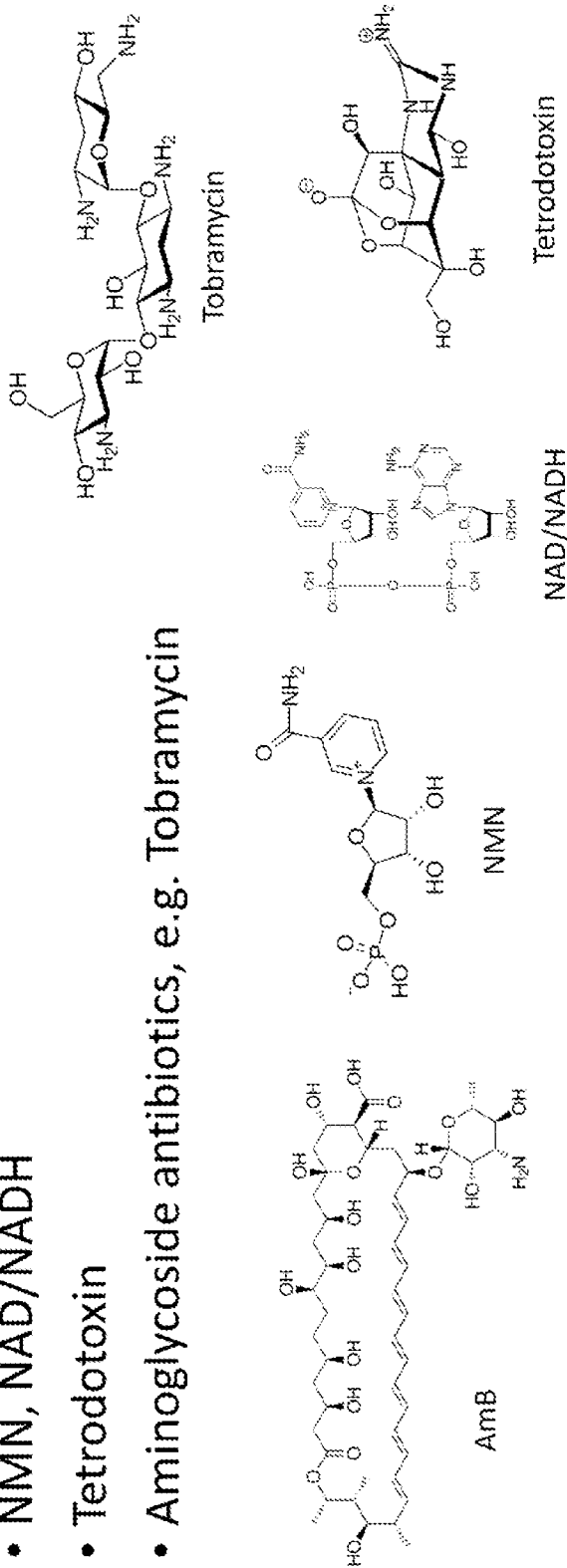


FIG. 10

Antibiotics: Tetracycline and other α -hydroxy ketone derivatives

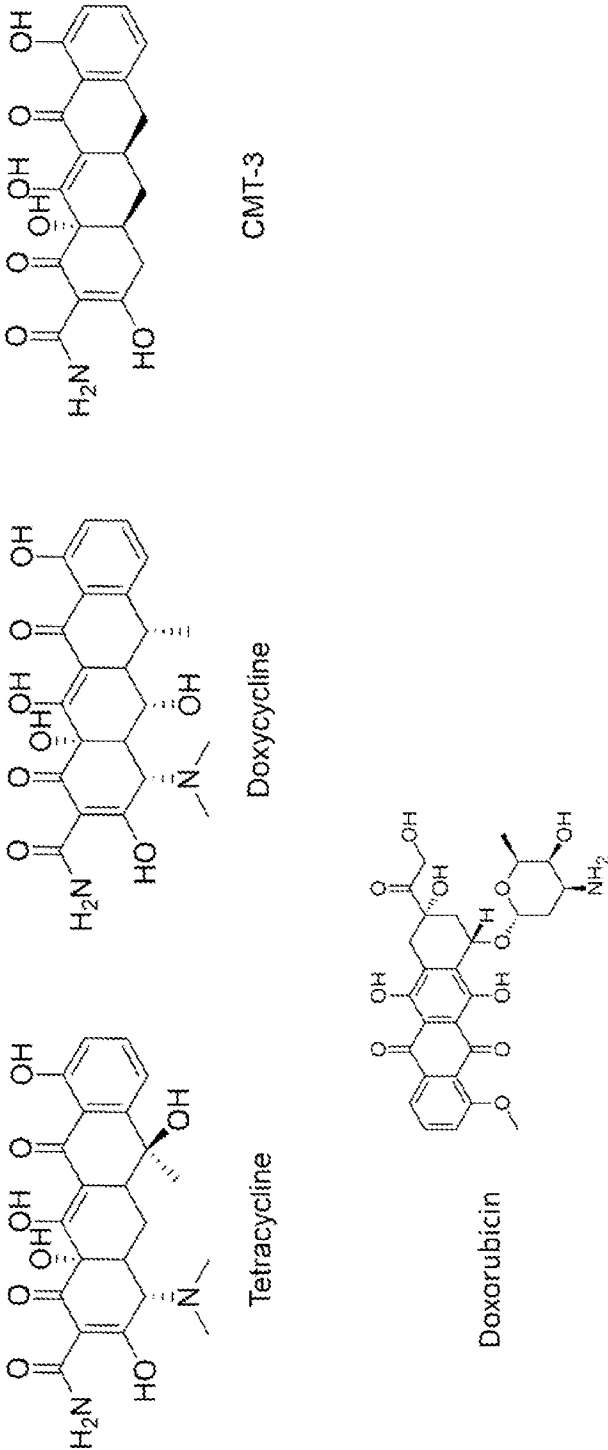
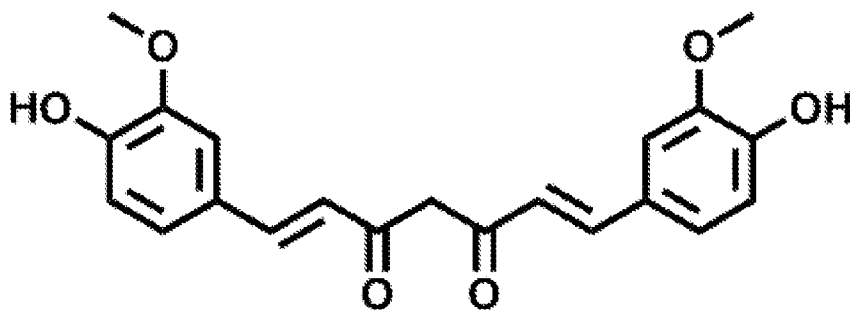
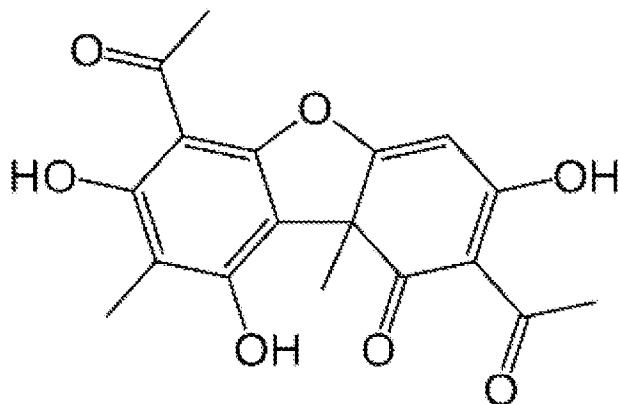


FIG. 11

dione structure



Curcumin



Usnic acid

FIG. 12

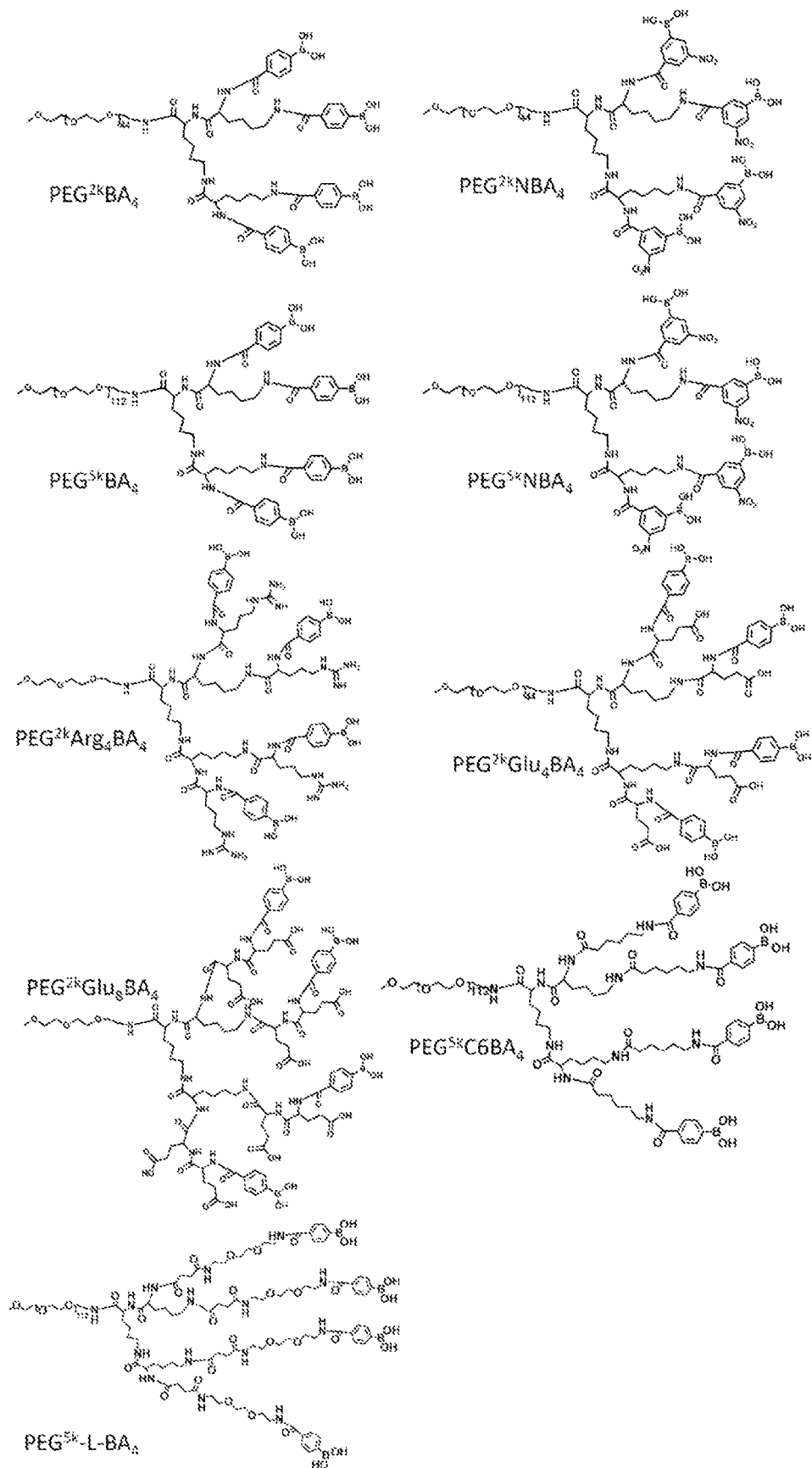


FIG. 14A

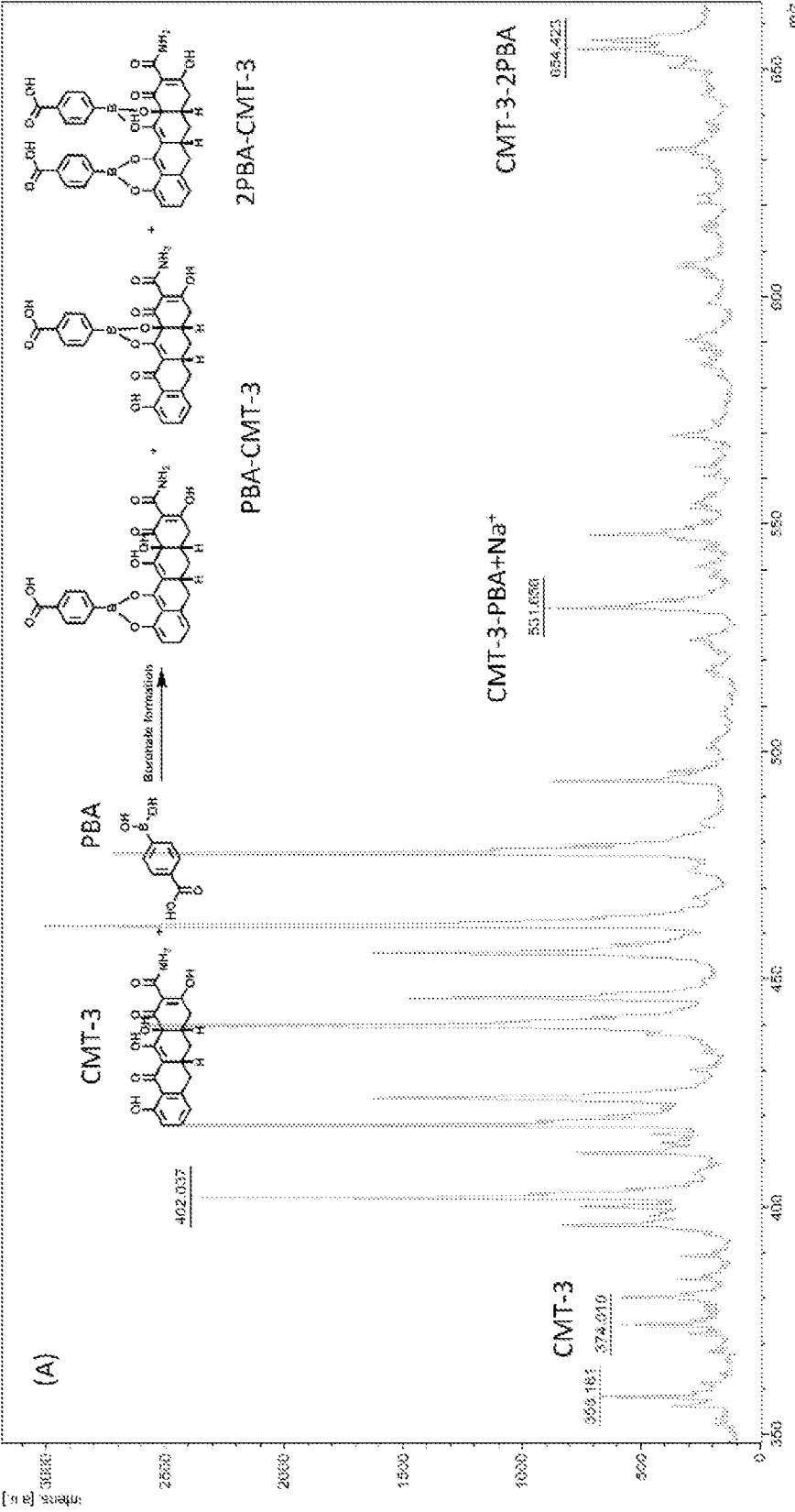


FIG. 14B

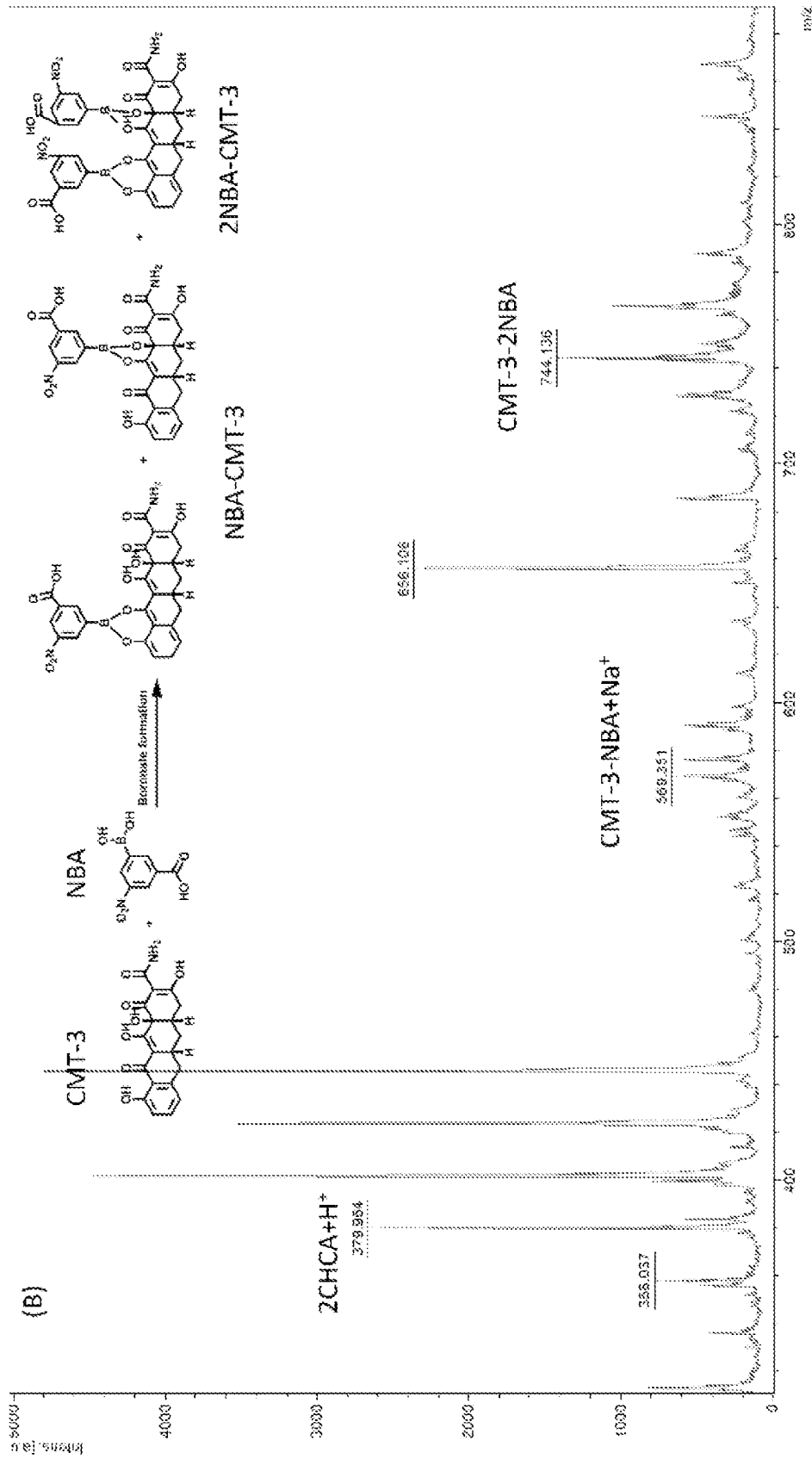


FIG. 15

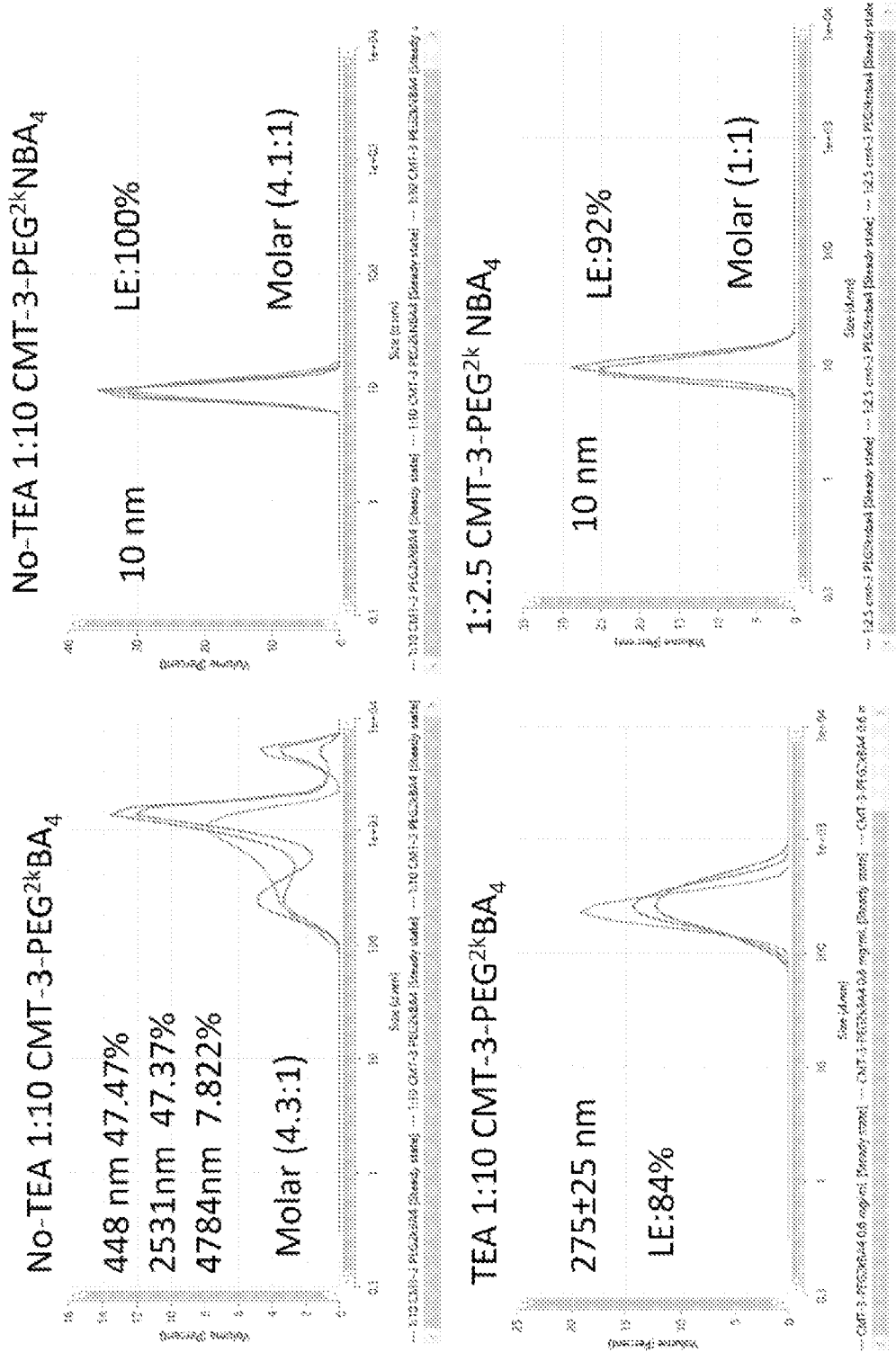


FIG. 16

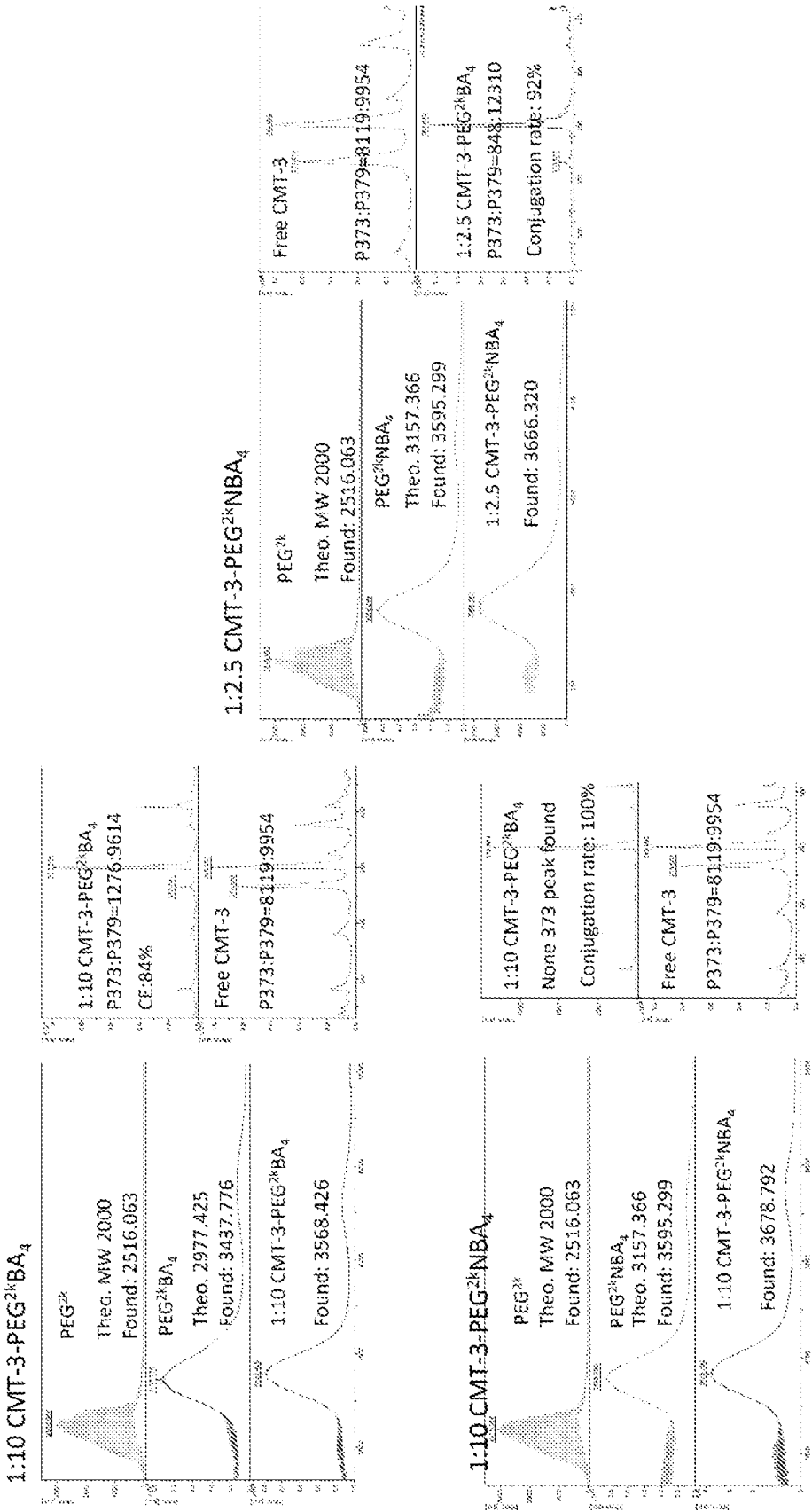


FIG. 17A

Formulations	360 nm	370 nm	390 nm
Free CMT-3	1.017	0.957	0.51
1:10 CMT-3- PEG ^{2k} BA ₄	0.709	0.874	1.067
1:10 CMT-3- PEG ^{7k} NBA ₄	0.937	1.027	1.002
1:10 CMT-3 PEG ^{5k} CA ₄ VE ₄	0.75	0.657	0.356

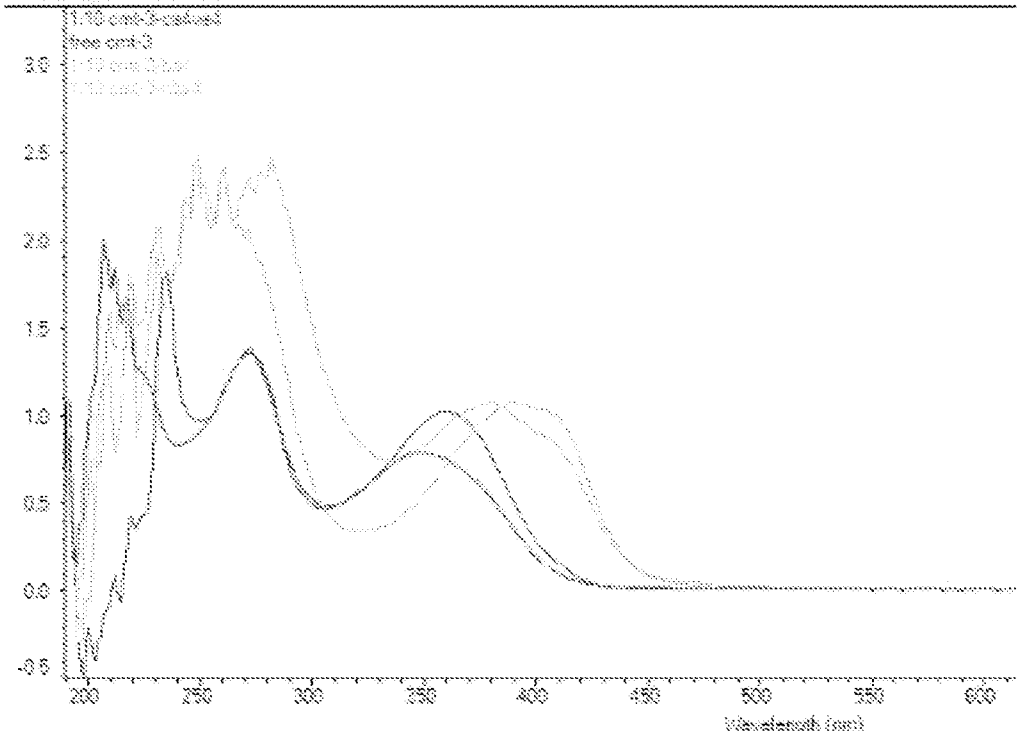


FIG. 17B

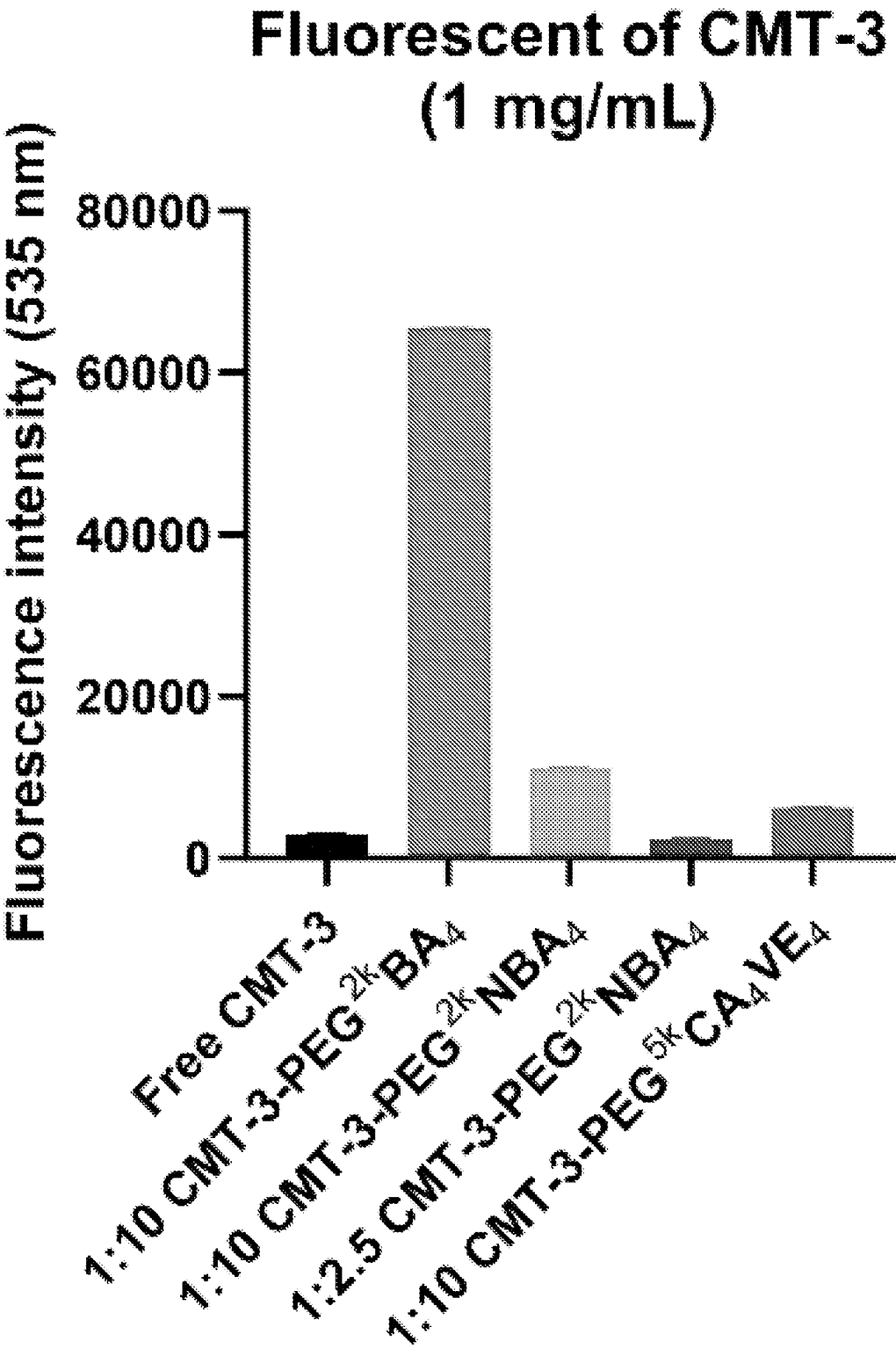


FIG. 17C

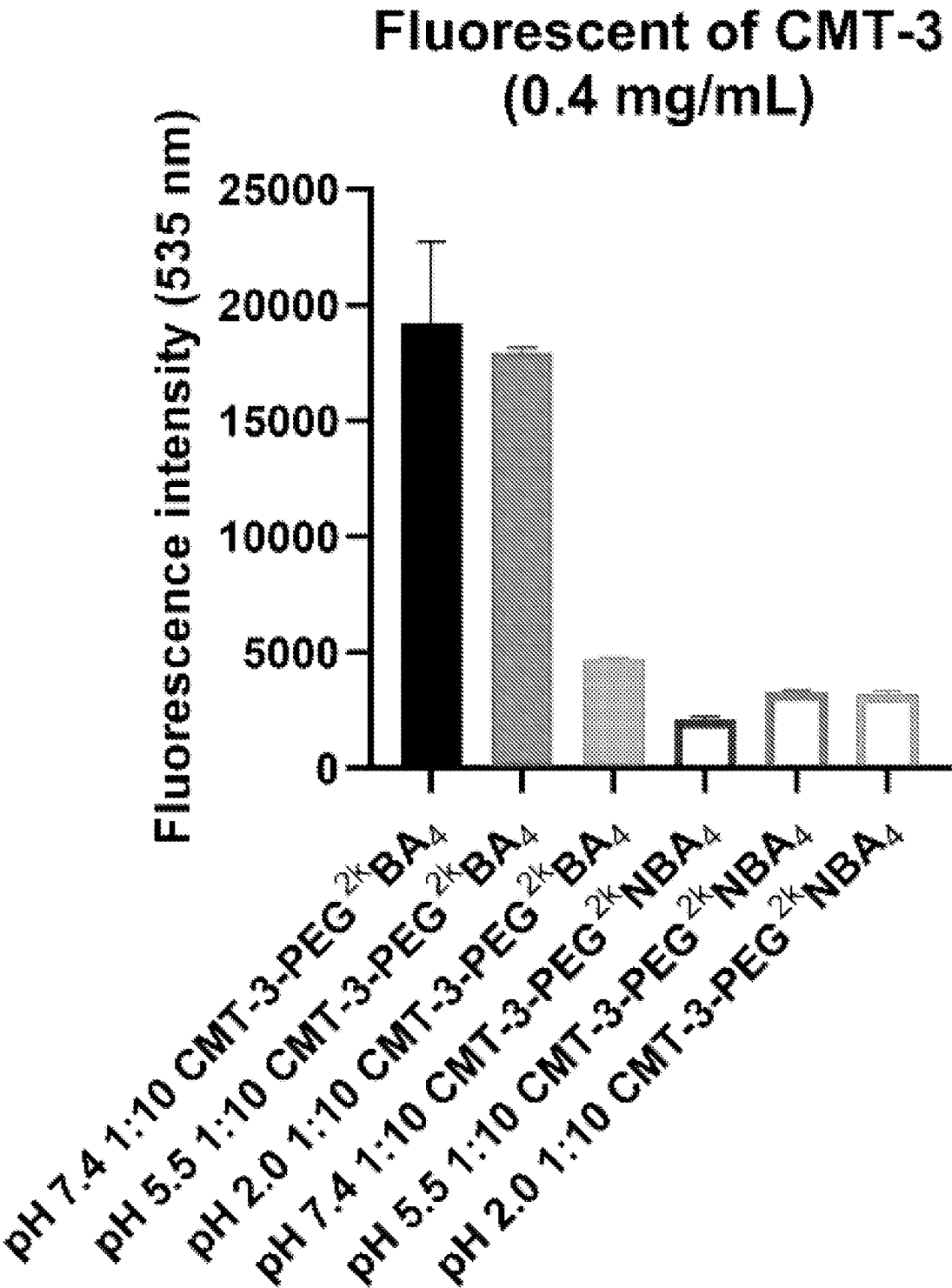


FIG. 18

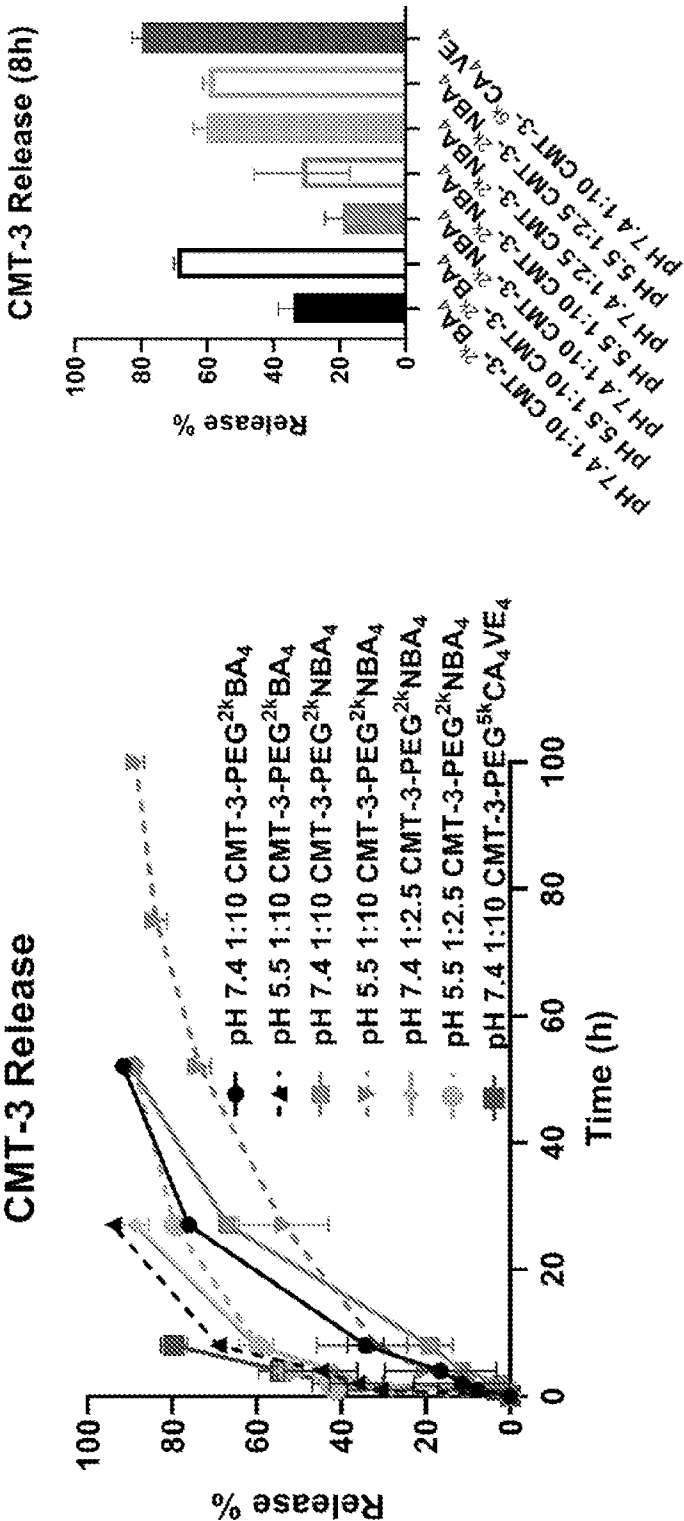


FIG. 19A

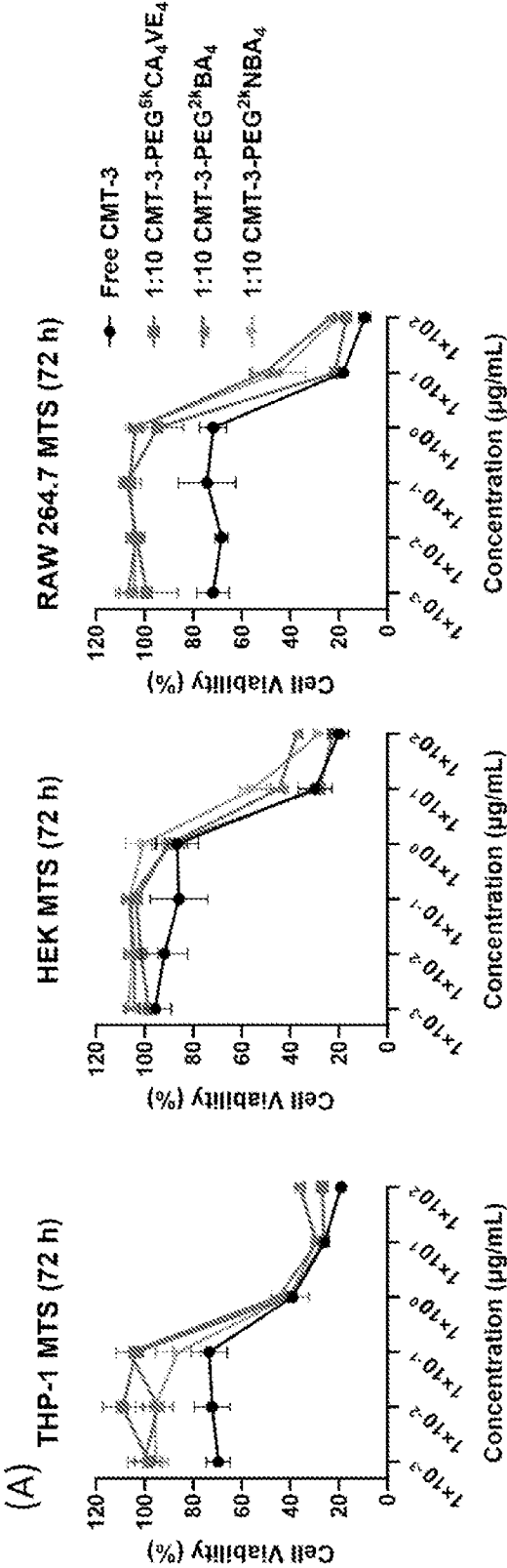


FIG. 19B

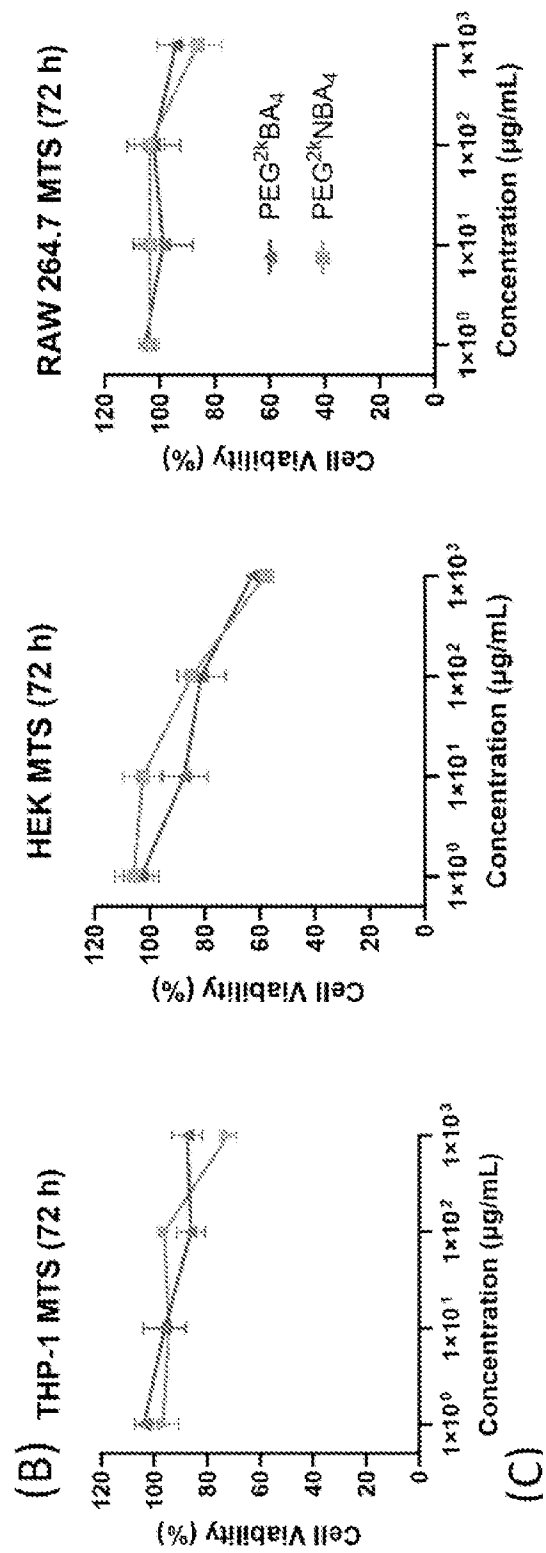


FIG. 19C

IC50 (µg/mL)	Free CMT-3	CMT-3- PEG ^{5k} CA ₄ VE ₄	CMT-3-PEG ^{2k} BA ₄	CMT-3-PEG ^{2k} NBA ₄
THP-1	0.37	1.61	3.19	2.07
HEK 293	5.32	5.41	18.02	20.30
RAW 264.7	0.80	4.84	14.99	10.81

