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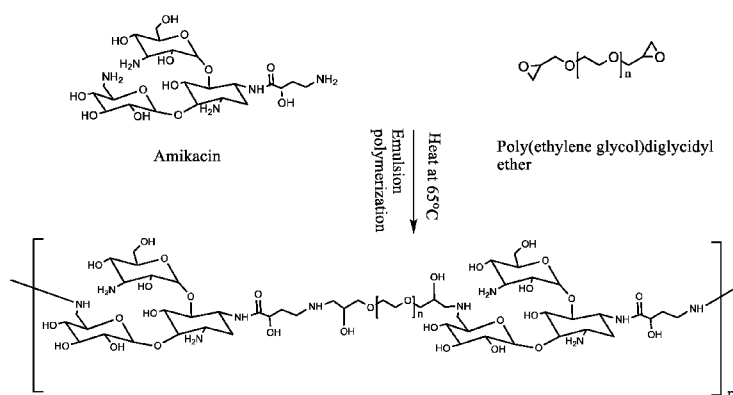
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(54) Title: AMINOGLYCOSIDE HYDROGEL MICROBEADS AND MACROPOROUS GELS WITH CHEMICAL CROSSLINK, METHOD OF PREPARATION AND USE THEREOF

**Fig. 3**

(57) Abstract: Methods and materials for the generation of amikacin antibiotic-derived microbeads, (Fig. 3). These beads may function as anion-exchange resins for use in pDNA purification as well as in situ capture of DNA from mammalian cells. New microresin and macroporous monolith based materials also are disclosed and may function for plasmid DNA binding and purification, mammalian whole cell genomic DNA extraction, and in-vitro cell culture.

**AMINOGLYCOSIDE HYDROGEL MICROBEADS AND MACROPOROUS
GELS WITH CHEMICAL CROSSLINK, METHOD OF PREPARATION AND
USE THEREOF**

Cross Reference

[0001] This application claims priority to U.S. provisional patent application 62/115,392 filed on February 12, 2015, which is incorporated by reference herein in its entirety.

Federally Sponsored Research Or Development

[0002] This invention was made with government support under 1067840 awarded by the National Science Foundation. The government has certain rights in the invention.

Field of the Invention

[0003] Aminoglycoside hydrogel based microbeads and monoliths with chemical cross-linkings are formed by mixing a polymer with a cross-linking agent. A method of preparing the same, and a use thereof, are also disclosed.

Background Of The Invention

[0004] Aminoglycoside antibiotics including neomycin, streptomycin, kanamycin, apramycin and paromomycin are known to prevent growth of gram-negative bacteria by inhibiting protein synthesis. The mode of action of aminoglycoside antibiotics involves binding and stabilizing 16s rRNA and complexing with 30S subunit of ribosome. This, in turn, inhibits protein synthesis and causes bactericidal activity. Aminoglycosides possess biocompatible sugar groups as well as multiple amines in the same molecule. Their natural affinity towards nucleic acids, makes them excellent candidates for generating diverse materials in nucleic acid biotechnology.

Brief Description Of The Drawings

[0005] The invention will be better understood from a reading of the following detailed description taken in conjunction with the drawings in which like reference designators are used to designate like elements, and in which:

[0006] FIG. 1 recites a list of antibiotic aminoglycosides that can be used for preparation of hydrogel microbeads and monoliths;

[0007] FIG. 2 recites a of crosslinkers that can be used to generate hydrogel microbeads and monoliths;

[0008] FIG. 3 recites a schematic of the reaction between amikacin hydrate and polyethylene glycol diglycidylether (PEGDE), resulting in the formation of Amikagel (hydrogel);

[0009] FIGs. 4a and 4b recite schematic overview of Amikabead-P synthesis. In certain embodiments, Amikabeads were prepared by pre-gelling the Amikagel solution for 4 minutes at 70 °C. The pre-gelled Amikagel solution was added to mineral oil bath supplemented with 1% Span 80 surfactant and maintained at 65 °C;

[0010] FIG. 4c comprises a phase-contrast image of Amikabeads-P generated after emulsion polymerization of Amikacin-PEGDE in mineral oil-1% Span 80 solution. Scale bar: 100 μm ;

[0011] FIG. 4d recites a scanning electron microscopy (SEM) image of Amikabeads indicates spherical particles with an average diameter of $\sim 9 \pm 4 \mu\text{m}$ (calculated over 50 beads). UV radiation can be used as photoinitiator for reactions using acrylates and amines;

[0012] FIG. 5a graphically shows average Amikabead-P diameter $\pm$ one standard deviation (in microns) of three consecutive batches in absence of additional Span-80. The p-values for the one-way ANOVA test are also shown for n=5 independent experiments;

[0013] FIG. 5b graphically shows average Amikabead-P diameter $\pm$ one standard deviation (in microns) of three consecutive batches in presence of Span-80. The p-values for the one-way ANOVA test are also shown for n=5 independent experiments;

[0014] FIG. 6(a) graphically illustrates Isotherm of pGL4.5 plasmid DNA adsorption on Amikabeads-P of diameter $11 \pm 4 \mu\text{m}$ in presence of (Buffer I) 10 mM, Tris.HCl buffer, pH 8.5, at 25°C for 24 hours (diamonds). Maximal adsorption (Q_{max}) is 44.5 μg of plasmid DNA / mg of Amikabeads-P (n=3) (diamonds). Effect of (Buffer II) 0.3 M salt on plasmid DNA adsorption on Amikabeads of average diameter $12 \pm 4 \mu\text{m}$ (squares). Reduced pDNA binding can be observed, indicating that electrostatic interactions drive adsorption;

[0015] FIG. 6(b) Desorption of plasmid DNA from Amikabeads-P (1 mg) using Tris-Cl buffer with 1 M salt (0.99 M NaCl, 10 mM Tris-Cl, pH 8.5) after 24 hours (diamonds), and Tris-Cl buffer with 1.3 M salt (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5) supplemented with 15% isopropanol (squares) at 25°C;

[0016] FIG. 7a graphically illustrates Adsorption isotherm of pGL4.5 plasmid DNA on quaternized Amikabeads-Q (Average diameter: $12 \pm 6 \mu\text{m}$) in (Buffer I) 10 mM, Tris.HCl buffer, pH 8.5, at 25°C following equilibration for 24 hours. Quaternization of Amikabeads greatly enhanced the plasmid DNA binding capacity compared to Amikabeads-Q ($p < 0.001$);

[0017] FIG. 7b recites Q_{max} calculated for three independent adsorption experiments in 10 mM, Tris.HCl buffer, pH 8.5, at 25°C. Average Q_{max} calculated for Amikabeads-Q was $\sim 300 \mu\text{g}$ of plasmid DNA / mg of Amikabeads-Q;

[0018] FIG. 7c graphically shows the percentage of bound pDNA desorbed with Tris-Cl buffer with 1 M salt (0.99 M NaCl, 10 mM Tris-Cl, pH 8.5), Tris-Cl buffer with 1.3 M salt (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5) and Tris-Cl buffer with 1.3 M salt (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5) supplemented with 15% isopropanol is shown. Significantly higher desorption of pDNA was recorded when isopropanol was used in the eluent ($p < 0.05^*$, Students' t-test);

[0019] FIG. 7d graphically shows desorption of pDNA from Amikabeads-Q at elevated temperature (50°C) and higher percentage of isopropanol (30%). Increasing the temperature from 25°C to 50°C significantly improved the amount of pDNA desorbed ($p < 0.05^*$, Students' t-test) while increasing the percentage isopropanol in the buffer from 15% to 30% had the opposite effect ($p < 0.001^{**}$, Students' t-test);

[0020] FIG. 8a comprises Confocal microscopy of pDNA loading on Amikabeads-P (average diameter: $\sim 9 \pm 4 \mu\text{m}$);

[0021] FIG. 8b comprises Confocal microscopy of pDNA loading on Amikabeads-Q (average diameter: $\sim 14 \pm 6 \mu\text{m}$) is shown. Amikabeads-P and Q (1 mg each) were incubated with 40,000 ng and 200,000 ng of pDNA respectively, in 10 mM Tris-Cl buffer, pH 8.5 at 25°C for 24 hours. Following washes, the beads were stained with 2 μM ethidium homodimer-1 for 20 minutes prior to imaging. Fluorescence of ethidium homodimer-1 was visualized using an excitation of 528 nm and emission of 617 nm (red color). Plasmid DNA adsorbed on the surface of both, Amikabeads-P and Q, with minimal penetration into the beads. Amikabead clusters were observed after addition of plasmid DNA, possibly due to the bridging;

[0022] FIG. 8c comprises Confocal microscopy of pDNA loading on Amikabeads not treated with pDNA did not demonstrate ethidium homodimer-1 fluorescence and

clustering. A representative image of Amikabeads-Q is shown. Scale bar = 100 μ m in all cases;

[0023] FIG. 9a graphically illustrates cell viability of PC3 human prostate cancer cells after exposure to different amounts of unmodified and quaternized Amikabeads for 24 hours as determined using the MTT assay (n=2). *1 – statistical significance p=0.003 between cell viability of cells exposed to 300 μ g Amikabeads-P and Q. *2 – statistical significance p=0.00002 between cell viability of cells exposed to 500 μ g Amikabeads-P and Q. Amikabeads-P (approximate diameter: $\sim 12 \pm 4 \mu$ m) and Amikabeads-Q (approximate diameter: $\sim 11 \pm 6 \mu$ m). Students' t-test was used to determine statistical significance;

[0024] FIG. 9b shows Amikabeads-P (500 μ g; approximate diameter: $\sim 12 \pm 4 \mu$ m) exposed to 10,000 PC3 prostate cancer cells for 24 hours followed by live (green)-dead (red) staining. Scale bar = 100 μ m. Green fluorescence emission of Calcein inside the live cells was detected using 38 HE filter set (Excitation: 470/40; Emission: 525/50) and red fluorescence of nucleic acid bound-EthD-1 was detected using a 43 HE filter set (Excitation: 550/25; Emission: 605/70);

[0025] FIG. 9c shows 500 μ g Amikabeads-Q (approximate diameter: $\sim 11 \pm 6 \mu$ m) exposed to 10,000 PC3 prostate cancer cells for 24 hours followed by live (green)-dead (red) staining. Scale bar = 100 μ m. Green fluorescence emission of Calcein inside the live cells was detected using 38 HE filter set (Excitation: 470/40; Emission: 525/50) and red fluorescence of nucleic acid bound-EthD-1 was detected using a 43 HE filter set (Excitation: 550/25; Emission: 605/70);

[0026] FIG. 10a shows in situ DNA capture using Amikabeads-P from mammalian cells and 500 μ g Amikabeads-P (approximate diameter: $\sim 12 \pm 4 \mu$ m) exposed to 10,000 PC3 prostate cancer cells for 24 hours followed staining with the nucleic acid binding dye, ethidium homodimer-1. Nucleic acids adsorbed to the beads were stained red;

[0027] FIG. 10b shows no red fluorescence was observed with ethidium homodimer-1 stained Amikabeads in the absence of bound DNA. Scale bar = 20 μ m;

[0028] FIG. 11 recites an optical image of Amikabeads-P prepared in silicone oil in absence of stabilizing surfactant. Scale bar = 100 μ m;

[0029] FIG. 12a recites an optical image of 0.5 mm model Amikabead-P indicated by an arrow;

[0030] FIG. 12b shows reaction with the ninhydrin assay reagent resulted in blue-purple color throughout the Amikabead (red arrow), indicating presence of reactive primary amines;

[0031] FIG. 13a shows Amikabeads-P reacted with excess glycidyl trimethyl ammonium chloride (GTMAC) at 70°C for 24 hours to generate quaternized Amikabeads-Q;

[0032] FIG. 13b. shows a Ninhydrin test on ~1 mg unmodified Amikabeads-P (left) and quaternized Amikabeads-Q (right). Formation of blue-purple color indicates presence of reactive (primary) amines;

[0033] FIG. 13c shows a fluorescein binding assay after incubating (i) Amikabeads-P and (ii) Amikabeads-Q with 200 μ L of 50 mg/mL of fluorescein sodium in 1% (v/v) NaOH solution (pH>12) in nanopure water for 10 minutes, followed by extensive washing. Formation of intense red color in the pellet indicates increased fluorescein retention by Amikabeads-Q, and thus, presence of quaternary amine groups. Lesser retention of fluorescein was observed in the case of the parental Amikabeads-P;

[0034] FIG. 14a shows representative images of Amikabeads $\sim 11 \pm 4$ μ m in diameter before quaternization using glycidyl trimethyl ammonium chloride (GTMAC). Scale bar = 100 μ m;

[0035] FIG. 14b shows representative images of Amikabeads $\sim 11 \pm 4$ μ m in diameter after quaternization using glycidyl trimethyl ammonium chloride (GTMAC). Scale bar = 100 μ m;

[0036] FIG. 14c graphically shows a diameter of Amikabeads-P and -Q. Quaternization did not change the spherical shape, but modestly increased the average diameter of Amikabeads by ~ 1.3 fold (* indicates $p < 0.005$);