FIG. 20A

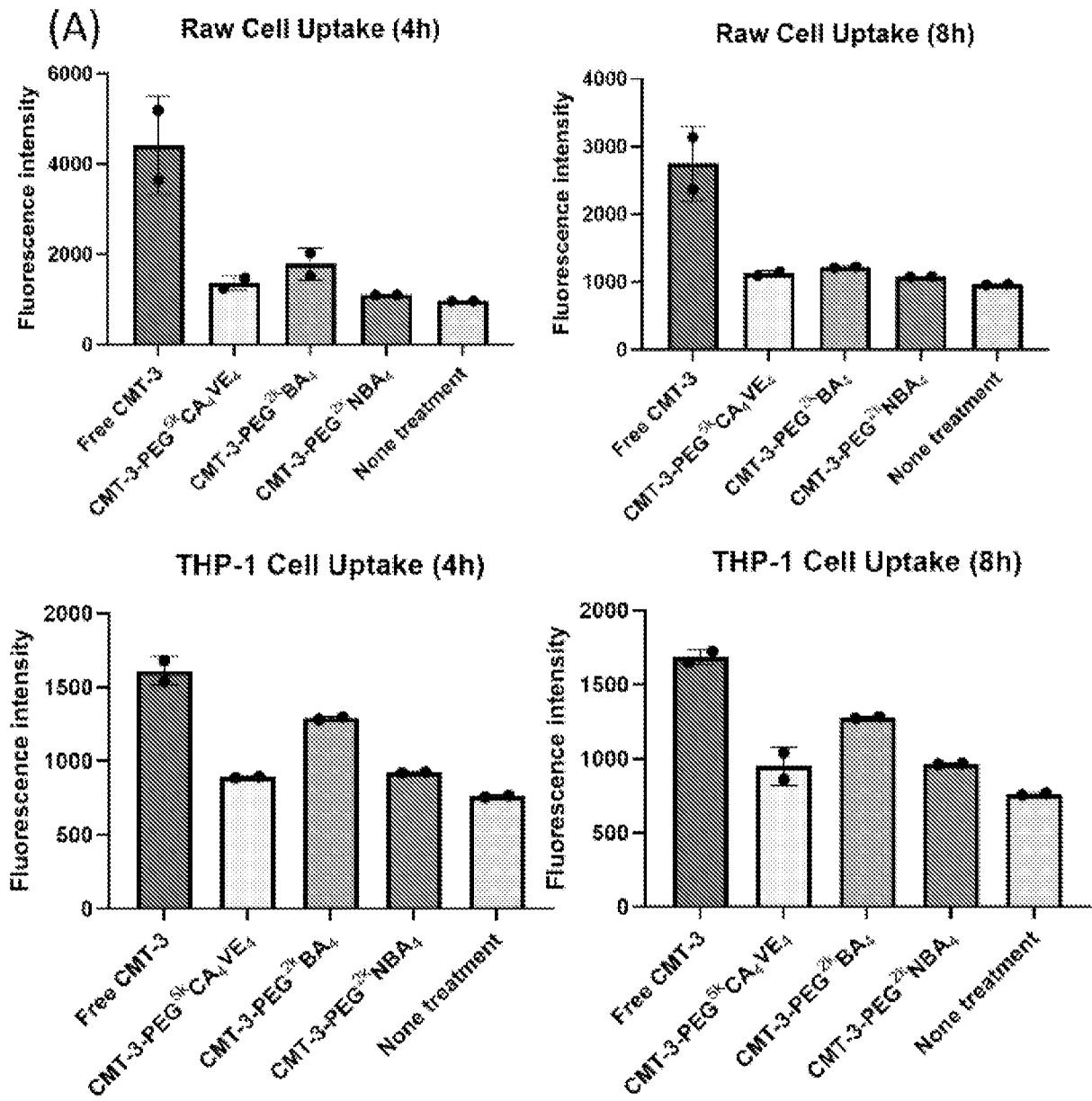


FIG. 20B

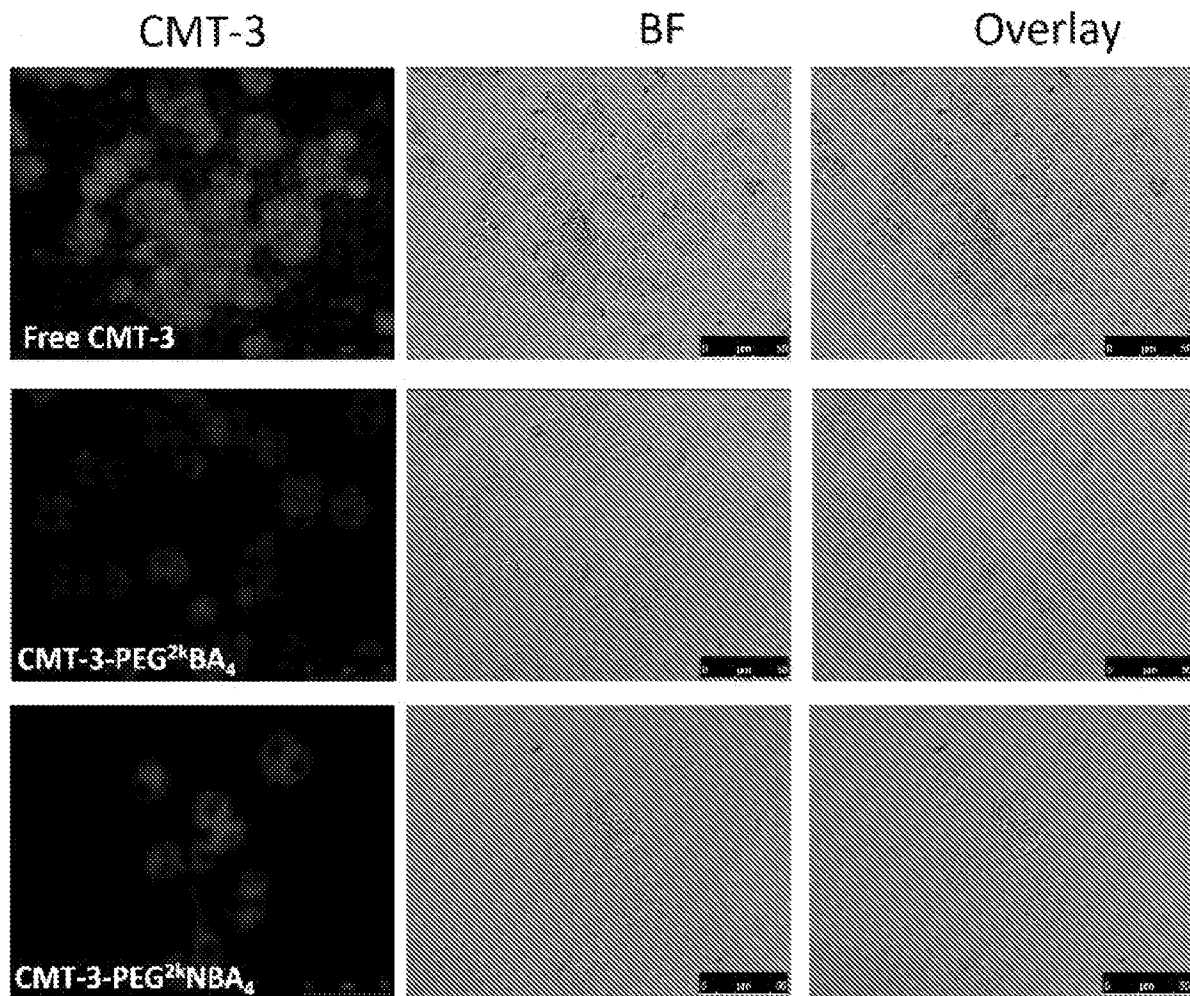


FIG. 21A

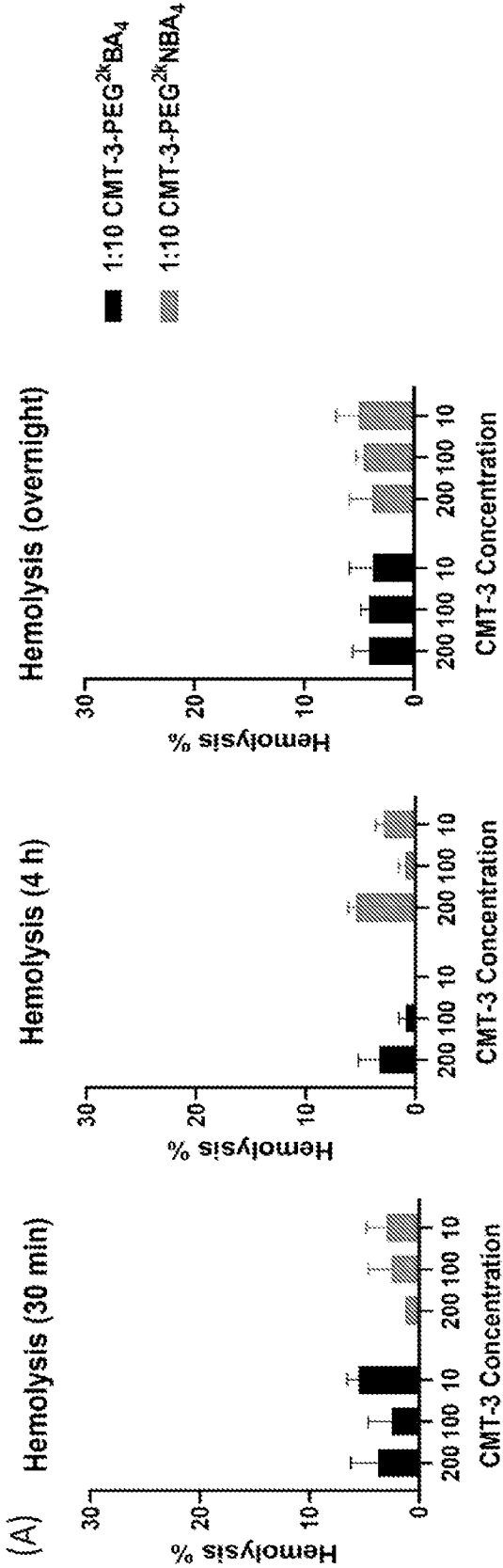


FIG. 21B

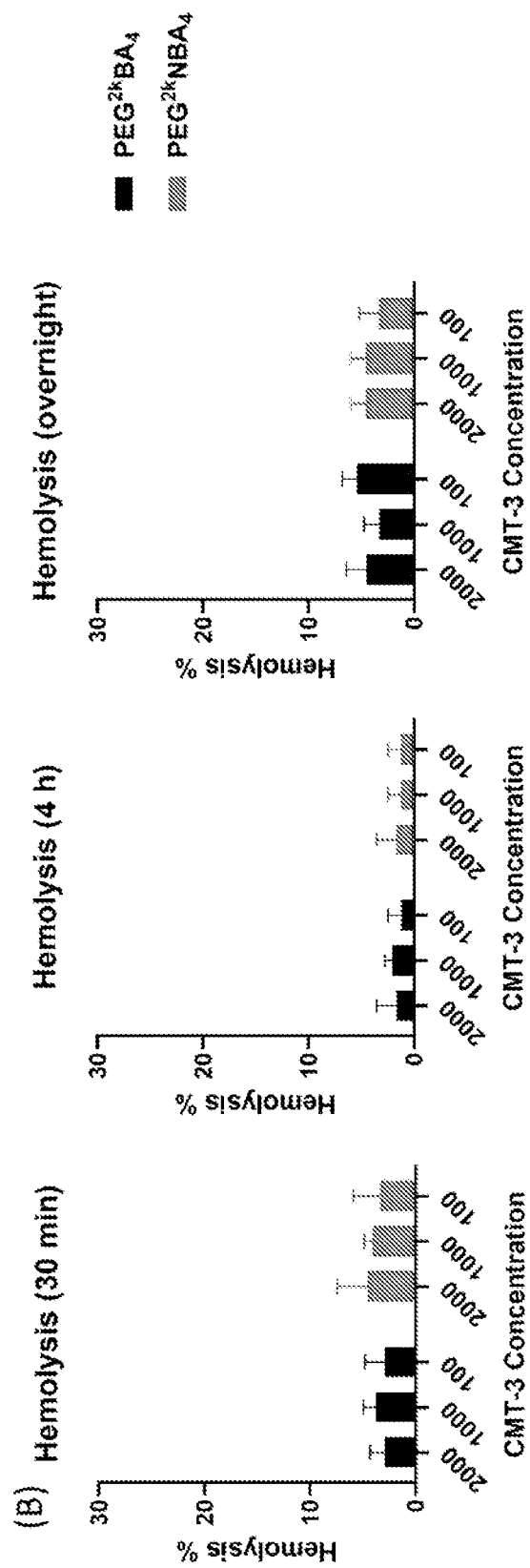


FIG. 22A

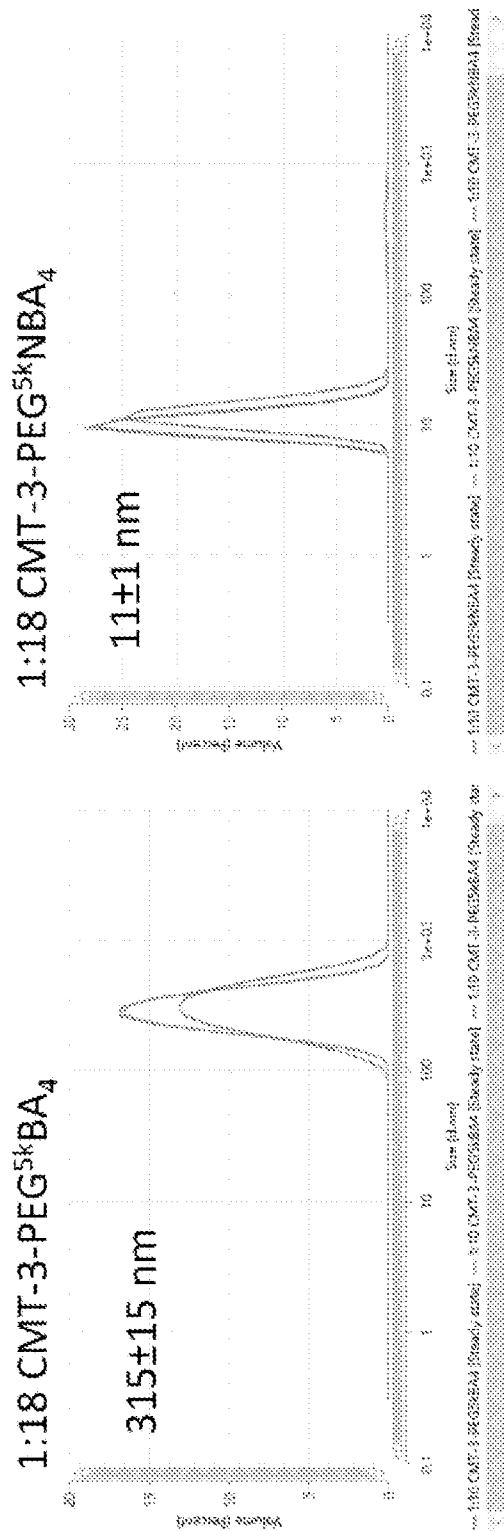


FIG. 22B

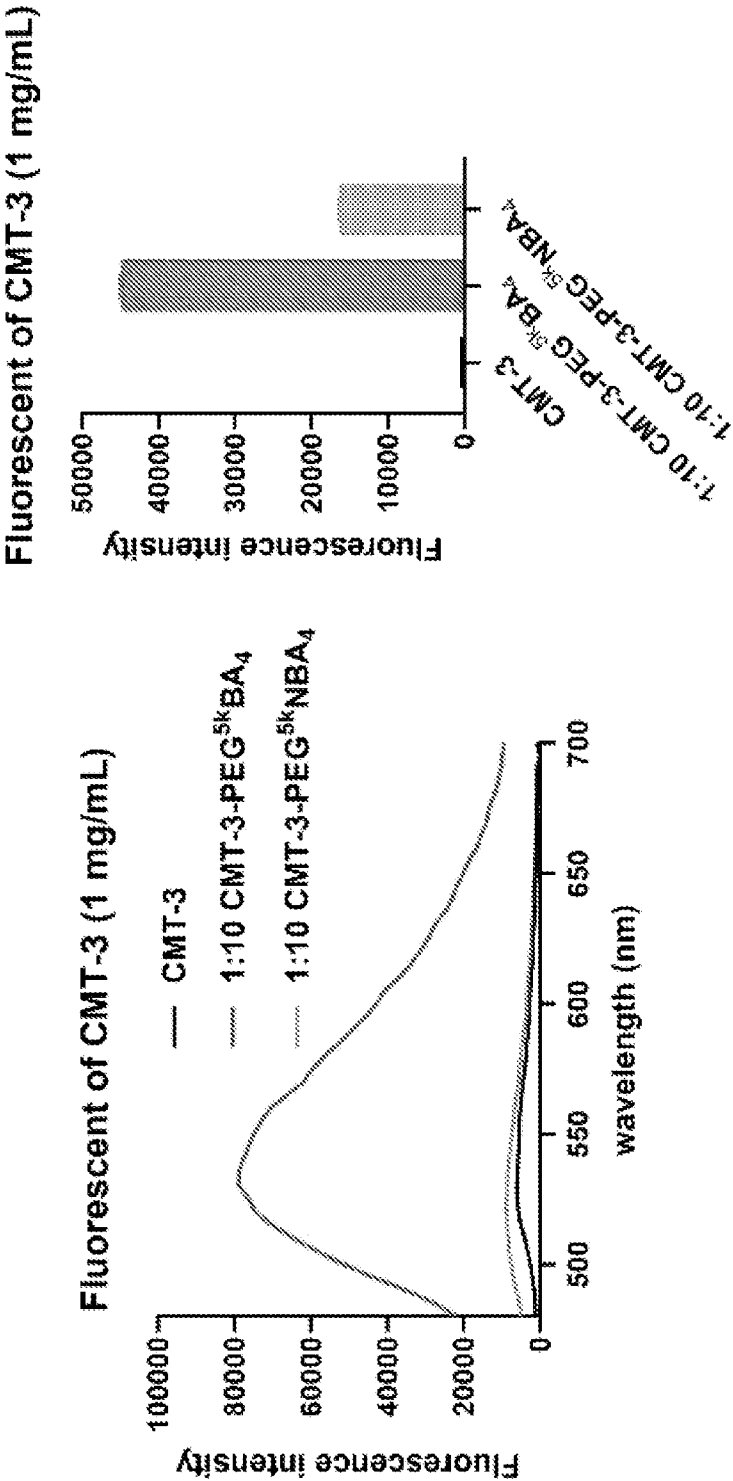


FIG. 22C

Formulations	360 nm	370 nm	390 nm
Free CMT-3	1.017	0.957	0.51
1:18 CMT-3- PEG ^{5k} BA ₄	0.633	0.79	0.989
1:18 CMT-3- PEG ^{5k} NBA ₄	0.933	1.02	0.957

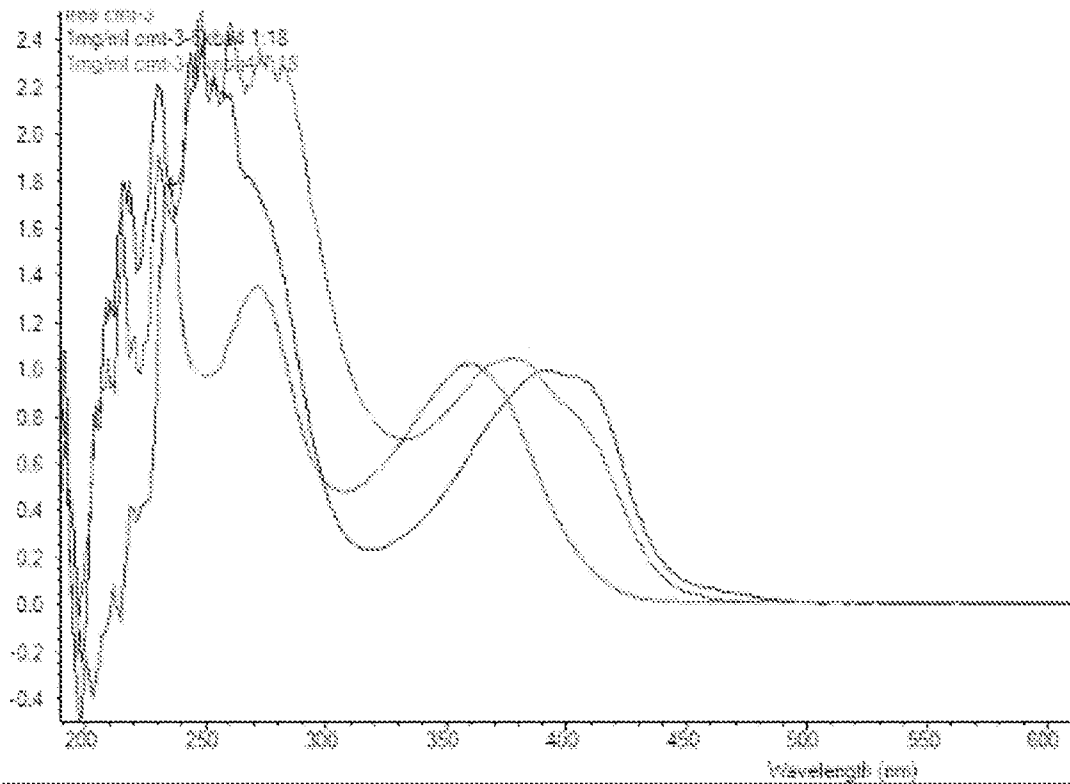


FIG. 23A

- Free CMT-3 DMSO
- CMT-3-PEG^{5k}BA₄
- CMT-3-PEG^{5k}NBA₄
- Free CMT-3 EtOH

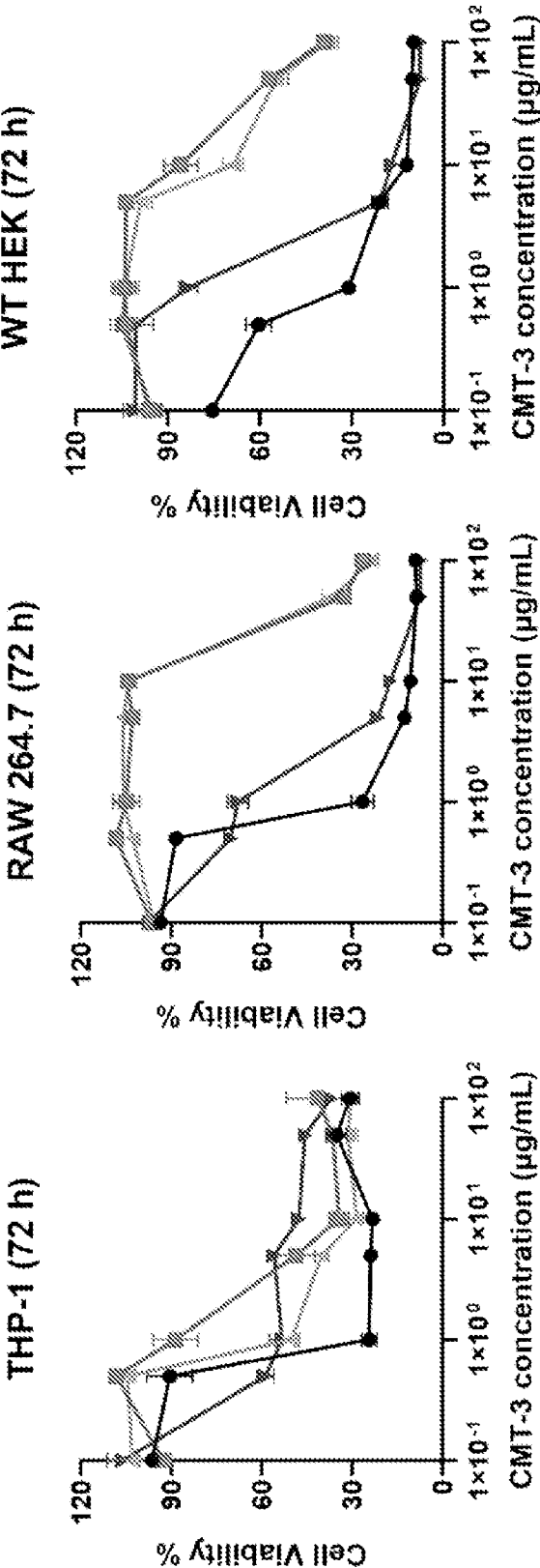


FIG. 23B

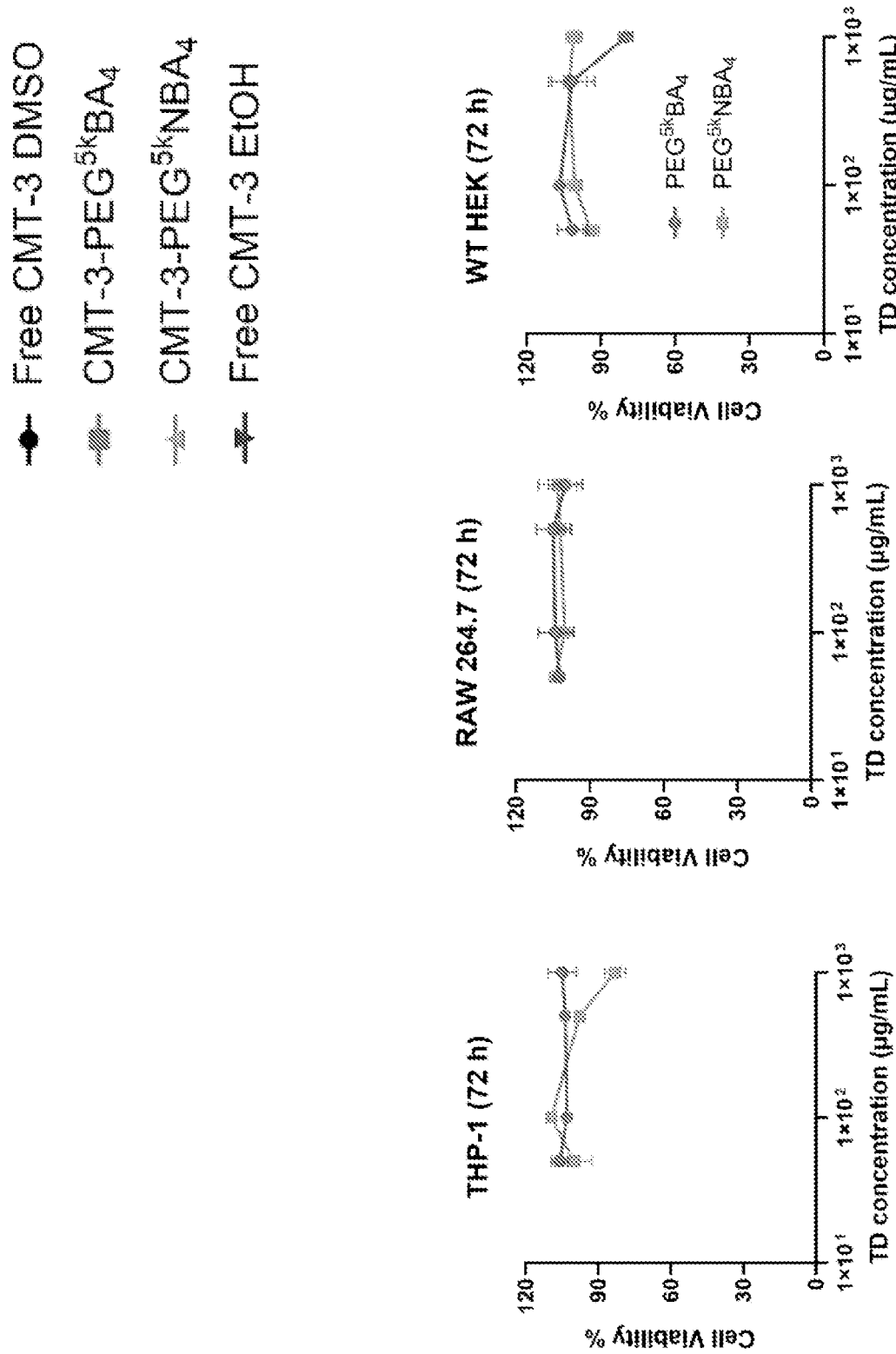


FIG. 23C

IC50 (µg/mL)	Free CMT-3	CMT-3-PEG ^{2k} BA ₄	CMT-3-PEG ^{2k} NBA ₄	Free CMT-3 (EtOH)
THP-1	1.83	12.98	4.82	1.97
RAW				
264.7	0.79	41.78	43.46	1.70
HEK293	0.57	65.99	55.43	2.68

FIG. 24A

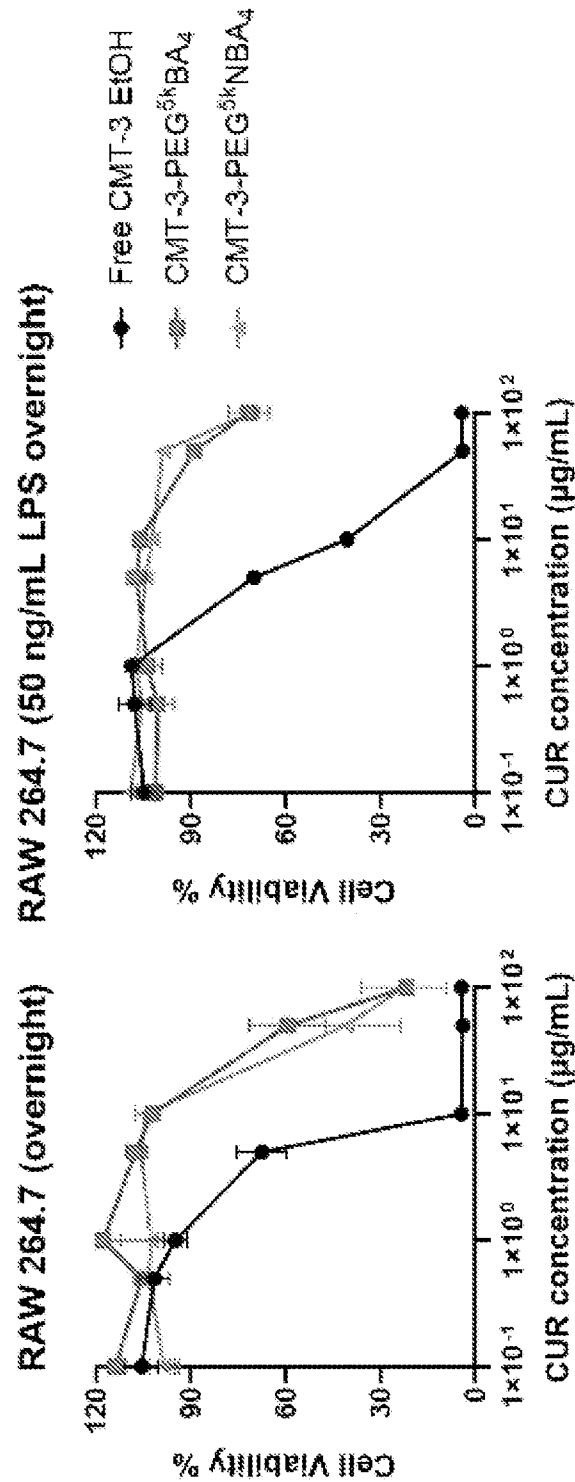


FIG. 24B

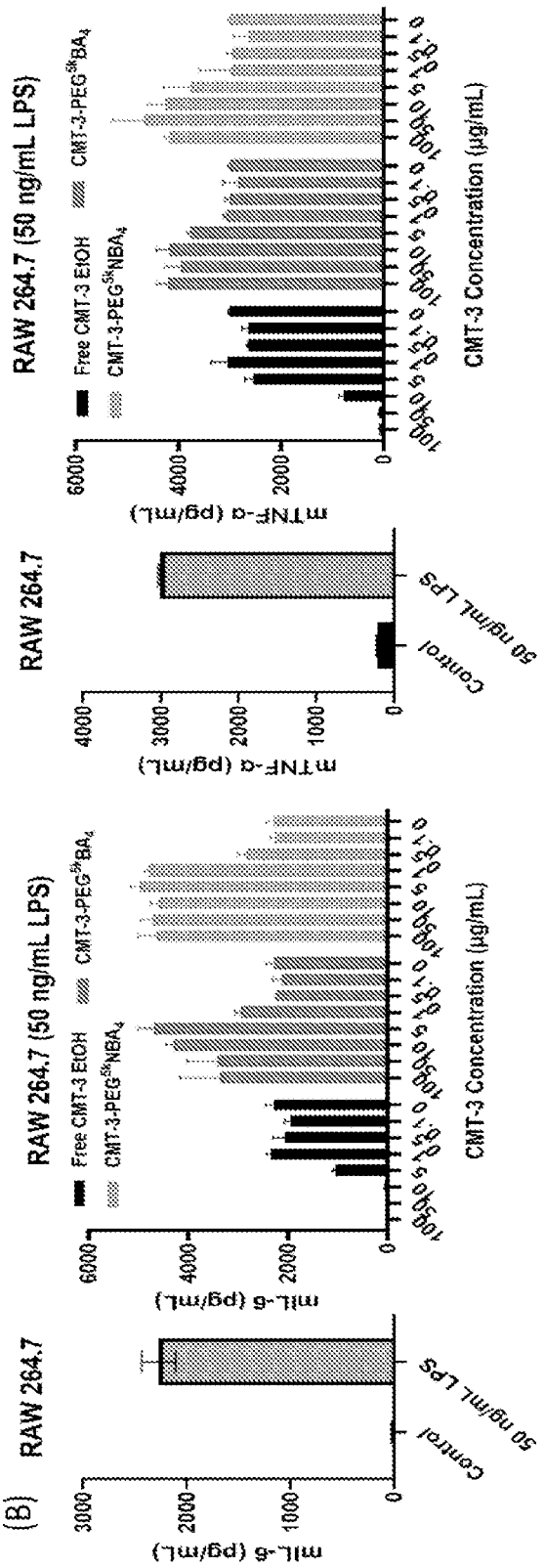


FIG. 25A

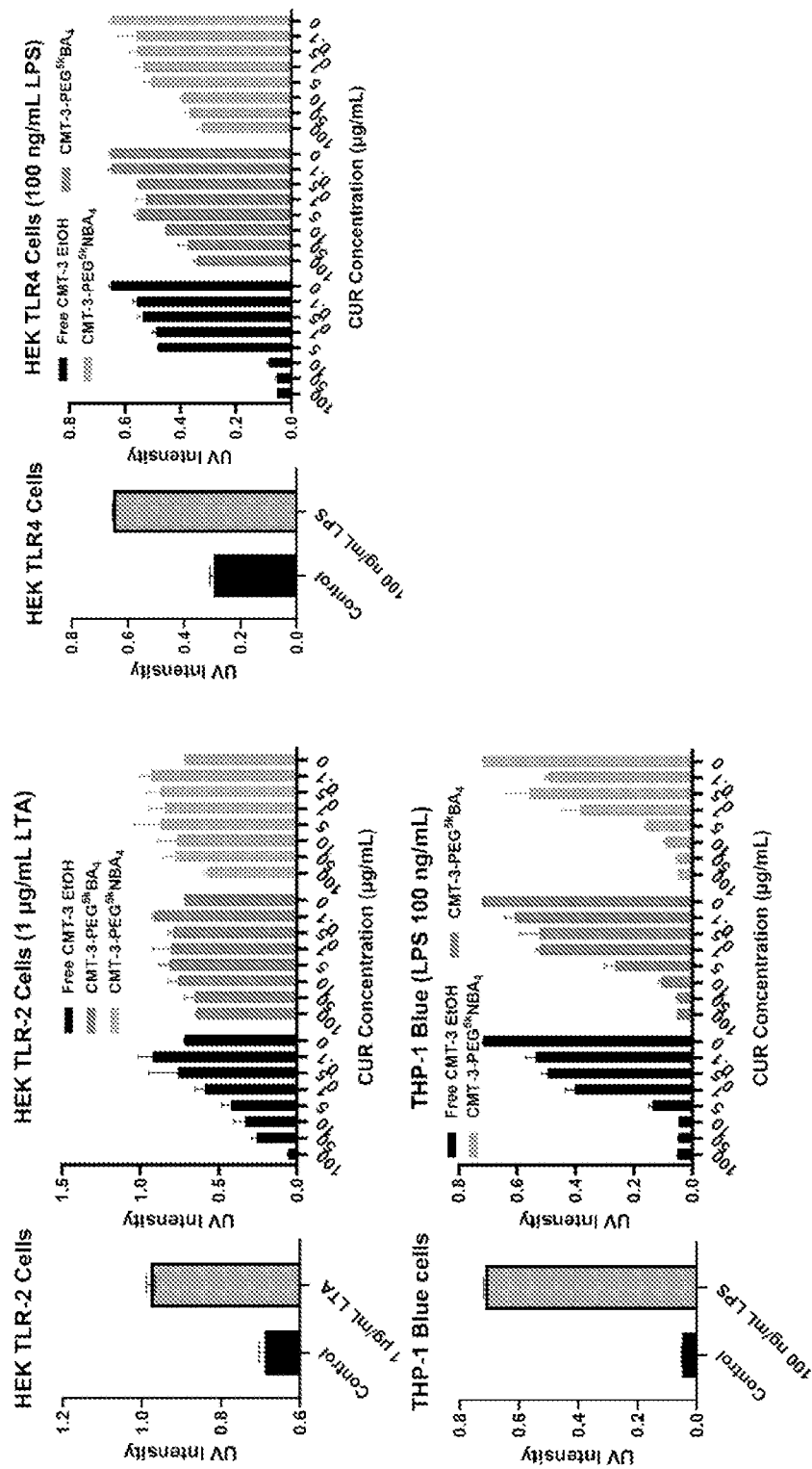


FIG. 25B

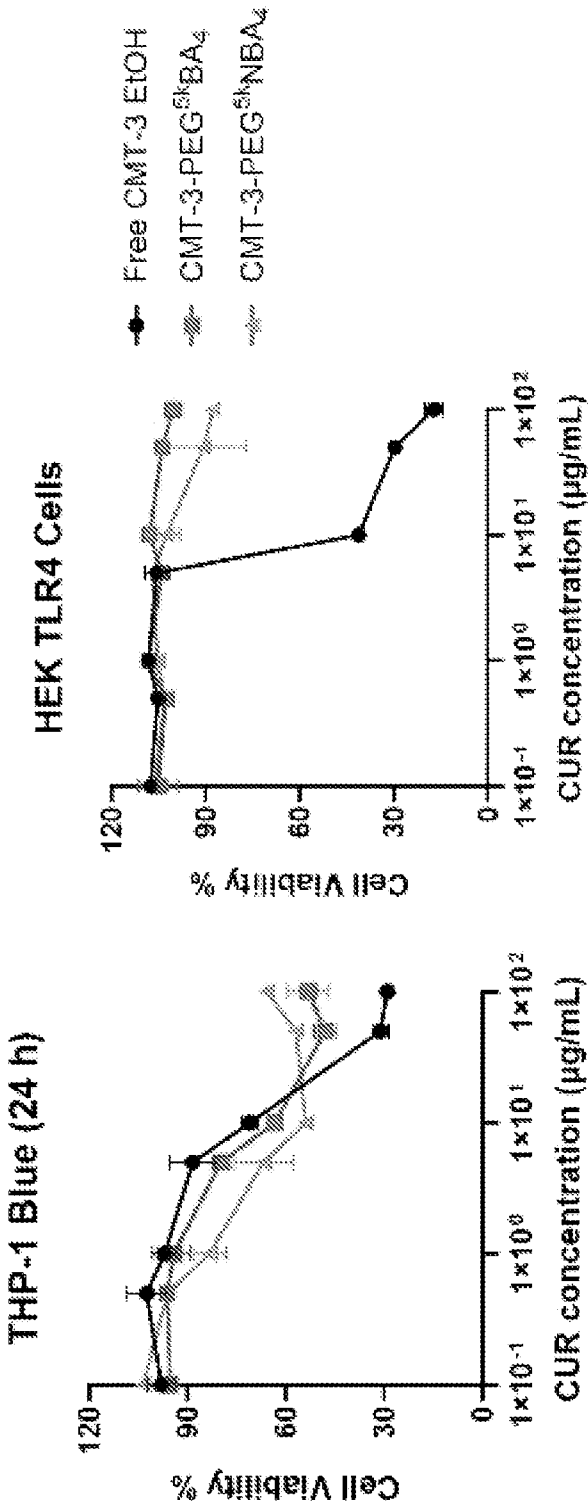


FIG. 26A

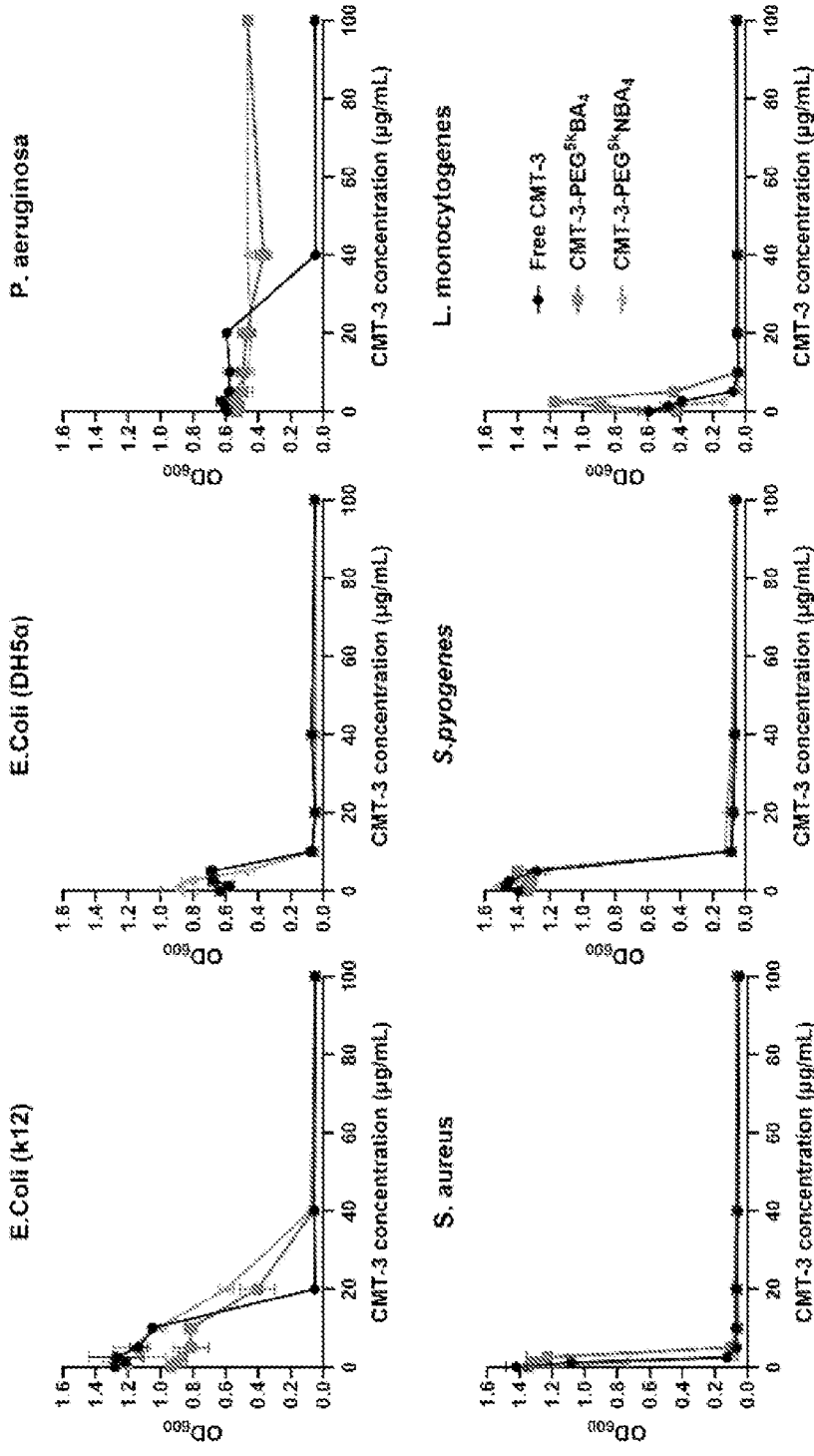


FIG. 26B

MIC (µg/ml)	Free CMT-3	CMT-3-PEG ^{5k} BA ₄	CMT-3-PEG ^{5k} NBA ₄
E.Coli. (K12)	20	40	40
E.Coli. (DH5α)	10	10	10
pseudomonas aeruginosa	40	ND	ND
staphylococcus aureus	5	10	5
streptococcus pneumoniae	10	10	10
listeria monocytogenes	5	10	5