[0037] FIG. 15 shows Agarose gel electrophoresis (AGE; 1% agarose gel) of desorbed plasmid DNA recovered from unmodified parental (P) and quaternized (Q) Amikabeads. Vertical Lanes 1 and 2 – Plasmid DNA desorbed from quaternized Amikabeads-Q (n=2). Lanes 3 and 4 - Plasmid DNA desorbed from unmodified Amikabeads-P (n=2). Lane 5 – Stock solution of plasmid DNA used in the binding studies. No visible differences can be seen between the desorbed pDNA and the stock pDNA, indicating the pDNA integrity is maintained upon binding and desorption from Amikabeads P and Q;

[0038] FIG. 16a shows an optical image Amikabeads-P before loading with (c) 40,000 ng and (d) 280,000 ng of pDNA/mg of beads;

[0039] FIG. 16b shows an optical image of Amikabeads-Q before loading with (c) 40,000 ng and (d) 280,000 ng of pDNA/mg of beads;

[0040] FIG. 16c shows an optical image Amikabeads-P after loading with (c) 40,000 ng and (d) 280,000 ng of pDNA/mg of beads;

[0041] FIG. 16d shows an optical image of Amikabeads-Q after loading with (c) 40,000 ng and (d) 280,000 ng of pDNA/mg of beads;

[0042] FIG. 16e graphically compares average aggregate sizes of Amikabeads P and Q after loading with pDNA. pDNA-Amikabeads-Q aggregates were significantly bigger than pDNA-Amikabeads-P aggregates (* $p < 0.001$, Students' t-test). Optical images of (f) Amikabeads-P and (g) Amikabeads-Q after desorption with Tris-Cl buffer with 1 M salt (10 mM Tris-Cl, 990 mM NaCl, pH 8.5) and Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) respectively. Scale bar = 100 μm ;

[0043] FIG. 16f recites an optical image of Amikabeads-P after desorption with Tris-Cl buffer with 1 M salt (10 mM Tris-Cl, 990 mM NaCl, pH 8.5) and Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) respectively. Scale bar = 100 μm ;

[0044] FIG. 16g recites an optical image of Amikabeads-Q after desorption with Tris-Cl buffer with 1 M salt (10 mM Tris-Cl, 990 mM NaCl, pH 8.5) and Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) respectively. Scale bar = 100 μm ;

[0045] FIG. 17a comprises a phase contrast image (10X zoom) of Amikabeads-Q loaded with 280,000 ng of pDNA/mg of beads treated with Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5);

[0046] FIG. 17b comprises a phase contrast image (10X zoom) of Amikabeads-Q loaded with 280,000 ng of pDNA/mg of beads treated with Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) supplemented with 15% isopropanol in absence;

[0047] FIG. 17c comprises a phase contrast image (10X zoom) of Amikabeads-Q loaded with 280,000 ng of pDNA/mg of beads treated with Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) after elevated temperature (50°C) for 36 hours;

[0048] FIG. 17d graphically shows average aggregate size of pDNA-Amikabeads-Q measured before and after treatment with different elution media. Average sizes of aggregates decreased following pDNA desorption (* $p < 0.001$). Equilibration with 15% isopropanol at elevated temperatures (50°C) significantly decreased the aggregate size

(** $p < 0.05$), which was consistent with highest amounts of pDNA desorption seen under these elution conditions;

[0049] FIG. 17e graphically shows pDNA-Amikabead-Q aggregate size decreased with increasing desorption of the biomacromolecule from the bead surface using different desorption buffers. (Square) – Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) (Diamond) - Tris-Cl buffer with 1.3 M salt (50 mM Tris-Cl, 1.25 M NaCl, pH 8.5) supplemented with 15% isopropanol in absence and (Triangle) - presence of elevated temperature (50°C) (f) Unadsorbed pDNA was found to be bound to the surface of Amikabeads-Q after desorption. Scale bar = 100 μm ;

[0050] FIG. 18 shows Amikabeads-Q (500 μg ; approximate diameter: $11 \pm 6 \mu\text{m}$) exposed to 10,000 PC3 prostate cancer cells for 24 hours, followed by staining with Calcein AM. The beads on the surface of the PC3 prostate cancer cells can be seen as dark spheres. Green fluorescence emission of calcein inside the live cells was detected using 38 HE filter set (Excitation: 470/40; Emission: 525/50). Scale bar = 100 μm ;

[0051] FIG. 19 graphically shows cell viability of PC3 human prostate cancer cells after treatment with different amounts of Amikabeads-P and -Q (Average diameters: Amikabeads-P: $12 \pm 4 \mu\text{m}$ Amikabeads-Q: $11 \pm 6 \mu\text{m}$) for 6 hours in serum-free media, followed by 18 hours in serum containing RPMI media as determined using the MTT assay. Statistical test (Students' t-test) was used to test the statistical difference between the unmodified and quaternized Amikabeads. At all concentrations, the parental Amikabeads-P (squares) were significantly more toxic than the quaternized Amikabeads-Q (diamonds) ($p < 0.05$); and

[0052] FIG. 20 shows doxorubicin (Red) conjugated Amikabeads (pellet).

[0053] FIGs. 21a-b illustrate a cylindrical macroporous Amikagel based monoliths for plasmid DNA extraction, bone mimetic 3D surfaces etc.

[0054] FIG. 21c illustrates a blue color which indicates positive for presence of amines on the surface of the gel (Ninhydrin assay). Scale bar = 1 cm

[0055] FIG. 22 illustrates the generation of Doxo-Beads.

[0056] FIG. 23A depicts pDNA absorbed per 1mg of doxo-beads against with pDNA in solution phase.

[0057] FIG. 23B depicts pDNA in equilibrium phase per amount of pDNA bound to the Amikabeads against with pDNA in equilibrium phase. The inverse of slope shows

the maximum pDNA binding capacity for doxo-beads. The maximum binding capacity of PGL 4.5 pDNA for doxo-beads is found out to be 200mg .

[0058] FIG. 24 depicts pDNA desorbed from doxo-beads in percentage against with pDNA absorbed on the surface of beads.

Detailed Description Of The Preferred Embodiments

[0059] This invention is described in preferred embodiments in the following description with reference to the FIGs, in which like numbers represent the same or similar elements. Reference throughout this specification to “one embodiment,” “an embodiment,” or similar language means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment,” “in an embodiment,” and similar language throughout this specification may, but do not necessarily, all refer to the same embodiment.

[0060] The described features, structures, or characteristics of the invention may be combined in any suitable manner in one or more embodiments. In the following description, numerous specific details are recited to provide a thorough understanding of embodiments of the invention. One skilled in the relevant art will recognize, however, that the invention may be practiced without one or more of the specific details, or with other methods, components, materials, and so forth. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the invention.

[0061] Applicants disclose the generation of amikacin antibiotic -derived microbeads, ‘Amikabeads’, as anion-exchange resins for potential use in pDNA purification as well as in situ capture of DNA from mammalian cells.

[0062] Applicants further disclose a new microresin and macroporous monolith based material for plasmid DNA binding and purification, mammalian whole cell genomic DNA extraction and in-vitro cell culture. The monolith and microresins are based on crosslinked aminoglycoside monomer. Aminoglycoside and a diepoxide crosslinker were emulsified into microdroplets and allowed to gel to generate the microbeads for plasmid DNA binding. In addition, the microbeads were then surface conjugated with multiple ligands to improve their plasmid DNA binding capabilities. The aminoglycoside after microbead formation contains multiple units of aminoglycosides rich in primary amines and hydroxyls. These primary amines and

hydroxyls can be further conjugated with multiple ligands useful for plasmid DNA binding. Aminoglycoside and crosslinker in organic solvent were used to wet a salt column before crosslinking. After crosslinking, the salt was dissolved away to leave a macroporous column that can be used as monoliths for plasmid DNA binding, 3D cell culture etc.

[0063] Reaction between amines present in amikacin with the epoxide groups in PEGDE resulted in the formation of a crosslinked hydrogel ('Amikagel') as shown in FIG. 1. Multiple amines in Amikagel, particularly in the form of microbeads, can be exploited for nucleic acid (pDNA) biotechnology. Parental Amikabeads-P were generated using an emulsion polymerization method (FIG. 2 a-b); a crosslinking ratio of 1:2 amikacin to PEGDE was used. The amikacin-PEGDE mixture formed a solid hydrogel within ~8 minutes when stirred at 100 rpm at 70°C. Hence, a pre-gelling time of 4 minutes was chosen, following which, the pre-gelled Amikagel solution was introduced into the heated mineral oil phase (65°C, constant stirring at 260 rpm) (FIG. 2). In all cases, mineral oil was supplemented with 1% Span-80 surfactant (w/v) in order to stabilize the water-in-oil microemulsion. Microbeads synthesized in absence of stabilizing surfactant were irregular and non-spherical (FIG. 11). Amikabeads-P formed in mineral oil were separated from the oil phase by centrifugation at 5000g for 10 minutes, following which, the microbeads were extensively washed with nanopure water supplemented with 1% (v/v) Tween 20. Addition of Tween 20 allowed the removal of remaining Span-80 and mineral oil. Tween and Span surfactants are often mixed together in order to generate surfactants of desired Hydrophilic : Lipophilic Balance (HLB) values (Note: An HLB value of more than 10 is necessary in order to ensure surfactant solubility in water). Given the insolubility of Span-80 in water (HLB value of 4.3), Tween-20 (HLB value of 16.3) was added to solubilize and remove any remaining Span-80 and mineral oil. The steps used to generate Amikabeads-P are shown in FIG. 2 a, b.

Characterization of Amikabeads

Shape and Particle Size of Amikabeads

[0064] Amikabeads-P generated using the above emulsion system were found to have spherical morphology and demonstrated minimal aggregation (FIG. 2c, FIG. S11). However, it is likely that in a chromatographic setting of a tightly packed column, Amikabeads will be in close contact with each other. Amikabeads-P were sputter-coated with 8 nm thick coating of Au-Pt in order to visualize them using scanning electron

microscopy (SEM). As shown in FIG. 2d, Amikagel-P microbeads had a predominantly spherical shape with smooth as well as rough surface morphology, which is similar to other hydrogel microbeads described previously 8. The average diameter of Amikabeads-P was $\sim 9 \pm 4$ microns (FIG. 2d), and was dependent on the number of times the mixture of mineral oil and span 80 was used in serial batches. Upon repeated usage of mineral oil and span 80, the diameters of Amikabeads demonstrated a modest increase ($p < 0.01$, one-way ANOVA) (FIG. 3a).

[0065] In certain embodiments, Applicants introduce additional fresh Span-80 after every batch of Amikabead synthesis to limit the batch-to-batch variation in the bead size. This supplementation accounts for any losses in Span-80 by thermal degradation during the preparation of each batch. As seen in FIG. 3b, the diameter of Amikabeads-P did not change significantly with addition of 400 mgs of fresh Span-80 after each batch of preparation. An observed p-value of 0.174 indicated that it is not possible to reject the null hypothesis that particle diameters in all three consecutive batches are the same in FIG. 3b (p-value threshold of 0.05 for statistical significance). Thus, addition of Span-80 indeed reduced the batch-to-batch variation in particle diameter / size. Following this observation, Applicants were able to mix different batches in order to obtain high amounts of Amikabeads-P for subsequent investigations.

[0066] Amikabeads-P demonstrated a swelling ratio of approximately 1.74 ± 0.2 (or 174%). This is similar to commercially available anion-exchange resins, which demonstrate swelling ratios of up to 200%. For example, polystyrene-divinylbenzene (PS-DVB) beads containing quaternary ammonium groups used as strong anion exchange resins demonstrated a swelling ratio of 1.7, which is very similar to that of Amikabeads.

Amine content of Amikabeads

[0067] The presence of accessible amine moieties is critical for the use of Amikabeads in anion-exchange applications. Furthermore, presence of reactive amines allows for subsequent conjugation chemistries if required. The reaction of ninhydrin reagent with reactive (primary and secondary amines) results in colorimetric changes; a bluish-purple color can be observed upon reaction with primary amines 11. Reaction of a 0.5 mm model Amikabead with 100 μ L of ninhydrin reagent at 70°C for ten minutes resulted in the formation of intense bluish-purple color throughout the bead (FIG. 12).

The amine content of lyophilized Amikabeads-P was estimated to be 1.8 ± 0.3 μ moles of amine per milligram of Amikabeads.

pDNA Binding to Amikabeads-P

[0068] The binding of pDNA to Amikabeads-P was determined using batch adsorption assays, in order to determine the potential use of these microbeads as anion-exchange resins; the pGL4.5 luciferase pDNA was used as a model plasmid in all batch binding experiments. The pGL4.5 plasmid codes for luciferase reporter protein, which is commonly used for analyzing the transfection efficacy of various non-viral vectors 12. The Langmuir adsorption isotherm was used to fit the experimental data and obtain parameters including the maximum binding capacity ($Q_{\max}$) and equilibrium binding constant (K). Tables 1A and 1B show the different parameters generated by fitting the experimental adsorption isotherm data to a Langmuir isotherm; the Langmuir adsorption equation was converted to its linear form in order to determine the $Q_{\max}$ value. The average ($n=3$) maximum adsorption capacity ($Q_{\max}$) calculated by fitting the adsorption isotherm of the Amikagel-P microbeads was approximately 44.5 μ g pDNA/mg of Amikabeads-P with an equilibrium constant K of 0.04-0.06 L/mg at an equilibrium pDNA concentration (solution phase) of 300-400 mg/L. Further increasing the concentration of pDNA in the solution phase did not result in any increase in the amount of DNA adsorbed on Amikabeads-P, indicating saturation (FIG. 5a, diamonds).

[0069] TABLE 1A recites adsorption of plasmid DNA to Amikabeads-P in presence of 10 mM Tris-Cl buffer (pH 8.5) (Buffer I)

TABLE 1A

ID	Average bead size (μm)	$Q_{\max}$ (μg/mg)	K (L/mg)	R-square
1	10 ± 3	56.0	0.01	0.97
2	11 ± 5	46.4	0.02	0.88
3	12 ± 4	31.5	0.05	0.96

[0070] pDNA binding to Amikabeads-P was significantly reduced in presence of 0.3 M (buffer II) and 0.6 M salt (buffer III). TABLE 1B recites adsorption of plasmid DNA to Amikabeads-P in presence of 300 mM Tris-Cl -NaCl buffer (pH 8.5) (Buffer II) and 600 mM Tris-Cl -NaCl buffer (pH 8.5) (Buffer III).

[0071] As expected, higher salt concentrations screen electrostatic charges and obviate interactions between negatively charged DNA molecules and the positively charged Amikabeads-P. The saturation amount of pDNA on Amikabeads-P was not calculated under these elevated salt concentrations, since the adsorption isotherm was found to be in the linear range, even for very high pDNA concentrations in the solution phase (FIG. 4a; squares).

[0072] Recovery of pDNA from Amikagels-P bound under low salt conditions (i.e. 10 mM Tris-Cl, pH 8.5) was investigated using Tris-Cl buffer with 1 M salt (0.99 M NaCl and 10 mM Tris-Cl, pH 8.5) and Tris-Cl buffer with an organic modifier (50 mM Tris-Cl and 1.25 M NaCl, with 15% isopropanol, pH 8.5). Approximately 70-100% of originally adsorbed pDNA was desorbed when a salt concentration of 1M was used. No improvement in desorption was noticed when a buffer with higher salt concentration and isopropanol was used (FIG. 4b), indicating that hydrophobic modifiers did not help recovery from Amikabeads-P. Higher percentages of desorption were observed when lesser amounts of pDNA were adsorbed onto the beads, indicating marginal losses in recovery at higher loadings (FIG. 4b).

Quaternization of Amikagel-P beads enhances pDNA binding

[0073] Amikacin has 4 primary amines, one secondary amine and 8 hydroxyl groups. Quaternization of amine groups can help increase the content of positive charges in the microbeads. This in turn, was anticipated to result in enhanced pDNA binding efficacy. The amines on the microbeads were therefore modified to quaternary amines using glycidyl trimethyl ammonium chloride (GTMAC), at neutral or acidic pH, only the amines of the amikacin react with the epoxide group of GTMAC as an addition reaction (FIG. 13(a)). Formation of quaternary amines on Amikabeads was verified using both, the ninhydrin assay as well as the fluorescein-binding assay. Ninhydrin reagent reacts with primary amines resulting in the formation of a blue-purple colored product. However, as expected, quaternized Amikabeads-Q did not demonstrate a blue color unlike unmodified Amikabeads-P (FIG. 13(b)). The fluorescein-binding assay was also able to ascertain quaternization in Amikabeads-Q; in presence of 1% (v/v) NaOH (pH > 12), the primary amines present on amikacin are no longer positively charged, whereas the quaternary amines on the quaternized beads retain the permanent positive charge. Hence, the negatively charged fluorescein can interact with and bind the quaternary

ammonium moieties in Amikabeads-Q, but not the primary amines in Amikabeads-P (FIG. 13(c)).

[0074] Adsorption experiments indicated that Amikabeads-Q demonstrated significantly higher ($p < 0.001$) pDNA loading capacities than Amikabeads-P (FIG. 5a-b). Average $Q_{\max}$ values, determined after $n=3$ independent experiments, were approximately 300 μg pDNA/mg of Amikabeads-Q at an equilibrium pDNA concentration of 150-200 mg/L. This pDNA loading was approximately 30% of the weight of the Amikabeads-Q and is almost 7-fold higher than that on Amikabeads-P. Interestingly, Amikabeads-Q demonstrated a slightly higher average diameter than Amikabeads-P (10.8 microns for Amikabeads-P and 13.9 microns for Amikabeads-Q; FIG. 14). This increase in average diameter of Amikabeads-Q could be due to repulsion between the quaternized charges, and may have a minimal contribution to the observed increase in pDNA binding capacity of Amikabeads-Q by increased surface area (~1.66 fold increase over Amikabeads-P).

[0075] The static binding capacities of commercially available resins range from 1 to 10 mg of pDNA/mL of resin slurry. Gigaporous rigid ceramic quaternary amine containing HyperD-Q polymerized hydrogel resins and quaternized polyethyleneimine-containing porous resins (POROS HQ resins) possess among the highest static binding capacities at ~10 mg of pDNA/mL of resin slurry. Static pDNA binding capacities of Amikabeads-Q and Amikabeads-P were estimated to be ~6 mg of pDNA/mL of resin slurry and ~1.2 mg of pDNA/mL of resin slurry, respectively, which is comparable to that of several commercially available resins. As described in the methods section, the static binding capacity of Amikabeads was estimated by extrapolating the pDNA binding on 1 mg or 50 μL of Amikabeads-Q to 1 mL.

Desorption of pDNA from Amikabeads-Q

[0076] Up to 40% of bound pDNA was desorbed from Amikabeads-Q when eluted with Tris-Cl buffer with 1M salt (0.99 M NaCl and 10 mM Tris-Cl, pH 8.5) at 25°C (FIG. 5c), which was significantly less in percentage than desorption from Amikabeads-P. Thus, while Amikabeads-Q were able to bind higher quantities of pDNA, complete recovery of the beads was not possible using 1 M salt. Improved recovery was seen when Tris-Cl buffer with 15% isopropanol (1.25 M NaCl and 50 mM Tris-Cl, pH 8.5) was employed for desorption at 25°C (FIG. 5c). Use of 1.25M NaCl did not enhance desorption of pDNA from Amikabeads-Q in absence of isopropanol (FIG. 5c), indicating

that the hydrophobic modifier was important for pDNA recovery from Amikabeads-Q. It is known that addition of small organic molecules to desorption buffers reduces the polarity of the solution, which can facilitate desorption of molecules from resins. It is likely that in addition to the anion-exchange character of the quaternary ammonium groups, the presence of three methyl groups in a GTMAC molecule, imparts a modest, but collective hydrophobic character to Amikabeads-Q. As a result, isopropanol may act as a hydrophobic modifier, resulting in an increase in desorption of pDNA from Amikabeads-Q. Interestingly, this opens up the possibility of screening a variety of different small-molecule organic modifiers for enhancing pDNA recovery from these resins, which clearly have the advantage of high binding capacities.

[0077] pDNA desorption was greatly increased when the temperature was raised from 25°C to 50°C ($p < 0.001$) (FIG. 5d) in presence of 15% isopropanol; a recovery of $\sim 70 \pm 6\%$ of initially adsorbed pDNA was obtained at 50°C. Higher temperatures are known to facilitate increased desorption of pDNA from chromatographic columns 17. Interestingly, increasing the isopropanol percentage beyond 15% did not improve recovery but rather decreased the pDNA content desorbed into the solution phase ($p < 0.05$) (FIG. 5d).

[0078] The quality of the recovered pDNA from the unmodified Amikabeads-P and the quaternized Amikabeads-Q was determined by running it on a 1% agarose gel. As shown in FIG. S5 (), the desorbed pDNA recovered by the addition of salt was of the same integrity as that of the pDNA that was loaded on the resin, indicating that Amikabeads did not induce any visible or gross damage to pDNA.

Comparison of pDNA Adsorption on and Desorption from Amikabeads-P vs. Amikabeads-Q

[0079] As described hereinabove, Amikabeads-Q demonstrated significantly higher ($p < 0.001$) pDNA loading capacities than Amikabeads-P (FIG. 5a-b) likely due to the higher positive charge of quaternary amines. It is well known that quaternary amine groups (present in Amikabeads-Q) are more positively charged and hydrophilic than primary amines (present in Amikabeads-P). It has been reported that methyl groups on a tetramethylammonium ion were found to promote hydrophilicity rather than hydrophobicity. However, the range for this effect was limited to 0-0.08 mole fraction of the solute. Presence of multiple copies of methyl groups from GTMAC on Amikabeads-Q is likely to impart these materials with a modest hydrophobic character, which is likely

absent in Amikabeads-P. However, this hydrophobic character will relatively be modest in comparison to its high hydrophilicity.

[0080] It has been reported that higher pDNA desorption from a substrate modified with tetraethyl quaternary ammonium groups in presence of lower amount of isopropanol (~20%), when compared to higher isopropanol amounts (~40%). It has been further suggested that the existence of a threshold alcohol percentage up to which the ability of the alcohol to overcome hydrophobic interactions is greater than the overall increase in the solution dielectric constant. This overcoming of hydrophobic interactions, in turn, allows for increased desorption of an adsorbed molecule from the surface. Once this threshold is crossed, it is thought that the decrease in dielectric constant can help electrostatic interactions dominate, which actually can result in lower desorption of pDNA from the adsorbent. Similar trends can be observed for pDNA desorption from Amikabeads-Q (FIG. 5). Addition of ~15% isopropanol (v/v) sufficiently increases the hydrophobic character of the solvent causing desorption of pDNA from the surface. Use of 30% isopropanol, however, can significantly modify the dielectric constant such that electrostatic interactions can dominate, resulting in lower pDNA desorption from Amikabeads-Q. Absence of such hydrophobic interactions in Amikabeads-P could be the reason behind no improvement in desorption after addition of organic modifier.

Visualization of Amikabeads before and after pDNA Binding

[0081] Confocal fluorescence microscopy was employed in order to visualize the localization of pDNA on the Amikabeads upon binding. As shown in FIG. 6, pDNA was predominantly found to adsorb on the surface of both, Amikabeads-P and -Q. BET analysis indicated that Amikabeads-P possessed a surface area of approximately 2.0 m²/g and a pore size of 4.0 nm. This pore size is consistent with that of several existing ion-exchange resins, but indicates challenges that may be associated with pDNA transport and penetration into the beads, given the larger size (70-100 nm) of super-coiled pDNA 22. These results can explain the observation that pDNA binding is primarily observed on the surface of Amikabeads.

[0082] Following binding of pDNA, Amikabeads-P and Q were found to aggregate, likely due to bridging of the biomacromolecule and the beads (FIG. 6a-b). Neither Amikabeads-P, nor Amikabeads-Q, were found to aggregate in absence of pDNA loading (FIG. 2c, FIG. 16 a-b). The average aggregate size of pDNA with Amikabeads-Q was significantly higher than that of Amikabeads-P ($p < 0.001$, Students' t-test; FIG. 16).

It is likely that higher positive charge on the Amikabeads-Q allows the formation of bigger aggregates upon pDNA binding, compared to those seen in case of Amikabeads-P. It was noticed that the aggregate size decreased after pDNA desorption. As shown in (FIG. 16 f-g), aggregate sizes of both Amikabeads-P and Q decreased after desorption. However, Amikabeads-Q showed lower pDNA desorption with 1 M salt than Amikabeads-P (~40% for Amikabeads-Q vs. ~75% for Amikabeads-P). It is likely that the higher size of aggregates in Amikabeads-Q could have played a role in this lower percent desorption.

[0083] The size of pDNA-Amikabead-Q aggregates were also determined after equilibration with different desorption buffers (listed in the Experimental section). Desorption resulted in lower aggregate sizes in all cases. In general, it was observed that the size of pDNA-Amikabead-Q aggregate decreased with increasing desorption of the biomacromolecule from the bead surface (FIG. 17e). Thus, desorption of the pDNA from Amikabead surface obviates extensive bridging, and therefore results in significant reduction in aggregate size. Undesorbed pDNA was found bound to the surface of Amikabeads (FIG. 17f). It is important to note that this aggregation behavior may be moot in case of well-packed chromatographic columns that may employ Amikabeads for pDNA separations.

In situ capture of DNA from Mammalian Cells

[0084] Cationic microparticles and membranes have been used for on-site capture of genomic DNA for polymerase chain reactions. It has been reported that chitosan coated beads that could extract DNA from lysed whole blood sample for PCR analyses. Here, Applicants investigated if Amikabeads P and Q could be employed for extracting DNA directly from mammalian cells.

[0085] PC3 human prostate cancer cells were incubated with different amounts of Amikabeads-P and -Q in order to investigate their effect on viability following cell lysis; direct lysis mediated by the microbeads can facilitate extraction of DNA from cells. Amikabeads-P (500 µg, 24 hour incubation) resulted in loss of viability of ~80% of the cell population, as determined by the MTT assay (FIG. 7a, diamonds). The LC50 (amount required to reduce cell viability to 50%) value of Amikabeads-P was approximately 400 µg for PC3 prostate cancer cells under these experimental conditions.