FIG. 27A

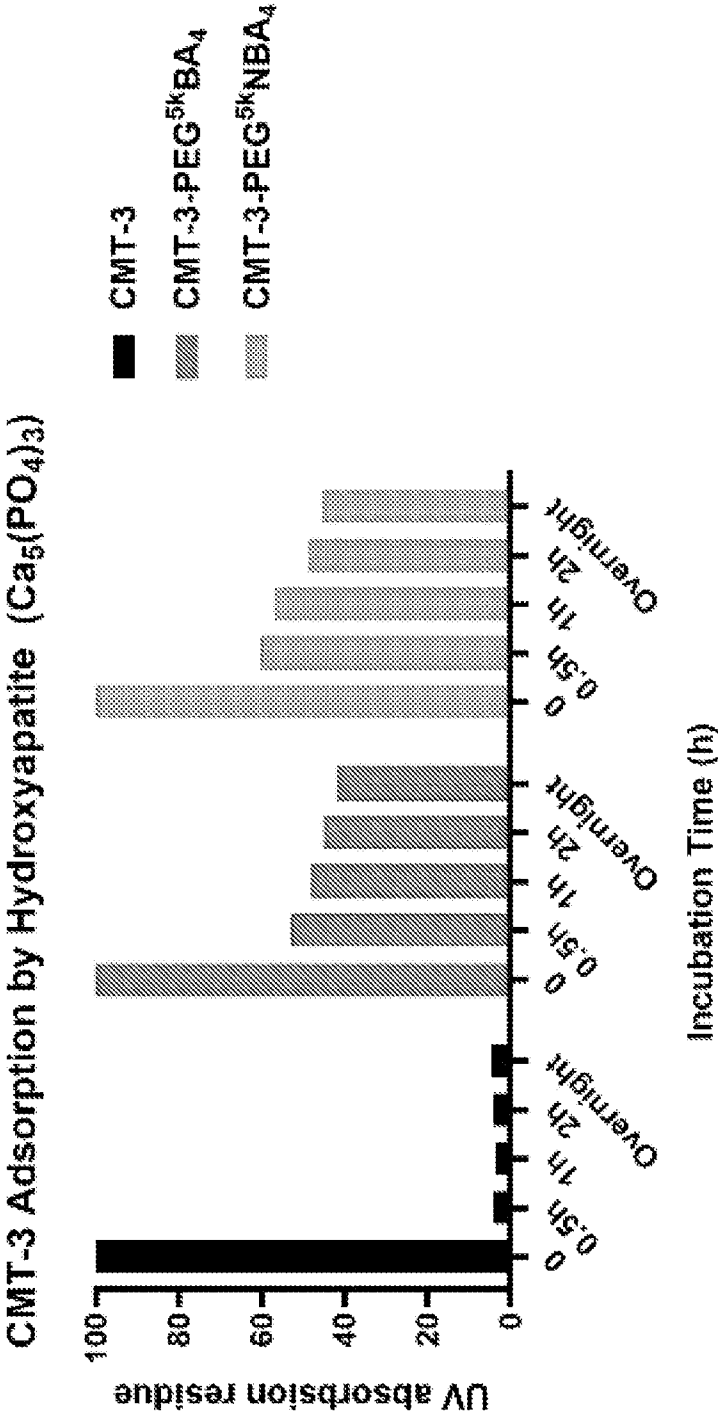


FIG. 27B

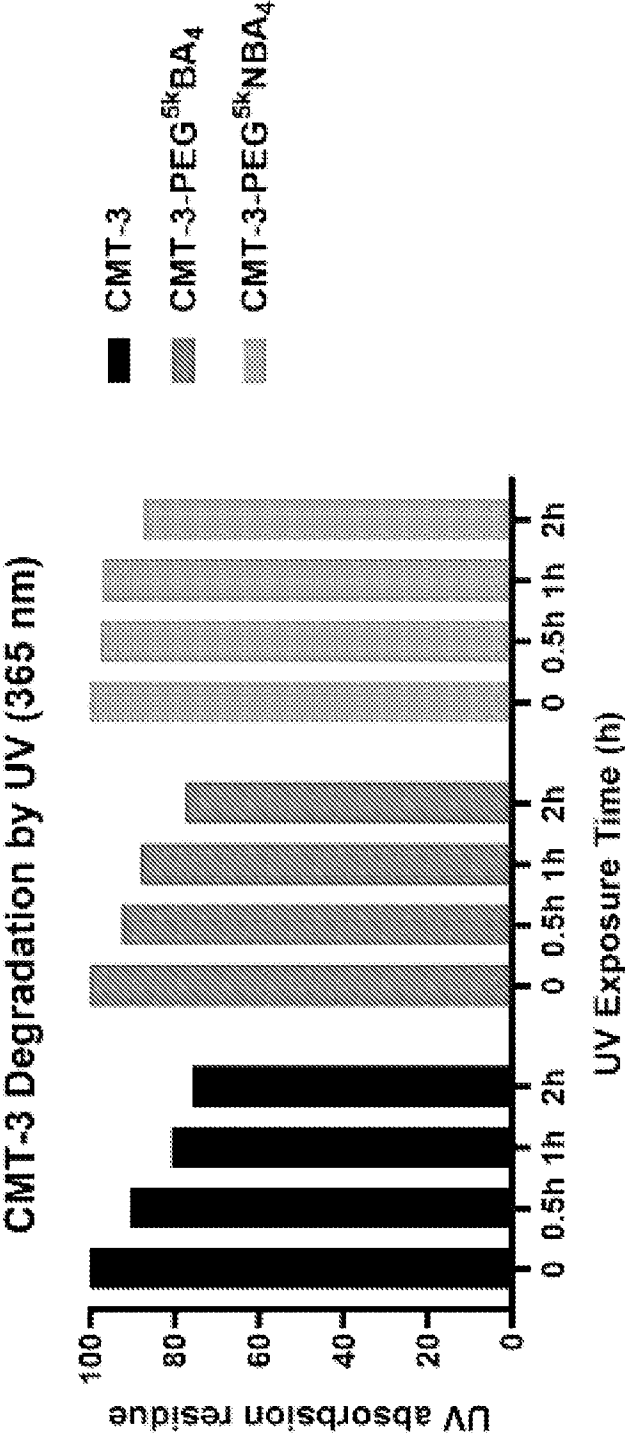


FIG. 27C

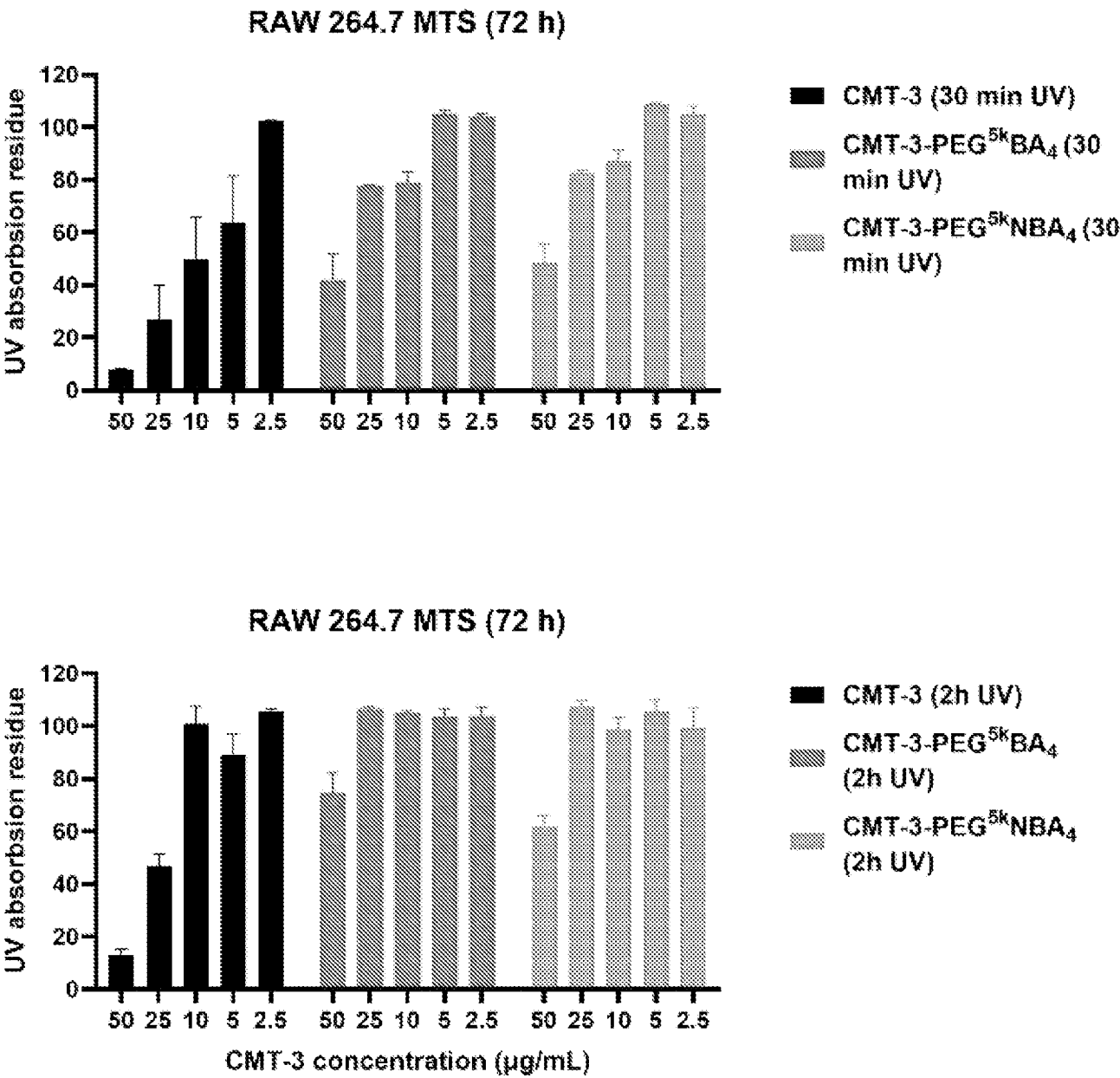


FIG. 28A

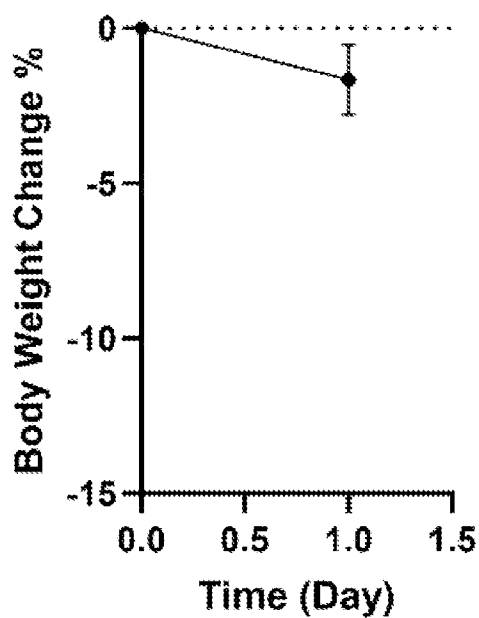
10 mg/kg CMT-3-PEG^{5k}BA₄

FIG. 28B

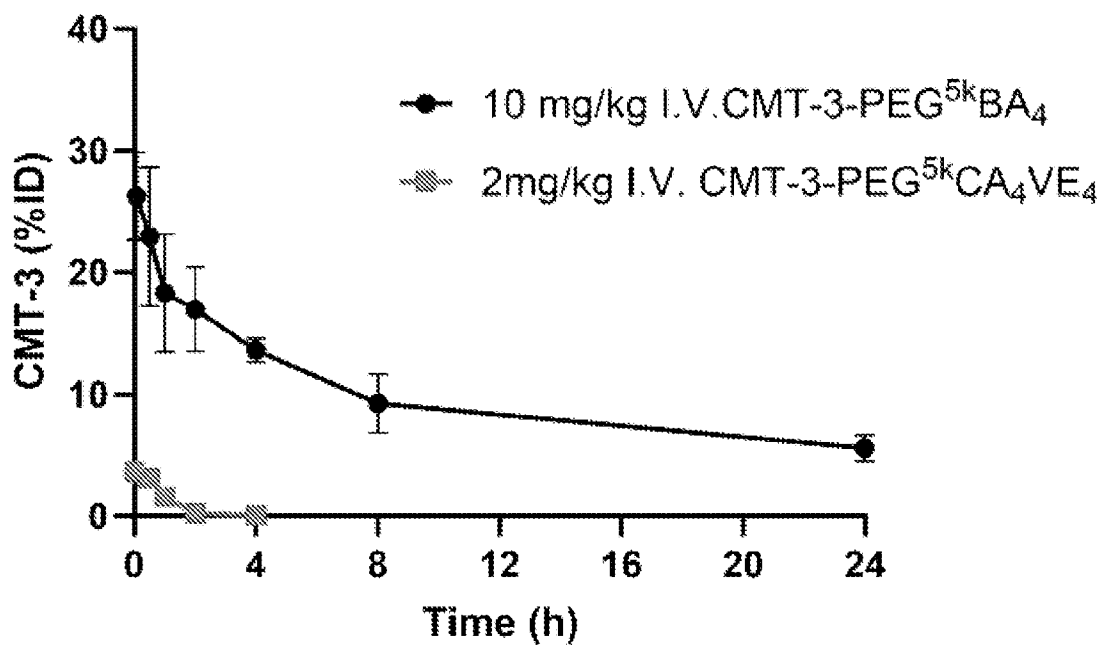
PK of CMT-3

FIG. 28C

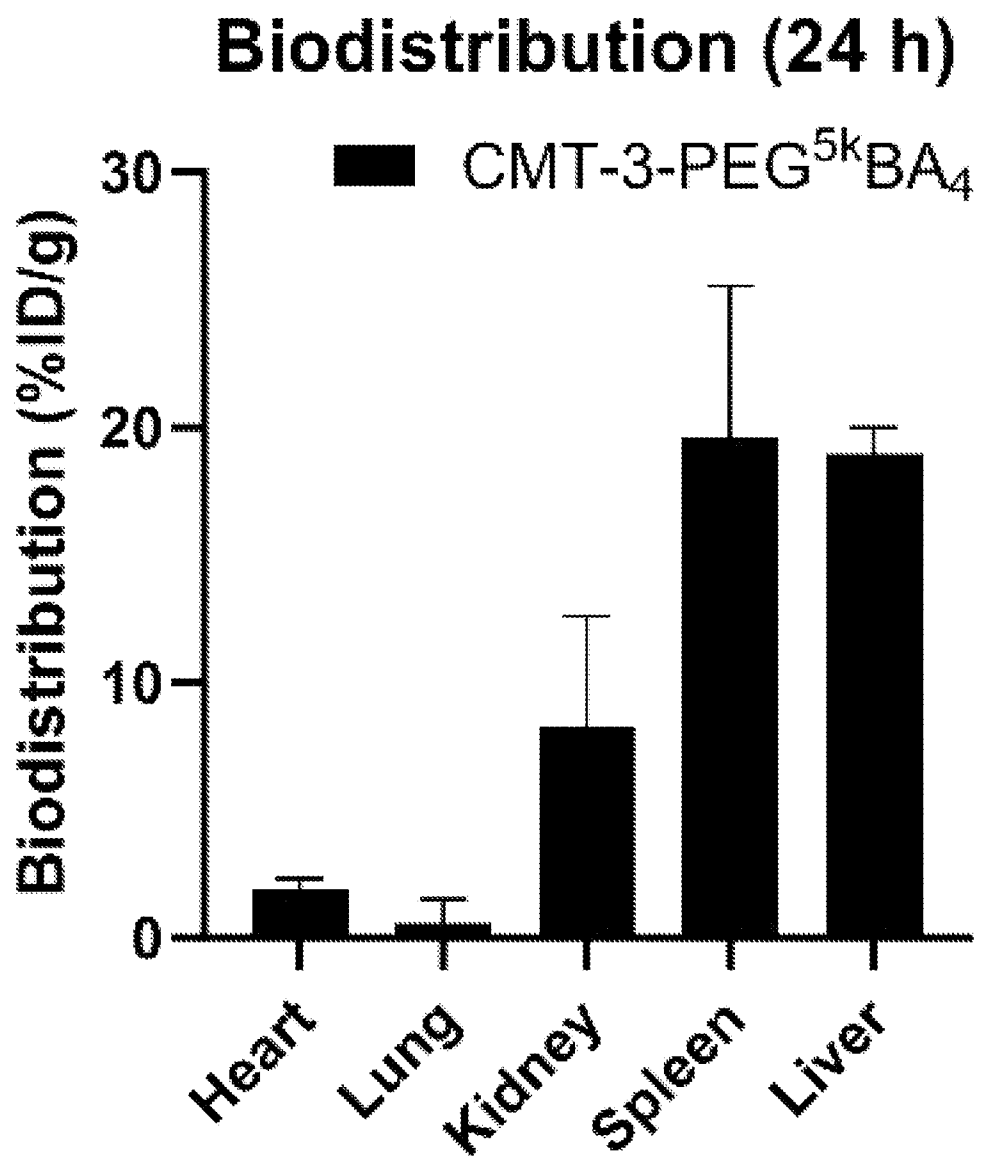


FIG. 29

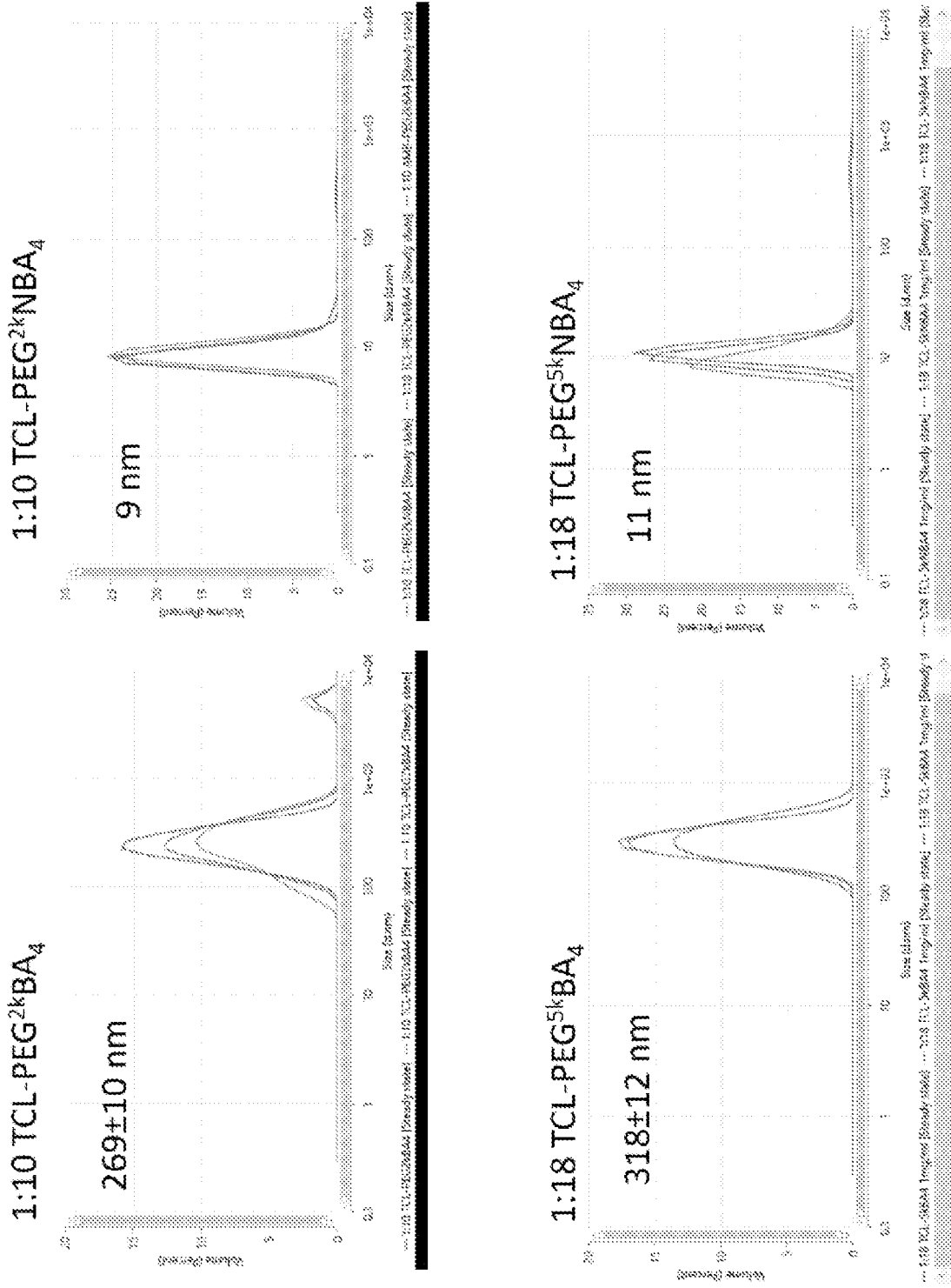


FIG. 30A

Formulations	361 nm	380 nm	408 nm
Free TCL	0.964	0.717	0.169
1:10 TCL-PEG ^{2k} BA ₃	0.762	0.943	1.2
1:10 TCL-PEG ^{2k} NBA ₃	0.966	0.991	0.912
1:10 TCL-PEG ^{5k} BA ₃	0.954	1.18	1.481
1:10 TCL-PEG ^{5k} NBA ₃	1.141	1.171	1.088

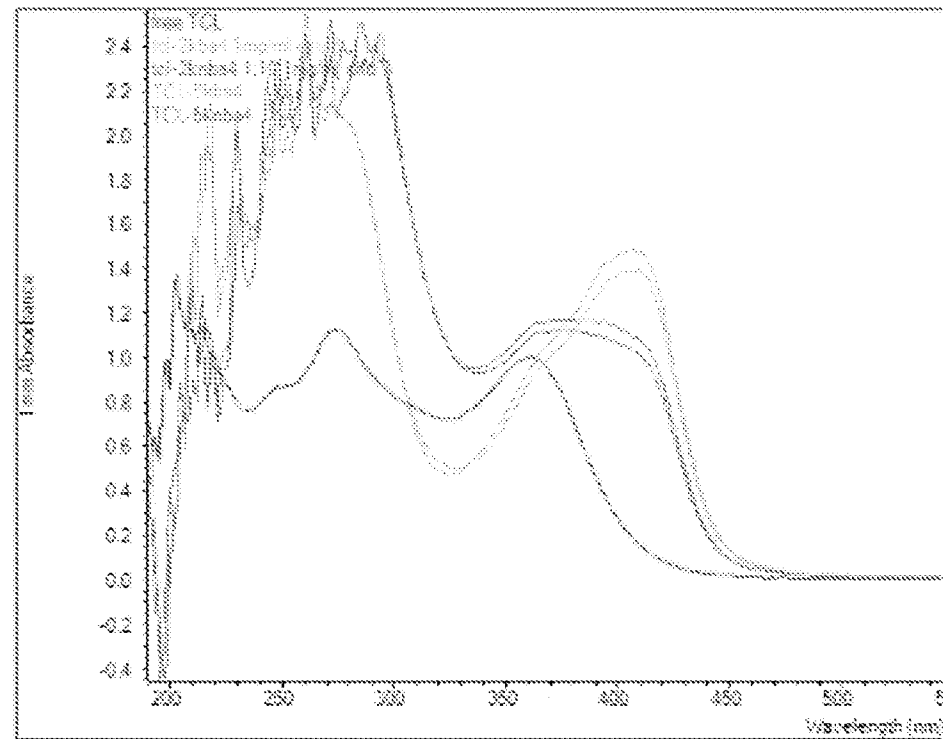


FIG. 30B

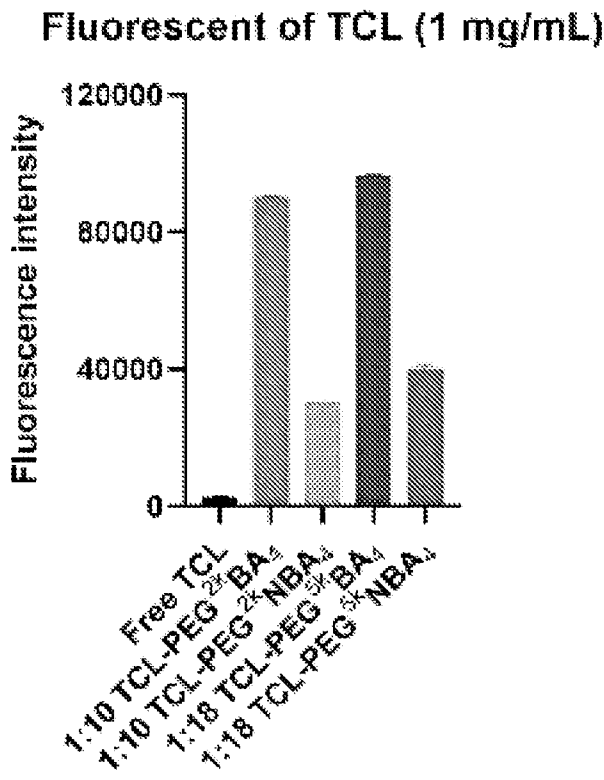
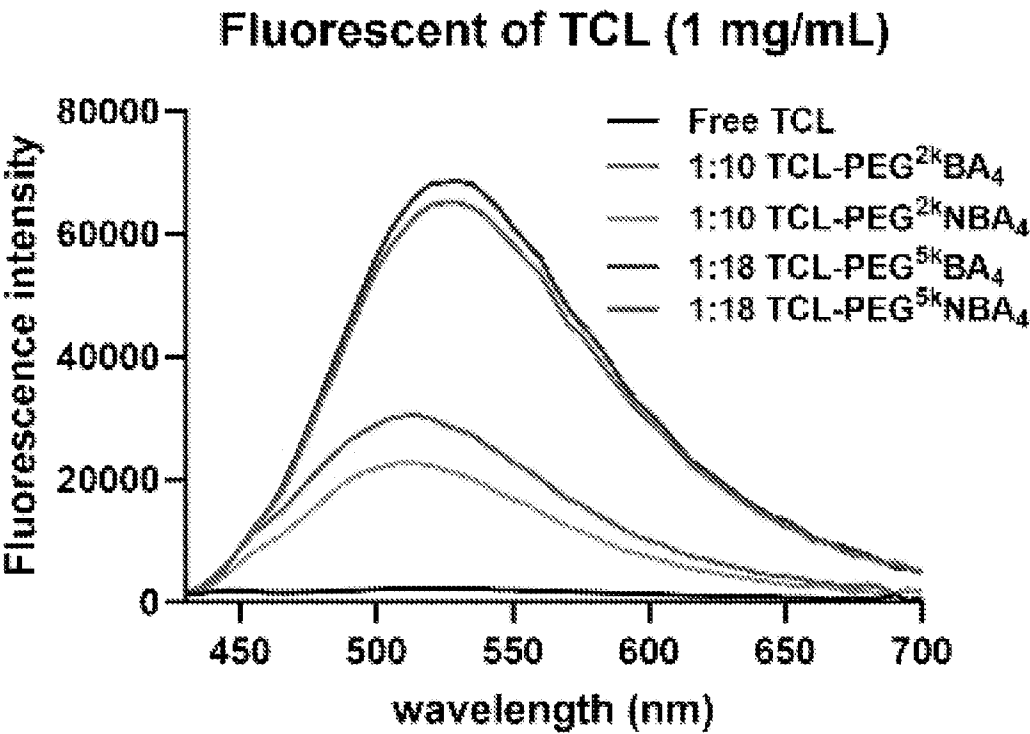


FIG. 30C

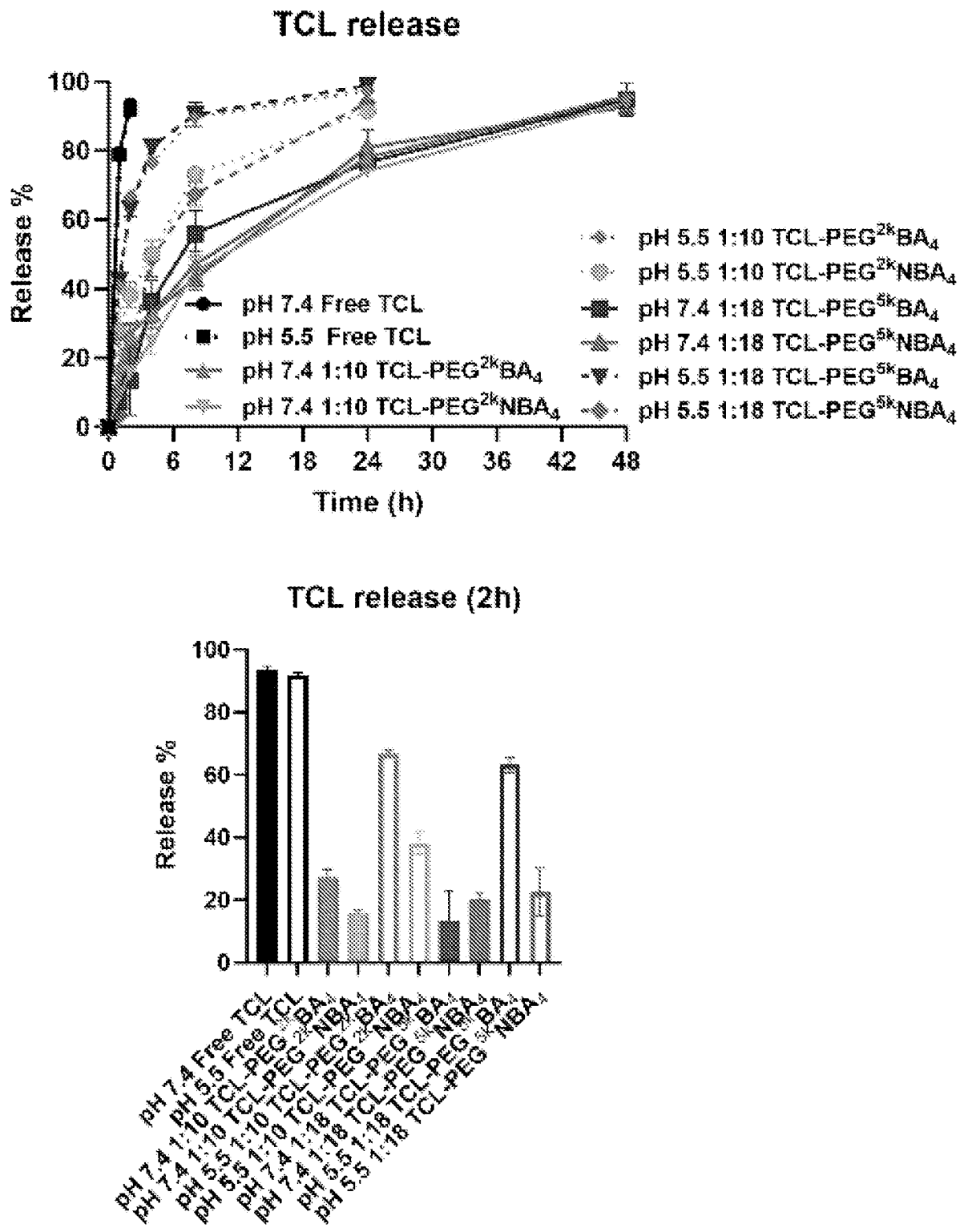


FIG. 31A

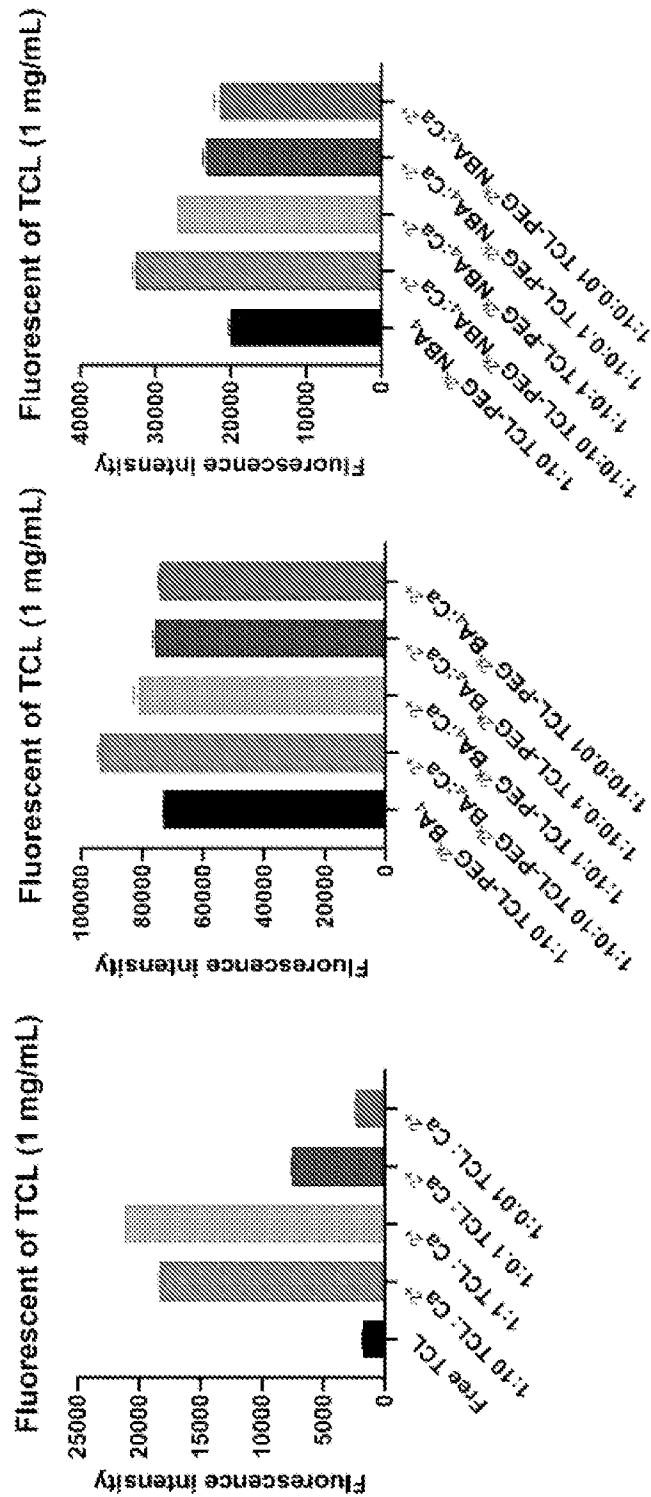


FIG. 32A

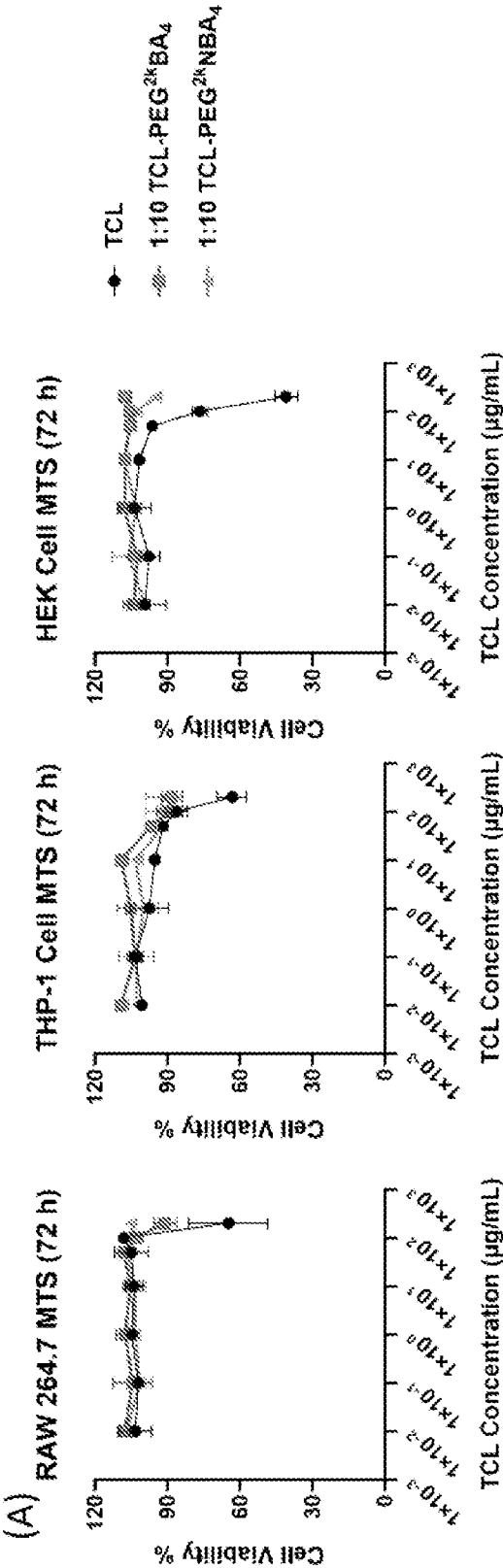


FIG. 32B

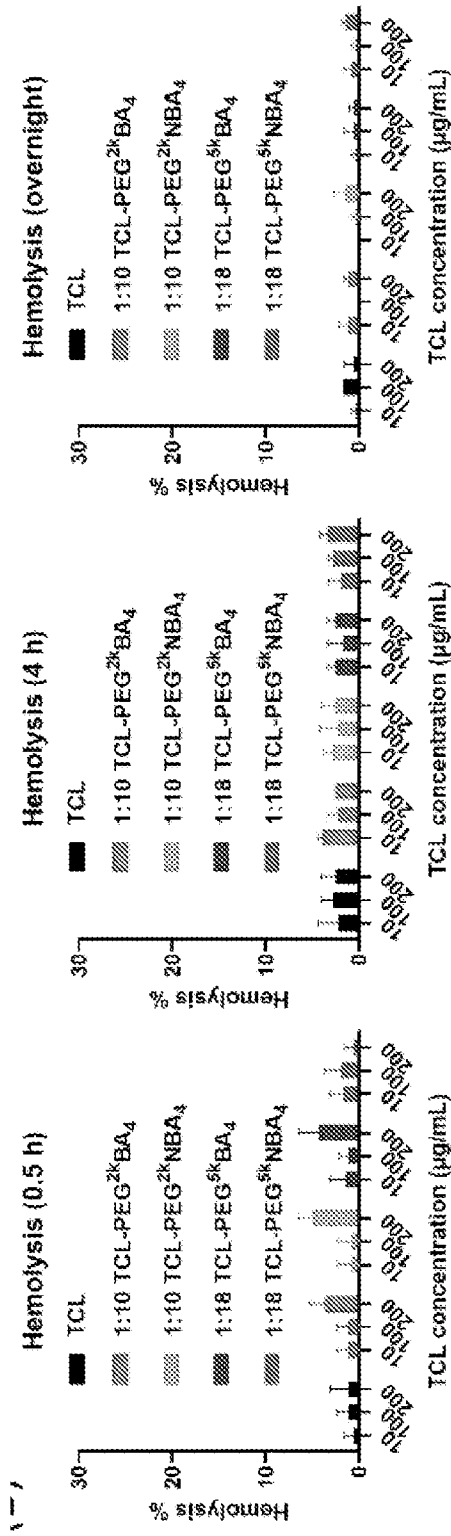


FIG. 33

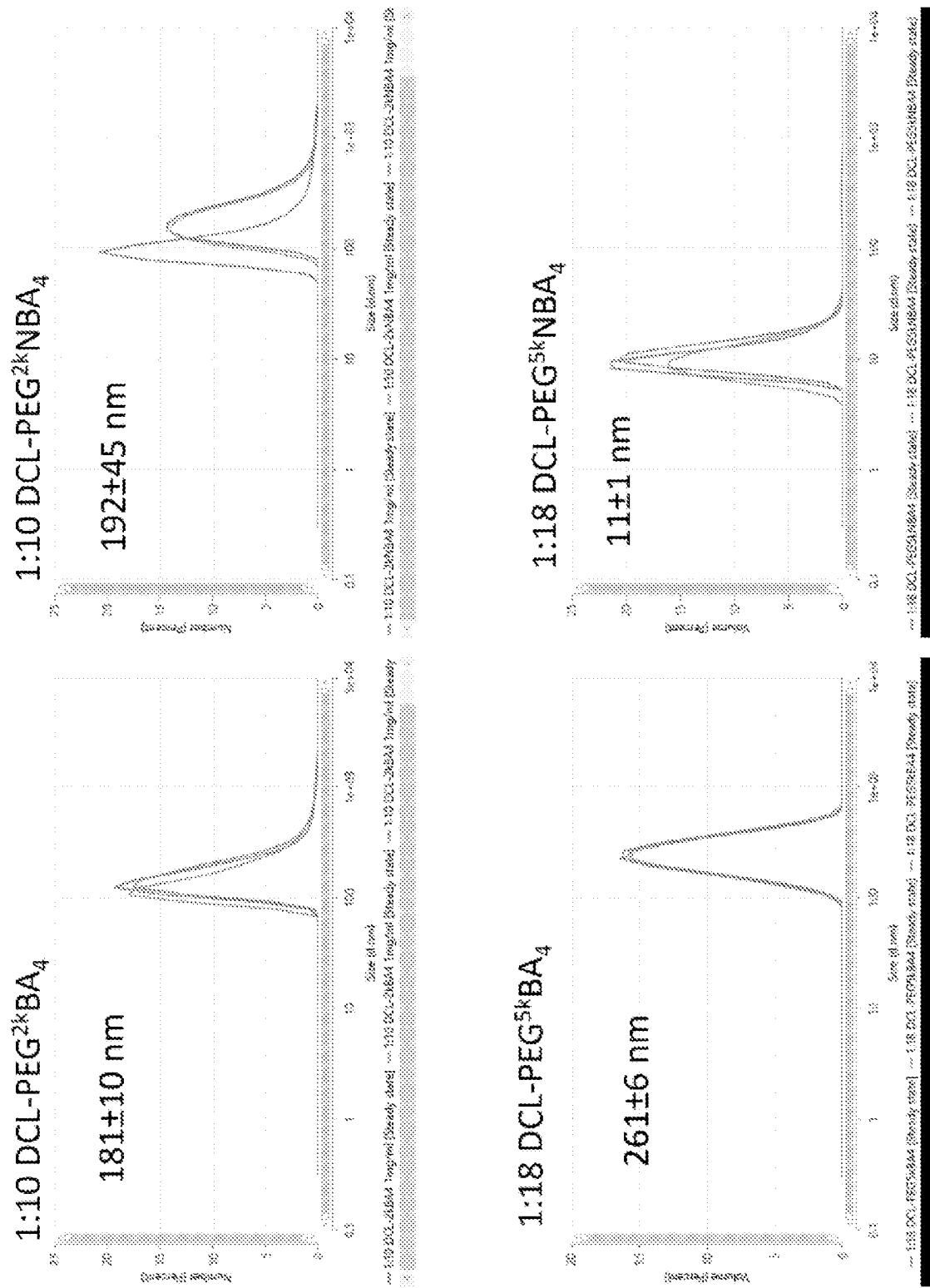


FIG. 34A

Formulations	350 nm	392 nm	371 nm
Free DCL	0.777	0.211	0.589
1:10 DCL-PEG ^{2k} BA ₄	0.715	1.232	1.088
1:10 DCL-PEG ^{2k} NBA ₄	1.034	1.019	1.142
1:10 DCL-PEG ^{5k} BA ₄	0.69	1.301	1.113
1:10 DCL-PEG ^{5k} NBA ₄	1.066	1.064	1.224

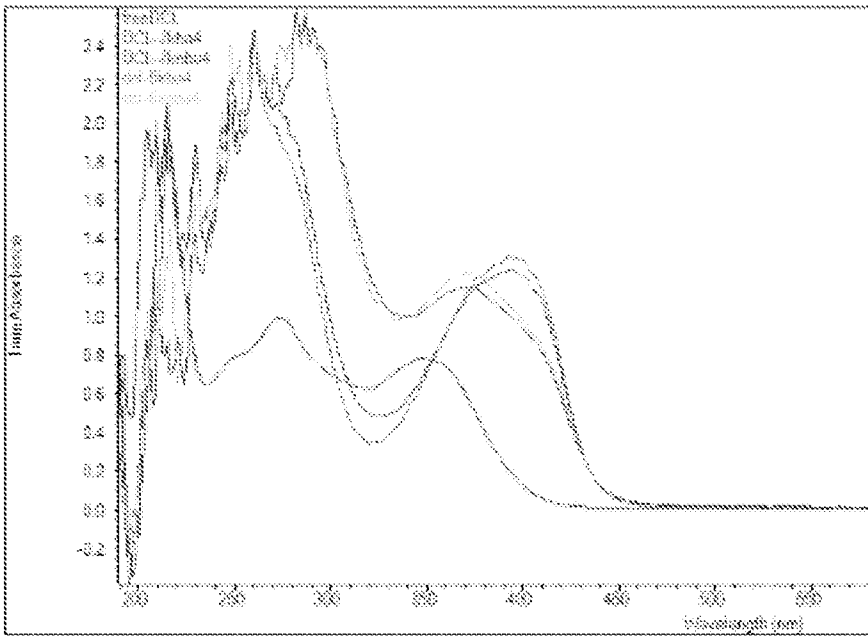


FIG. 34B

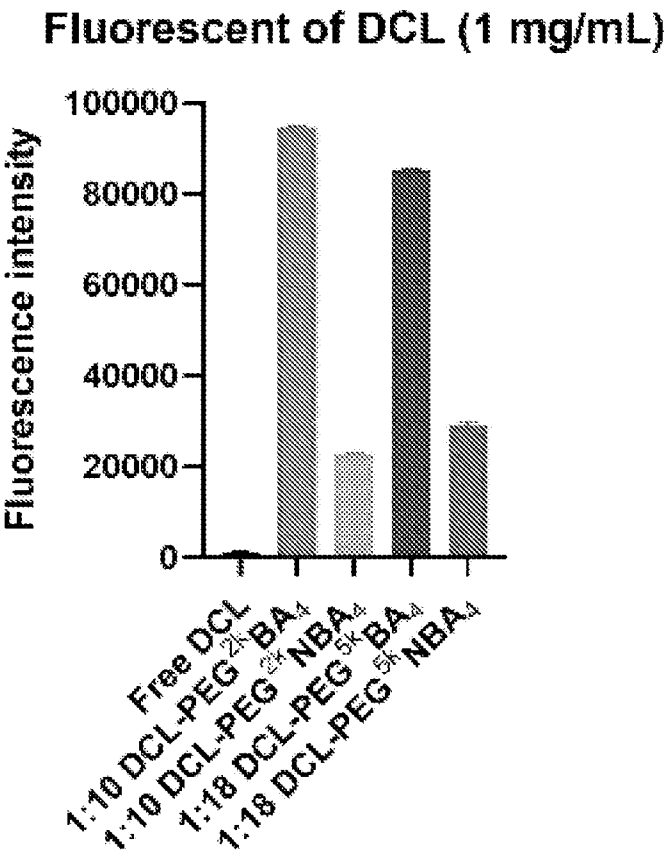
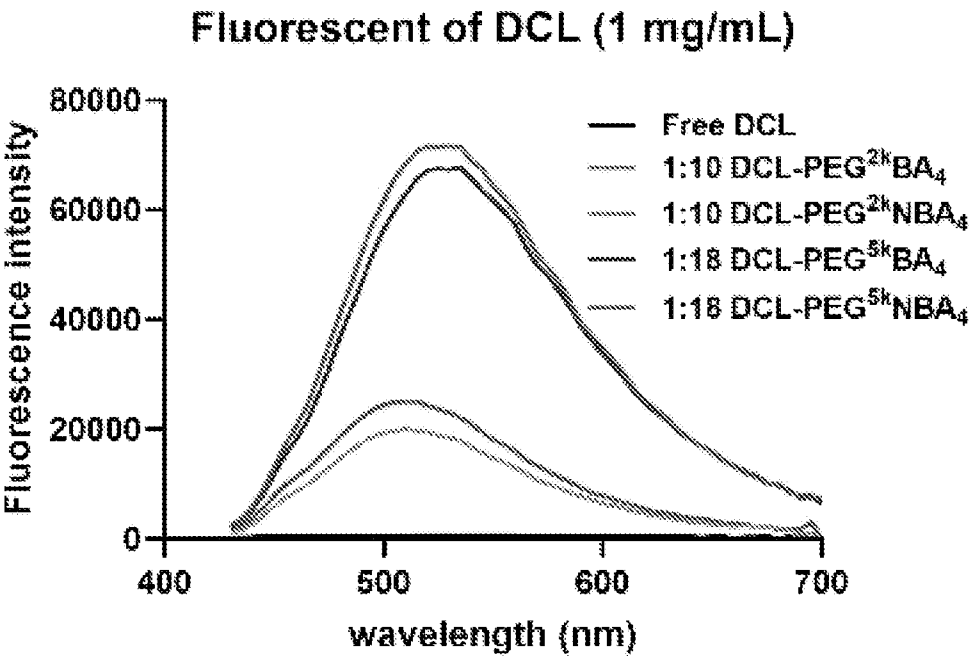