[0086] In contrast, unlike Amikabeads-P, Amikabeads-Q did not induce significant cell death in PC3 cells even when amounts as high as 500 µg were employed (FIG. 7b,

squares). These results from the MTT assay were further confirmed using LIVE/DEAD analyses, in concert with fluorescence microscopy (FIG.s 7a-b and FIG.s 18).

Amikabeads-P demonstrated significantly higher levels of red fluorescence compared to Amikabeads-Q, since cell lysis by the former results in damage and exposure of cellular DNA to the red-fluorescent ethidium homodimer-I dye. In all cases, the microbeads were seen in close proximity with the cells (FIG. 7c and 19). Incubation of Amikabeads-P and -Q with PC3 cells in absence of serum proteins for 6 hours did not change the toxicity of parental (P) or quaternized (Q) Amikabeads (FIG. 19), indicating that serum proteins have minimal or no role in determining Amikabead cytotoxicity.

[0087] These results are along the lines of previous reports in literature that indicate reduced cytotoxicity of polymers after quaternization. For example, Brownlie et al. showed that quaternization of amines in polyethyleneimine (PEI) reduced its cytotoxicity by almost 4 fold in A431 lung cancer cells. It is understood that permanent positive charge of quaternized nitrogens unlike primary amines creates a solubilizing and hydrophilic environment that reduces interaction with the negative charges of the cell surface. Palermo et al. suggested that the interaction between polymers containing primary (1°), tertiary (3°) and quaternary ammonium groups (4°) with lipid membranes is dependent upon a combination of hydrophilicity combined with hydrogen-bonding effects. 4° ammonium containing compounds were found to be most hydrophilic, ineffective in partitioning into the hydrophobic core of the lipid-bilayer, less hemolytic than the other two polymers that caused dye leakage from dye-filled liposomes. Effective solvation on the 4° ammonium groups were deemed the reason for its hydrophilic behavior. Applicants believe similar interaction could be responsible for cytotoxicity of Amikabeads-P and not Q. Thus, although quaternization of Amikabeads-P led to microbeads with significantly lower toxicities, Amikabeads-P were employed for directly sequestering DNA following lysis of mammalian cells.

[0088] Fluorescence microscopy indicated in situ capture of cellular DNA in case of PC3 cells treated with $12 \pm 4 \mu\text{m}$ Amikabeads-P for 24 h (FIG. 8a). As seen in FIG. 8a, Amikabeads-P were able to simultaneously lyse cells, extract DNA molecules, and bind them. This activity of Amikabeads-P can have direct application in point-of-care testing 28, on-chip nucleic acid extraction and detection, and on-site/on-chip whole cell lysis and DNA/RNA capture for PCR reactions. Amikabeads-P, in absence of bound DNA, did not demonstrate any red fluorescence, which is along expected lines (FIG. 8b).

Amikabead drug conjugation

[0089] Primary amines and hydroxyls of Amikabeads-P were reacted with crosslinker 1,4-Cyclohexane dimethanol diglycidyl ether in excess to generate free epoxide groups. These free epoxide groups were further reacted and quenched with anticancer chemotherapeutic drug doxorubicin. Doxorubicin was found to bind to the beads and pellet down upon centrifugation (FIG. 20). Red color pellet was further analyzed using fluorescence microscopy to identify the location of the drug on the resin (data not shown). Table 2. shows the list of small molecules and anticancer drugs that can be attached to Amikabeads and Amikagel monolithic columns for novel mixed-mode pDNA chromatographic resins.

TABLE 2

ID	Small molecules
1	Doxorubicin
2	Mitoxantrone
3	Daunomycin
4	Amonafide
5	Etoposide
6	Adenine
7	Guanine
8	Cyclosporamide
9	Vincristine
10	Netropsin
11	Furamidines
12	Ethidium bromide
13	Proflavine
14	Epirubicin
15	8-Aminoacridine
16	Mitomycin
17	Distamycin
18	Idarubicin
19	Valrubicin
20	Pixantrone
21	Bleomycin
22	Methotrexate

Amikagel monolith design

[0090] Solution of Amikacin Hydrate was dissolved in DMSO and PEGDE was applied to a salt NaCl column and incubated at 40°C for 24 hours. After 24 hours, the salt was dissolved in water leaving the macroporous monolith column as shown in FIG. 21 (a-c).

[0091] Applicants have developed a novel anion-exchange resin material based on hydrogel microbeads ('Amikabeads') generated from amikacin and poly(ethylene glycol) diglycidyl ether, with an eye towards applications in DNA biotechnology. Parental (P) Amikabeads demonstrated a $Q_{\max}$ of 44.5 $\mu\text{g pDNA} / \text{mg}$ of the microbeads as determined from the Langmuir isotherm.

[0092] Near-complete recovery of pDNA was possible from Amikabeads-P using high salt concentrations, indicating electrostatic binding between the biomolecule and the microbeads. Quaternization of amines present in parental Amikabeads resulted in the formation of microbeads (Amikagels-Q), which demonstrated $Q_{\max}$ values approximately 7-fold higher than those for Amikagels-P.

[0093] Desorption of the pDNA from these beads was not as efficient as Amikabeads-P, although, recovery could be significantly improved by using an organic modifier, isopropanol. Amikabeads-P were able to extract and bind cellular DNA following lysis of mammalian cells, indicating their use for in situ DNA extraction; Amikabeads-Q however, were not able to lyse cells and demonstrated lower cytotoxicities.

[0094] Applicants' results indicate that Amikabeads are a versatile platform, with multiple easily conjugable groups for several applications in DNA biotechnology ranging from purification to cellular DNA recovery.

[0095] Antibiotic aminoglycosides were mixed with cross-linker poly(ethyleneglycol) diglycidyl ether and were emulsified in mineral oil and detergent to yield parental amikagel microbeads (Amikabeads-P) of $\sim 10 \mu\text{m}$ after 25 minutes of mixing. Synthesized Amikabeads-P were extensively characterized using SEM, BET surface area and porosity measurement, optical microscopy, ninhydrin assay for amine quantification etc. After preparation, pGL4.5 plasmid was used to study its ability to bind plasmid DNA. Amikabeads-P were quaternized using glycidyl trimethyl ammonium chloride (GTMAC) to yield Amikabeads-Q to improve their cationicity towards plasmid DNA binding. Multiple chemotherapeutic anticancer drugs were conjugated to Amikabeads-P using crosslinkers to improve its plasmid DNA binding abilities. Amikabeads-P and Q were exposed to PC3 prostate cancer cells (ATCC - CRL 1435) and were used to extract their genomic DNA in-situ.

[0096] Pre-packed salt columns were wetted with aminoglycoside-PEGDE pre-gel DMSO solutions which were allowed to crosslink at 40 °C for 24 hours. After

crosslinking, the salt was dissolved away to leave macroporous amikagels/amikagel monoliths. Macroporous amikagels/amikagel monoliths will be used for plasmid DNA binding, in-vitro models for prostate cancer cell culture in dormancy and relapse, mechanical reinforcement to mimic trabecular bone tissue, photothermally responsive material for wound healing etc.

[0097] In addition, amikabeads can be used as efficient tools for endotoxin binding and removal, substrate for peptide synthesis, substrate for chemical conjugation for ligands for pollutant removal, biomolecule binding and detection etc. Macroporous amikagels/amikagel monoliths can also be used for the same with increased surface area. In addition, they can be used for cell culture and tissue grafts to regenerate and replace tissues.

[0098] Gene therapy through plasmid DNA (pDNA hereafter) is being increasingly explored as a suitable therapeutic biologic for multiple diseases such as cancer, AIDS, Cystic fibrosis and DNA based vaccinations. Plasmid DNA is an extrachromosomal double stranded genetic material responsible for conferring selective advantages to bacteria such as antibiotic resistance and adaptability. These plasmid DNAs can be engineered to code for selective anticancer proteins that can induce death in cancer cells. Engineered plasmid DNA can then be grown in large quantities in bacterial cells before being extracted to pure form for therapeutic purposes. Extraction of pharmaceutical grade plasmid DNA requires multiple downstream processing steps to get rid of unwanted cellular materials and genomic DNA. Anion-exchange chromatography, affinity-based chromatography, hydrophobic interaction chromatography, and size-exclusion and perfusion chromatography have been explored for purification of pDNA.

[0099] Two critical components of these chromatographic resins are the ligands used for binding the pDNA and the substrate to which the ligands are attached. Multiple ligands such as arginine, histidine, zinc fingers, triple-helix forming nucleotide sequences etc. have been explored as pseudo-affinity ligands for plasmid DNA binding. Stable substrates such as agarose and cross-linked agarose are commonly used. But these substrates are abundant in hydroxyl groups which are not as reactive as amine groups. To overcome these current challenges, here Applicants describe an invention of a completely new chromatographic resin where a crosslinked aminoglycoside-PEGDE hydrogel microbead and monolith substrate material and anticancer chemotherapeutic based ligands. Anticancer chemotherapeutic ligands interact with DNA via multiple

modes such as electrostatic and hydrophobic interactions. Incorporation of such chemotherapeutics as ligands is hypothesized to improve binding capabilities of plasmid DNA via multi-modal interactions.

[00100] Applicants' chromatographic beads provide a high density amine rich resin with high cationicity. In addition, it has abundant highly conjugable groups such as hydroxyls and amines on its surface for further modification. Aminoglycoside-PEGDE hydrogel material was emulsified to create microbeads with extensive surface area for plasmid DNA binding. In addition, the primary amines were quaternized to generate a highly cationic substrate that could bind very high amounts of the cargo. Although microbeads provides a unique geometry for binding and extraction of plasmid DNA, numerous challenges such as efficient packing of chromatographic column and high fluid pressure during operation have to be dealt with. In order to overcome these challenges, Applicants designed macroporous amikagels/ amikagel monoliths with varying pore sizes. These monoliths equipped with similar highly conjugable groups allows conjugation of pseudo affinity and multi-modal ligands that can be used for selective binding and purification of plasmid DNA.

[00101] The invention relates to a novel aminoglycoside based anion exchange chromatographic resin for plasmid DNA purification with a binding capacity of ~6 mg of plasmid DNA/mL of the resin. Abundant presence of easily conjugable groups make the resin highly desirable for further conjugations and modifications. The resin has been developed in two architectures namely microbeads and monolithic columns. Multiple ligands such as quaternary ammonium and mixed- mode ligands have been successfully attached to the resin for improvements in plasmid DNA binding.

[00102] The invention also relates to utilization of these novel beads for whole mammalian cell lysis for on-chip PCR reaction and point-of-care diagnostics.

[00103] A novel aminoglycoside based hydrogels have been generated in two different architectures of microbeads and monoliths for enhancing plasmid DNA binding. These resins provide a potential for further modifications with other ligands by the availability of multiple easily conjugable sites.

[00104] These resins have also been used for mammalian cell lysis and extraction of whole cell DNA. These can be used for on- chip PCR reactions and point-of-care diagnostics.

[00105] The material also provides a macroporous 3D substrate for cell culture and *in vitro* models of bone trabeculae etc.

[00106] The same material has been developed in multiple architectures for different applications. The presence of abundant easily conjugable groups such as amines and hydroxyls allows for further modification of the resin to cater the specific need of the application.

[00107] The invention also relates to the development of a macroporous hydrogel substrate for bone tissue engineering for prostate cancer dormancy and relapse. These macroporous hydrogels can be used for multiple applications including, but not limited to 3D *in vitro* model for cell culture for drug screening and discovery, stem cell differentiation substrates, tissue welding constructs and regenerative medicine.

[00108] Presence of a macroporous structure also potentiates its use towards bone mimetic material where macroporous amikagel can be used as bone trabecular mimetic. Macroporous amikagels can be strengthened using different reinforcing agents such as but not limited to carbon nanotubes, gold nanorods, hydroxyapatite to match the mechanical stiffness of bone trabecular region. These three dimensional macroporous gels provide unique substrate for binding of cells in their natural environment which two-dimensional substrates fail to provide. Applicants' mechanically reinforced macroporous gel will be used to study how the relapsing prostate cancer cells from dormancy will interact with the system. Co-culture of prostate cancer cells with osteoblasts and osteoclasts could provide a window to study unique tumor-osteal interactions in 3D environment, not possible with monolayer cells.

[00109] Synthetic procedure: Aminoglycoside amikacin and Poly (ethylene glycol) diglycidyl ether (PEGDE) mixture in 1:2 mole ratio was emulsified in Mineral oil and Span-80 surfactant (1% w/w) to give rise to ~10 um diameter microparticles/microbeads.

[00110] After crosslinking between Amikacin and PEGDE, the microbeads were collected and washed extensively to get rid of the mineral oil.

[00111] Washed microbeads were further conjugated with anticancer chemotherapeutic drug doxorubicin and quaternary ammonium group containing Glycidyl trimethyl ammonium chloride (GTMAC) for plasmid DNA binding.

[00112] Washed microbeads were also used for whole cell lysis and genomic DNA extraction by incubation of the microbeads with the cells. This invention provides a new

tool for whole cell DNA extraction for on-chip PCR reactions and point-of-care diagnostics.

[00113] A cylindrical column filled with NaCl was wet with a solution containing Amikacin and PEGDE in DMSO. The salt column was left at 37 °C for 24 hours to allow gelation. After gelation, the construct was submerged in water to get rid of salt leaving behind a macroporous/monolithic amikagel construct. Macroporous monolith amikagel construct was further used for plasmid DNA binding, cell culture and surface conjugation of drugs.