FIG. 34C

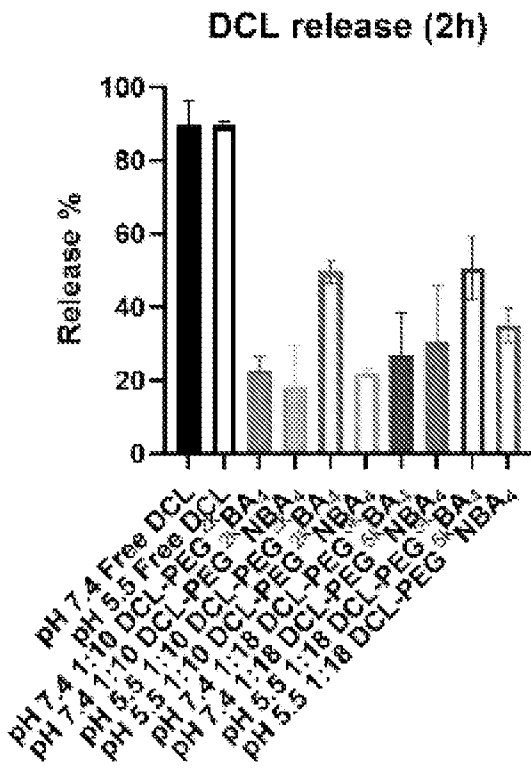
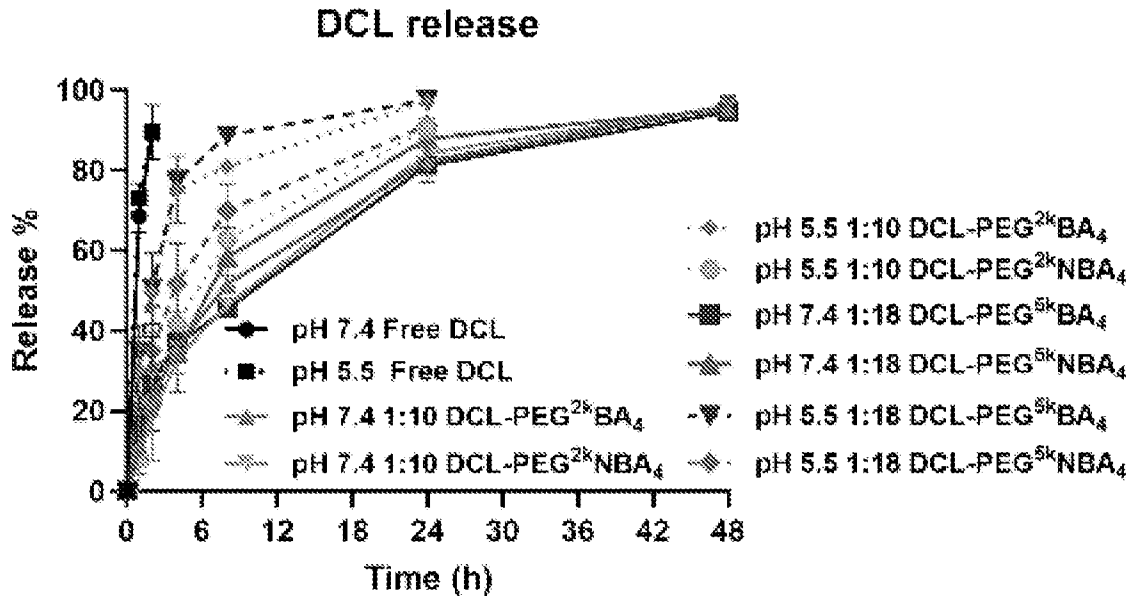


FIG. 35A

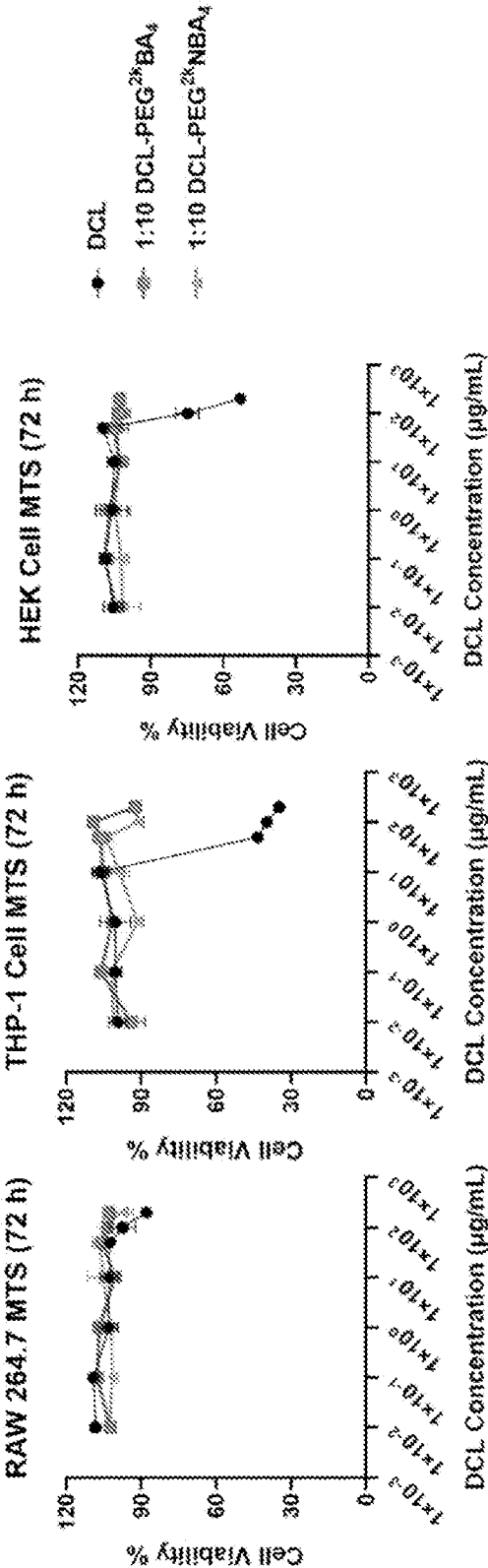


FIG. 35B

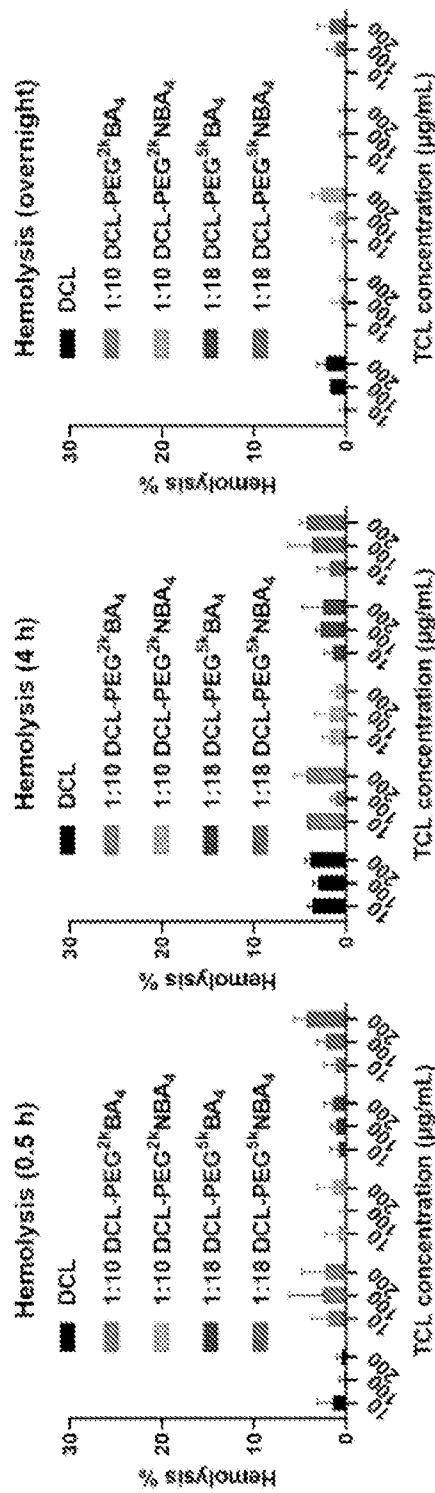


FIG. 35C

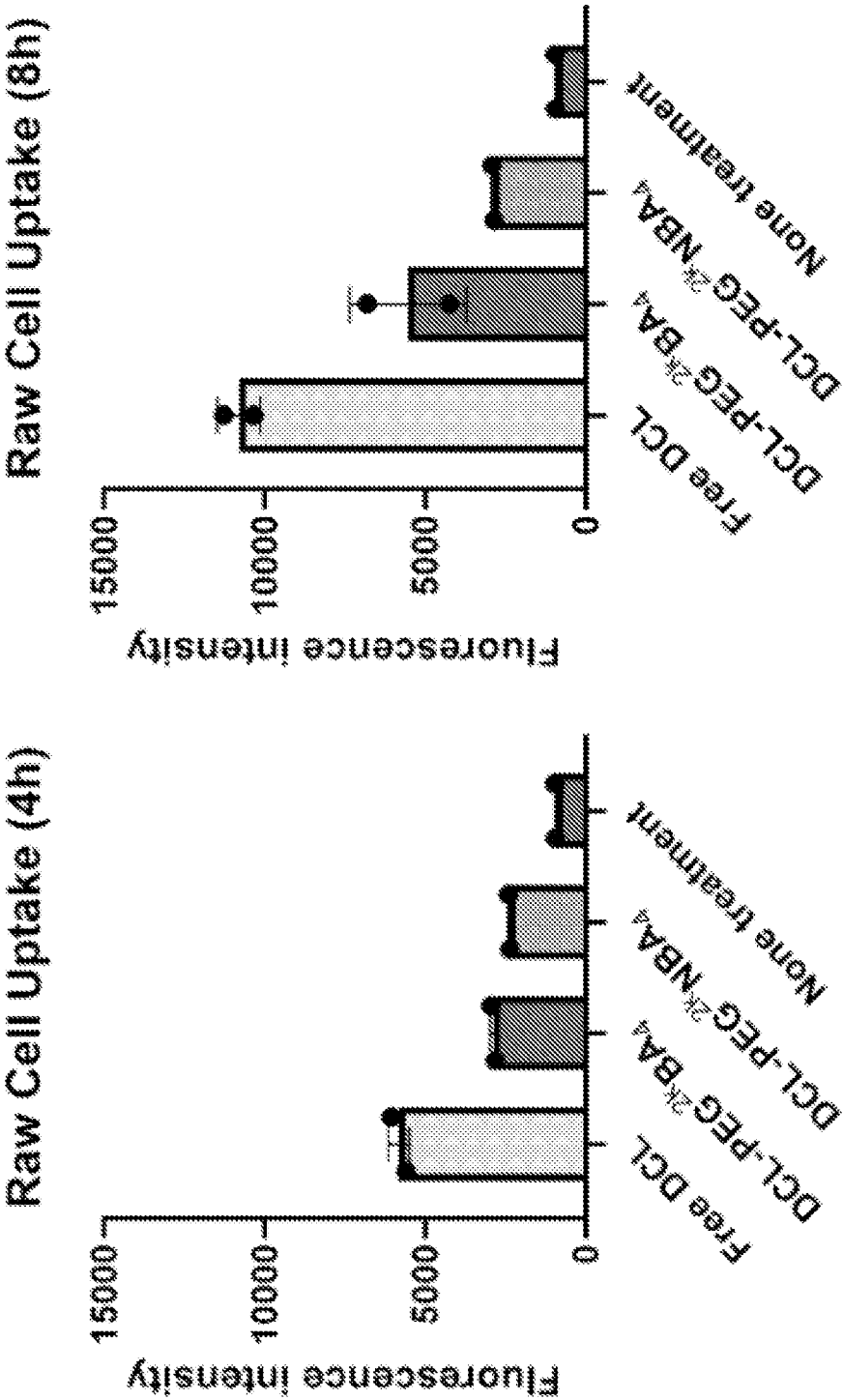


FIG. 36A

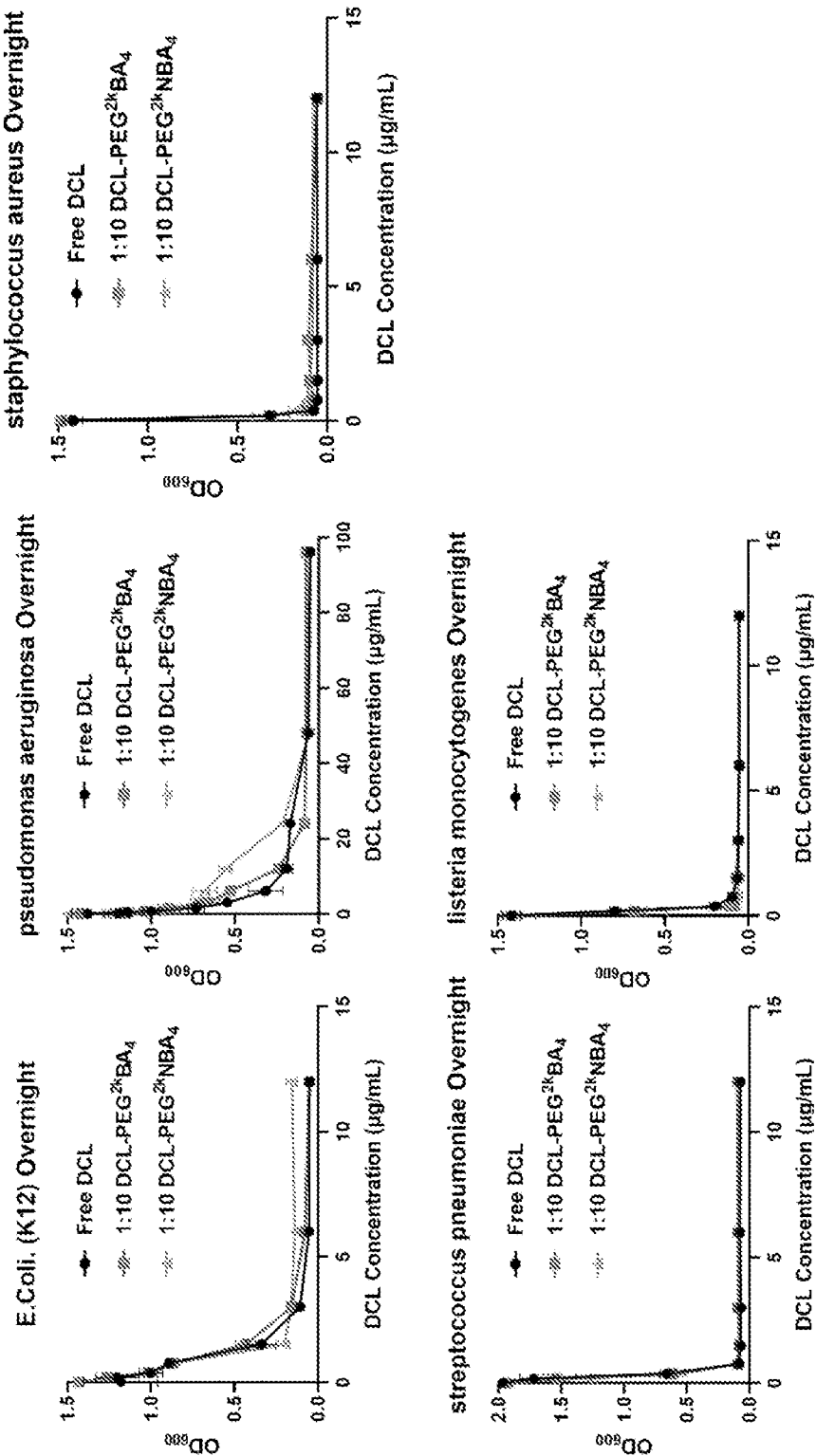


FIG. 36B

MIC (µg/mL)	Free DCL	1:10 DCL-PEG ^{2k} BA ₄	1:10 DCL-PEG ^{2k} NBA ₄
E.Coli. (K12)	3	3	3
pseudomonas aeruginosa	24	24	48
staphylococcus aureus	0.375	0.75	0.75
streptococcus pneumoniae	0.75	0.75	0.75
listeria monocytogenes	0.75	0.75	0.75

FIG. 37A

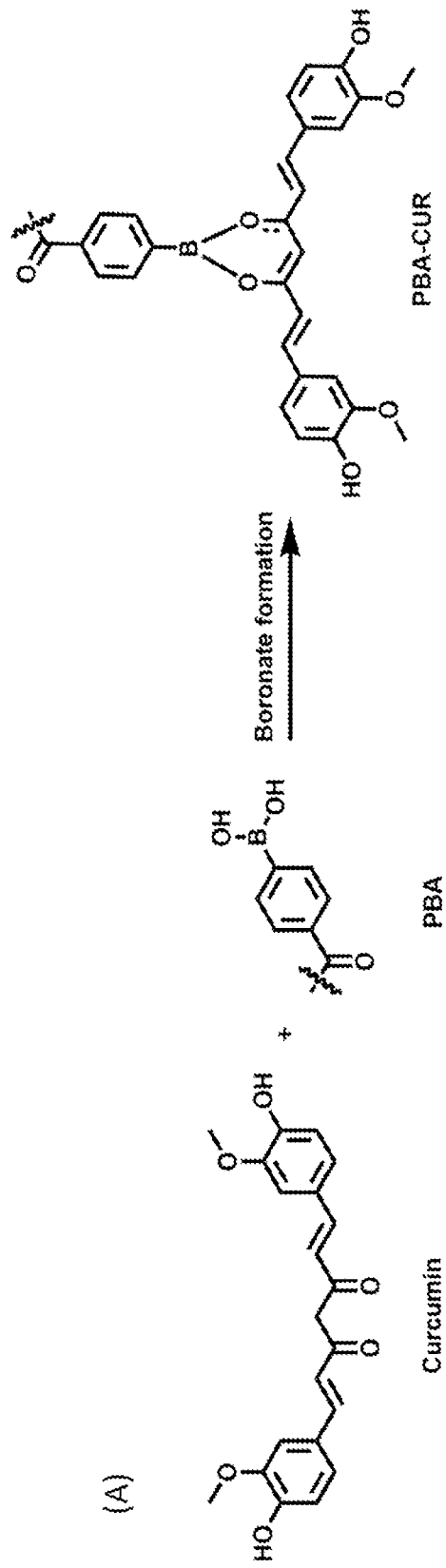


FIG. 37B

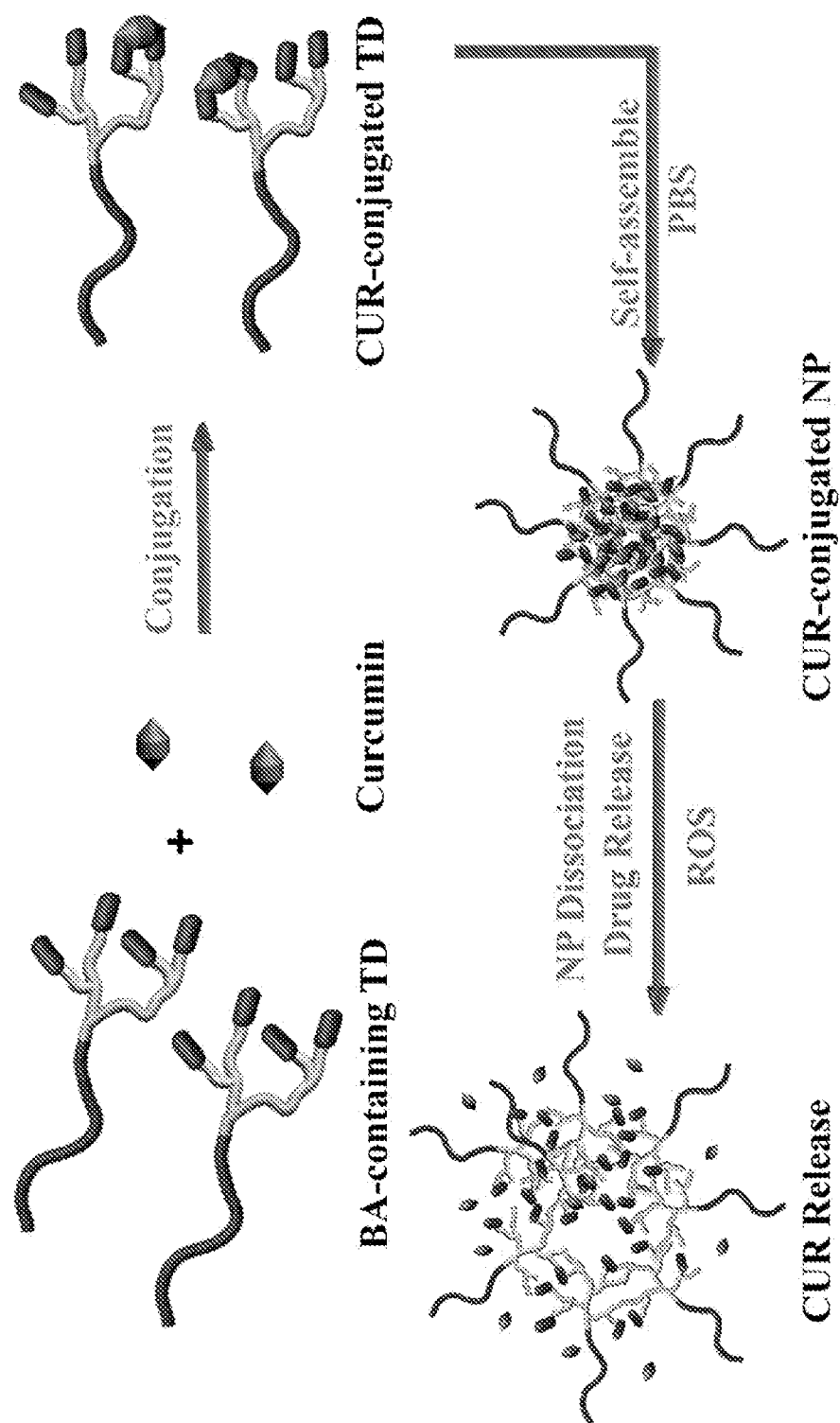


FIG. 38



FIG. 39A

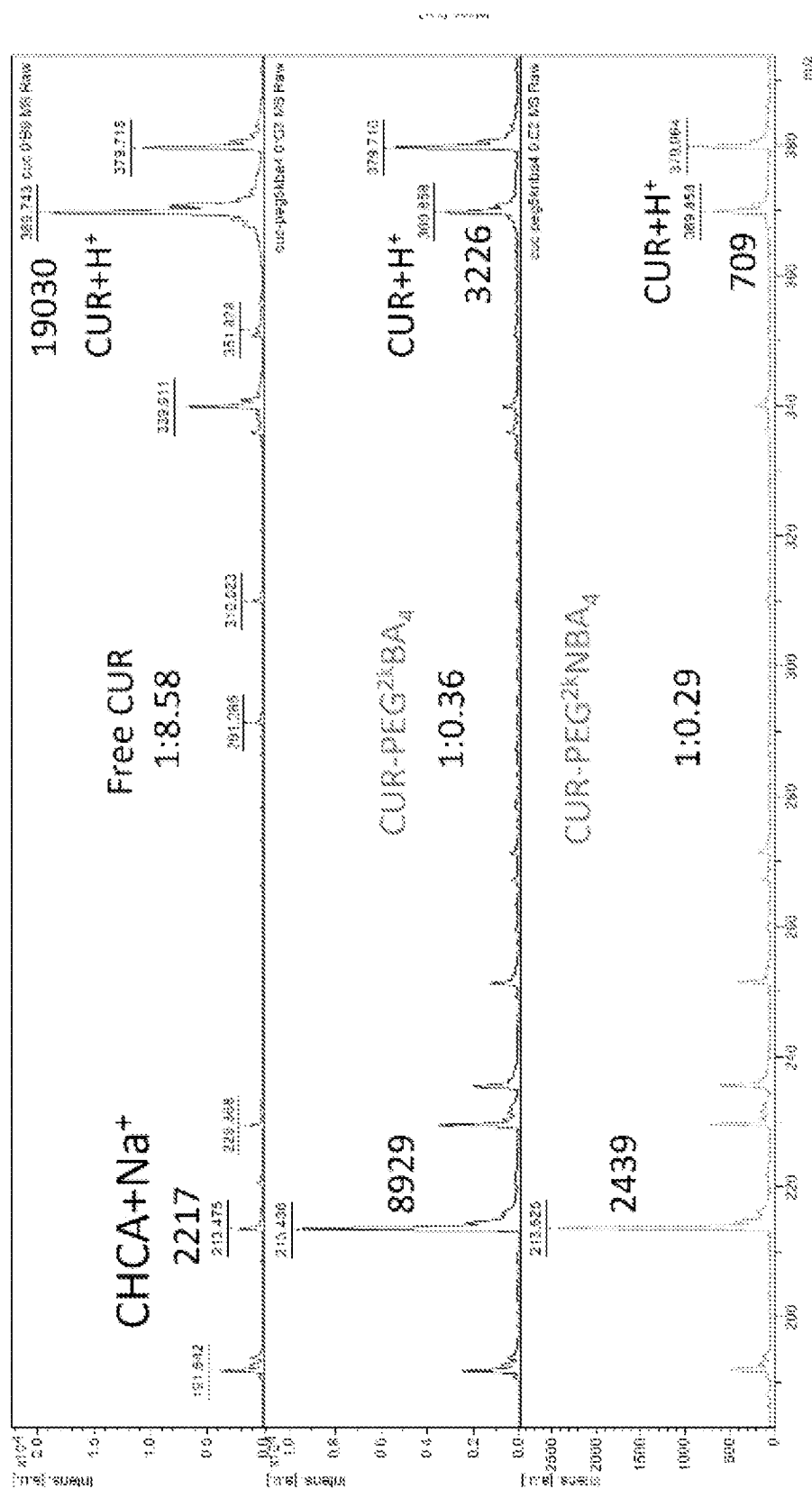


FIG. 39B

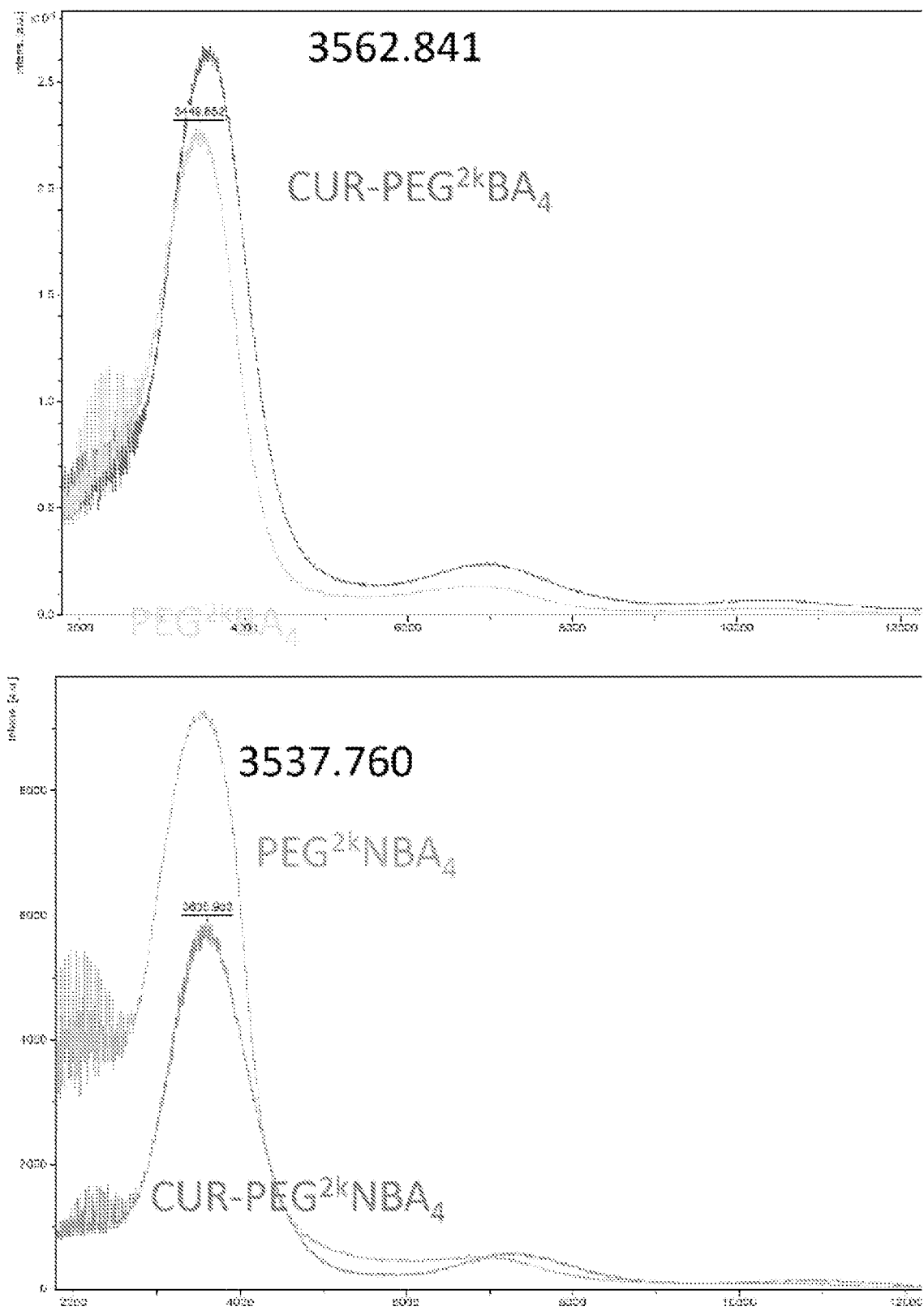
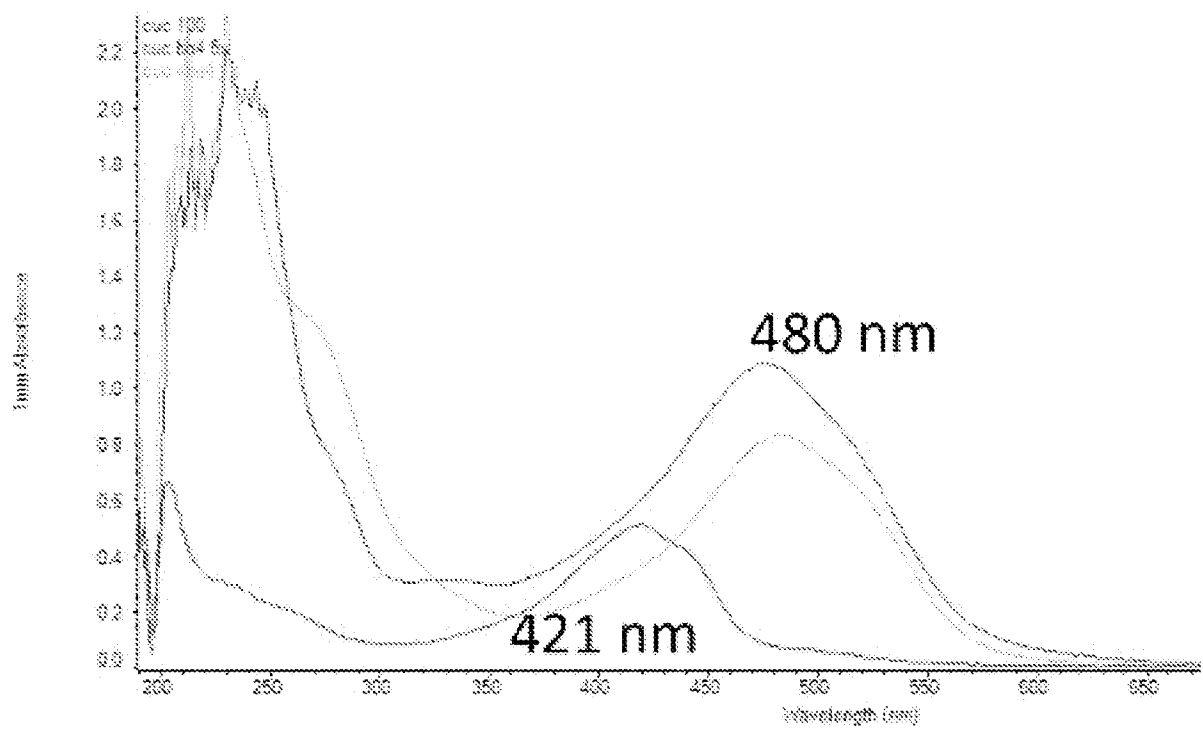


FIG. 40A



	421 nm	480 nm
Free Cur	0.452	0.168
Cur-PEG ^{2k} BA ₄	0.61	1.15
Cur-PEG ^{2k} NBA ₄	0.366	0.805

FIG. 40B

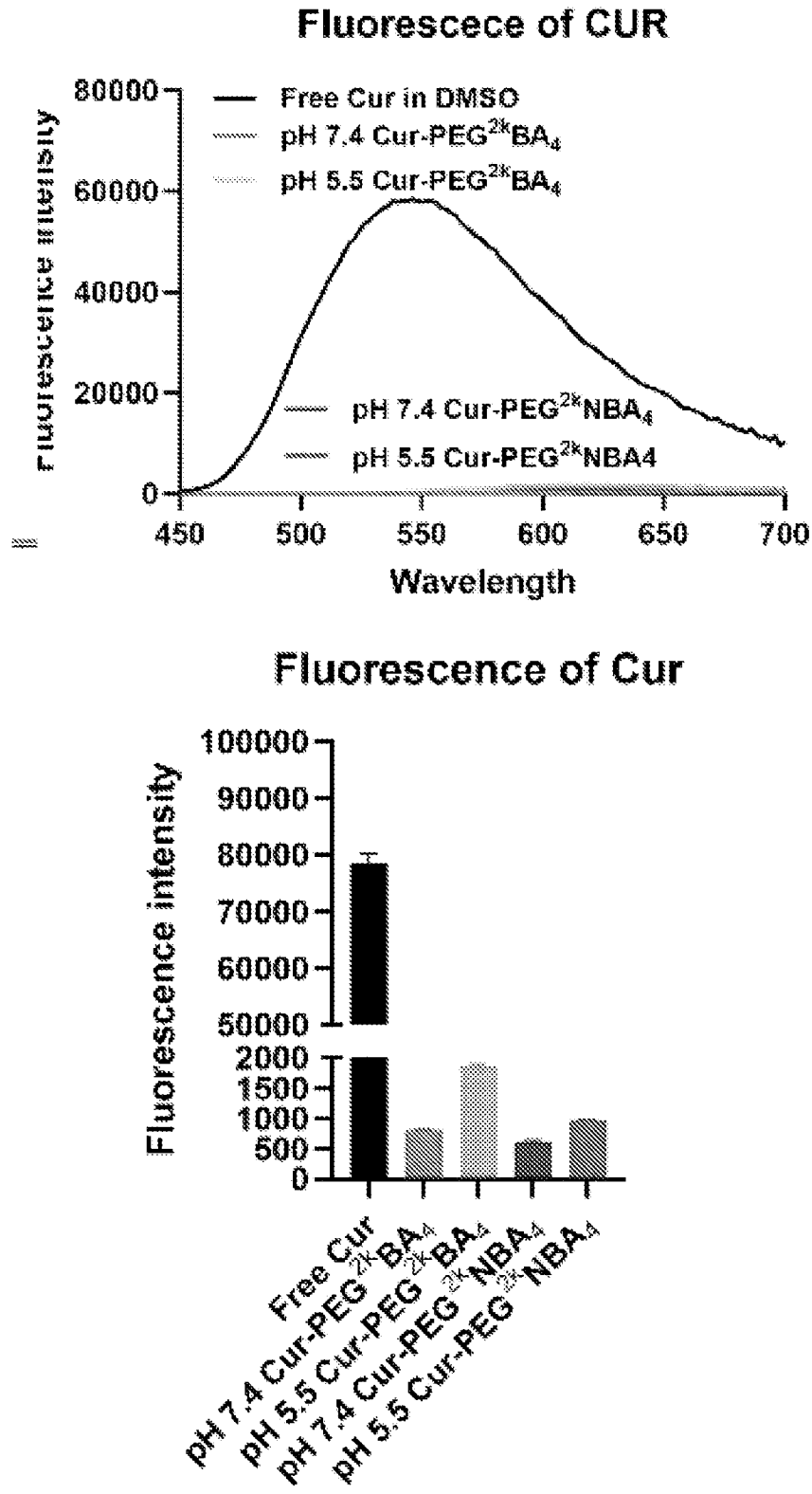


FIG. 40C

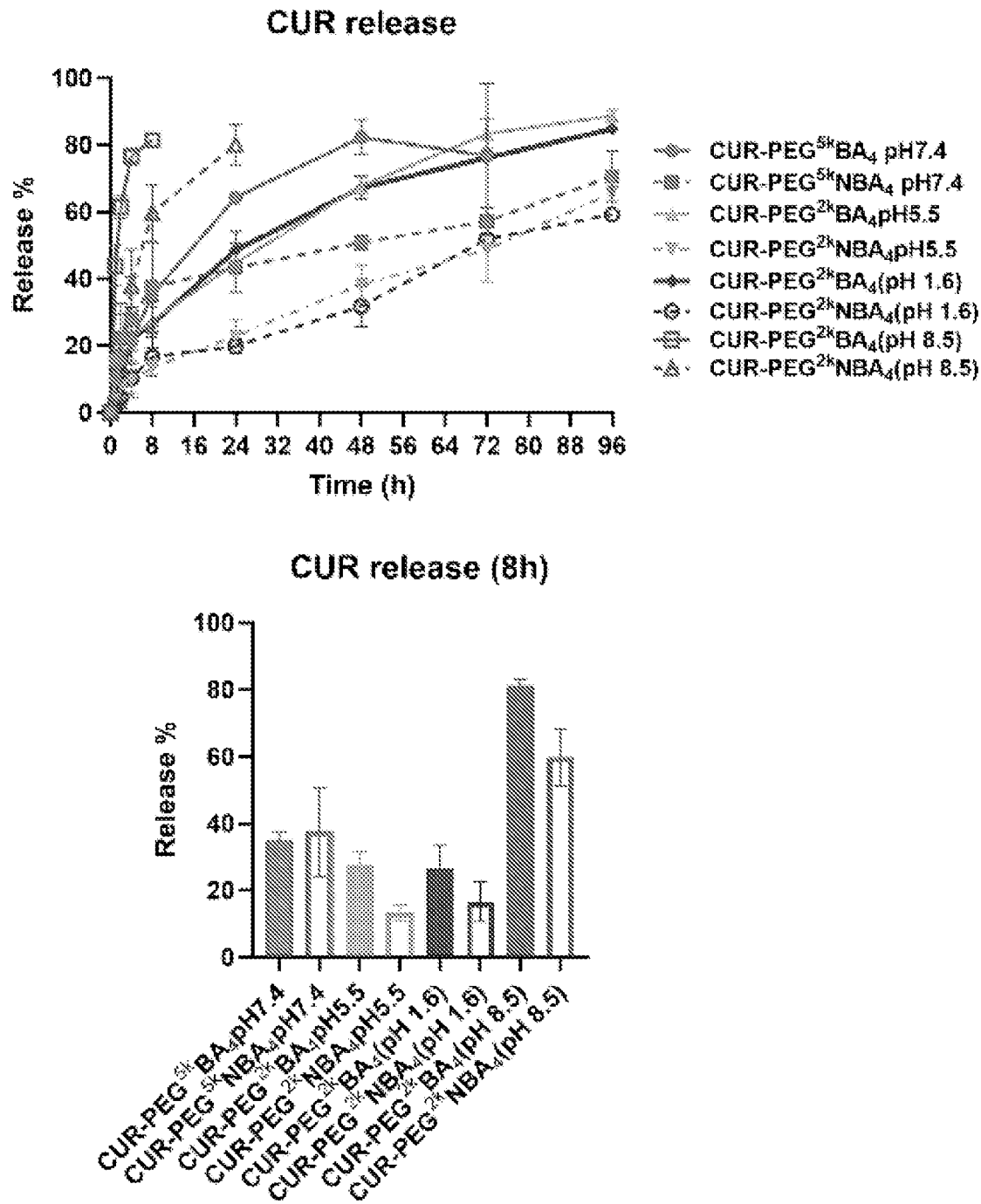


FIG. 41A

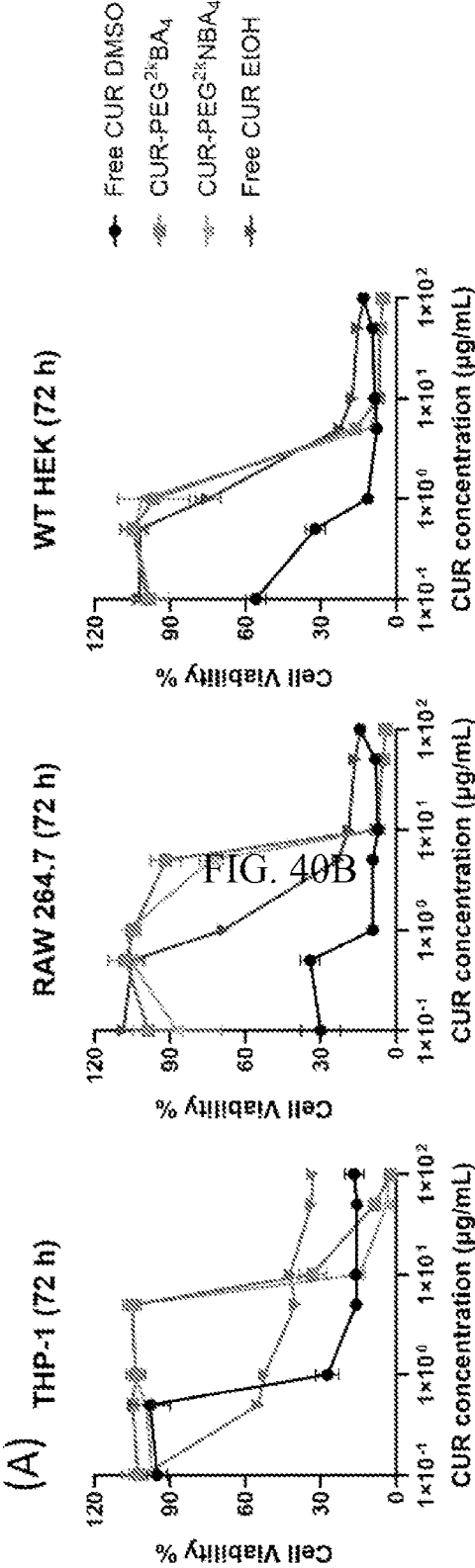


FIG. 41B

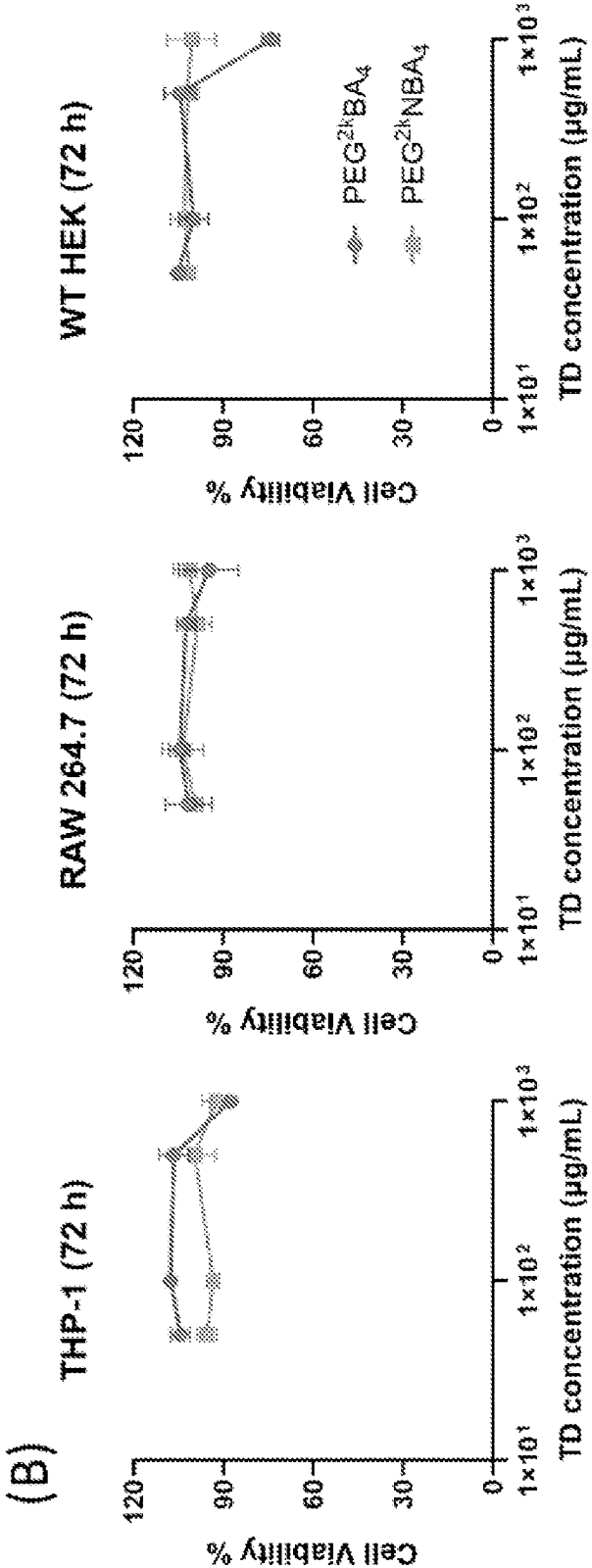


FIG. 41C

IC50 (µg/mL)	Free CUR	CUR-PEG ^{2k} BA ₄	CUR-PEG ^{2k} NBA ₄	Free CUR (EtOH)
THP-1	0.87	9.69	9.27	3.79
RAW				
264.7	0.006	7.06	6.07	2.49
HEK293	0.13	2.96	3.10	2.55

FIG. 42A

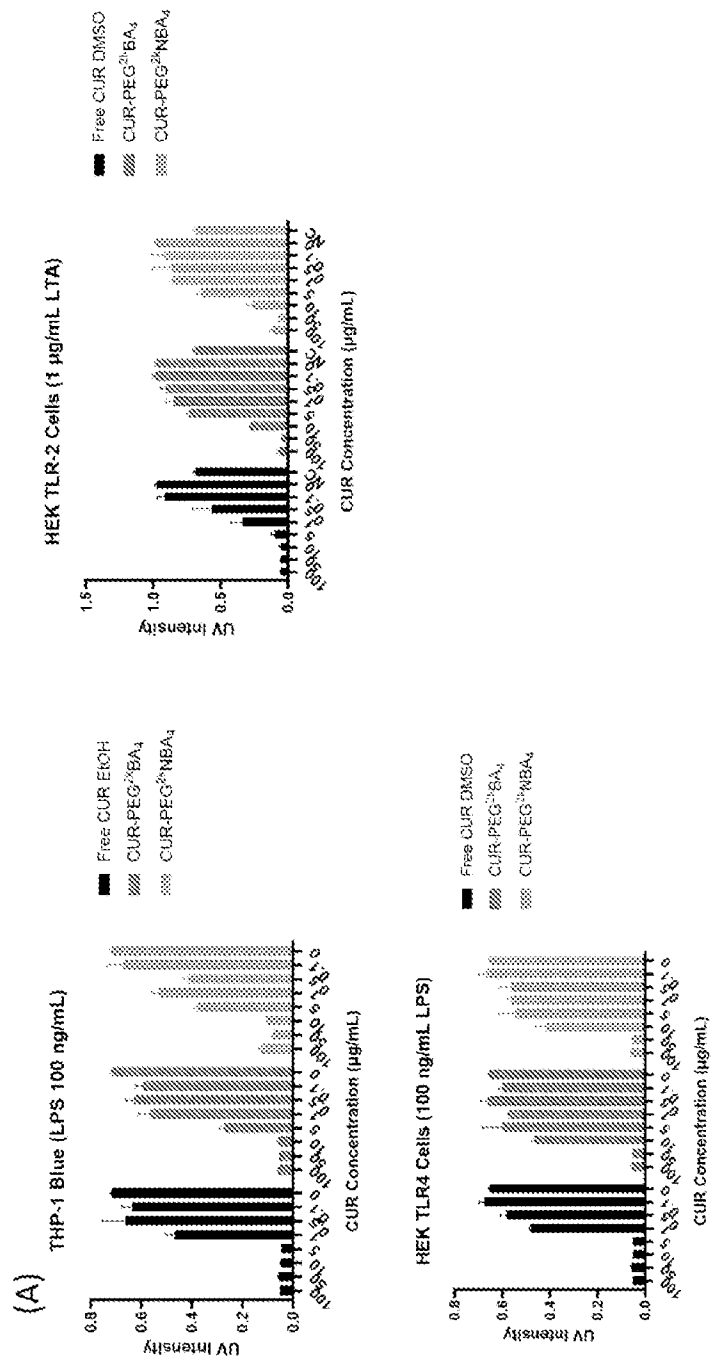
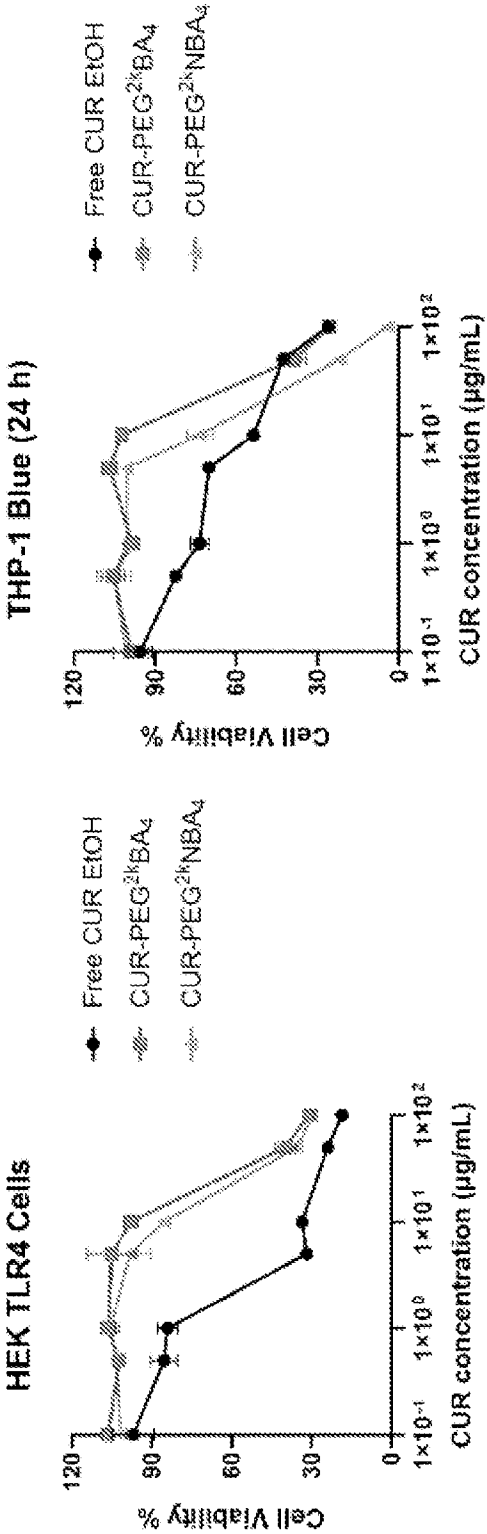


FIG. 42B



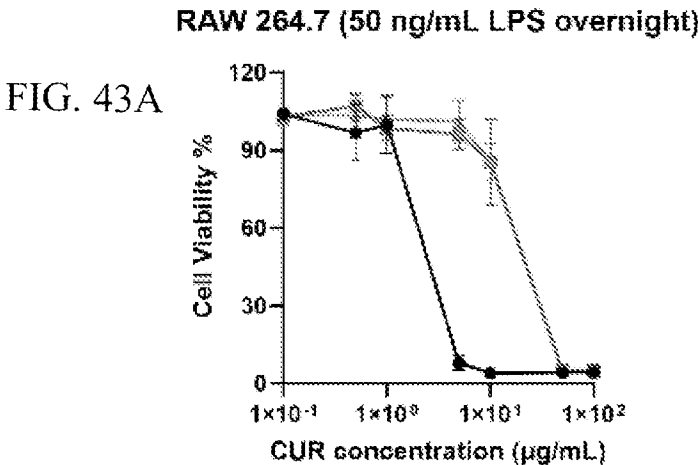


FIG. 43B

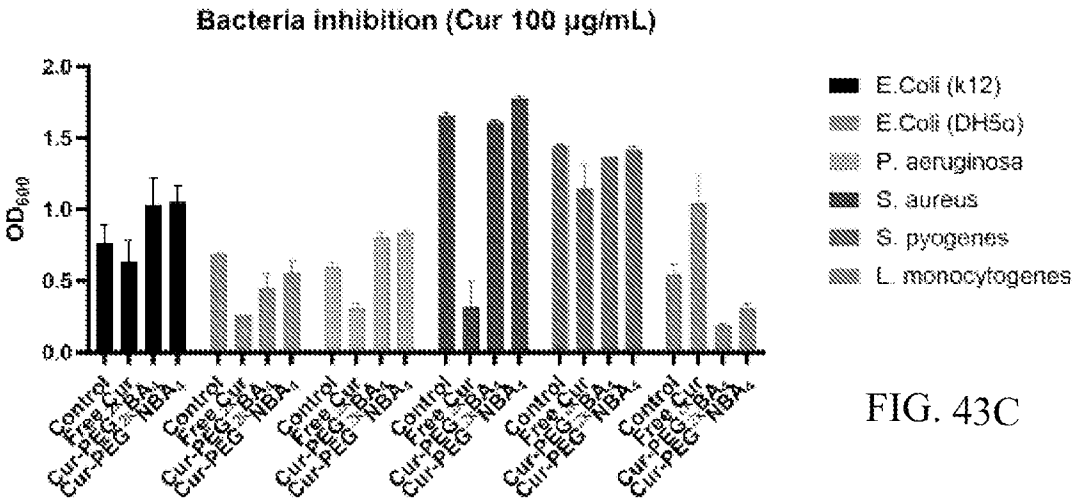
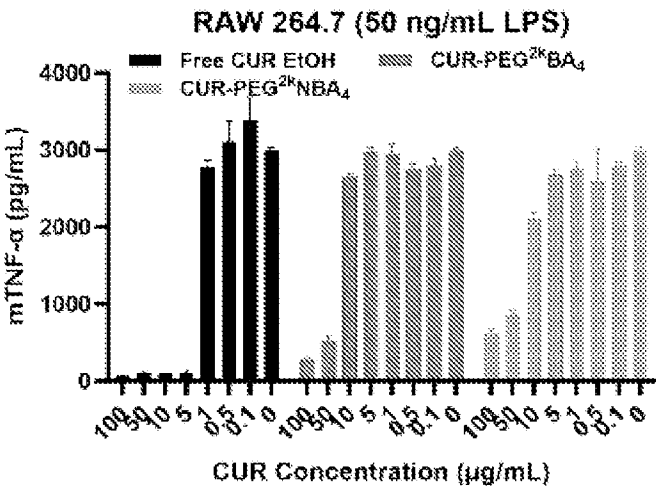
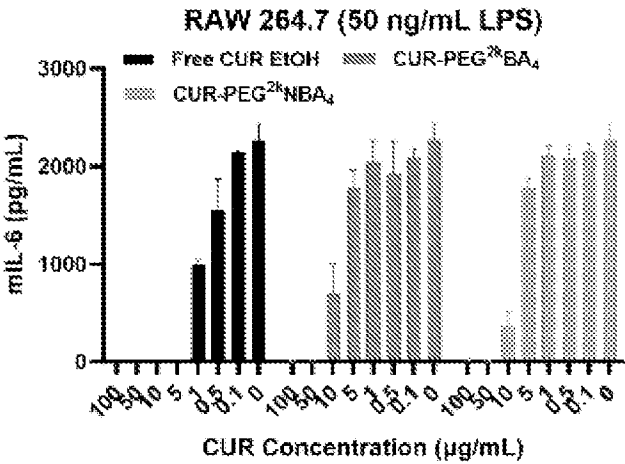
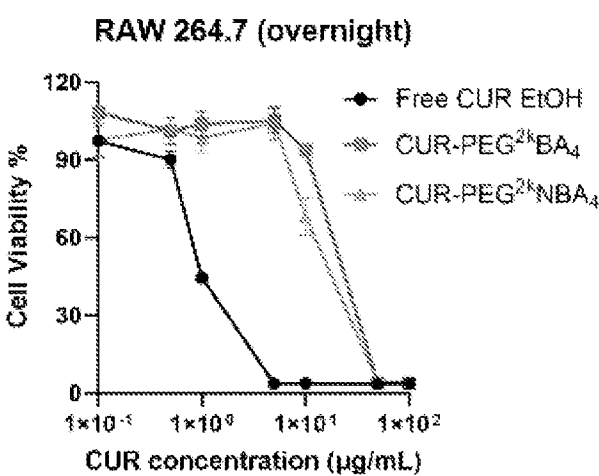


FIG. 43C

FIG. 44A

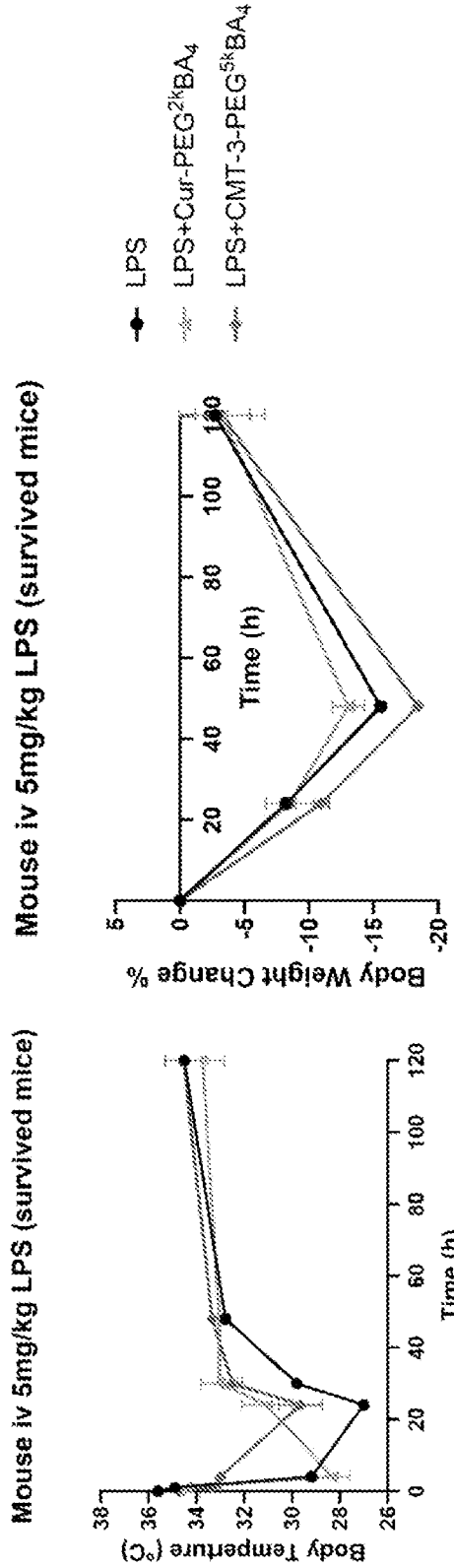


FIG. 44B

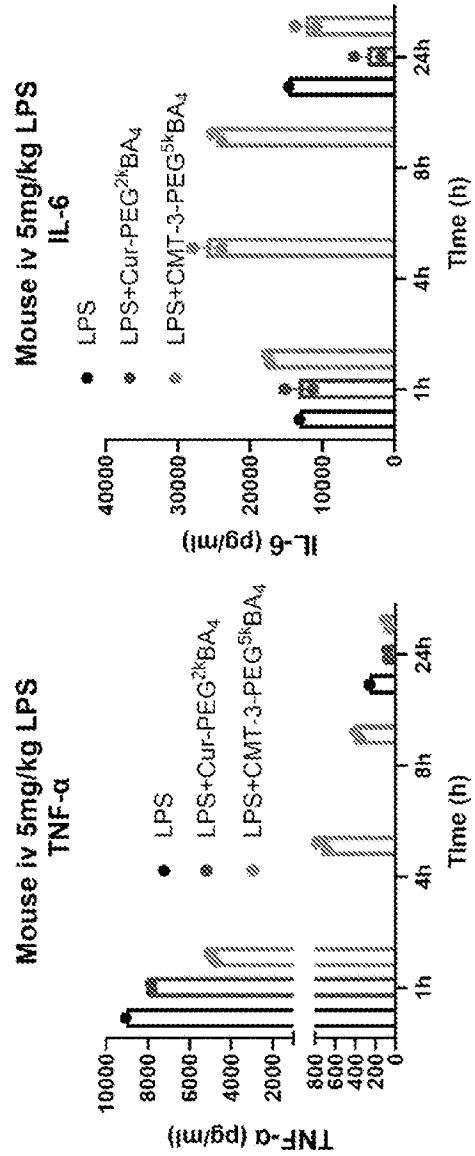


FIG. 45

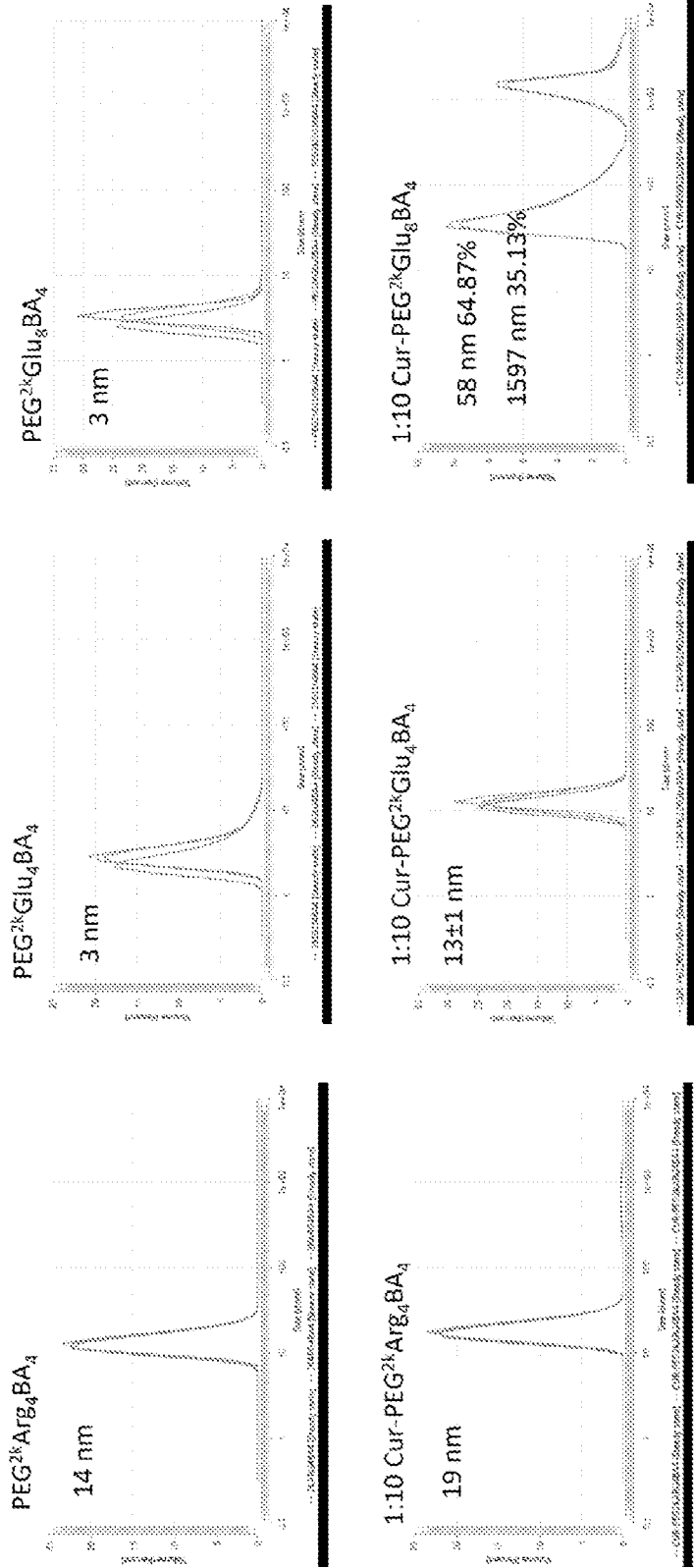


FIG. 46A

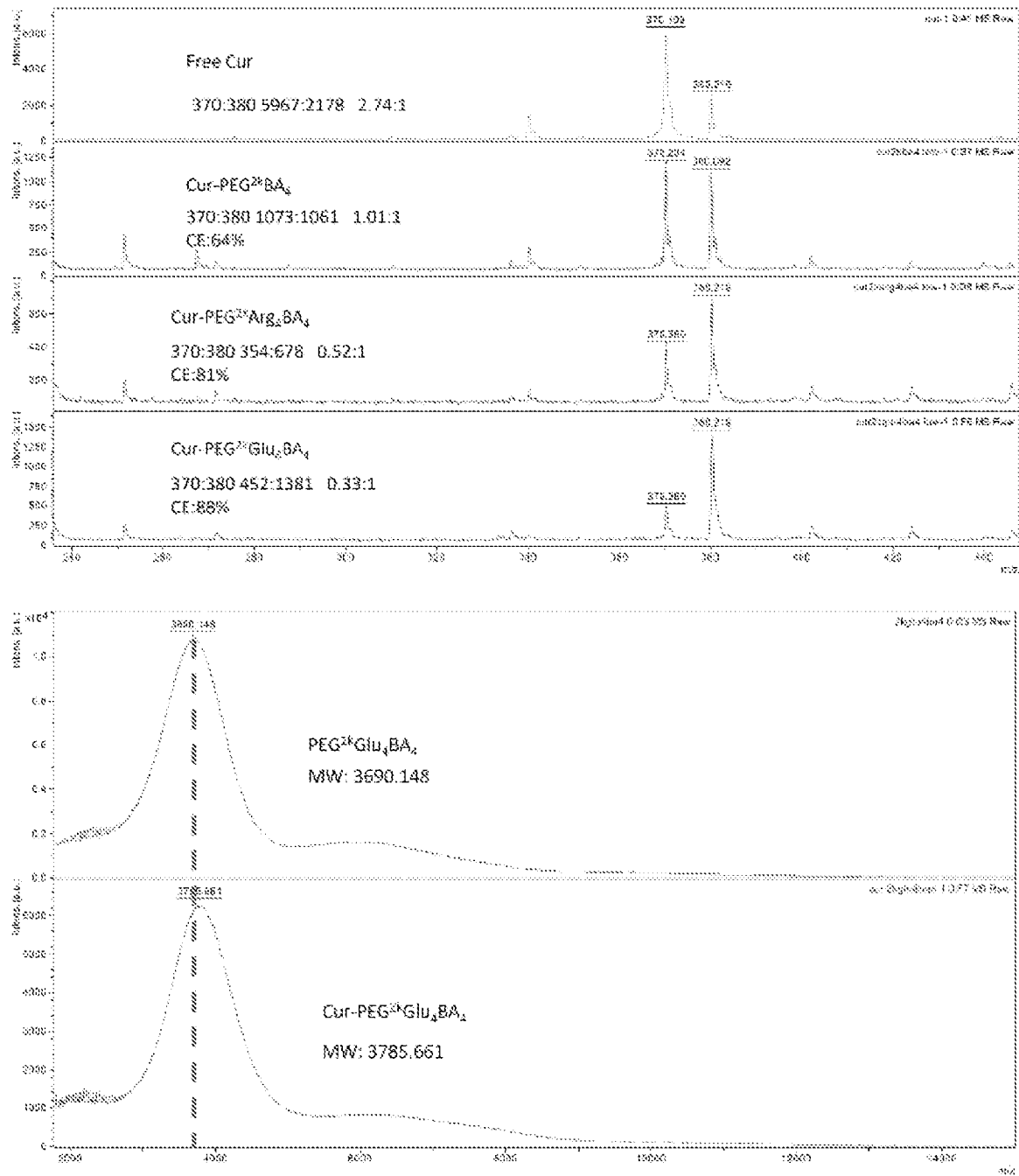


FIG. 46B

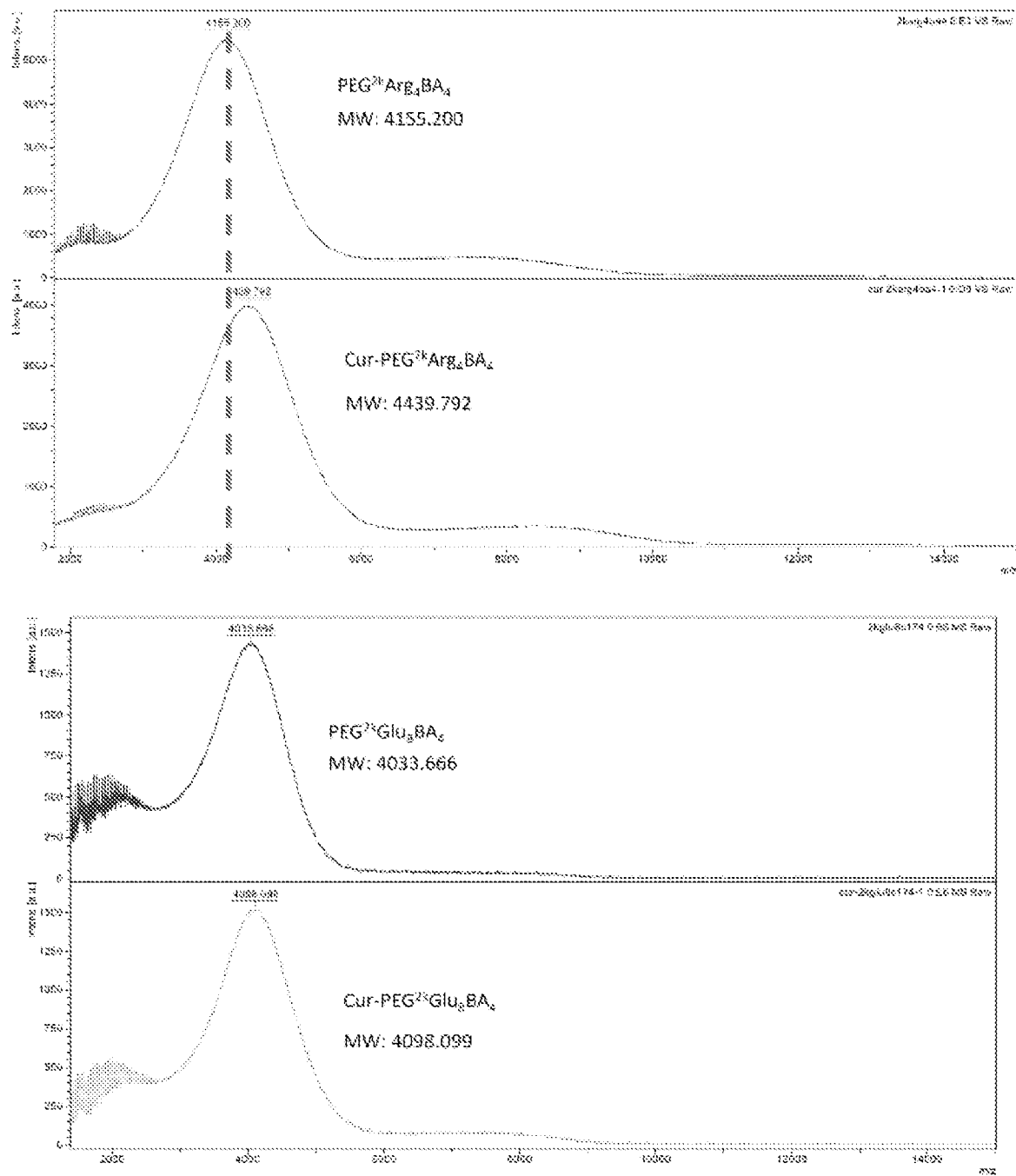


FIG. 47A

	421 nm	442 nm	470 nm	480 nm
Cur-PEG ^{2k} Arg ₄ BA ₄	0.733	0.873	1.017	1.039
Cur-PEG ^{2k} Glu ₄ BA ₄	0.908	0.981	0.896	0.828
Cur-PEG ^{2k} Glu ₈ BA ₄	0.737	0.68	0.537	0.455

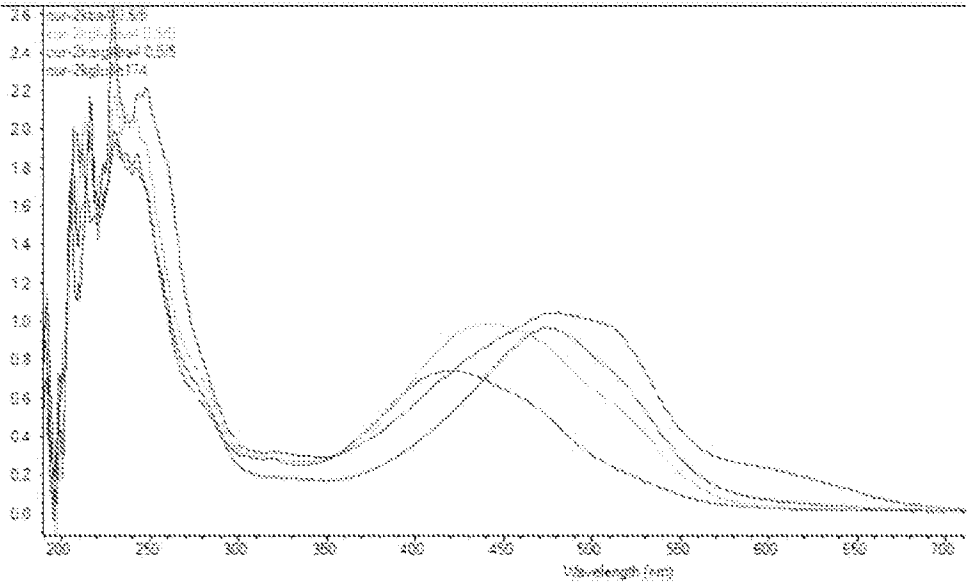


FIG. 47B

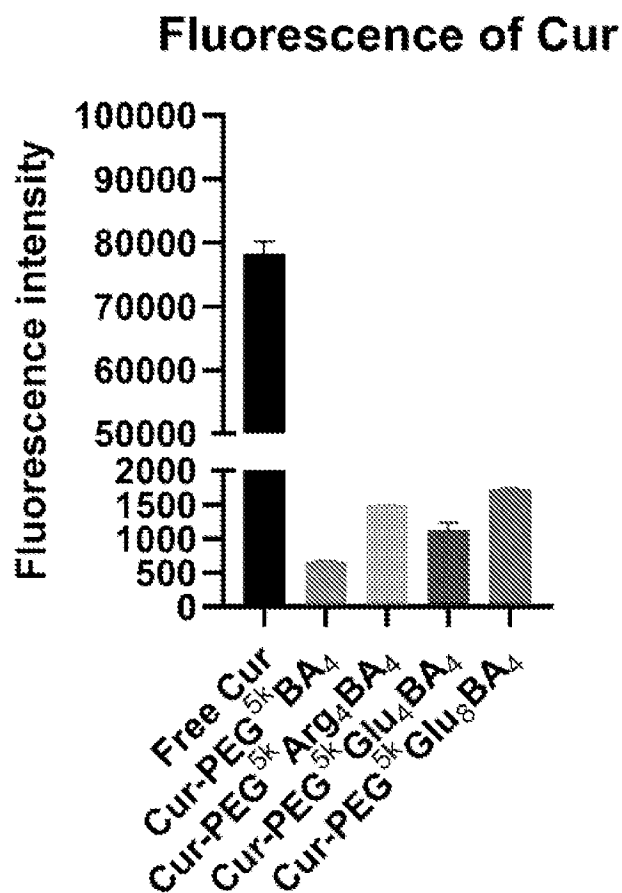
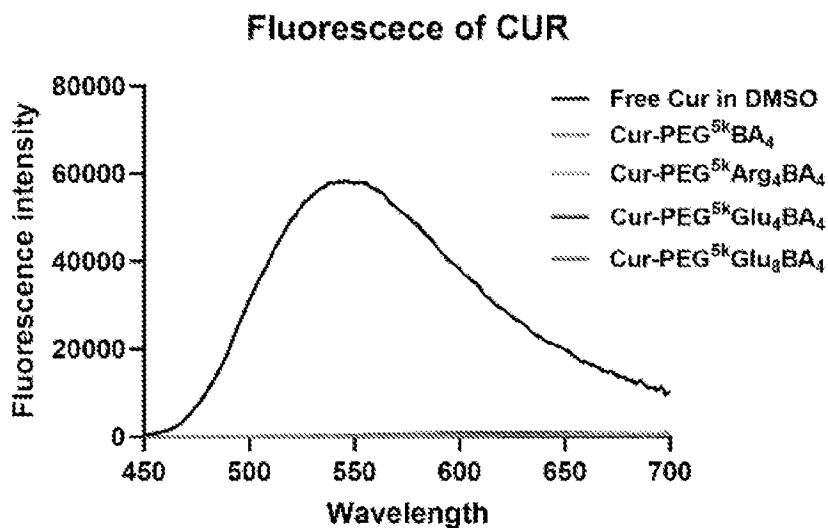
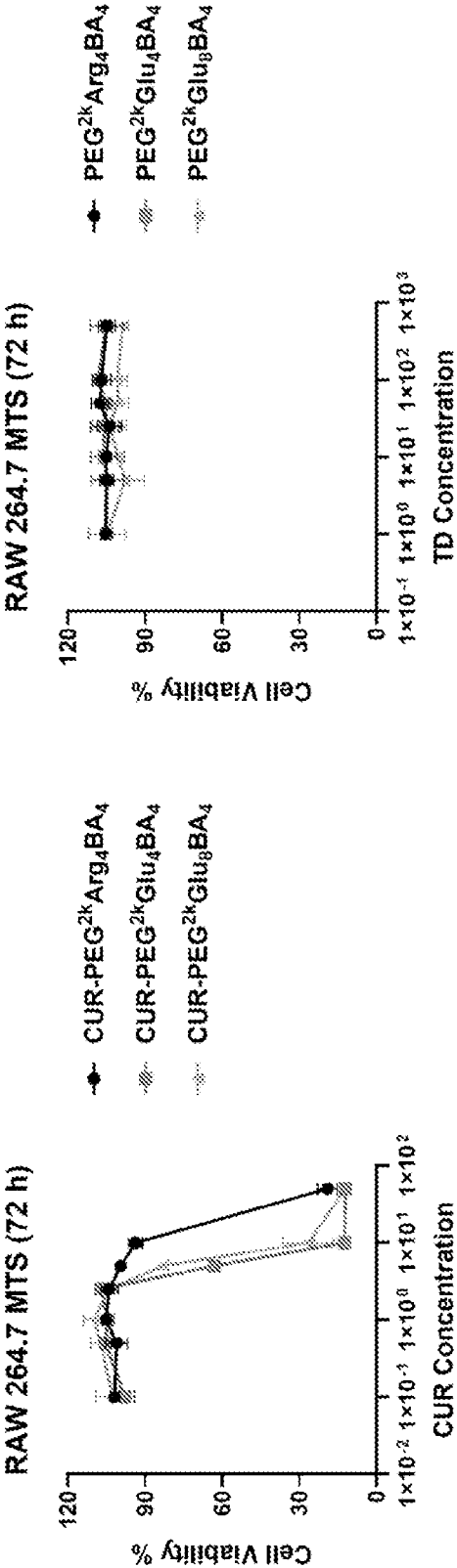


FIG. 48



IC50 (µg/mL)	CUR-PEG ^{2k} Arg ₄ BA ₄	CUR-PEG ^{2k} Glu ₄ BA ₄	CUR-PEG ^{2k} Glu ₆ BA ₄
RAW 264.7	28.76	5.826	7.667