[00114] The present invention also provides the methods of characterization, methods include at SEM, BET, Ninydrin assay, plasmid DNA adsorption and desorption etc, *in vitro* mammalian cell lysis and genomic DNA extraction.

[00115] The present invention has several advantages compare to the existing products. The merits include: High density amines on monoliths and microbeads for plasmid DNA binding, presence of multiple conjugable sites such as hydroxyls and amines for further modification, Chemotherapeutic drugs to bind plasmid DNA via electrostatic and hydrophobic interactions, Antibiotic based Macroporous gels for DNA binding

[00116] Novel macroporous gels as bone mimetic surfaces for novel 3D cell culture of cancer cells (breast and prostate cancer) and stem cells (Embryonic stem cells and pluripotent stem cells).

[00117] In addition, Applicants have shown that the Amikabeads can be conjugated with Doxorubicin drug towards the development of anticancer chemotherapeutic based mixed mode chromatographic resins. Amikagel was also morphed into macroporous monolithic design to generate 3D scaffold for enhanced plasmid DNA binding, bone mimetic cell culture.

[00118] *Preparation of Pseudo-Affinity Ligands from Anti-Cancer Drug:*

Preparation of Anti-Cancer Drug Solution. Doxorubicin (100mg, 543g/mol) was dissolved in 10mL of Dimethyl sulfoxide (DMSO) followed by addition of 50uL of triethylamine (TEA). The stock mixture of doxorubicin, DMSO and TEA (10mg/mL) was stored in a 20mL glass via at 4°C and covered by aluminum foil for minimizing photoreaction.

[00119] *Preparation of Pseudo-Affinity Ligands.* Doxorubicin was obtained from 4°C storage and preheated at 35°C for 5 mins. 10mg of Doxorubicin was collected from

the stock solution followed by addition of 1, 4-Cyclohexanediol diglycidyl ether (CHDDE) and TEA with a mole ratio of 1:3:5 in 20mL glass vial. A certain amount of DMSO was added to the mixture in order to fulfill the total volume to be 2mL. The glass container was covered by aluminum foil for minimizing photoreaction and the mixture was stirred with magnetic beads for 8 hours at 320rpm to form pseudo-affinity ligands.

[00120] Formation of Conjugated Doxo-Beads. 1mg of Amikabeads was collected from Amikabeads stock solution and washed by DMSO twice. Supernatant was decanted and microbeads were transferred to the prepared pseudo-affinity ligands solution. The solution was placed away from light and stirred at 320rpm for 18 hours at 70°C. The diameter of conjugated doxo-beads were measured through microscope device and the image of beads were taken.

[00121] pDNA Binding to Conjugated Doxo-Beads. 1mg of conjugated doxo-beads were incubated with 10,000ng-120,000ng of plasmid DNA (PGL 4.5) in 1mL of 1.3M buffer I solution ((NH)₄SO₄ and 10mM Tris-Cl) at room temperature (25°C) for 24 hours. All conjugated doxo-beads were first incubated with 10M HCl solution for 5 hours and washed with buffer I twice prior to binding with plasmid DNA. NanoDrop spectrophotometer was utilized to measure the pDNA content in the supernatant. Mass balance was performed to calculate the amount of pDNA adsorbed by conjugated doxo-beads. The data containing amount of pDNA adsorbed on the beads surface along with the pDNA content in equilibrium supernatant was fitted into linearized Langmuir Isotherm in order to determine the maximum binding capacity (Q_{max}) and Langmuir adsorption constant (K_a) for the conjugated doxo-beads. The linearized Langmuir Isotherm was shown below:

$$\frac{C_e}{Q_e} = \frac{1}{Q_{\max}} C_e + \frac{1}{Q_{\max} * K_a}$$

where Q_e=amount of pDNA bound to the conjugated doxo-beads at equilibrium (ug/mg), C_e=concentration of pDNA in the solution at equilibrium (mg/L), K_a=Langmuir adsorption constant (L/mg), Q_{max}=maximum amount of pDNA bound to the conjugated doxo-beads (ug/mg). 1/Q_{max} vs C_e/Q_e was plotted in order to calculate the maximum binding capacity (Q_{max}) from slope and the Langmuir adsorption constant from intercept.

[00122] Desorption of Bound pDNA from Conjugated Doxo-Beads. Conjugated doxo-beads (~1mg) were loaded with 75,000ng-120,000ng to ensure the beads was fully bound with pDNA. The desorption process was through two steps. Prior to the first step,

the pDNA bound doxo-beads were firstly washed by buffer I in order to remove all the free pDNA content from supernatant. In the first step, the pDNA bound doxo-beads were immersed in 1mL of buffer II solution (500ul of buffer I and 500ul of 10mM Tris-Cl) for 24 hours; whereas in the second step, the pDNA bound doxo-beads were immersed in 1mL of buffer III solution (10mM Tris-CL) for 24 hours. The buffer III was refreshed after every 24 hours until no further pDNA was desorbed from doxo-beads. The amount of pDNA desorbed from both steps was measured via NanoDrop spectrophotometer.

[00123] Generation of Doxorubicin conjugated Amikabeads. We have previously demonstrated the development of parental aminoglycoside microbeads using emulsion polymerization. Next, we hypothesized that the conjugation of DNA binding anticancer drugs on parental aminoglycoside microbeads could improve their selectivity towards plasmid DNA in a mixture of pDNA and RNA. Doxorubicin a widely used anthracycline antibiotic anticancer drug which inhibits actively dividing cancer cells by interacting with the phosphate backbone of the DNA and the hydrophobic core. In order to conjugate doxorubicin on to the parental microbeads, we firstly conjugated the 1,4-butanediol diglycidyl ether and doxorubicin with excess of 1,4 butane diol diglycidyl ether (1:3) (Figure 3.1 a-b).

[00124] In our attempts to conjugate 1,4-butane diol diglycidyl ether on to the microbeads directly led to large scale aggregation of the microbeads (Figure 3.2 a). Hence, we firstly conjugated the pseudoaffinity ligand (doxorubicin in this case) to the crosslinker (1,4-butanediol diglycidyl ether in this case) and then conjugated the doxorubicin-1,4-butanediol diglycidyl ether moiety to the parental Amikabeads (Figure 3.2 b-c).

[00125] We believe our novel strategy of firstly linking pseudoaffinity ligand (doxorubicin in this case) to the crosslinker followed by their reaction to the microbeads can be used for multiple other applications of attaching ligands to the polymeric microbead surface while preventing large scale aggregation of the microbeads.

[00126] Using this strategy, we could generate large amounts of unaggregated doxorubicin conjugated aminoglycoside microbeads (amikabeads).

[00127] Loading plasmid DNA on doxorubicin conjugated Amikabeads. Loading plasmid DNA using 10 mM Tris-Cl did not cause any significant loading of plasmid DNA. Hence, we switched to Hydrophobic interaction loading of the plasmid DNA on to the resin. It is likely that after doxorubicin conjugation to the microbead surface,

electrostatic interactions no longer dominate the binding. In HIC based loading of pDNA, high salt content forces pDNA unwinding and interaction of the hydrophobic nitrogenous bases of pDNA with the hydrophobic aminoglycoside resin. Under, 1.3M buffer I solution ($(\text{NH}_4)_2\text{SO}_4$ and 10mM Tris-Cl), pH 8.5 at 25C, we noticed a very high binding capacity of pDNA on doxorubicin conjugated microbeads (Q-max = 200,000 ng of pDNA/mg of resin) which was significantly higher than the parental beads (Q-max = 40,000 ng of pDNA/mg of resin).

Elution of pDNA with 24 hour washes of 50% mixture of 1.3M buffer I solution ($(\text{NH}_4)_2\text{SO}_4$ and 10mM Tris-Cl) and 10 mM Tris-Cl solution each followed with 100% Tris-Cl allowed desorption and recovery of approximately 80-90% of loaded pDNA.

[00128] We show a novel strategy to bind anticancer drugs to the aminoglycoside microbeads without causing largescale aggregation of the beads. Doxorubicin conjugated aminoglycoside microbeads were found to load pDNA under high salt conditions (1.3M buffer I solution ($(\text{NH}_4)_2\text{SO}_4$ and 10mM Tris-Cl)). High recovery of pDNA was possible using low salt solutions. Our results show that anticancer drugs can be used as promising agents for pDNA binding and recovery.

[00129] While the preferred embodiments of the present invention have been illustrated in detail, it should be apparent that modifications and adaptations to those embodiments may occur to one skilled in the art without departing from the scope of the present invention.

CLAIMS

1. A method to prepare a macroporous column, comprising:
 - providing a chromatographic column packed with salt;
 - mixing an aminoglycoside and a cross-linker in an organic solvent;
 - disposing said aminoglycoside / cross-linker / organic solvent in said chromatographic column;
 - after said aminoglycoside reacts with said cross-linker to form a macroporous porous, cross-linked resin, adding water to said column to dissolve said salt;
 - draining said water from said chromatographic column.
2. The method of claim 1, wherein said aminoglycoside is selected from the group consisting of Amikacin, Neomycin, Streptomycin, Tobramycin, Sisomicin, Paromomycin, Apramycin, Framecycin, Ribostamycin, Kanamycin, Arbekacin, Beckanamycin, Dibekacin, Astromicin, Spectinomycin, Hygromycin b, Gentamicin, Netilmicin, Isepamicin, and Verdamycin.
3. The method of claim 1, wherein said cross-linker comprises a di-epoxide.
4. The method of claim 1, wherein said cross-linker is selected from the group consisting of Poly (ethylene glycol) diglycidyl ether, Ethylene glycol diglycidyl ether, 1,4-Cyclohexane dimethanol diglycidyl ether, Neopentyl glycol diglycidyl ether, 1,4-Butanediol diglycidyl ether, Resorcinol diglycidyl ether, Poly (propylene glycol) diglycidyl ether, Glycerol diglycidyl ether, Poly(ethylene glycol) diacrylate, Hexamethylene diacrylate, Neopentyl glycol diacrylate, 1,3-Butanediol diacrylate, 1,6-Hexanediol diacrylate, Bisphenol A ethoxylate diacrylate, Ethylene glycol diacrylate, 1,4-Butanediol diacrylate, Glycerol 1,3-diglycerolate diacrylate, Neopentyl glycol diacrylate, Tetra(ethylene glycol) diacrylate, Poly(propylene glycol) diacrylate, Tri(ethyleneglycol) diacrylate, 1,6-Hexanediol ethoxylate diacrylate, and Neopentyl glycol propoxylate diacrylate.
5. A macroporous, cross-linked resin, formed by the process of:
 - providing a chromatographic column packed with salt;
 - mixing an aminoglycoside and a cross-linker in an organic solvent;
 - disposing said aminoglycoside / cross-linker / organic solvent in said chromatographic column;

after said aminoglycoside reacts with said cross-linker to form a macroporous porous, cross-linked resin, adding water to said column to dissolve said salt;

draining said water from said chromatographic column.

6. The macroporous, cross-linked resin of claim 5, wherein said aminoglycoside is selected from the group consisting of Amikacin, Neomycin, Streptomycin, Tobramycin, Sisomicin, Paromomycin, Apramycin, Framecycin, Ribostamycin, Kanamycin, Arbekacin, Beckanamycin, Dibekacin, Astromicin, Spectinomycin, Hygromycin b, Gentamicin, Netilmicin, Isepamicin, and Verdamicin.

7. The macroporous, cross-linked resin of claim 5, wherein said cross-linker comprises a di-epoxide.

8. The macroporous, cross-linked resin of claim 5, wherein said cross-linker is selected from the group consisting of Poly (ethylene glycol) diglycidyl ether, Ethylene glycol diglycidyl ether, 1, 4-Cyclohexane dimethanol diglycidyl ether, Neopentyl glycol diglycidyl ether, 1,4- Butanediol diglycidyl ether, Resorcinol diglycidyl ether, Poly (propylene glycol) diglycidyl ether, Glycerol diglycidyl ether, Poly(ethylene glycol) diacrylate, Hexamethylene diacrylate, Neopentyl glycol diacrylate, 1,3-Butanediol diacrylate, 1,6-Hexanediol diacrylate, Bisphenol A ethoxylate diacrylate, Ethylene glycol diacrylate, 1,4-Butanediol diacrylate, Glycerol 1,3-diglycerolate diacrylate, Neopentyl glycol diacrylate, Tetra(ethylene glycol) diacrylate, Poly(propylene glycol) diacrylate, Tri(ethyleneglycol) diacrylate, 1,6-Hexanediol ethoxylate diacrylate, and Neopentyl glycol propoxylate diacrylate.

9. A method to form microbeads comprising an aminoglycoside moiety, comprising:

polymerizing a reaction mixture comprising an aminoglycoside and a cross-linker;

prior to gellation, disposing said reaction mixture into heated mineral oil;

collecting said microbeads by centrifugation.

10. The method of claim 9 wherein said aminoglycoside comprises amikacin.

11. The method of claim 9, wherein said cross-linker comprises polyethylene glycol diexpoxide.

12. The method of claim 9, wherein said reaction mixture comprises a weight ratio of 1:2 amikacin to PEGDE.
13. The method of claim 8, wherein said heated mineral oil is maintained at a temperature of 65 °C with constant stirring at 260 rpm.
14. The method of claim 5, wherein said reaction mixture comprises a surfactant.
15. The method of claim 6, further comprising prior to forming said reaction mixture, reacting said amikacin with glycidyl trimethyl ammonium chloride.
16. Microbeads comprising an aminoglycoside moiety, formed by the process of:
polymerizing a reaction mixture comprising an aminoglycoside and a cross-linker;
prior to gellation, disposing said reaction mixture into heated mineral oil;
collecting said microbeads by centrifugation.
17. The microbeads of claim 16, wherein said aminoglycoside comprises amikacin.
18. The microbeads of claim 16, wherein said cross-linker comprises polyethylene glycol diexoxide (PEGDE).
19. The microbeads of claim 16, wherein said reaction mixture comprises a weight ratio of 1:2 amikacin to PEGDE.
20. The microbeads of claim 16, wherein said heated mineral oil is maintained at a temperature of 65 °C with constant stirring at 260 rpm.
21. The method of claim 16, wherein said reaction mixture comprises a surfactant.
22. The method of claim 16, further comprising prior to forming said reaction mixture, reacting said amikacin with glycidyl trimethyl ammonium chloride.

ID	Aminoglycoside
1	Amikacin
2	Neomycin
3	Streptomycin
4	Tobramycin
5	Sisomicin
6	Paromomycin
7	Apramycin
8	Framecytin
9	Ribostamycin
10	Kanamycin
11	Arbekacin
12	Beckanamycin
13	Dibekacin
14	Astromicin
15	Spectinomycin
16	Hygromycin b
17	Gentamicin
18	Netilmicin
19	Isepamicin
20	Verdamycin

Fig. 1

ID	Crosslinker
1	Poly (ethylene glycol) diglycidyl ether
2	Ethylene glycol diglycidyl ether
3	1,4-Cyclohexane dimethanol diglycidyl ether
4	Neopentyl glycol diglycidyl ether
5	1,4- Butanediol diglycidyl ether
6	Resorcinol diglycidyl ether
7	Poly (propylene glycol) diglycidyl ether
8	Glycerol diglycidyl ether
9	Poly(ethylene glycol) diacrylate
10	Hexamethylene diacrylate
11	Neopentyl glycol diacrylate
12	1,3-Butanediol diacrylate
13	1,6-Hexanediol diacrylate
14	Bisphenol A ethoxylate diacrylate
15	Ethylene glycol diacrylate
16	1,4-Butanediol diacrylate
17	Glycerol 1,3-diglycerolate diacrylate
18	Neopentyl glycol diacrylate
19	Tetra(ethylene glycol) diacrylate
20	Poly(propylene glycol) diacrylate
21	Tri(ethyleneglycol) diacrylate
22	1,6-Hexanediol ethoxylate diacrylate
23	Neopentyl glycol propoxylate (1 PO/OH) diacrylate

Fig. 2

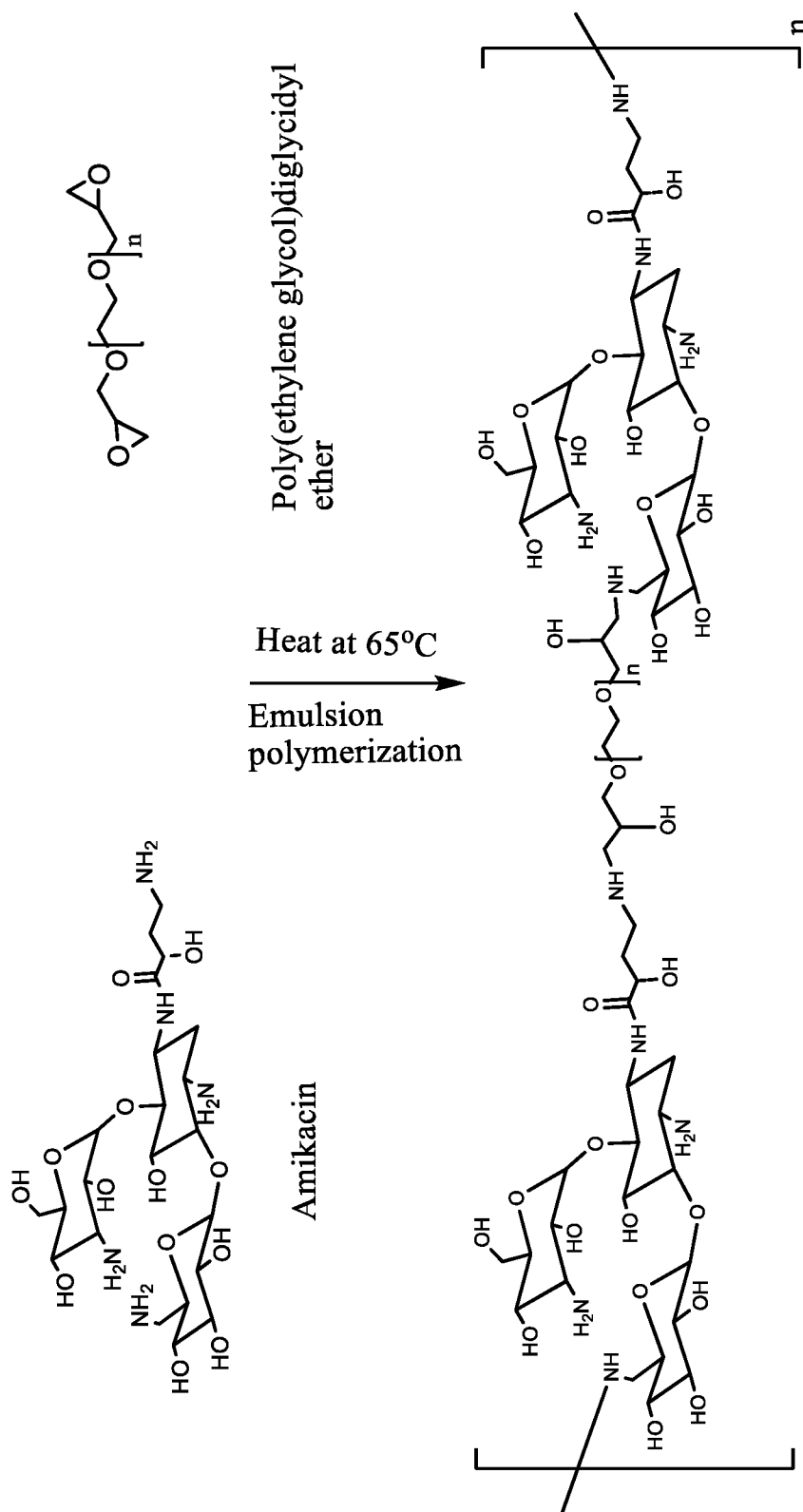
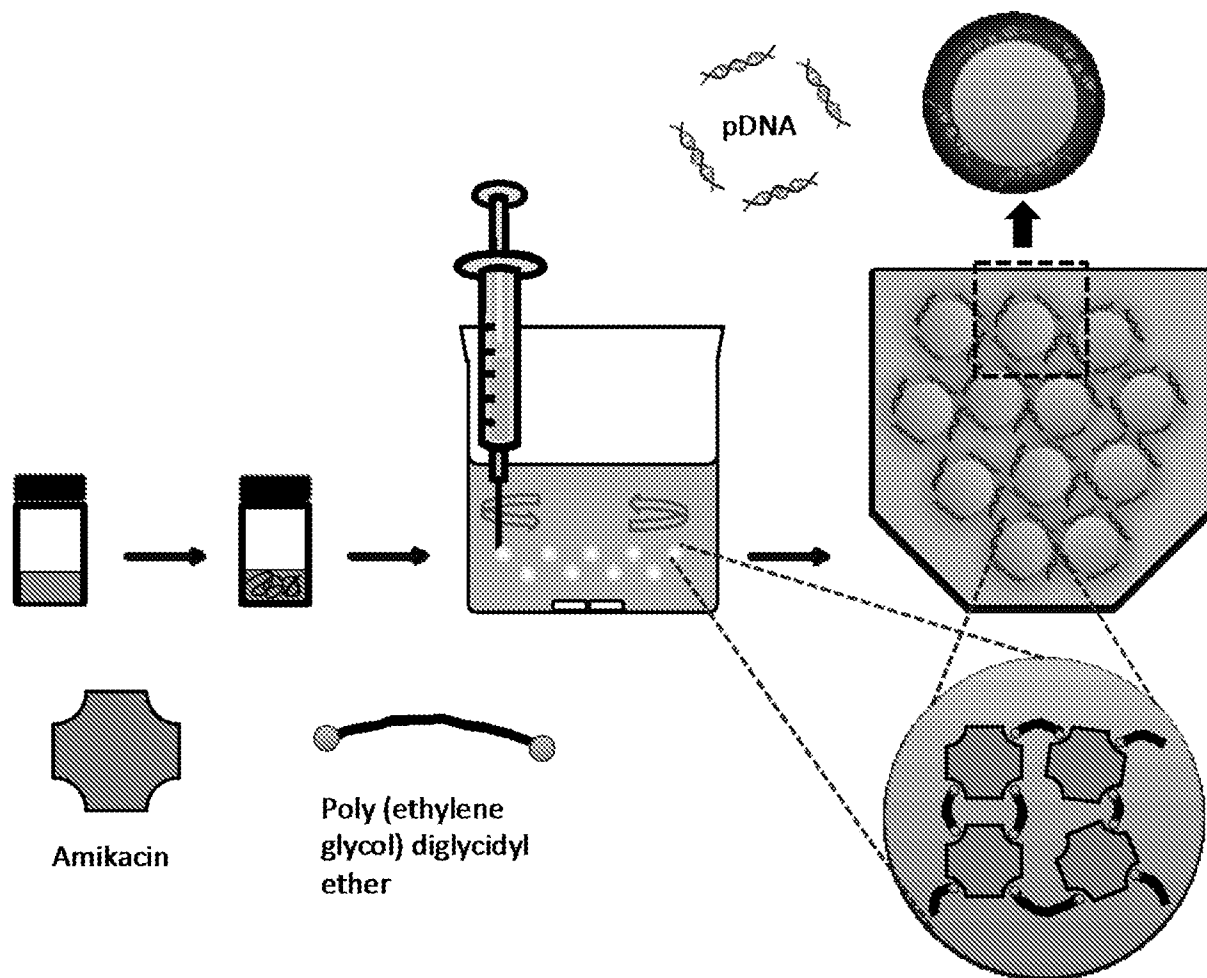
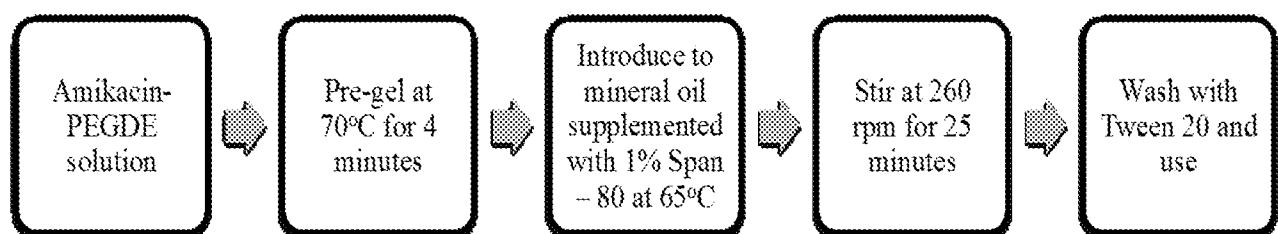
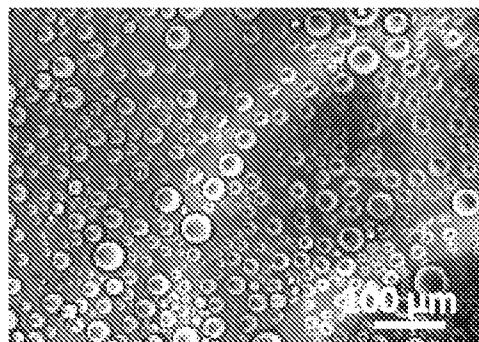
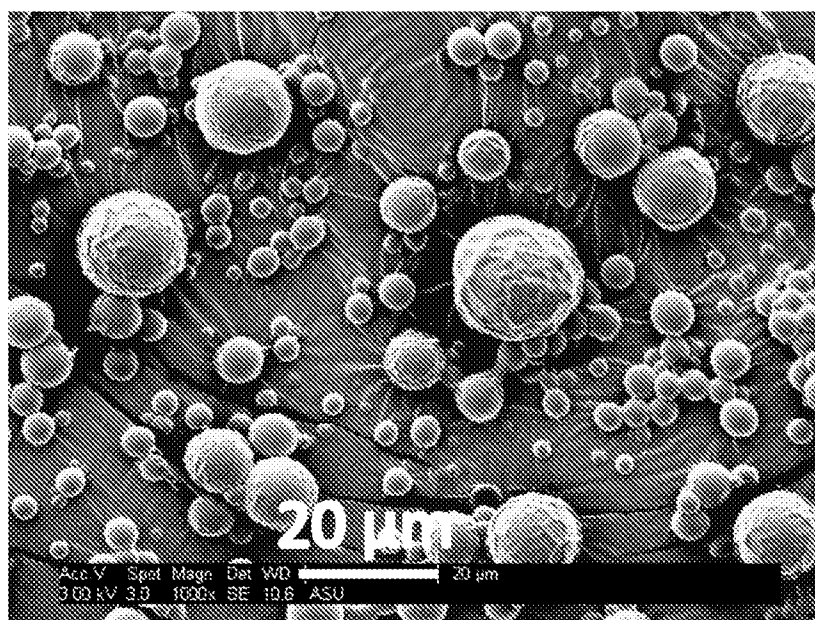
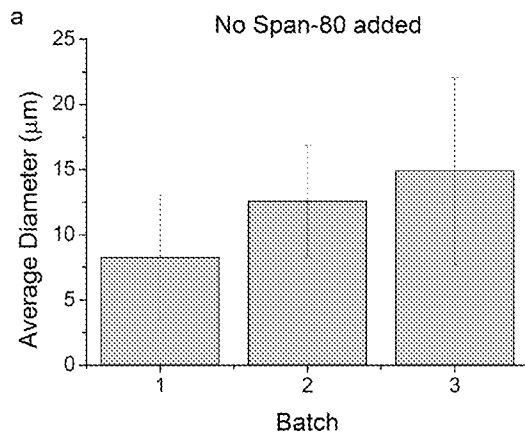
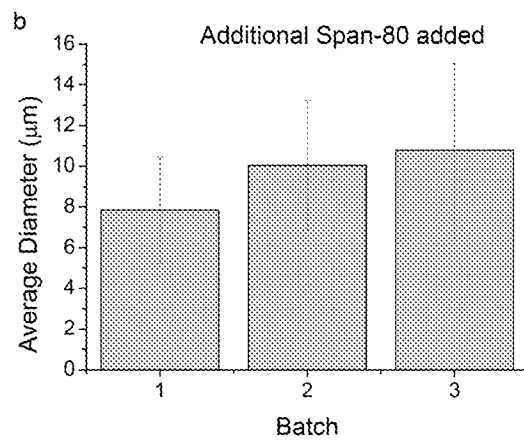