FIG. 49A

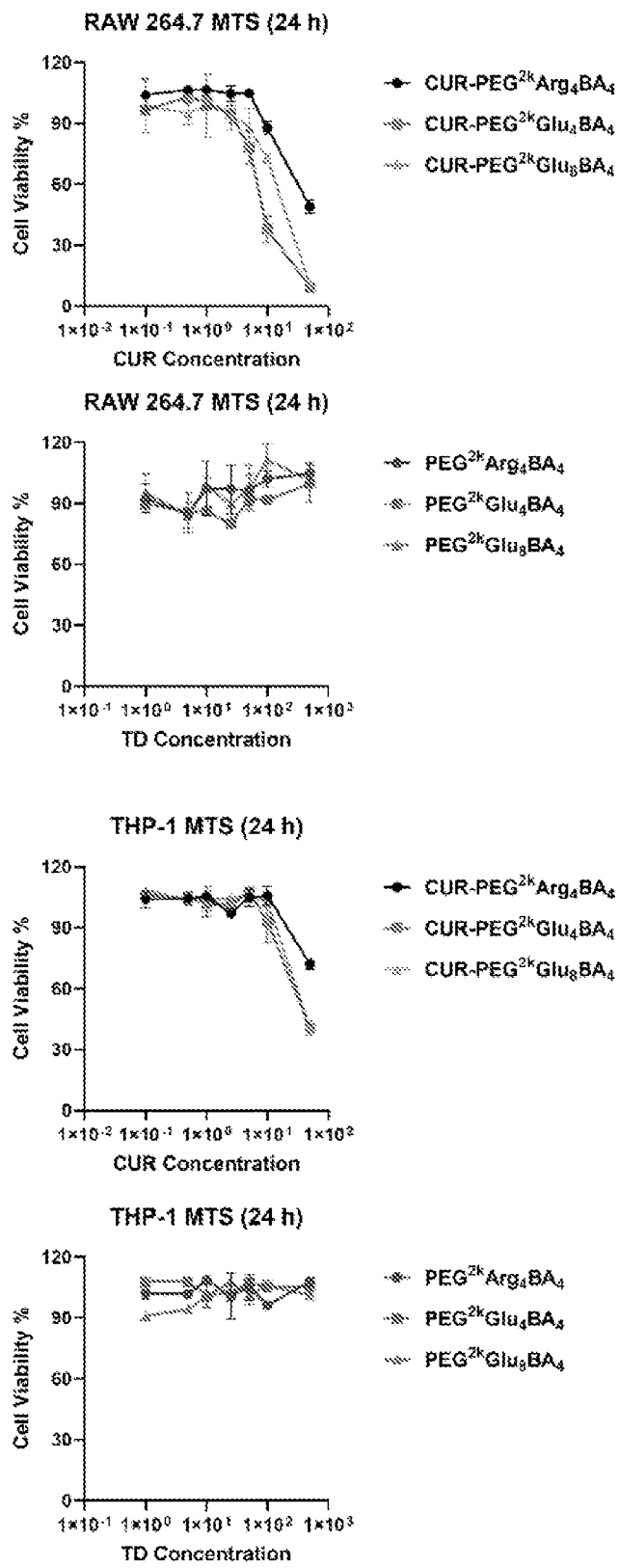


FIG. 49B

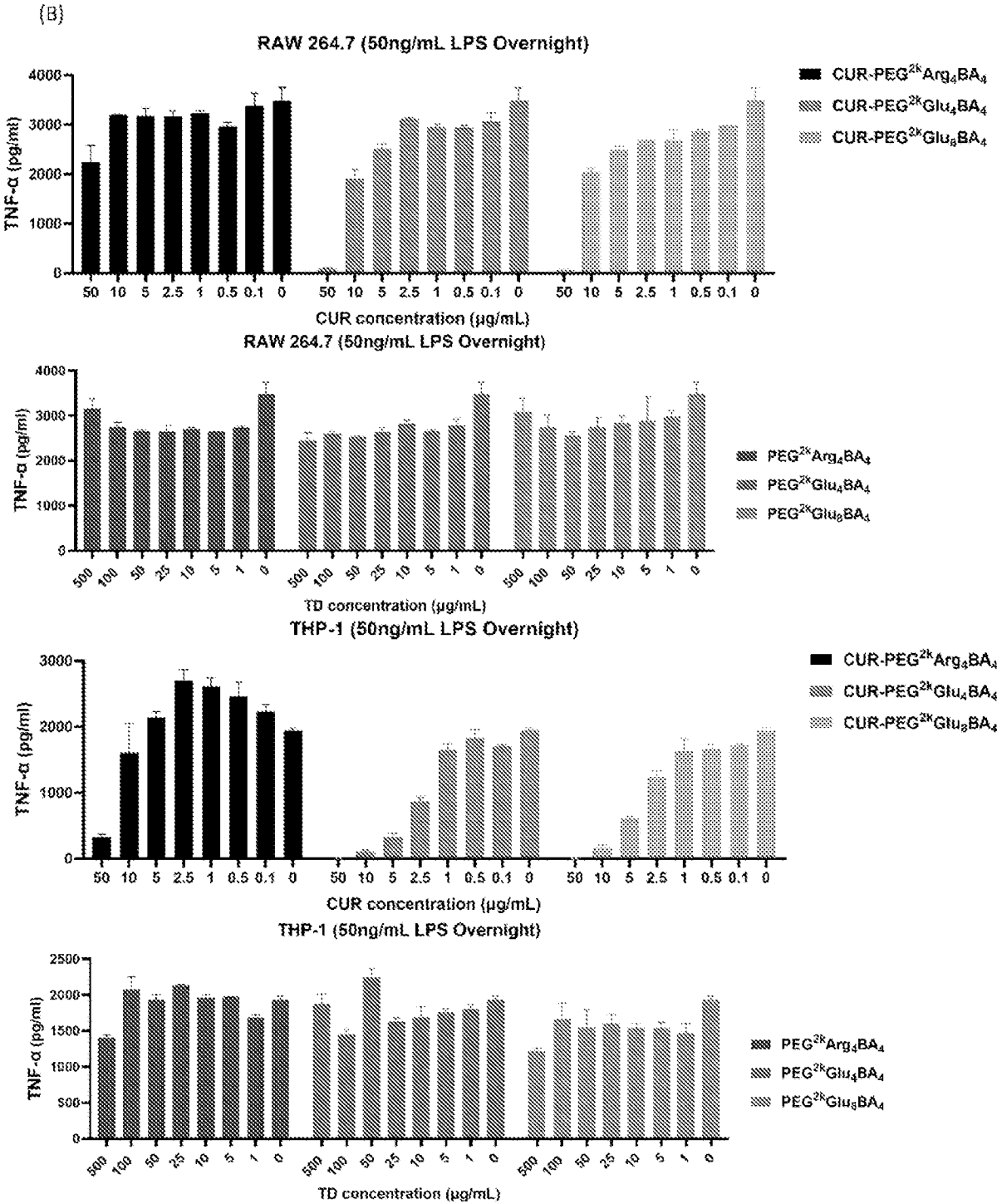


FIG. 50

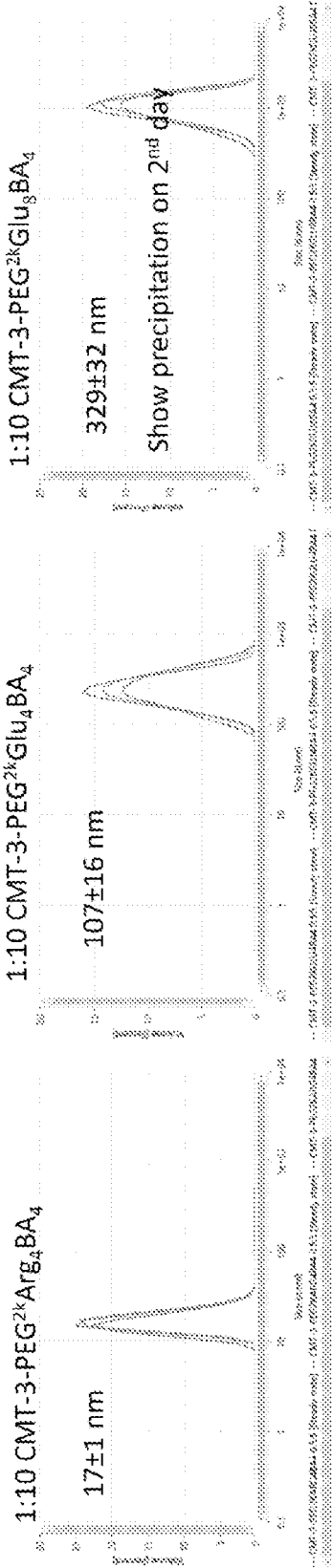


FIG. 51A

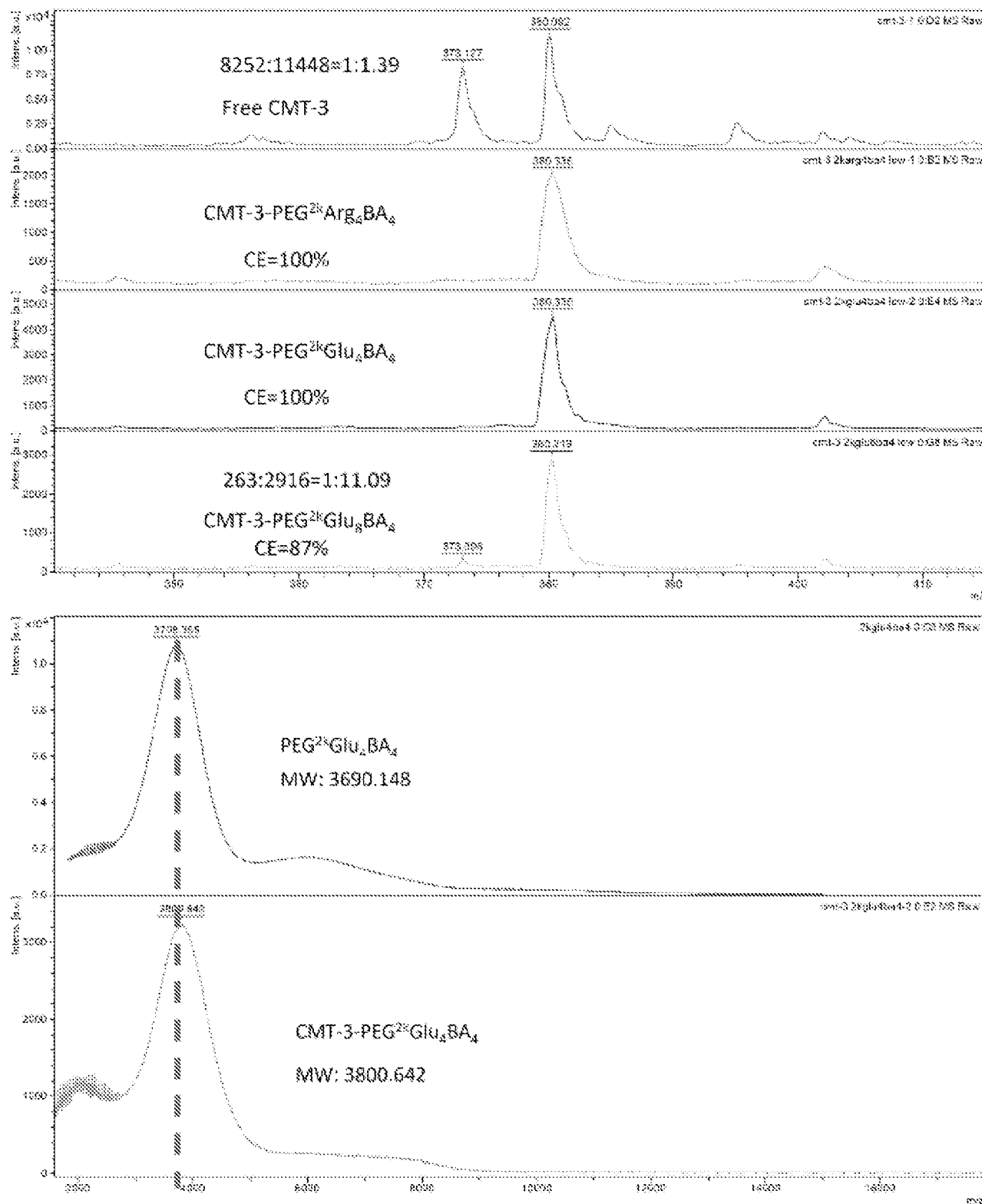


FIG. 51B

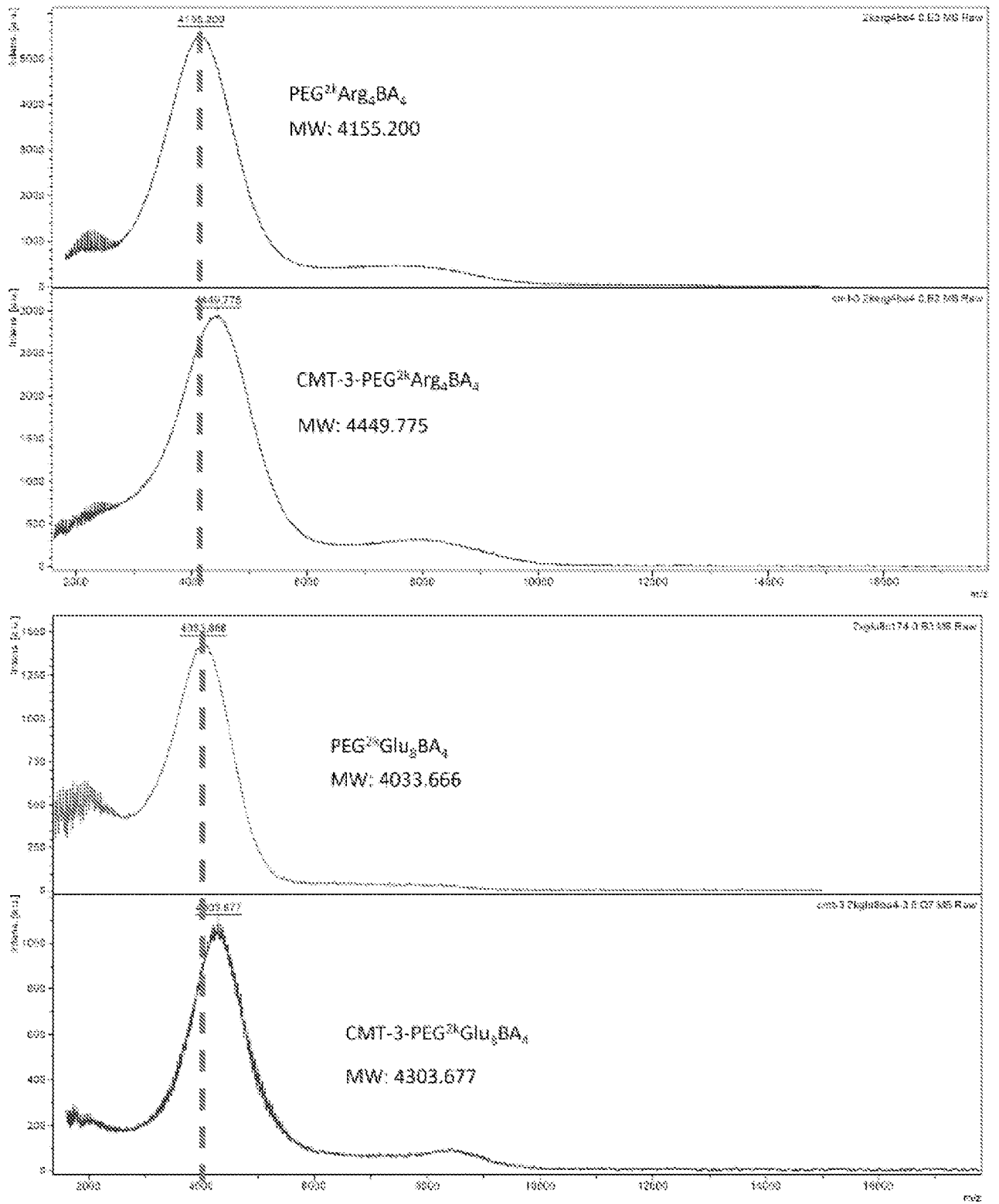


FIG. 52

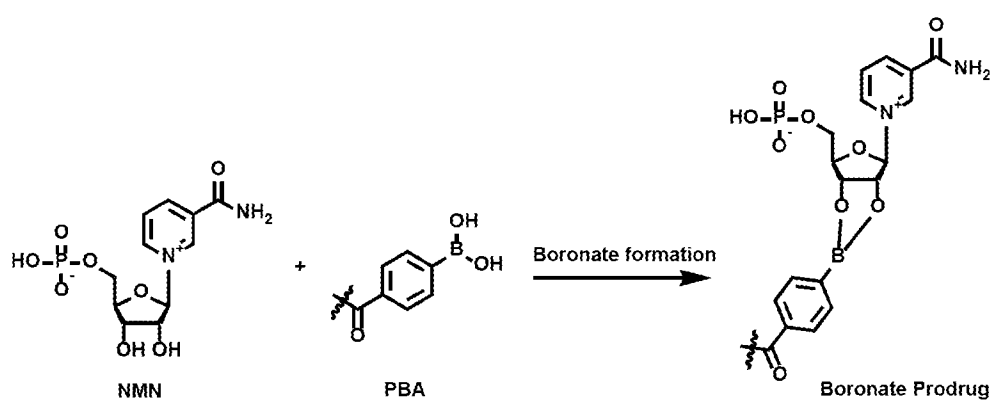


FIG. 53

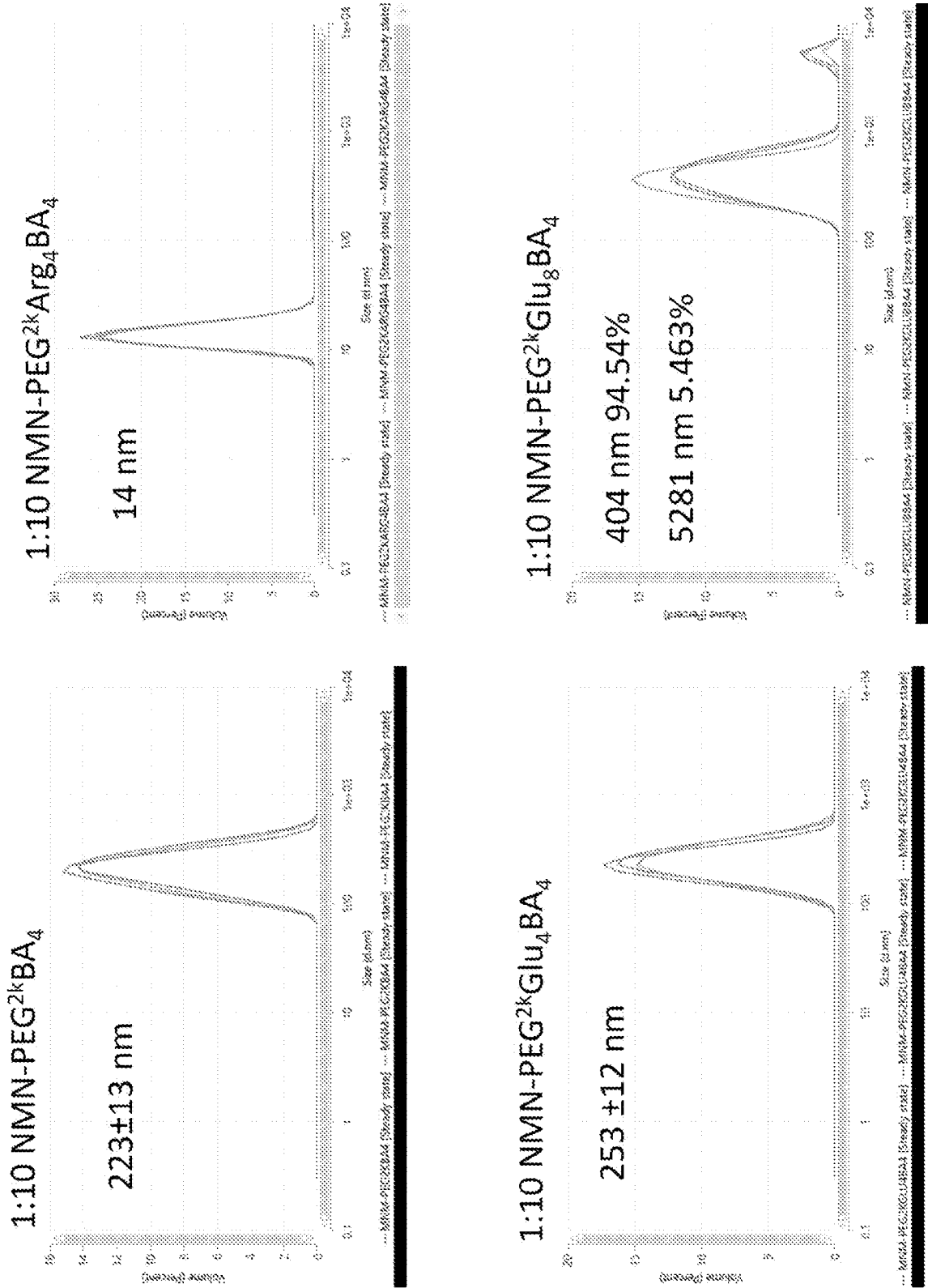


FIG. 54A

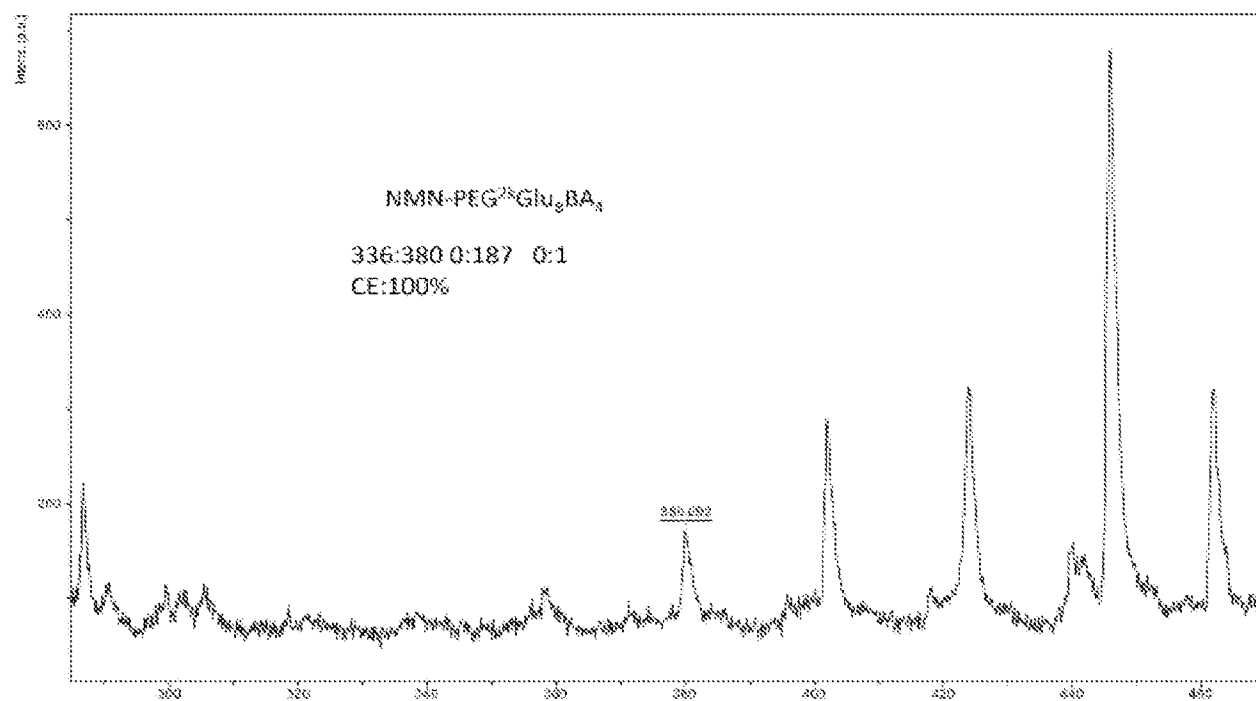
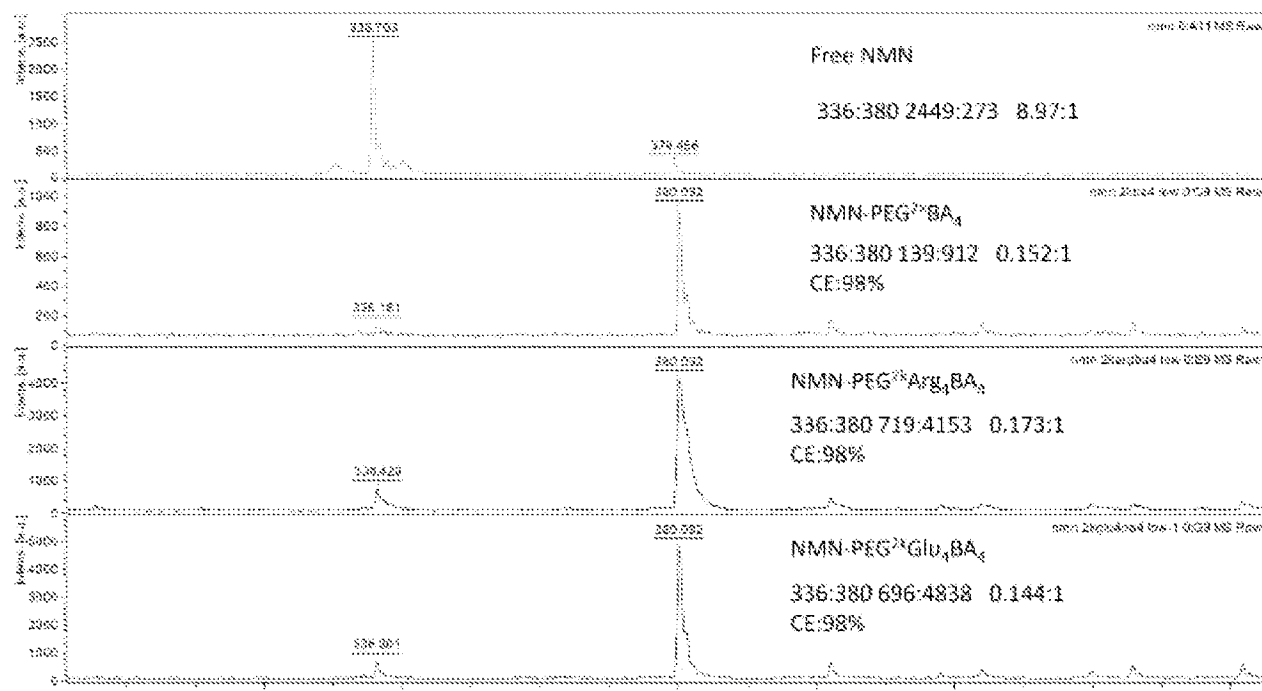


FIG. 54B

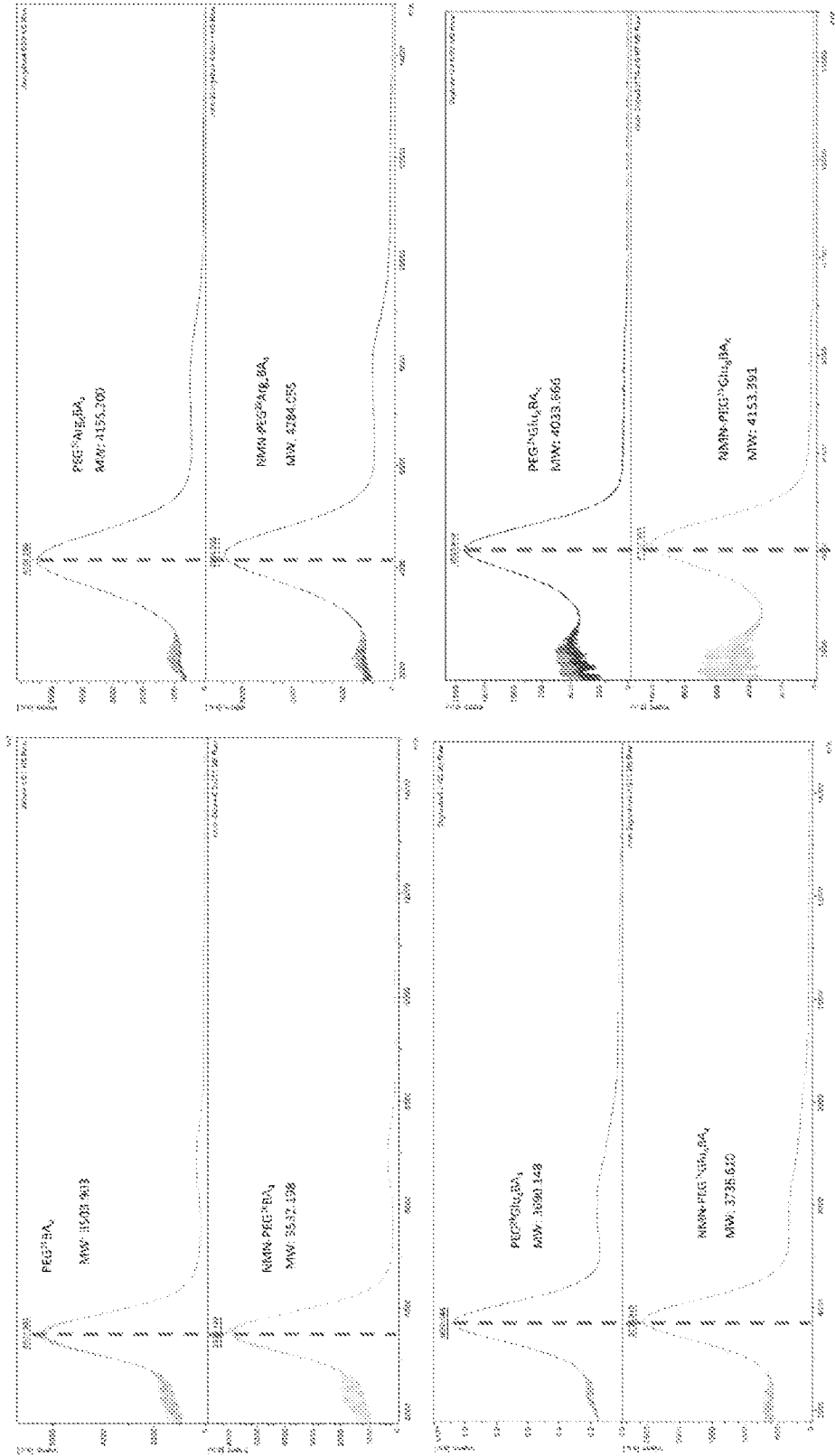


FIG. 55A

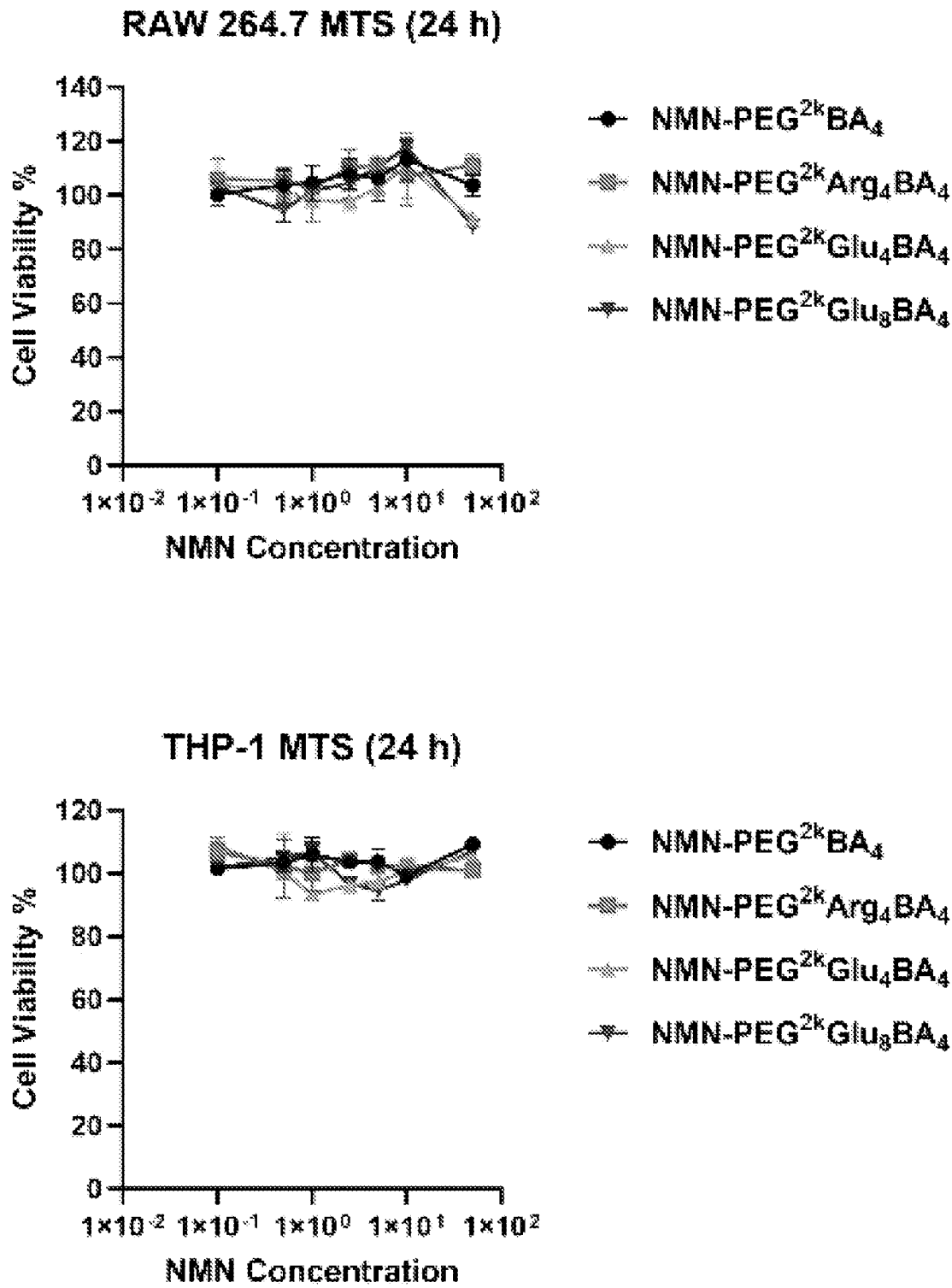


FIG. 55B

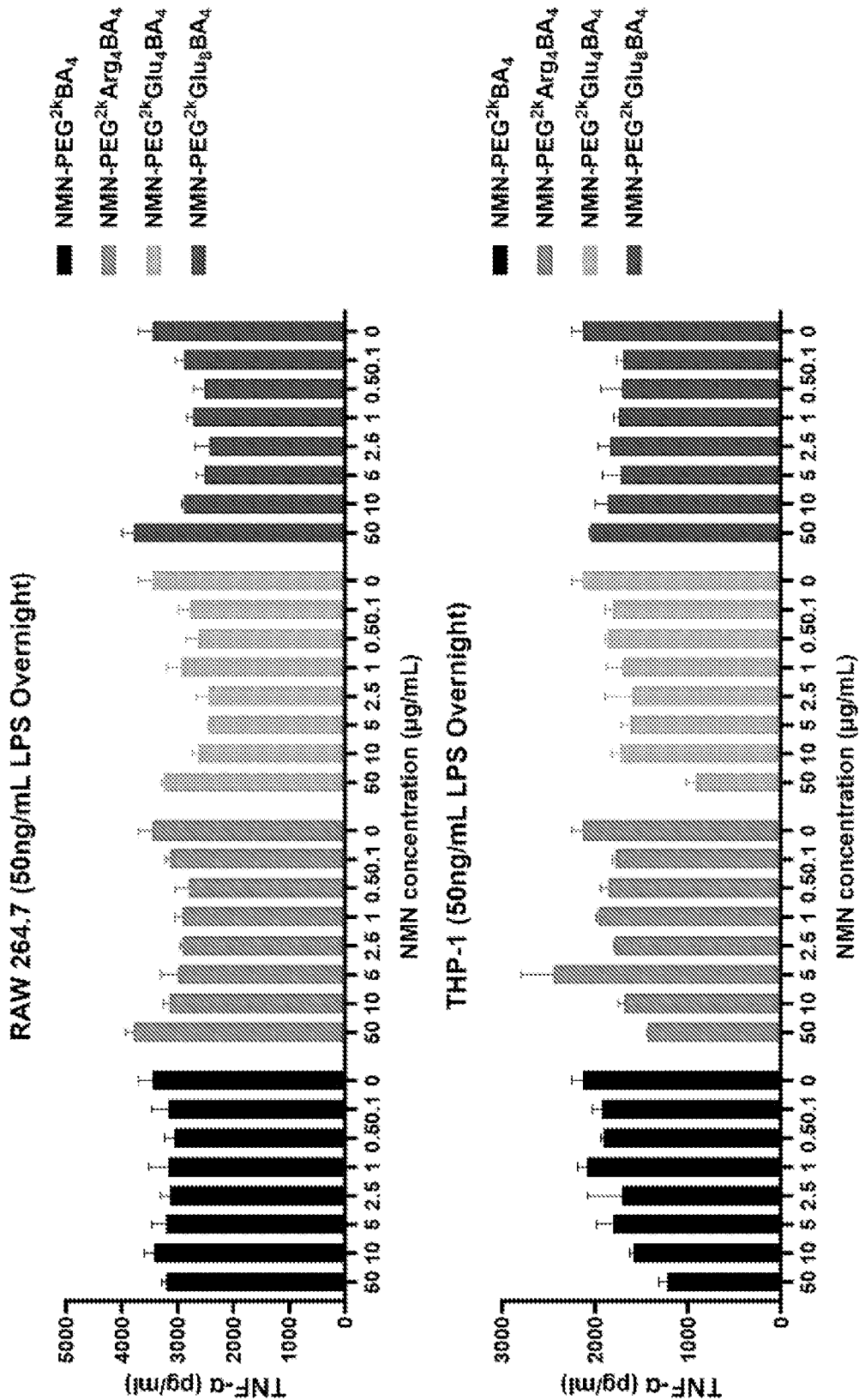


FIG. 55C

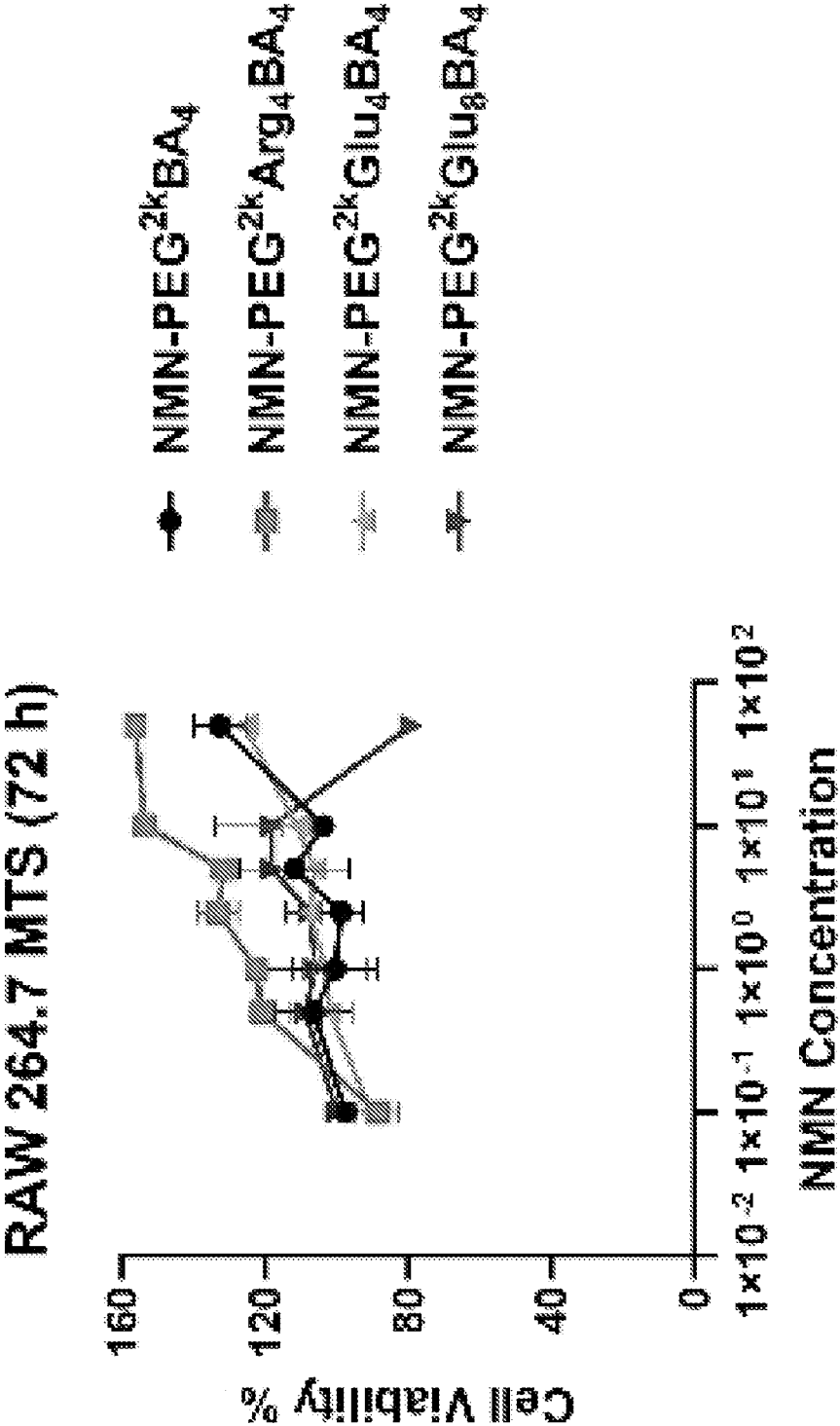


FIG. S1A

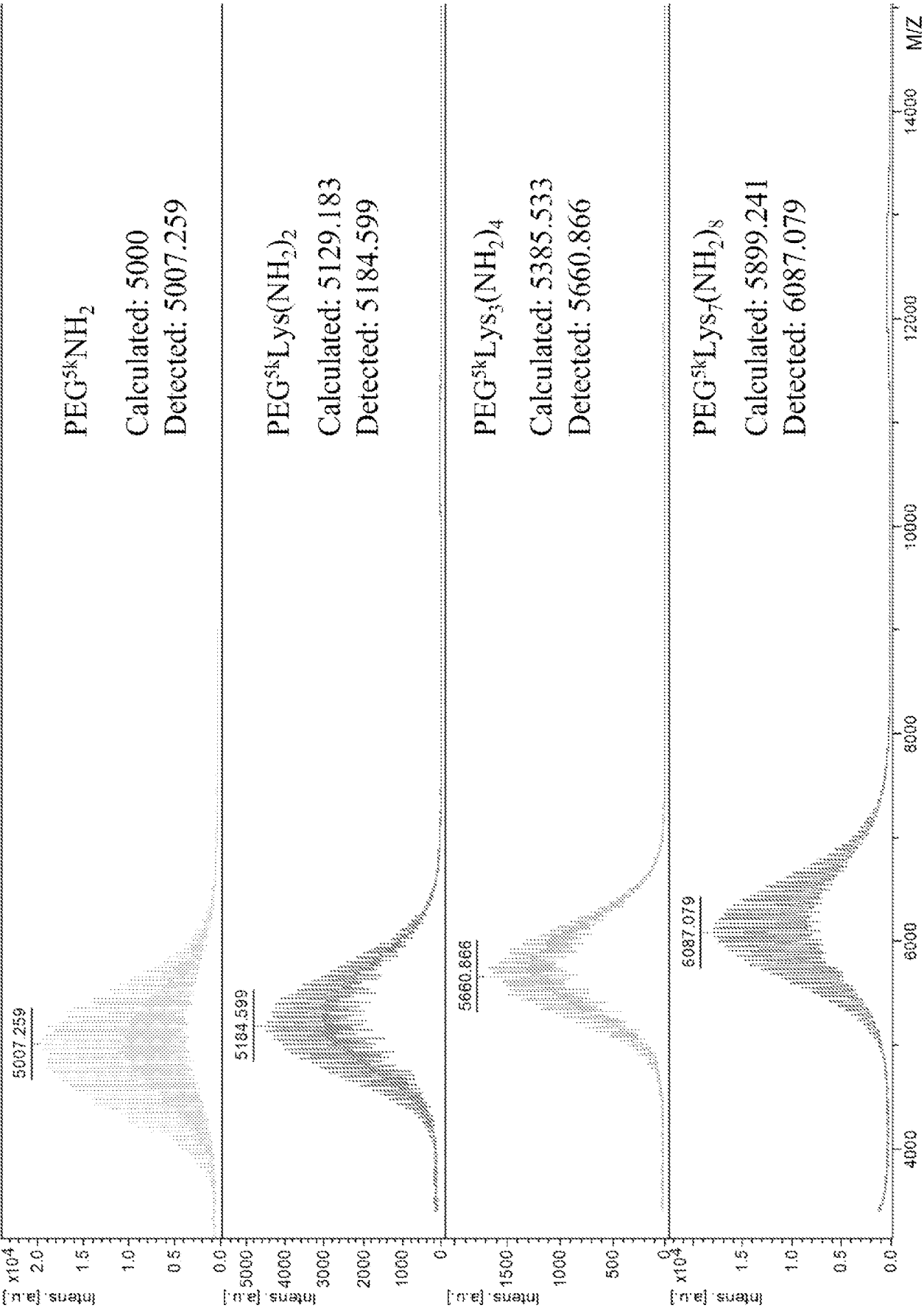


FIG. S1A

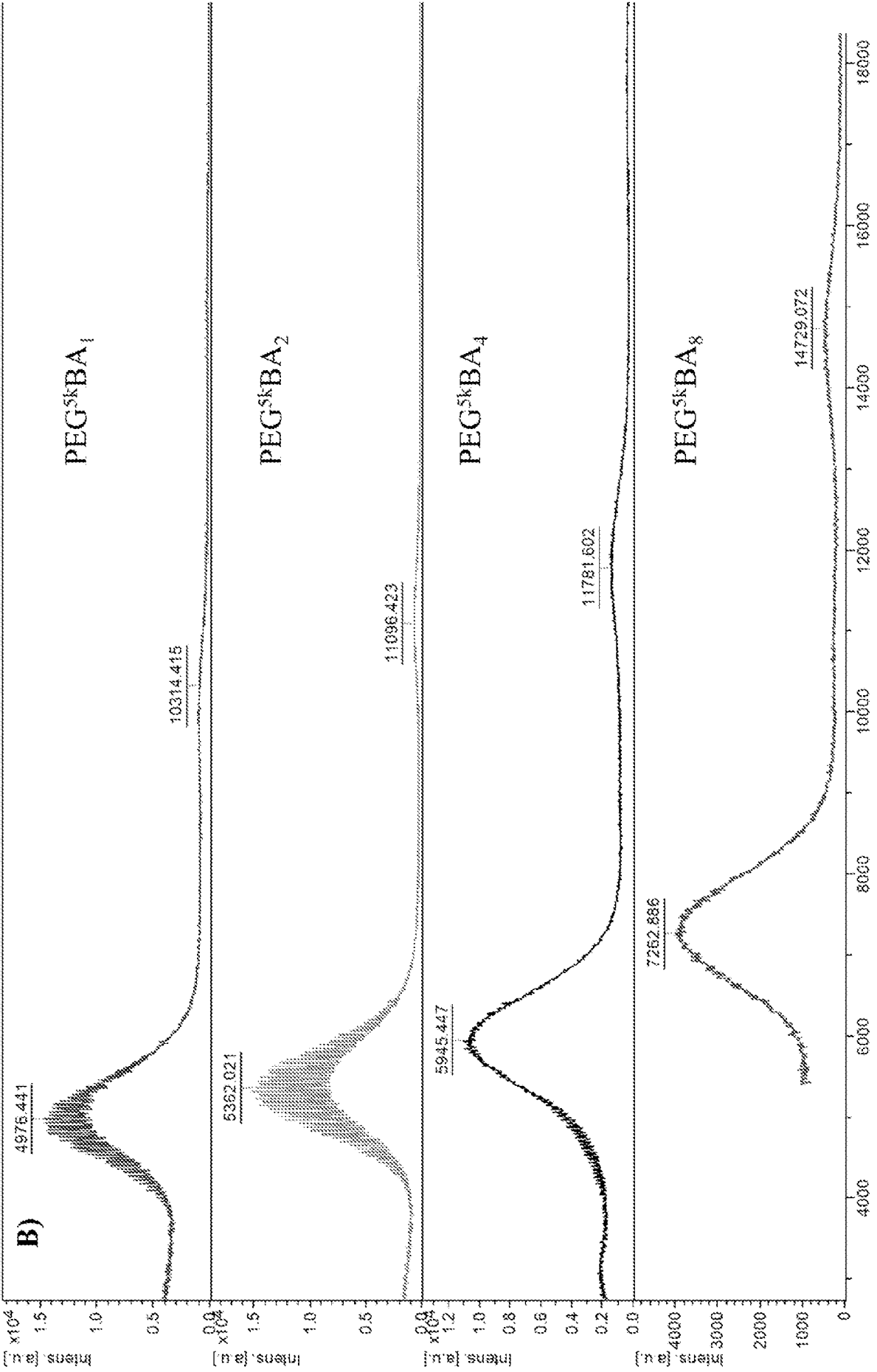


FIG. S2

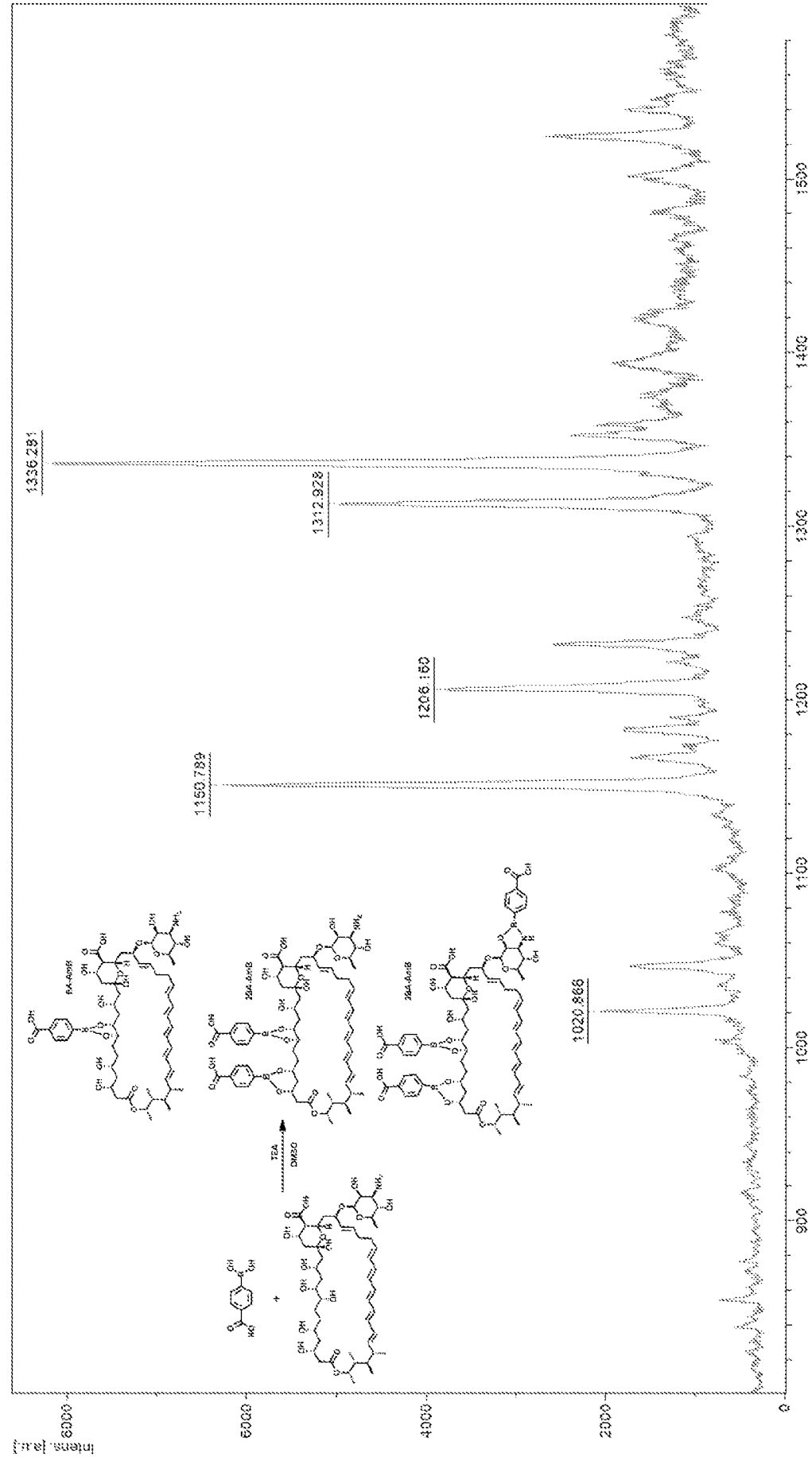
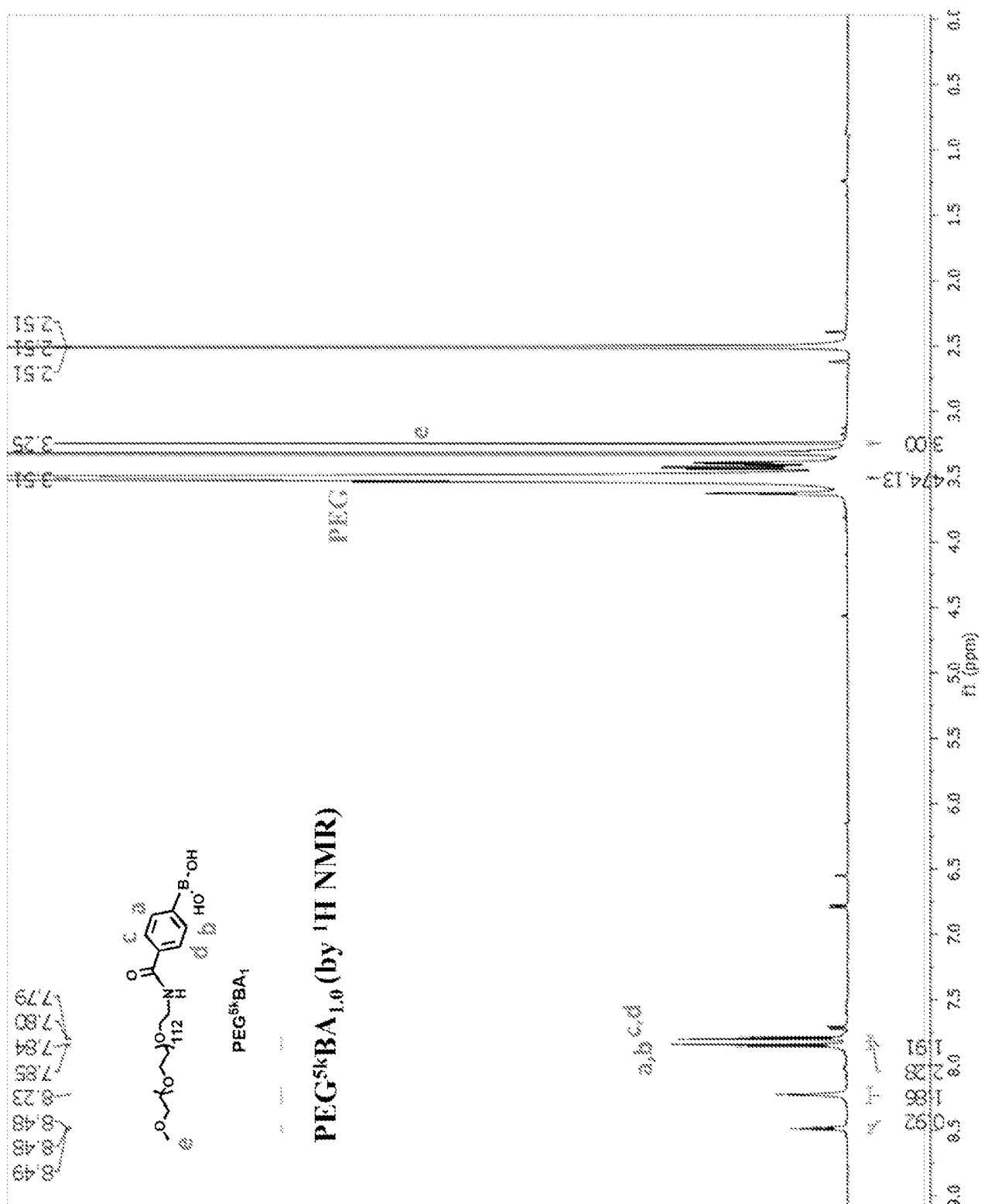


FIG. S3A



[illegible]