Fig. 3

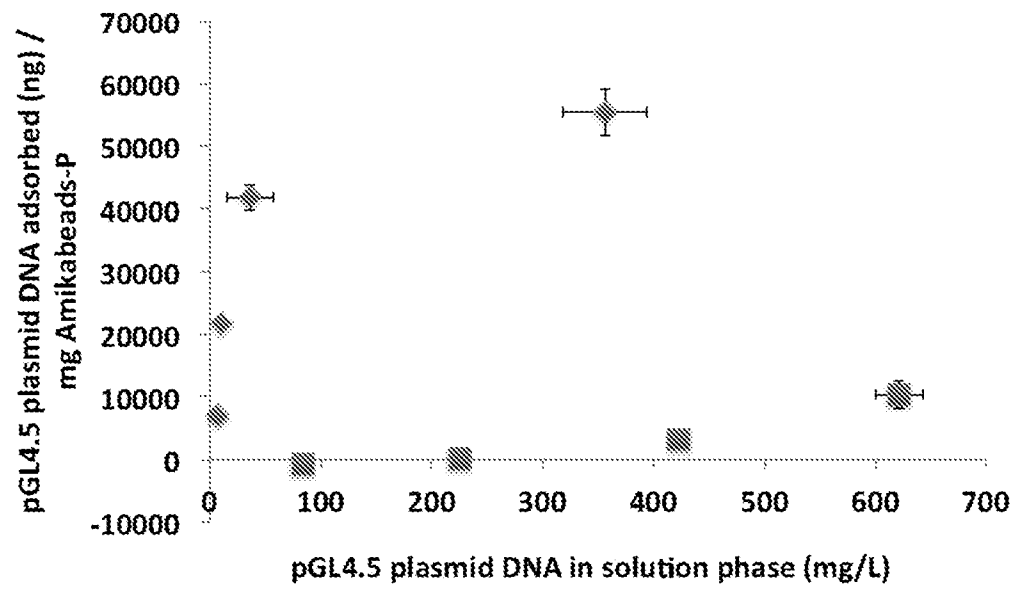
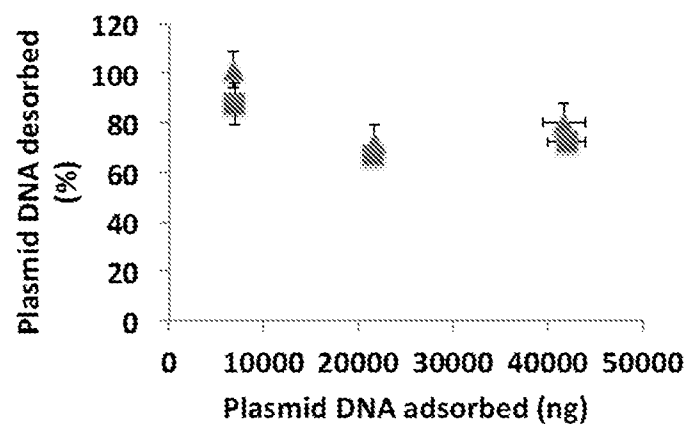
**Fig. 4A****Fig. 4B**

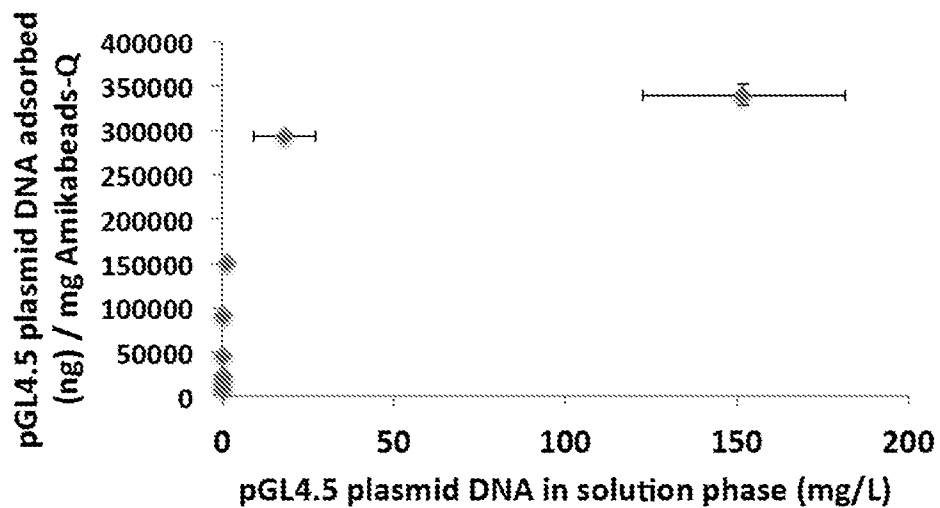
**Fig. 4C****Fig. 4D**

**Fig. 5A**

	No Span-80 added	Span-80 added
p-value of One-way ANOVA	0.00162	0.174

Fig. 5B

**Fig. 6A****Fig. 6B**

**Fig. 7A**

Batch of Amikabeads-Q	Average Diameter (μm) $\pm$ 1 S.D (Standard Deviation)	Q_{max} DNA Binding Capacity (μg pDNA bound/mg of beads)
n=1	12 ± 6	333.3
n=2	15 ± 7	277
n=3	10 ± 6	296

Fig. 7B

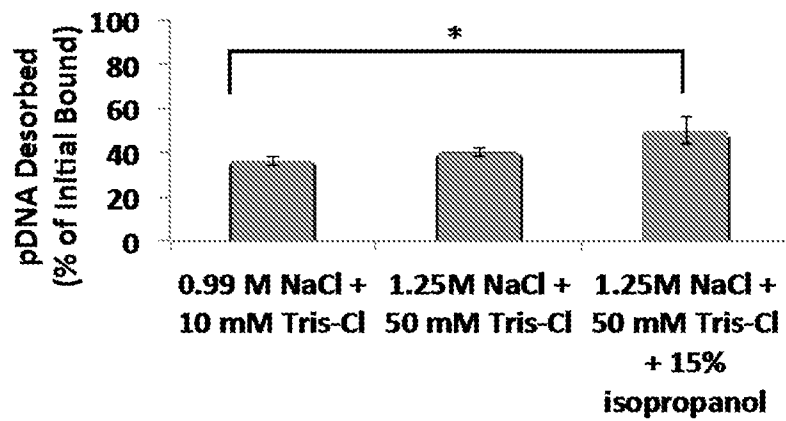


Fig. 7C

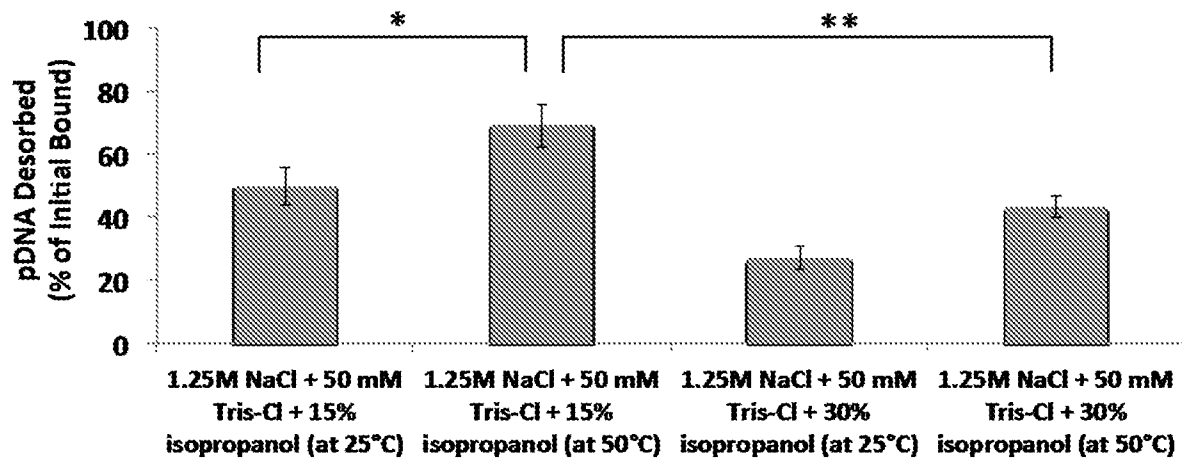
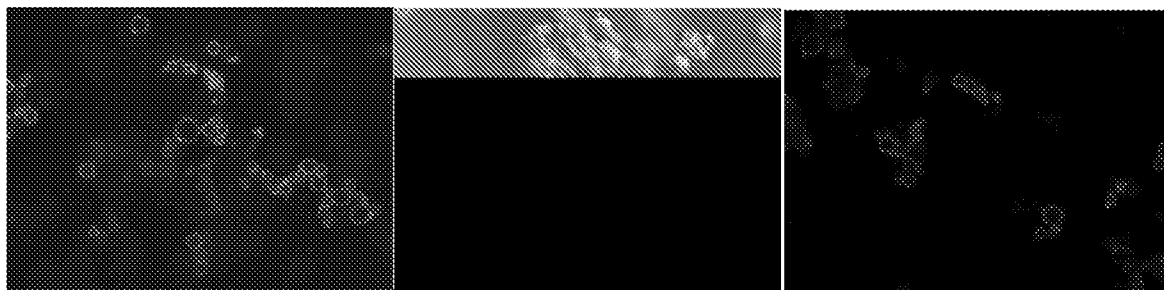
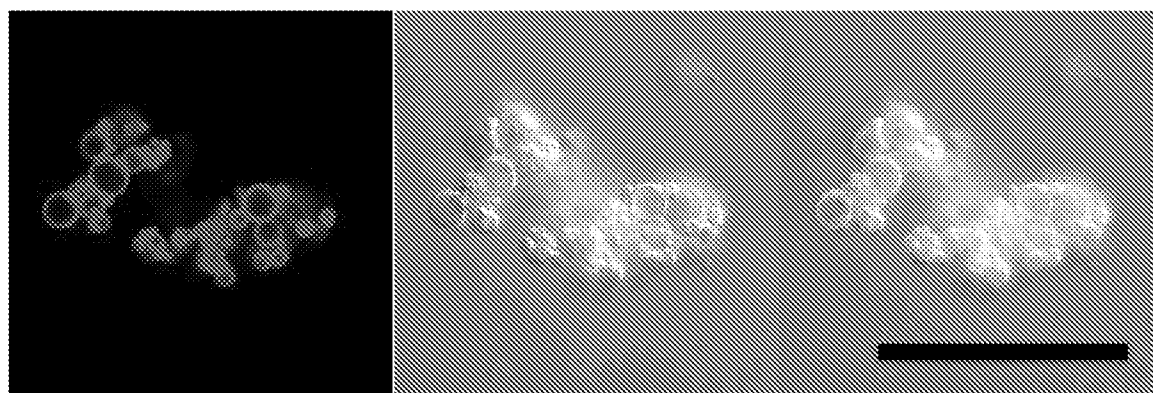
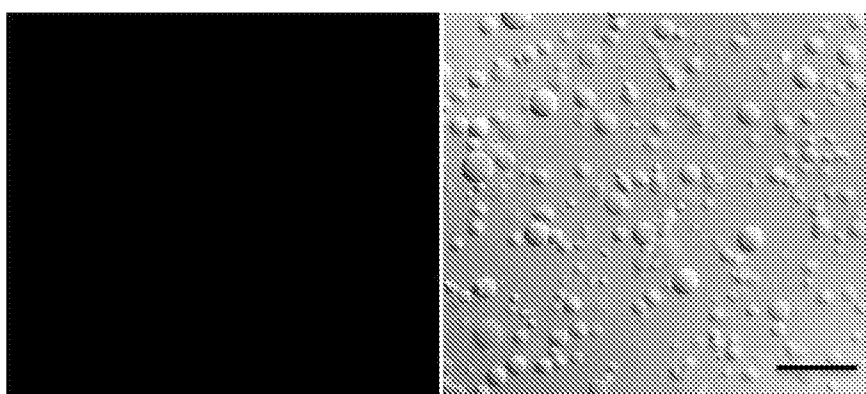