FIG. S3D

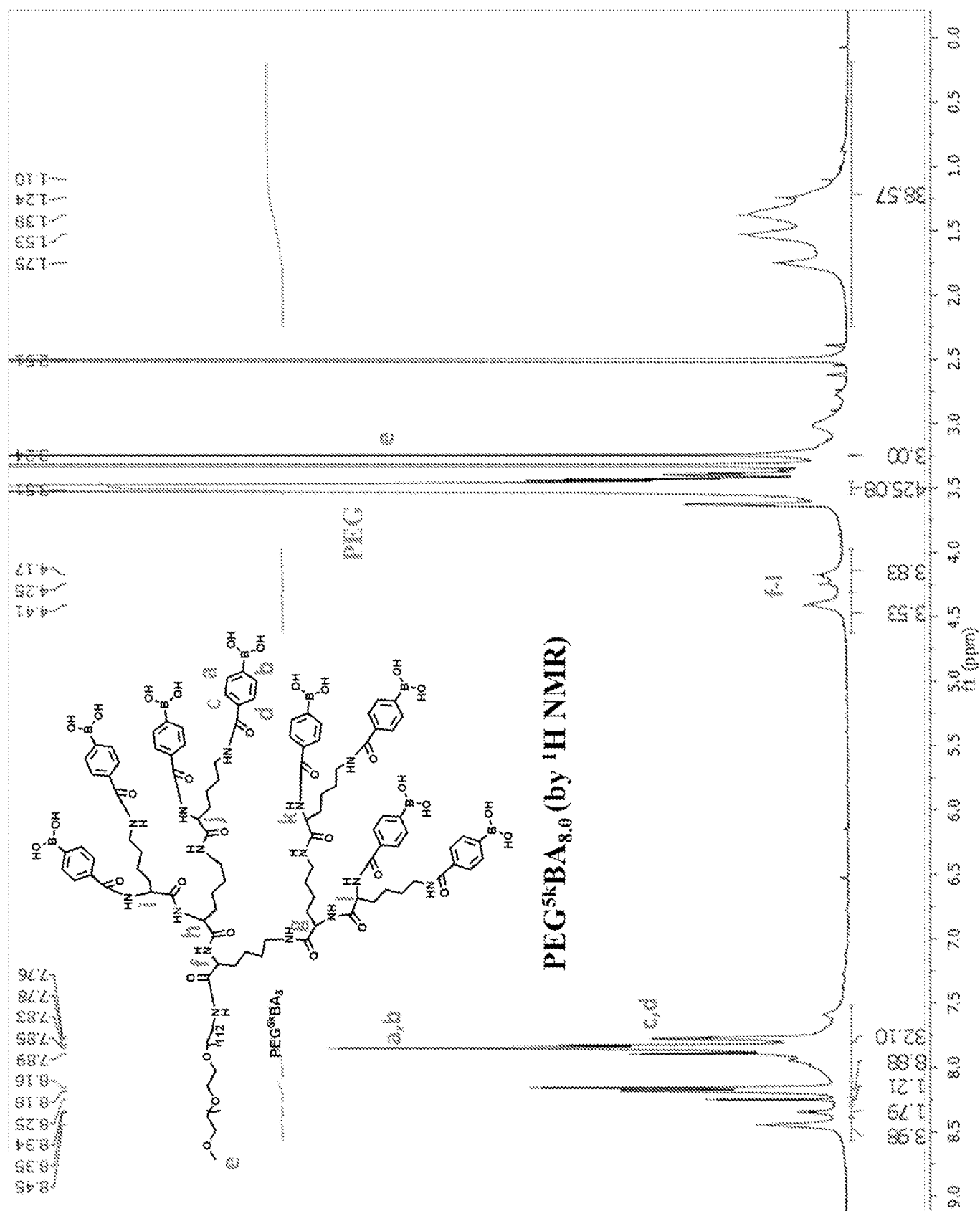


FIG. S4A

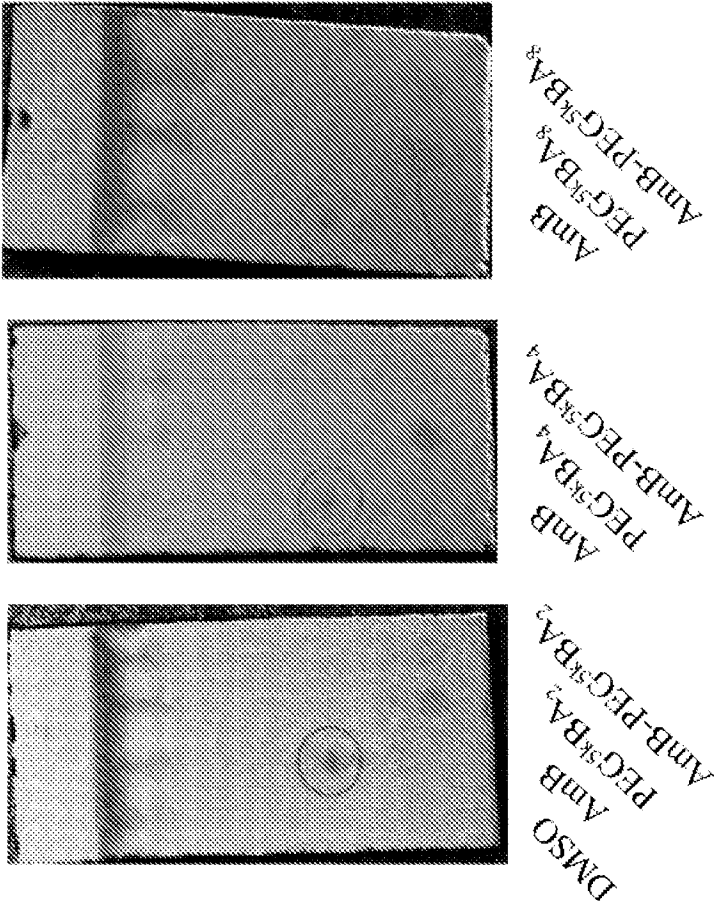


FIG. S4B

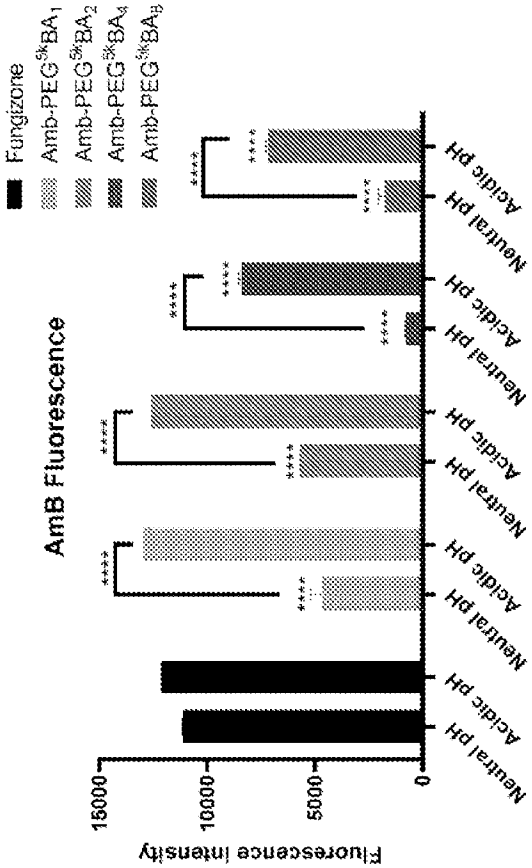


FIG. S5A

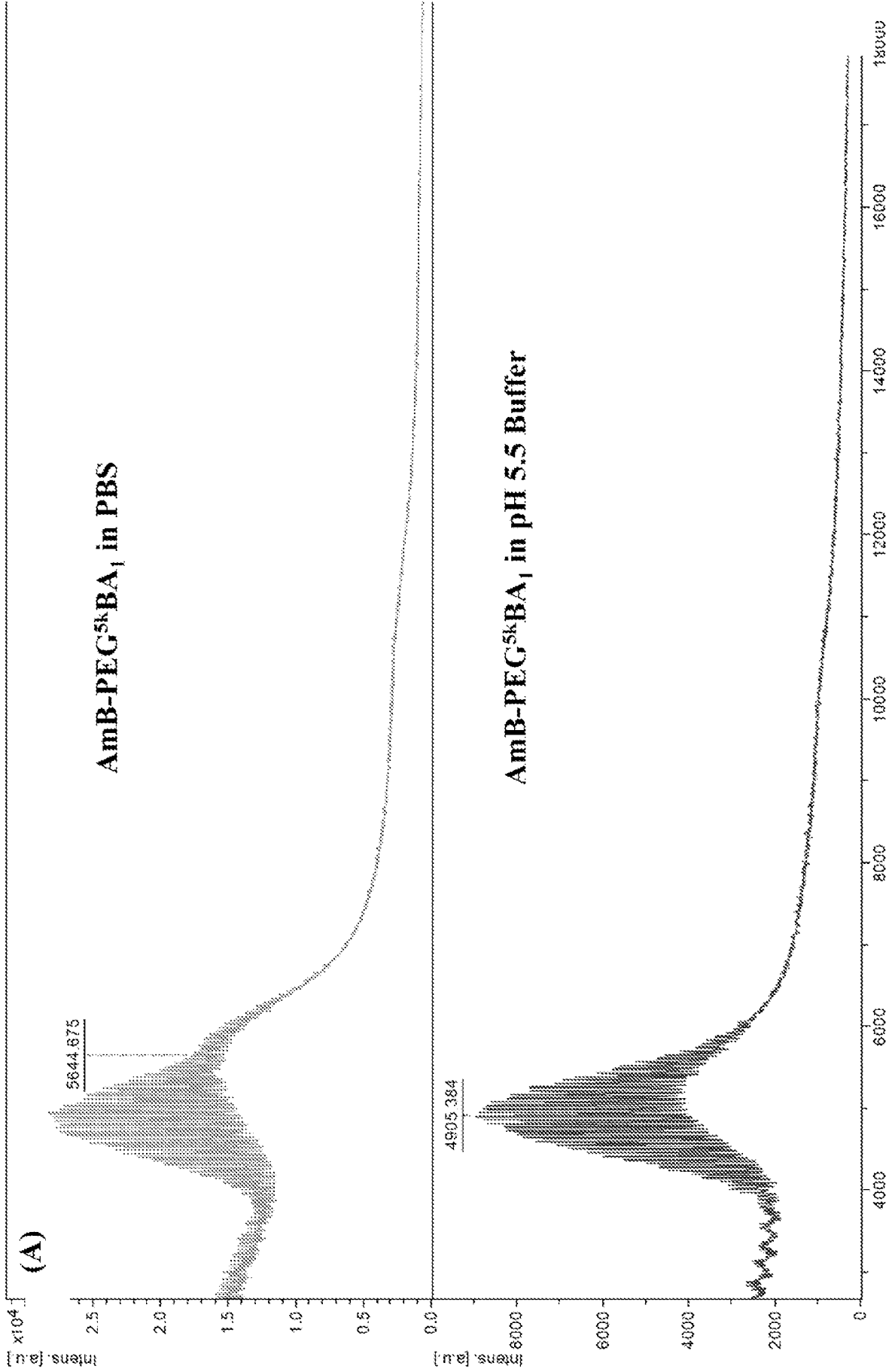


FIG. S5B

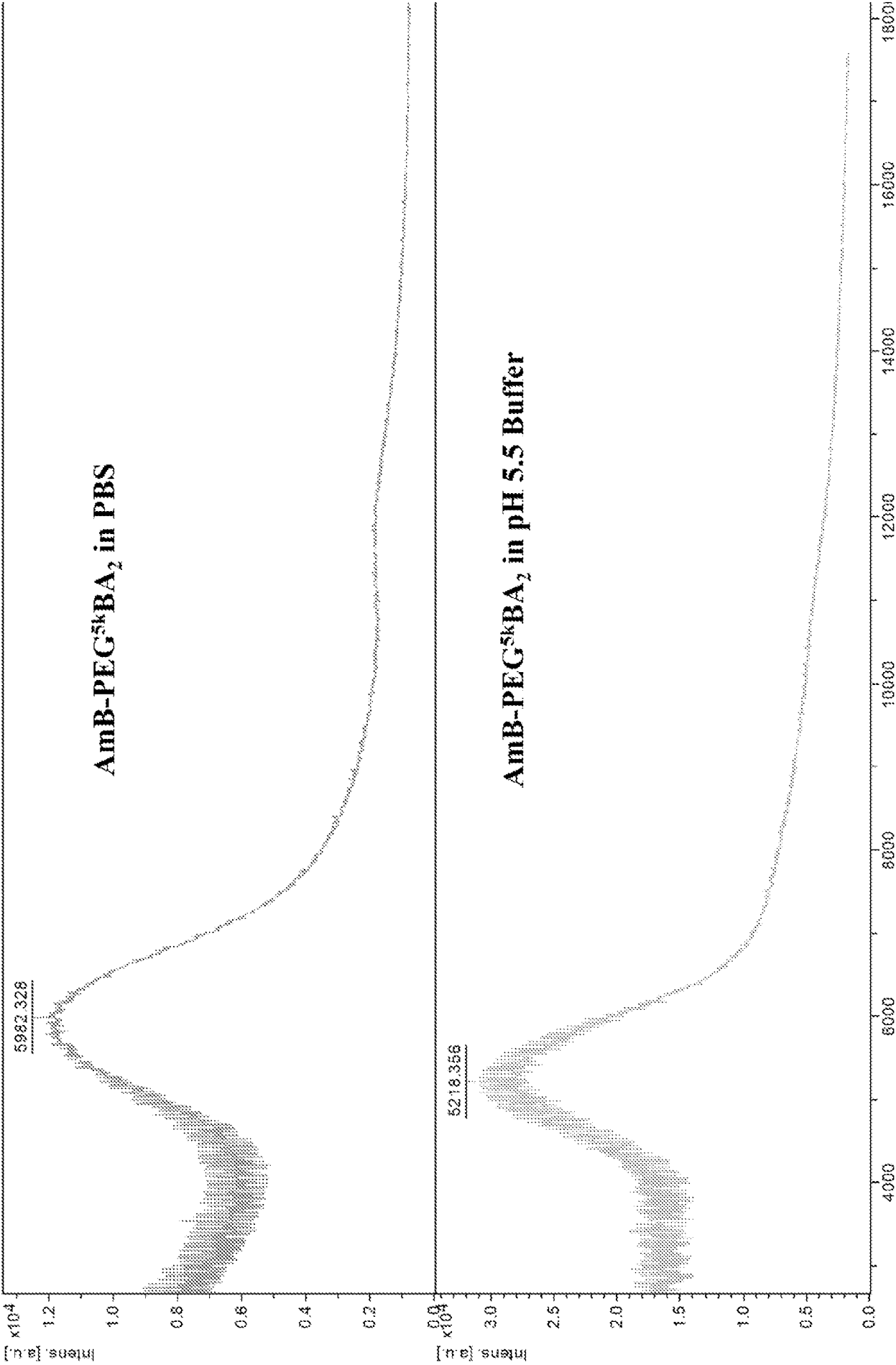


FIG. S5C

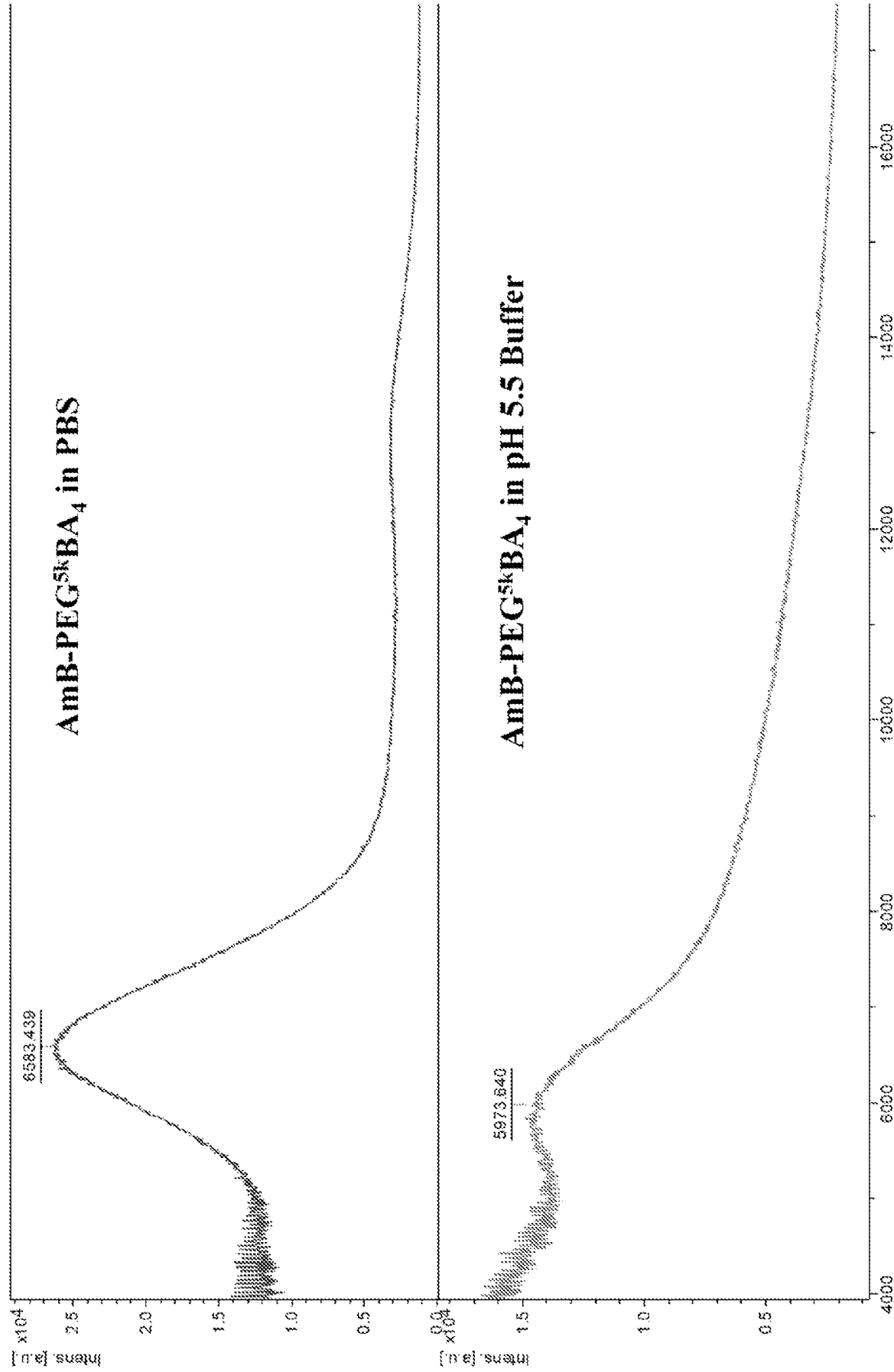


FIG. S5D

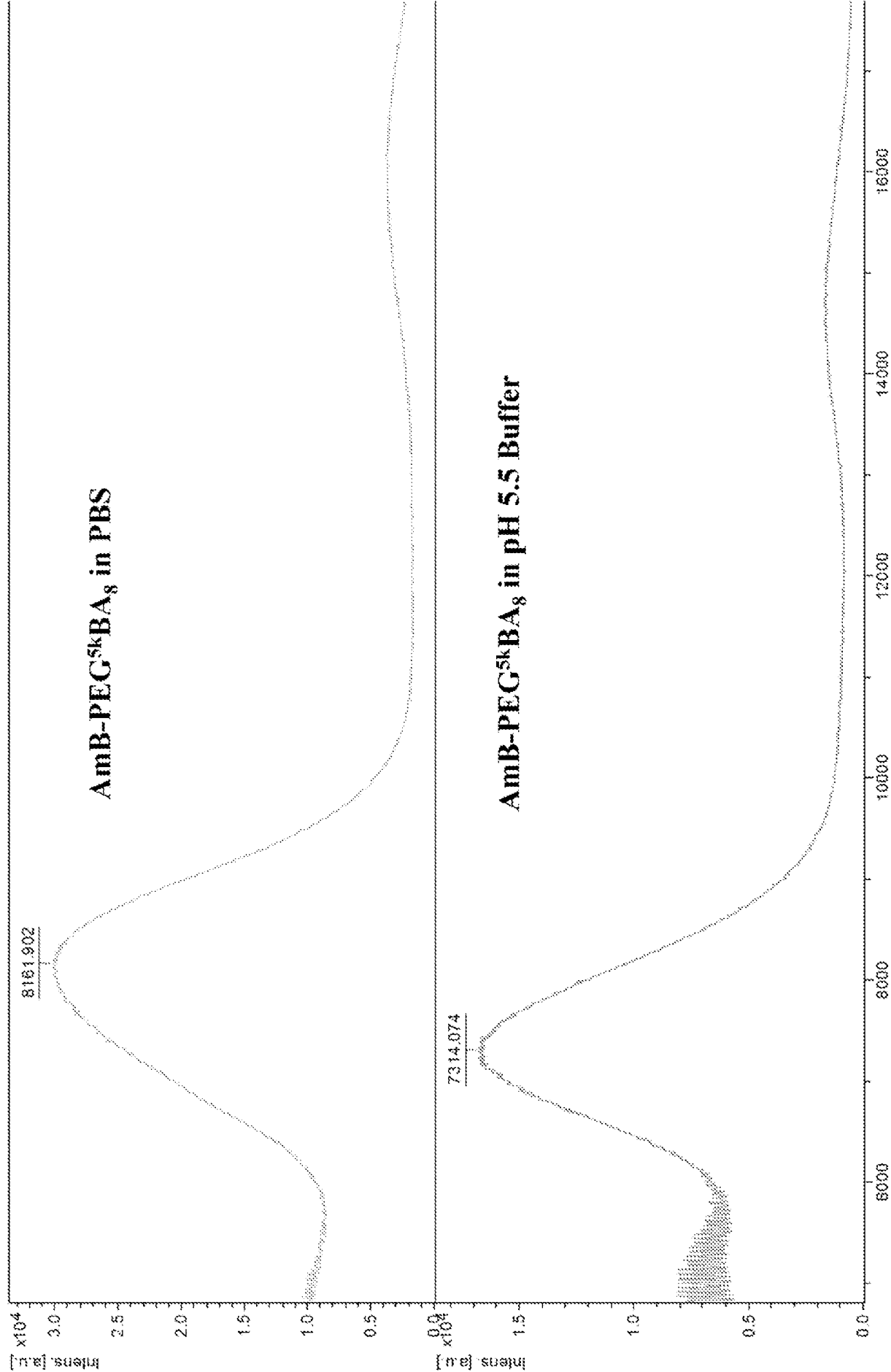


FIG. S6A

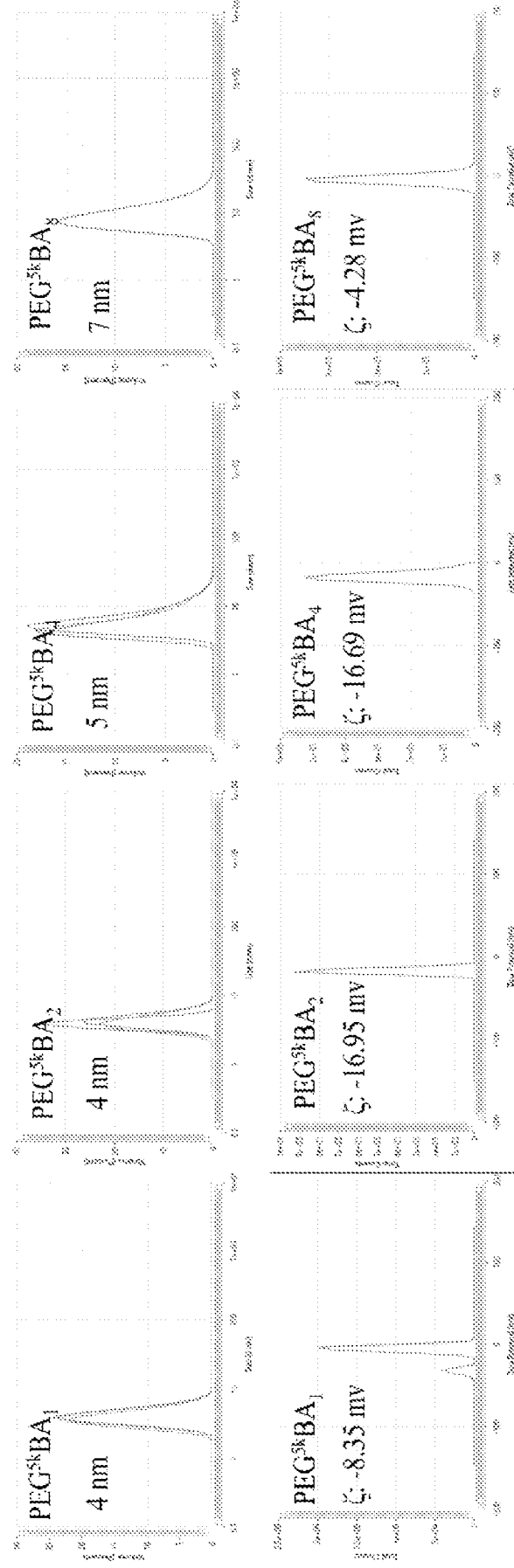


FIG. S6B

FIG. S6C

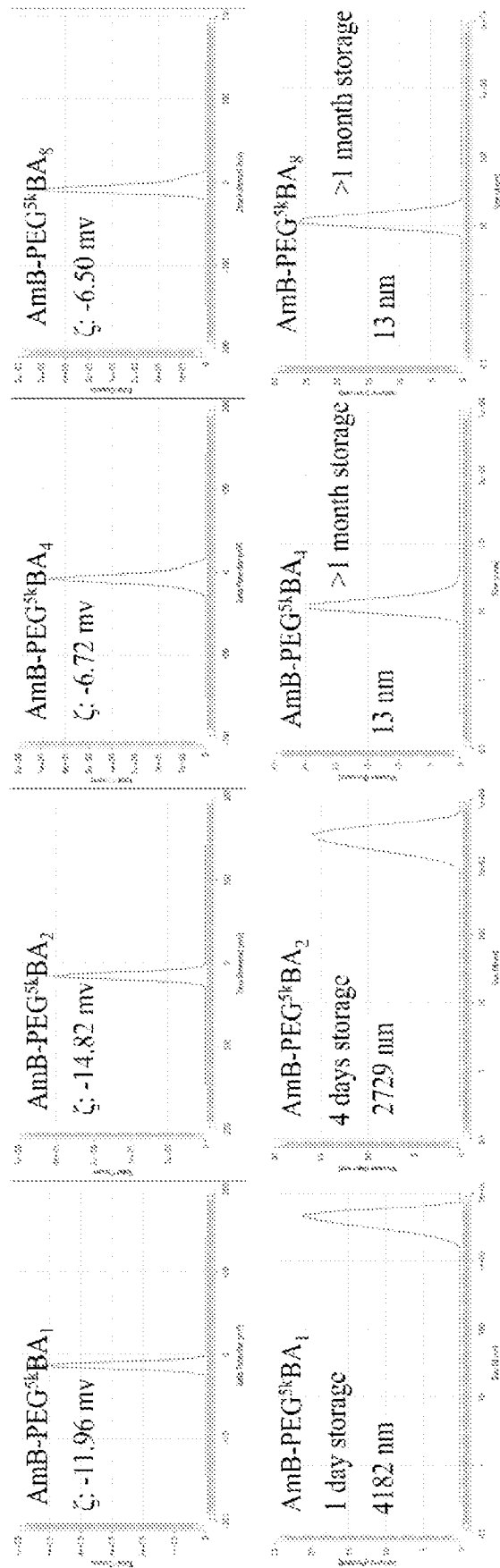


FIG. S6D

FIG. S7A

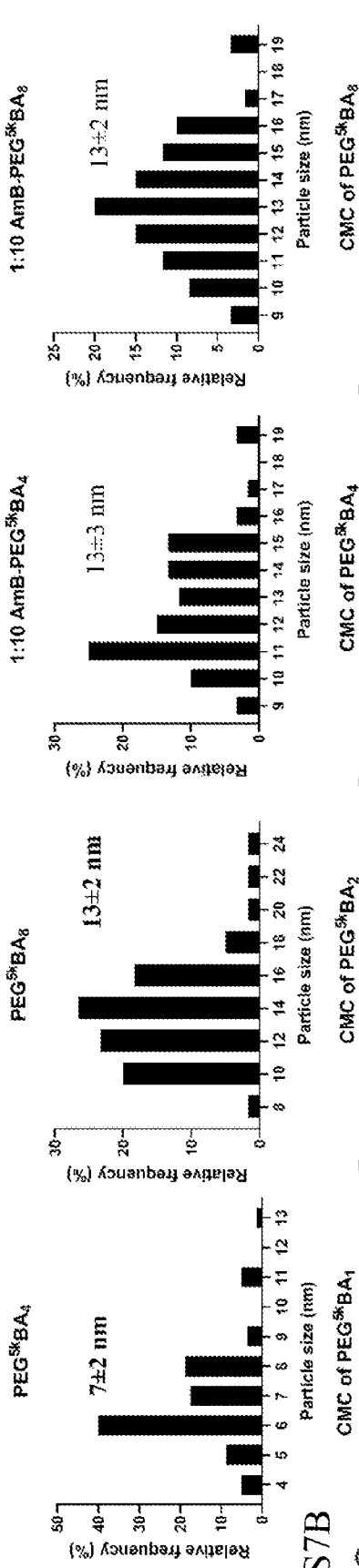


FIG. S7B

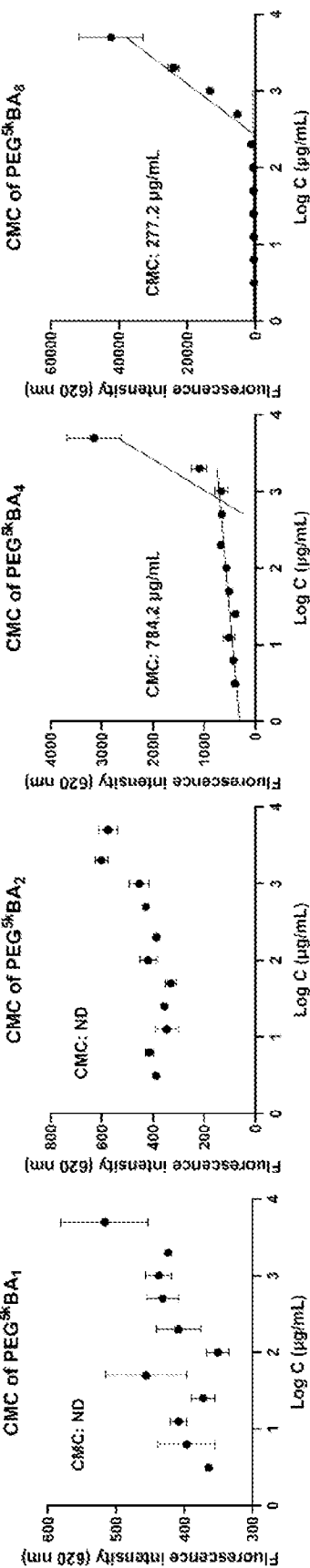


FIG. S7C

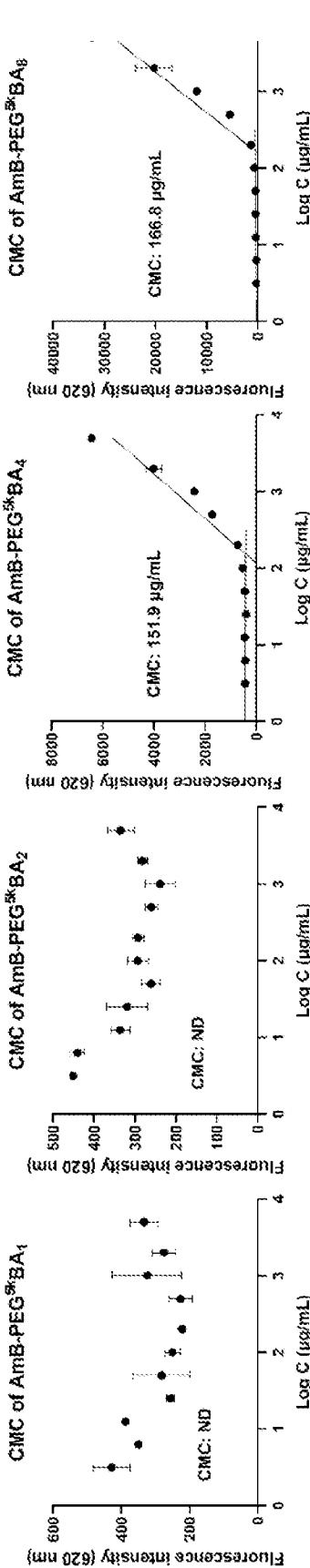


FIG. S8

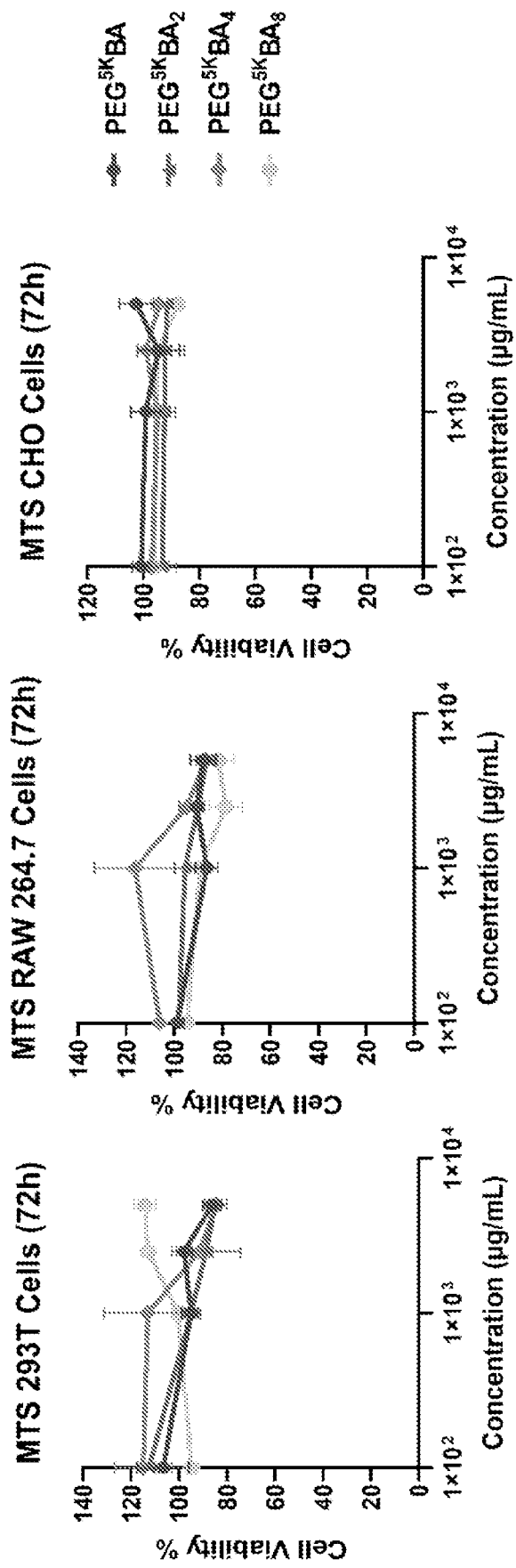


Figure 1: Hemolysis of S9B cells by various formulations.

The figure consists of six bar charts arranged in a 3x2 grid. The left column shows hemolysis percentage for different formulations, and the right column shows the corresponding TD concentration (µg/mL).

Legend:

- Free Amb (white bar)
- Fungizone (diagonal lines)
- Ambisome (cross-hatch)
- Amb-PEG^{5k}BA₁ (solid black)
- Amb-PEG^{5k}BA₂ (diagonal lines)
- Amb-PEG^{5k}BA₄ (cross-hatch)
- Amb-PEG^{5k}BA₈ (solid black)

Top Row: Hemolysis (30 min)

Left Chart: Hemolysis % vs. Amb concentration (µg/mL)

Amb concentration (µg/mL)	Free Amb	Fungizone	Ambisome	Amb-PEG ^{5k} BA ₁	Amb-PEG ^{5k} BA ₂	Amb-PEG ^{5k} BA ₄	Amb-PEG ^{5k} BA ₈
100	100	100	100	100	100	100	100
500	100	100	100	100	100	100	100
1000	100	100	100	100	100	100	100
5000	100	100	100	100	100	100	100
10000	100	100	100	100	100	100	100
50000	100	100	100	100	100	100	100
100000	100	100	100	100	100	100	100
500000	100	100	100	100	100	100	100
1000000	100	100	100	100	100	100	100
5000000	100	100	100	100	100	100	100
10000000	100	100	100	100	100	100	100
50000000	100	100	100	100	100	100	100
100000000	100	100	100	100	100	100	100
500000000	100	100	100	100	100	100	100
1000000000	100	100	100	100	100	100	100
5000000000	100	100	100	100	100	100	100
10000000000	100	100	100	100	100	100	100
50000000000	100	100	100	100	100	100	100
100000000000	100	100	100	100	100	100	100
500000000000	100	100	100	100	100	100	100
1000000000000	100	100	100	100	100	100	100
5000000000000	100	100	100	100	100	100	100
10000000000000	100	100	100	100	100	100	100
50000000000000	100	100	100	100	100	100	100
100000000000000	100	100	100	100	100	100	100
500000000000000	100	100	100	100	100	100	100
1000000000000000	100	100	100	100	100	100	100
5000000000000000	100	100	100	100	100	100	100
10000000000000000	100	100	100	100	100	100	100
50000000000000000	100	100	100	100	100	100	100
100000000000000000	100	100	100	100	100	100	100
500000000000000000	100	100	100	100	100	100	100
1000000000000000000	100	100	100	100	100	100	100
5000000000000000000	100	100	100	100	100	100	100
10000000000000000000	100	100	100	100	100	100	100
50000000000000000000	100	100	100	100	100	100	100
100000000000000000000	100	100	100	100	100	100	100
500000000000000000000	100	100	100	100	100	100	100
1000000000000000000000	100	100	100	100	100	100	100
5000000000000000000000	100	100	100	100	100	100	100
10000000000000000000000	100	100	100	100	100	100	100
50000000000000000000000	100	100	100	100	100	100	100
100000000000000000000000	100	100	100	100	100	100	100
500000000000000000000000	100	100	100	100	100	100	100
1000000000000000000000000	100	100					

Hemolysis (30 min)

Liposome	100	500	1000
PEG ^{5k} BA ₁	~2.5	~2.5	~2.5
PEG ^{5k} BA ₂	~2.5	~2.5	~2.5
PEG ^{5k} BA ₄	~2.5	~2.5	~2.5
PEG ^{5k} BA ₈	~2.5	~2.5	~2.5

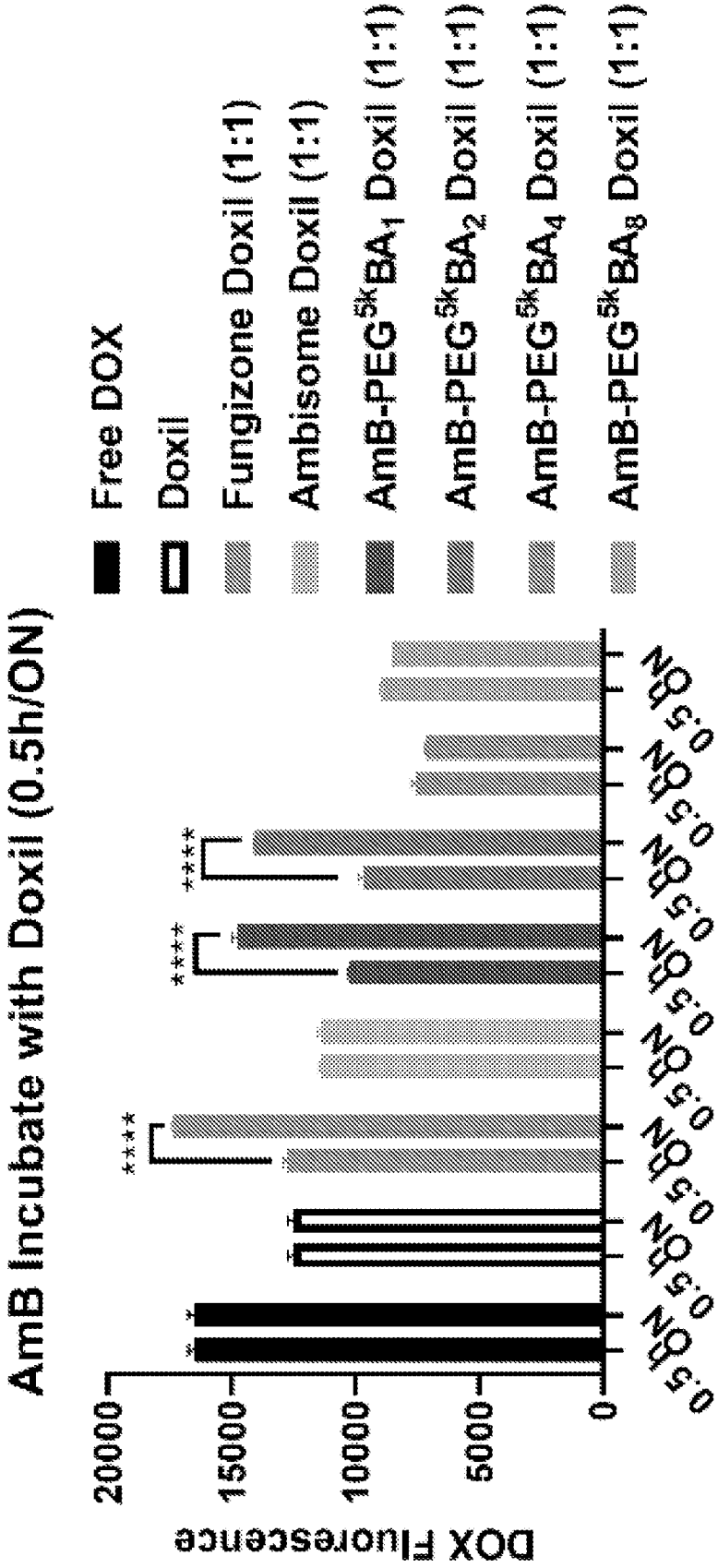
Hemolysis (4 h)

Liposome	100	500	1000
PEG ^{5k} BA ₁	~2.5	~2.5	~2.5
PEG ^{5k} BA ₂	~2.5	~2.5	~2.5
PEG ^{5k} BA ₄	~2.5	~2.5	~2.5
PEG ^{5k} BA ₈	~2.5	~2.5	~2.5

Hemolysis (overnight)

Liposome	100	500	1000
PEG ^{5k} BA ₁	~2.5	~2.5	~2.5
PEG ^{5k} BA ₂	~2.5	~2.5	~2.5
PEG ^{5k} BA ₄	~2.5	~2.5	~2.5
PEG ^{5k} BA ₈	~2.5	~2.5	~2.5

FIG. S10



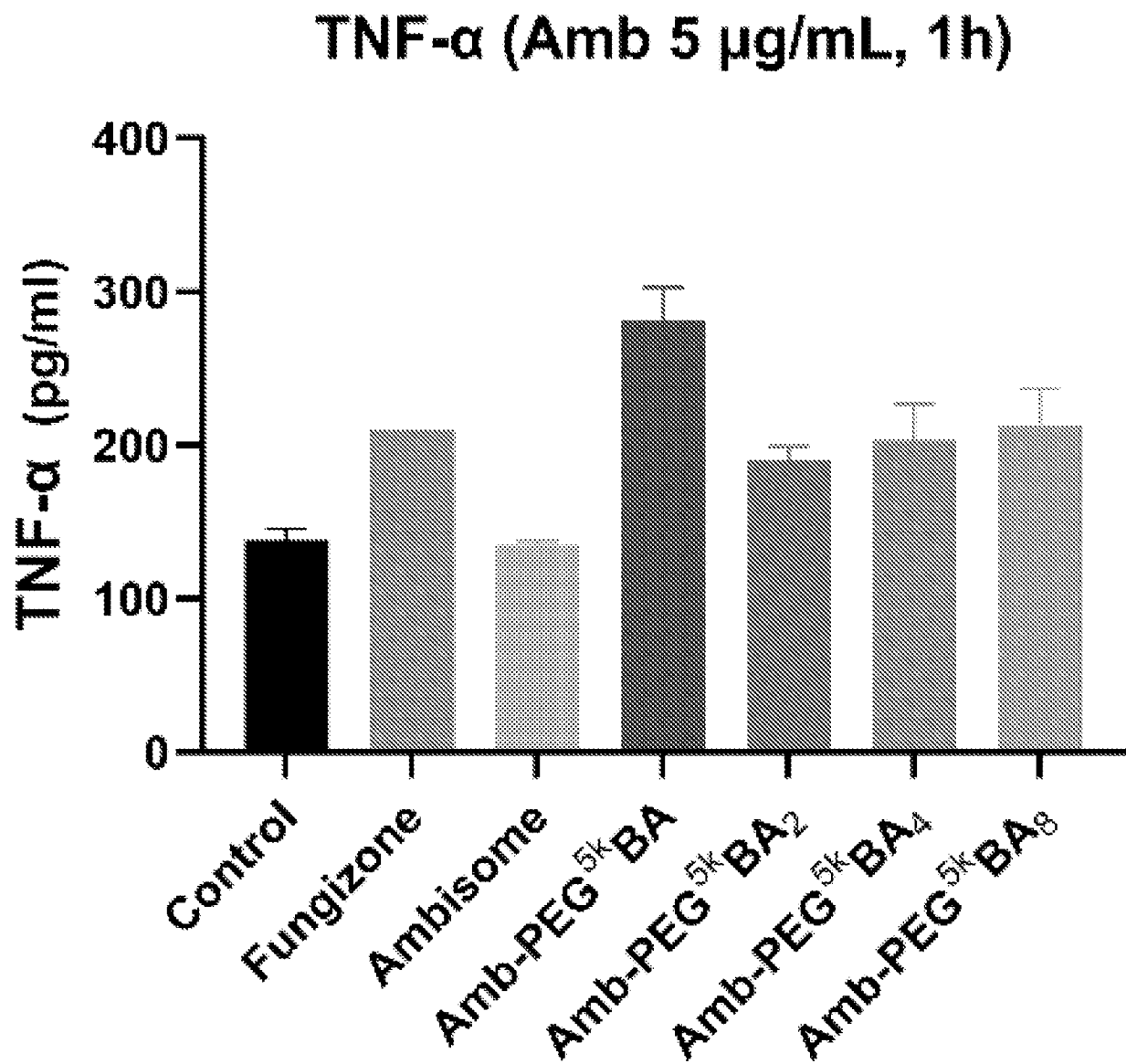


FIG. 11

FIG. S12A

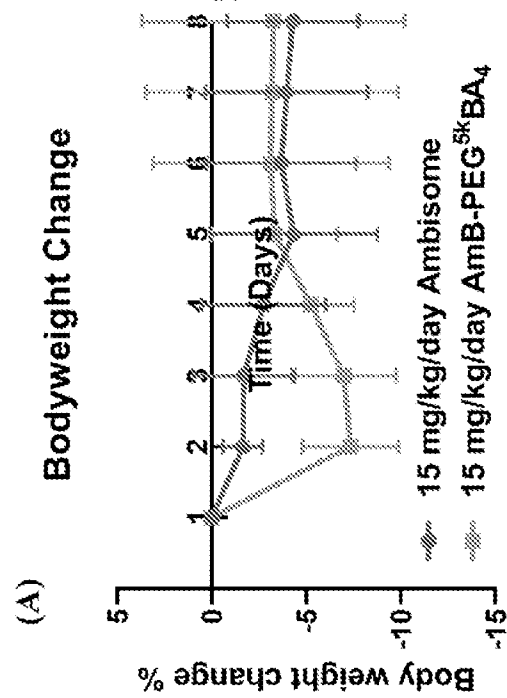


FIG. S12B

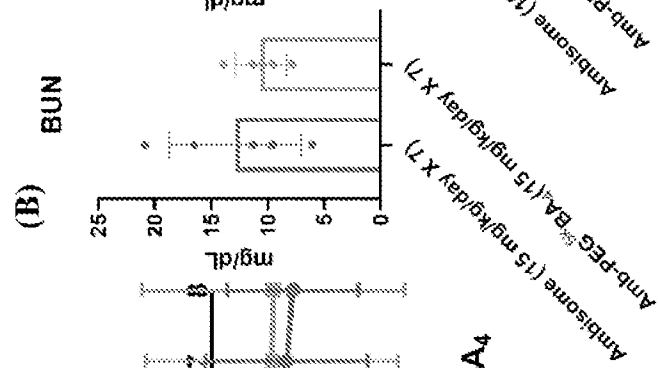


FIG. S12C

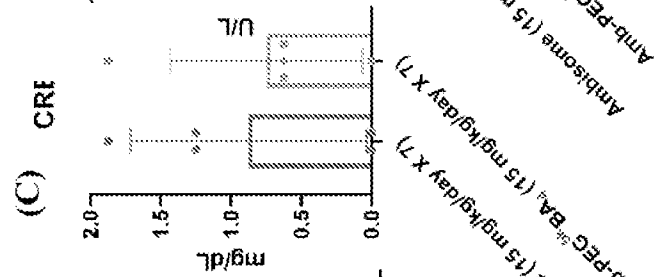


FIG. S12D

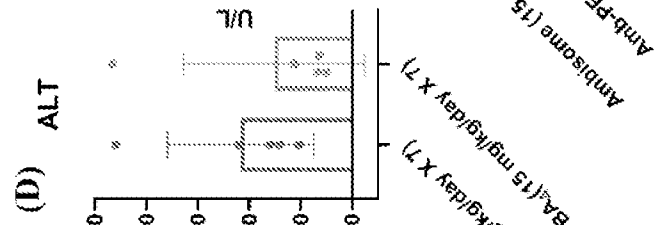


FIG. S12E

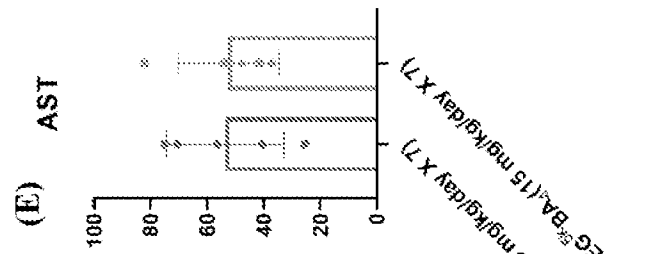


FIG. S13

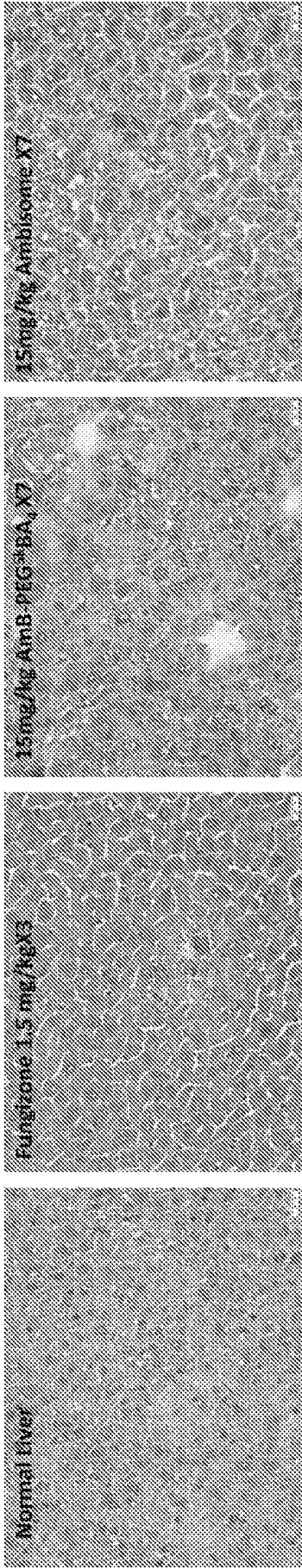


FIG. S14

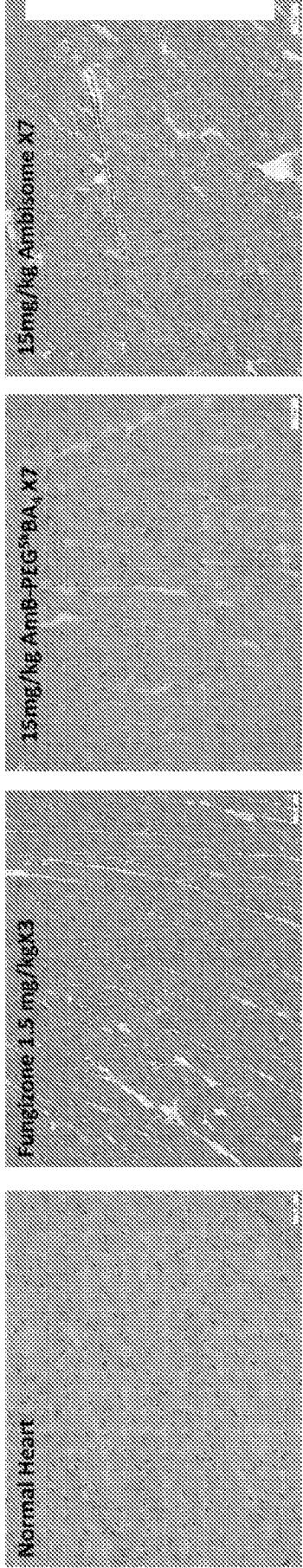


FIG. S15A

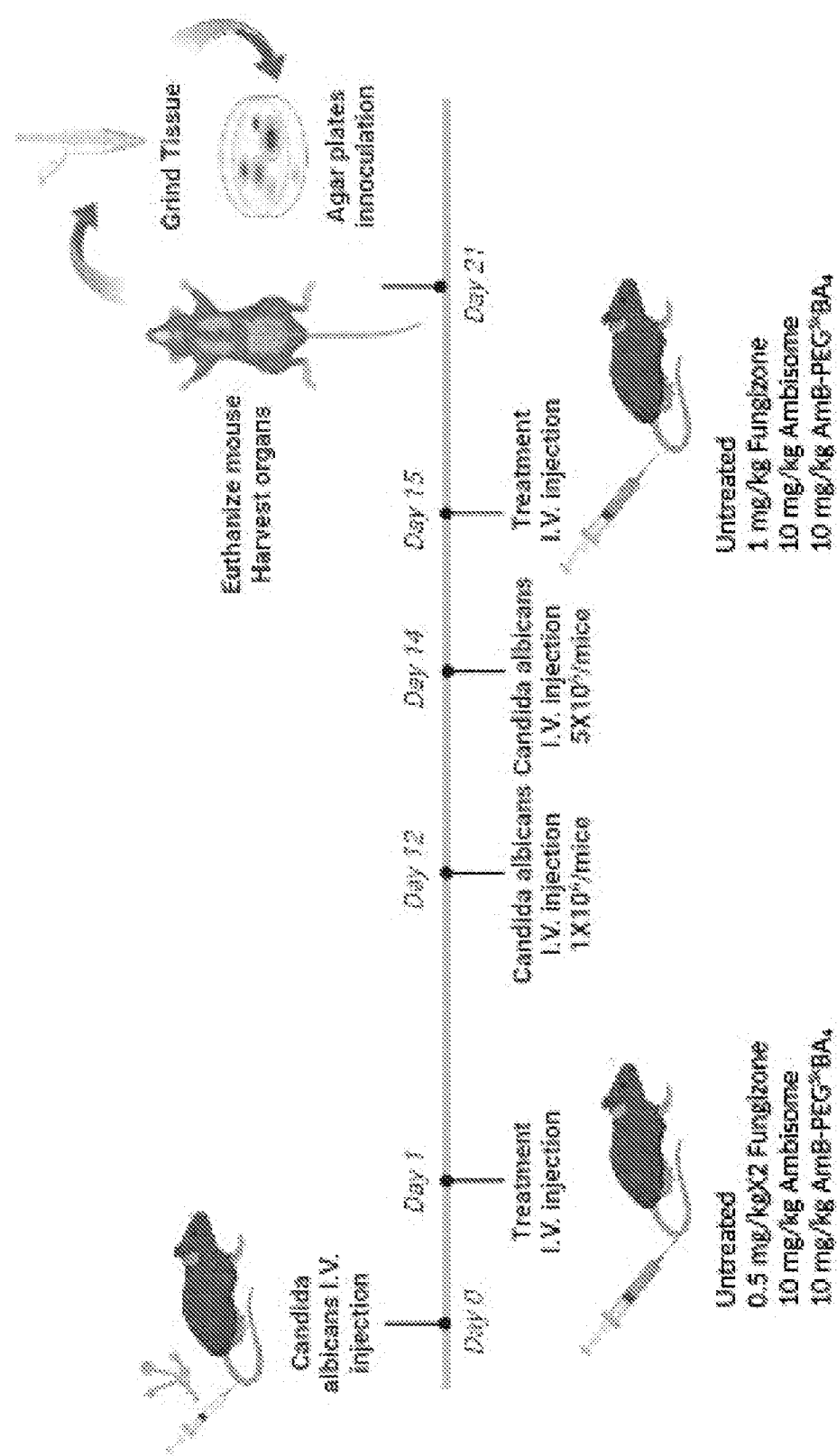


FIG. S15B

Survival of Mice infected with *Candida Albicans*

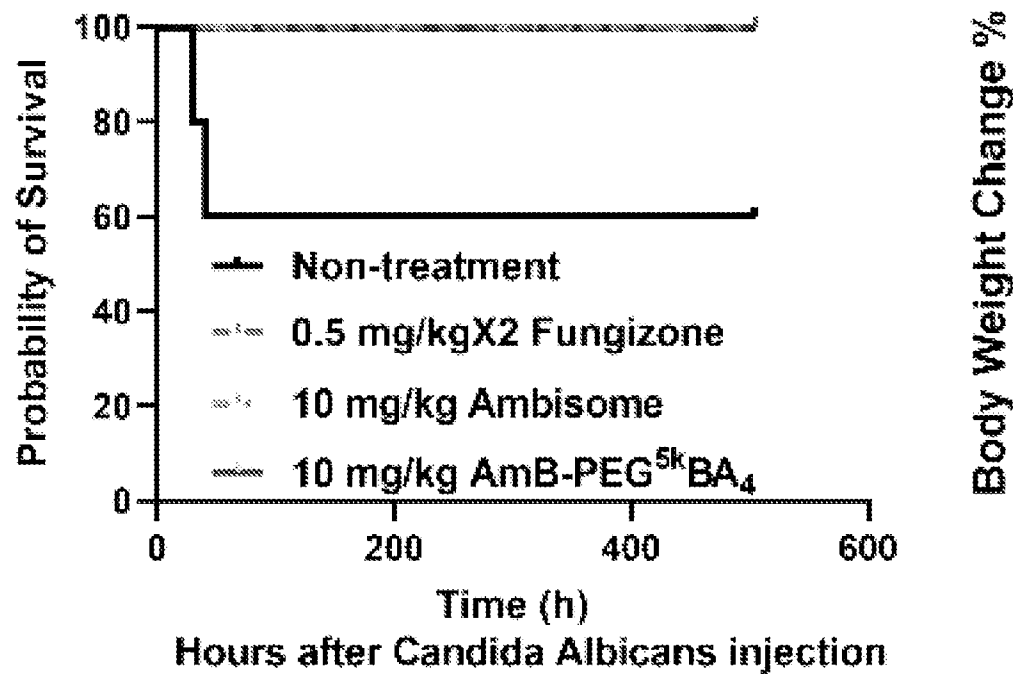


FIG. S15C

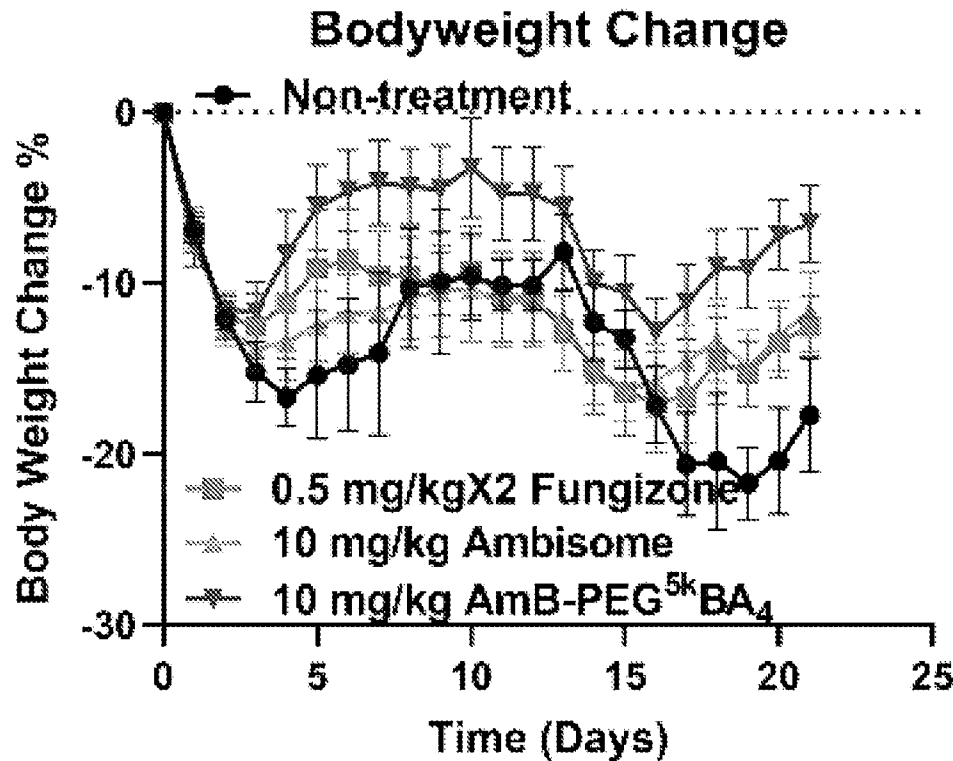


FIG. S15D

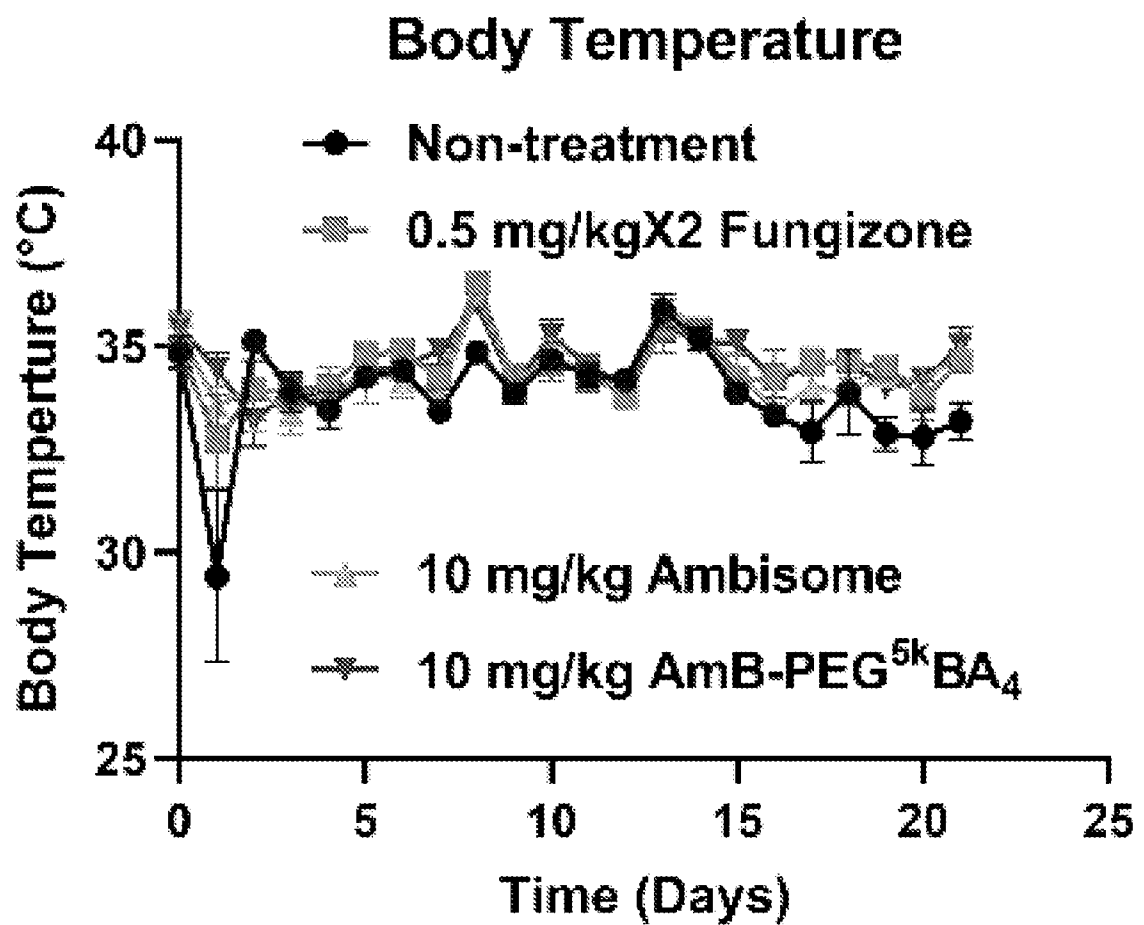


FIG. S15E

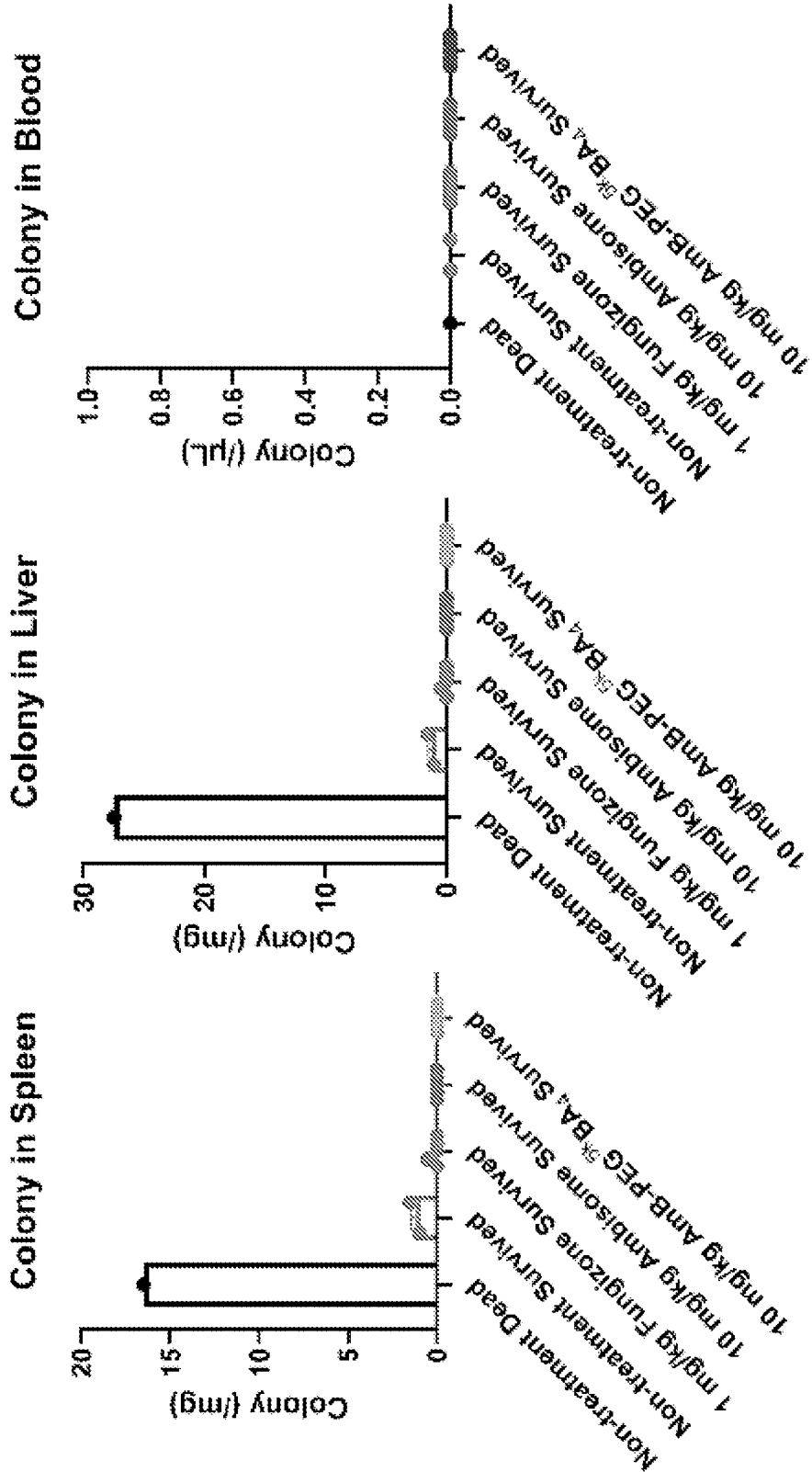


FIG. S15F

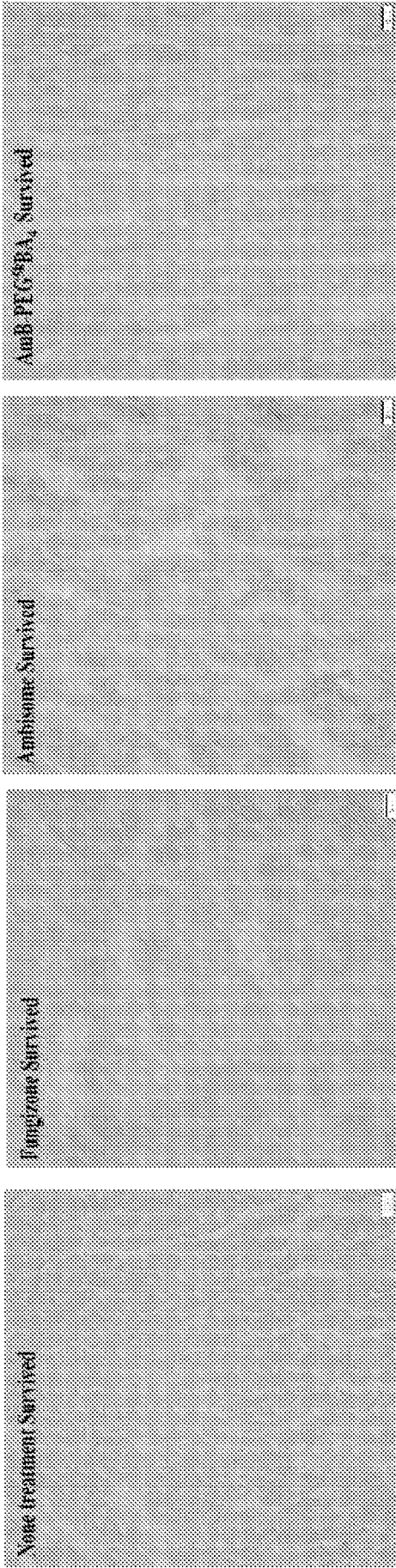


FIG. S15F

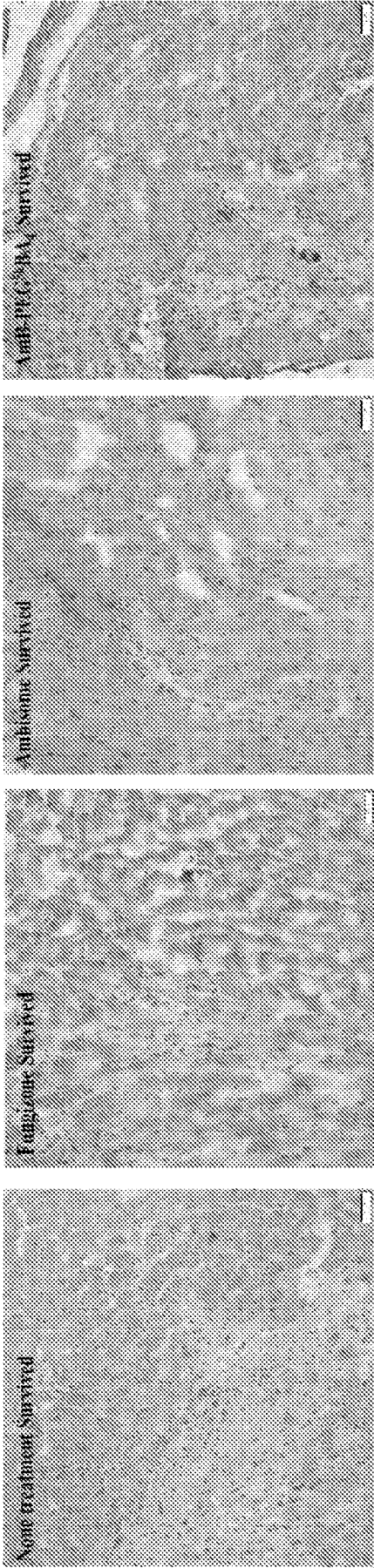


FIG. S16

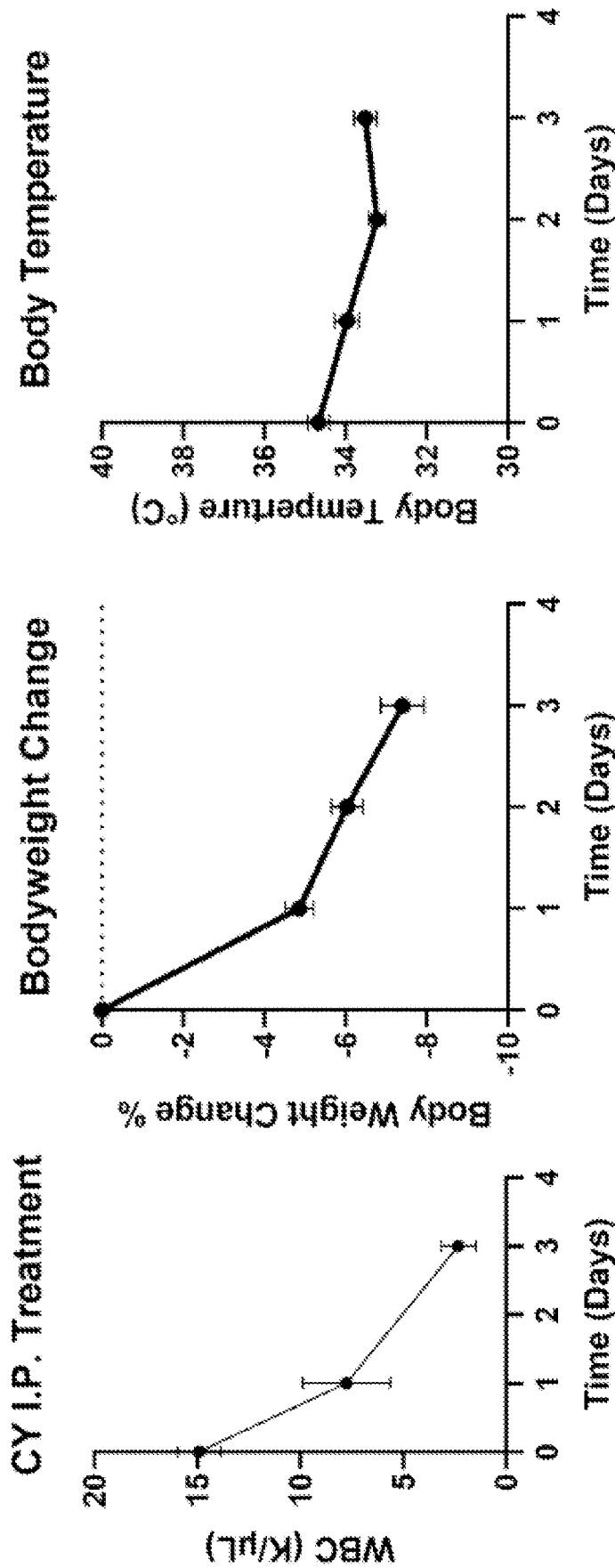


FIG. S17A

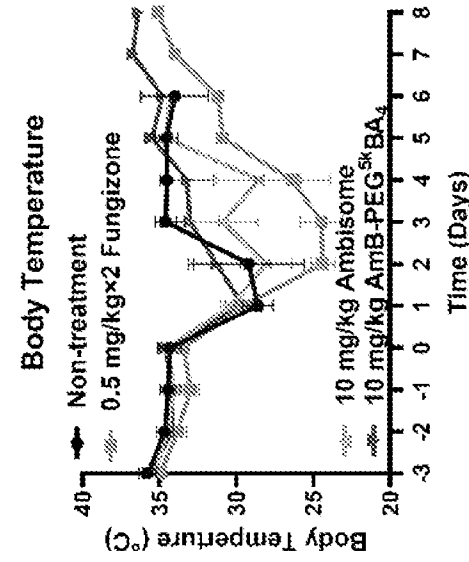
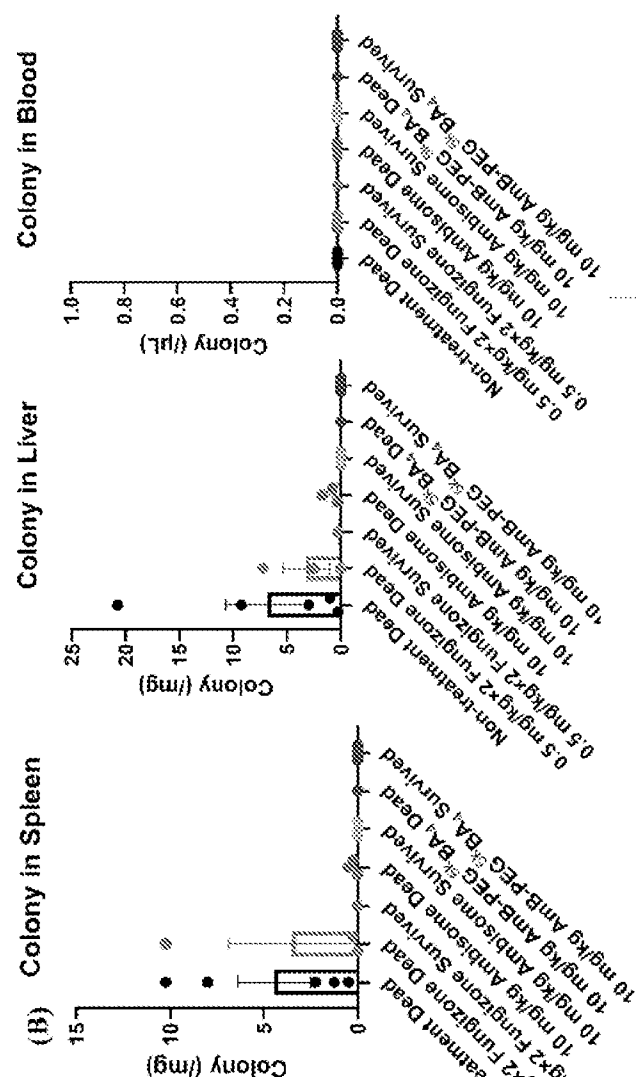


FIG. S17B



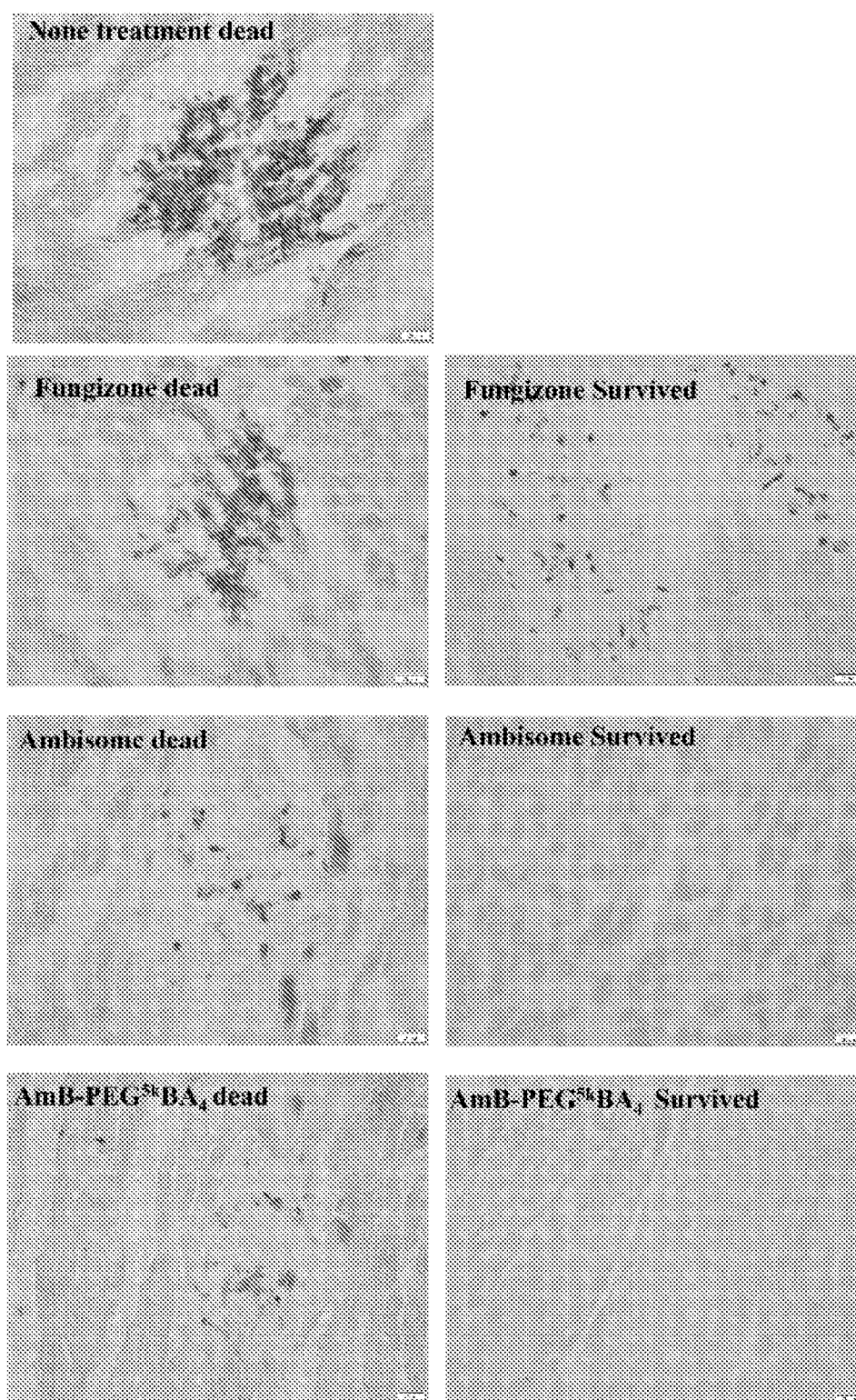


FIG. S17C

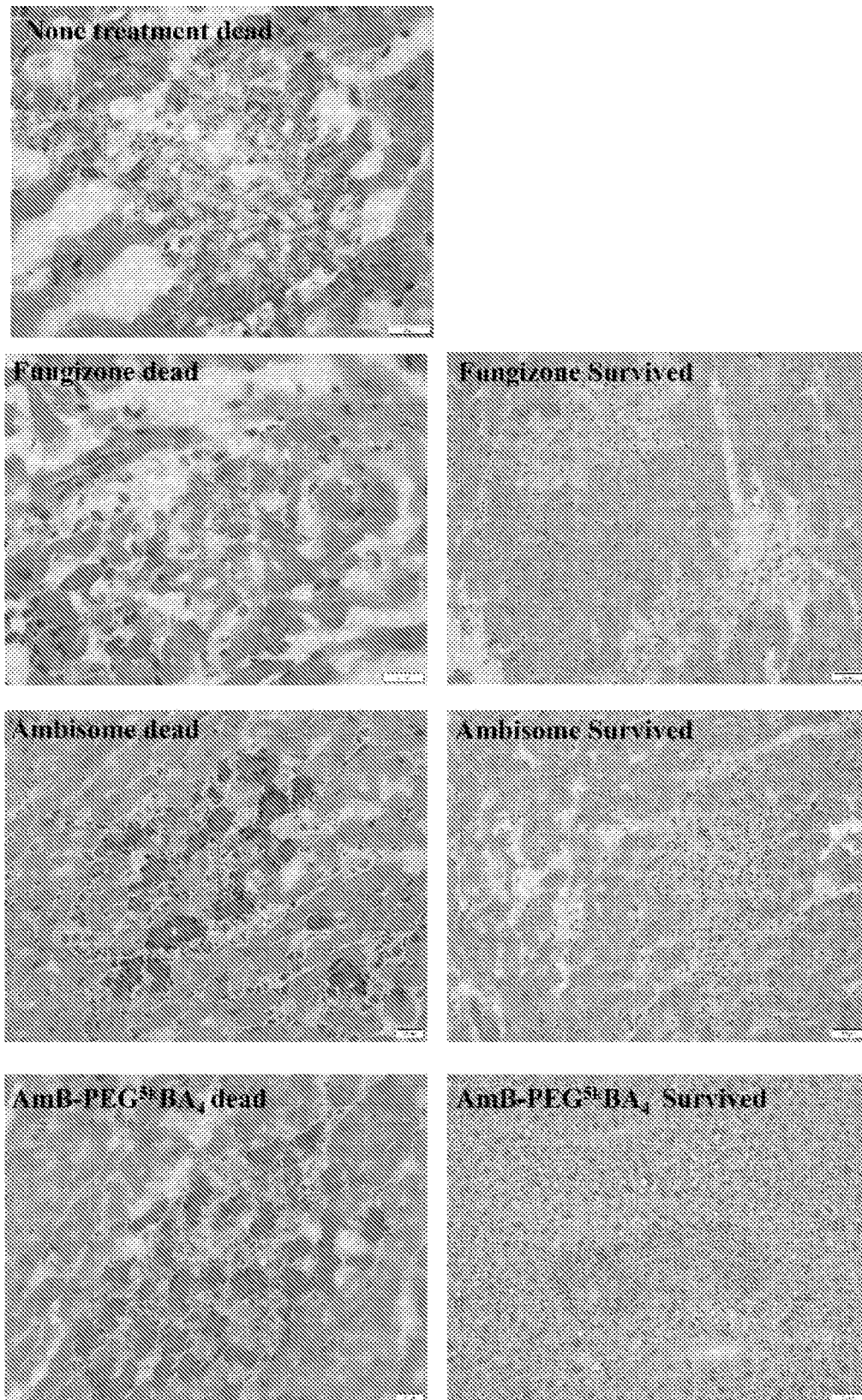


FIG. 17D

FIG. S18A

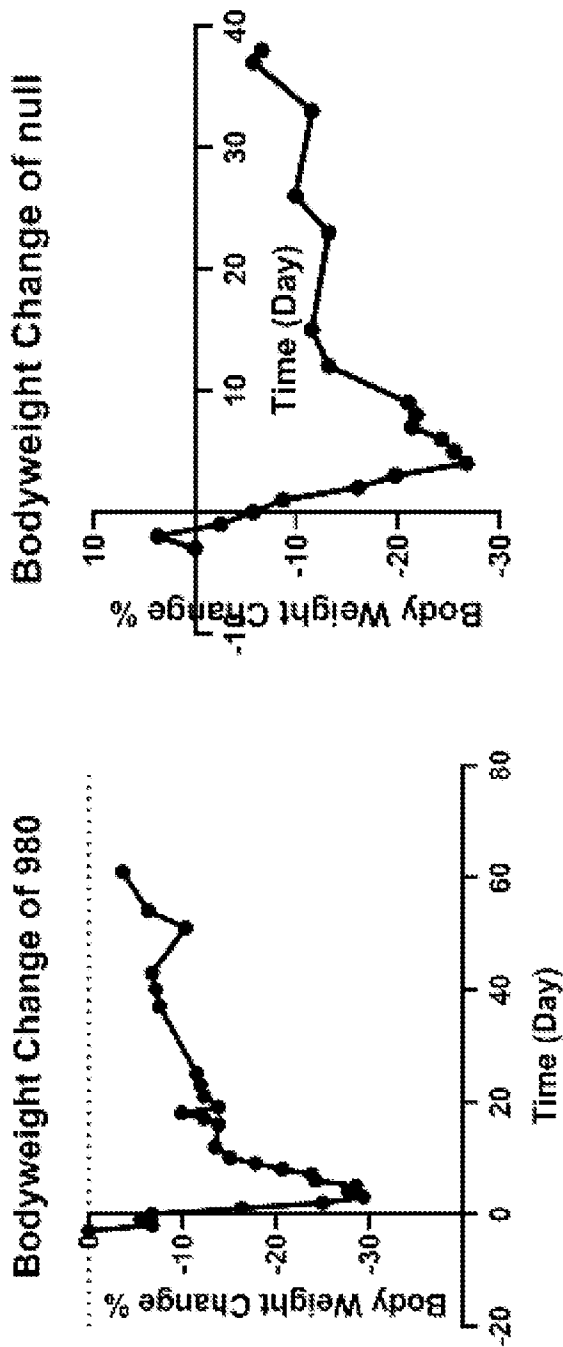


FIG. S18B

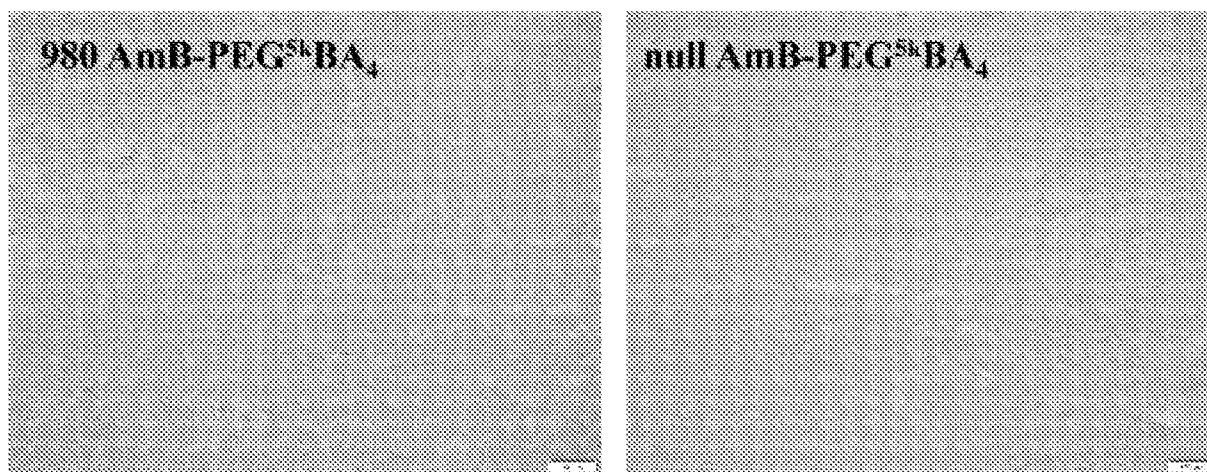


FIG. S18C

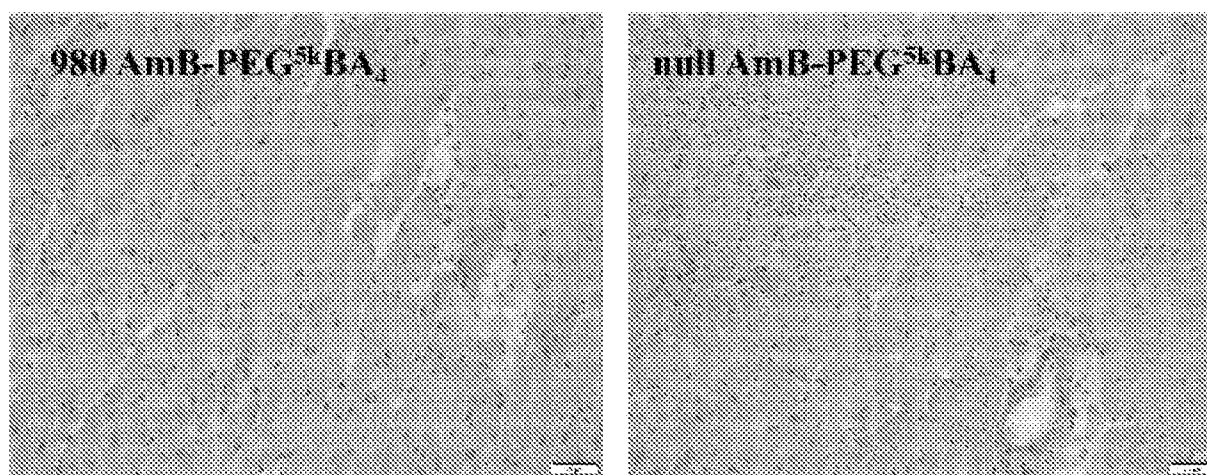


FIG. S18D

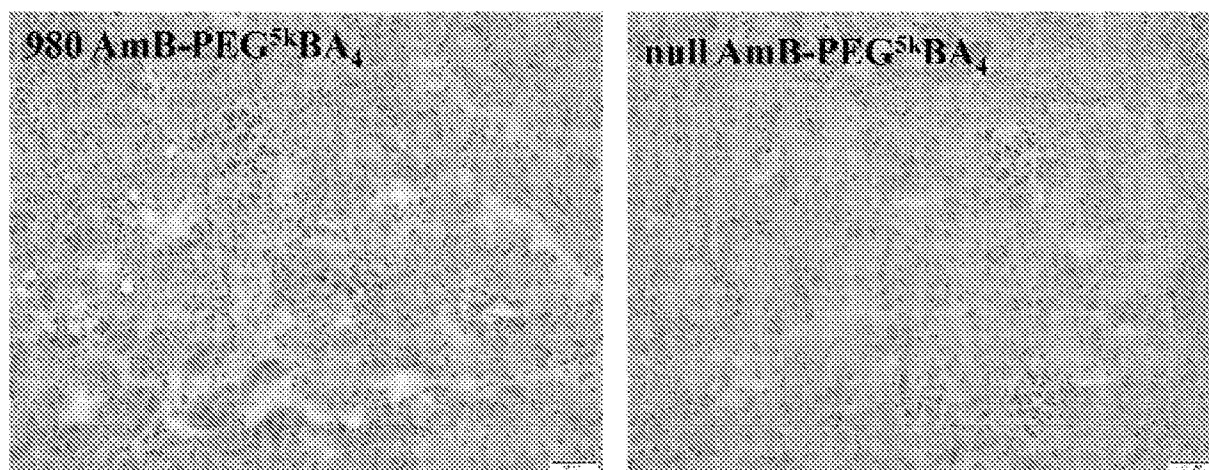


FIG. S18E

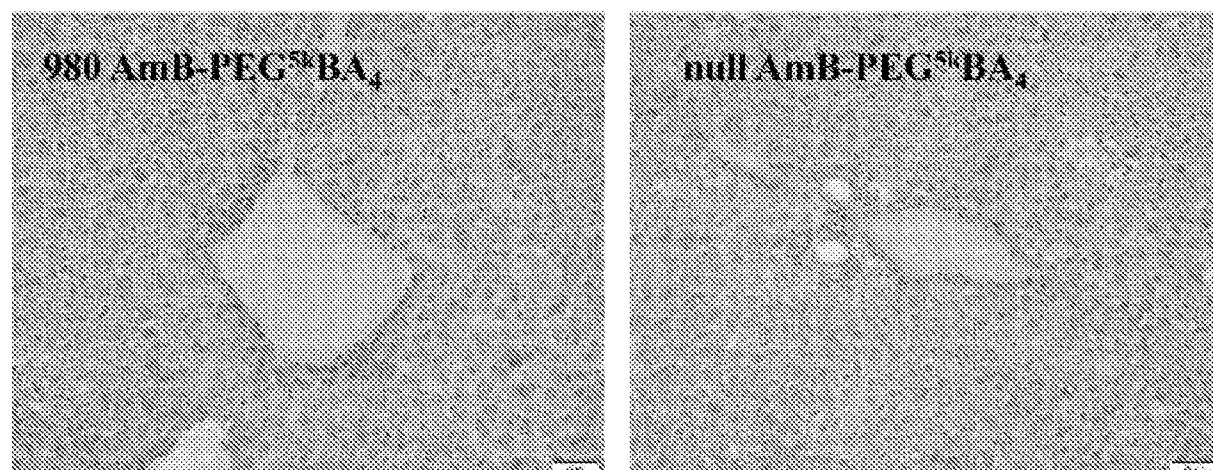


FIG. S19