Fig. 7D

**Fig. 8A****Fig. 8B****Fig. 8C**

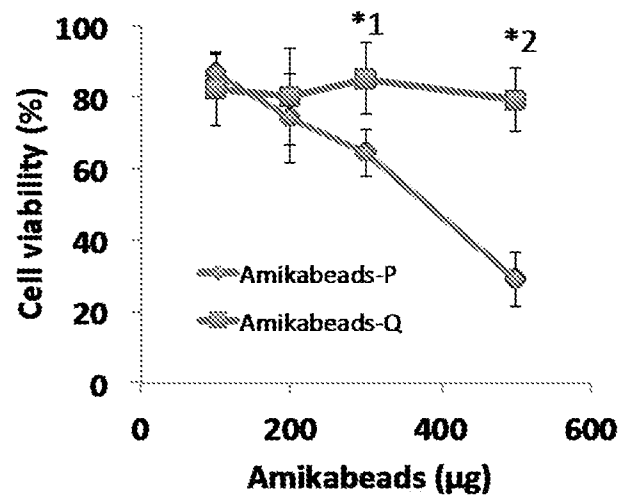


Fig. 9A

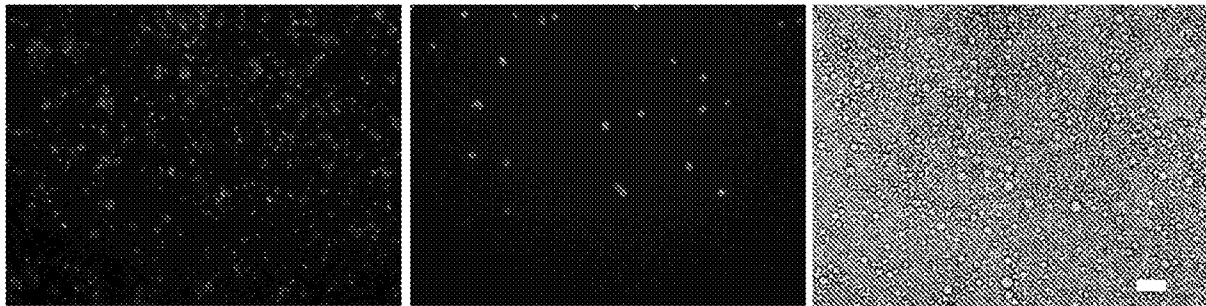


Fig. 9B

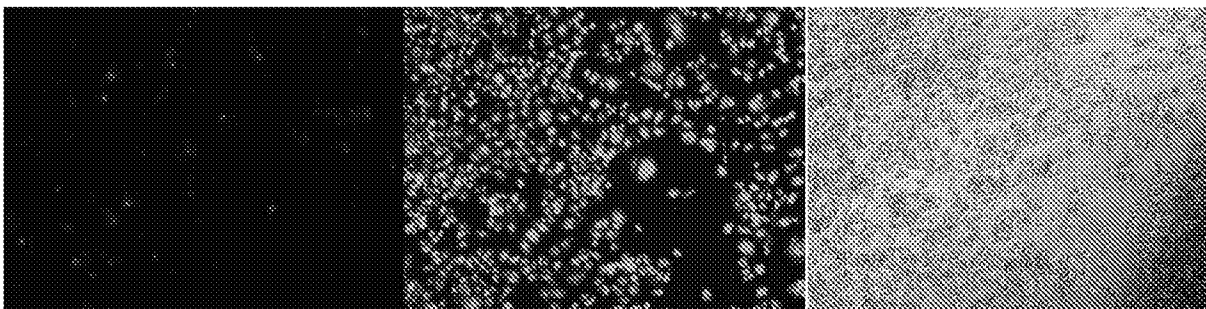
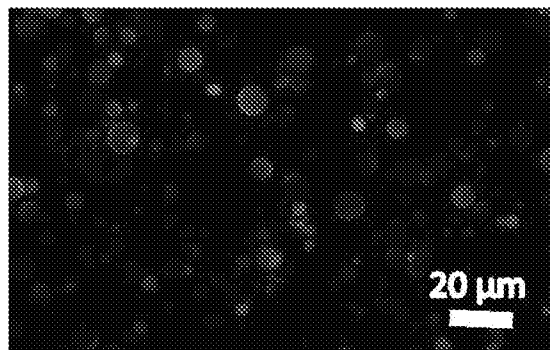
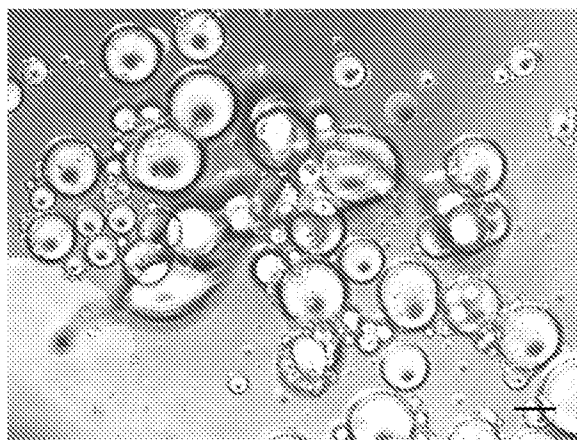
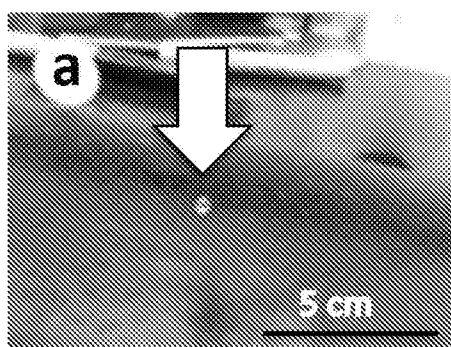
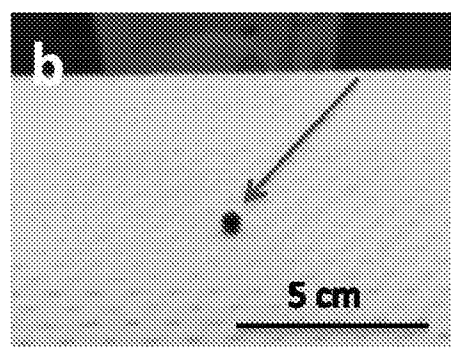


Fig. 9C

**Fig. 10A****Fig. 10B****Fig. 11****Fig. 12A****Fig. 12B**

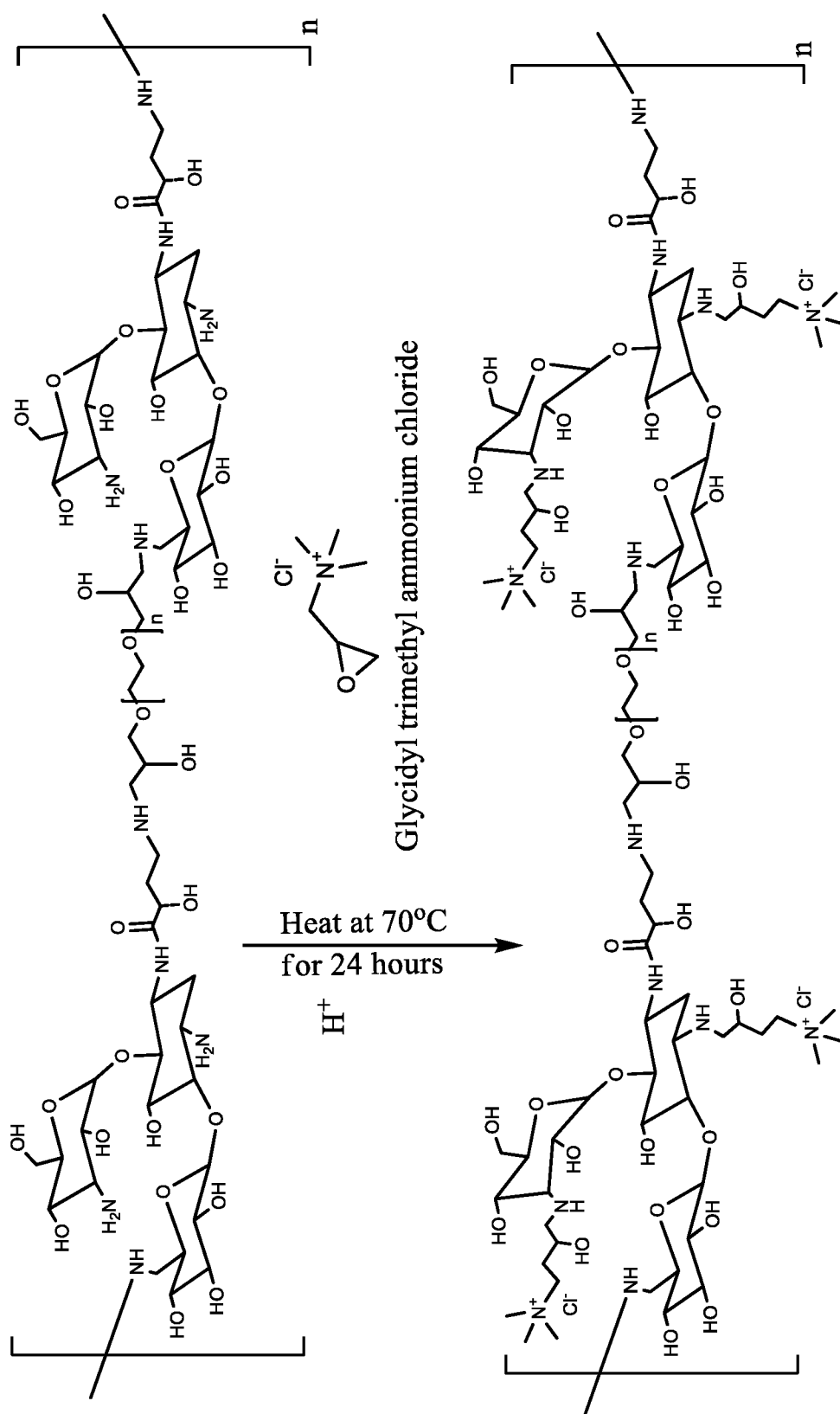


Fig. 13A

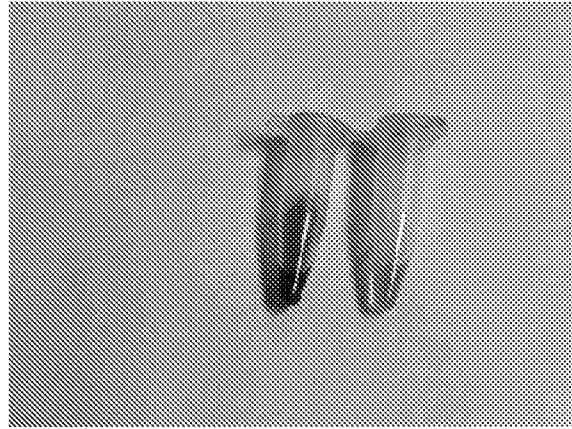


Fig. 13B

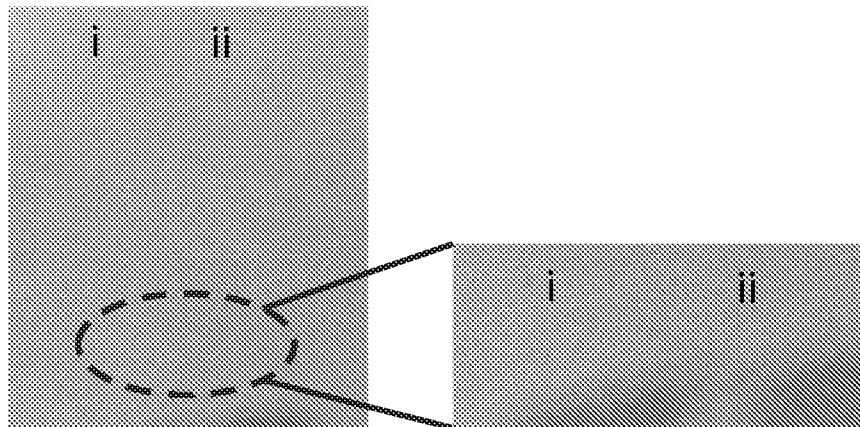
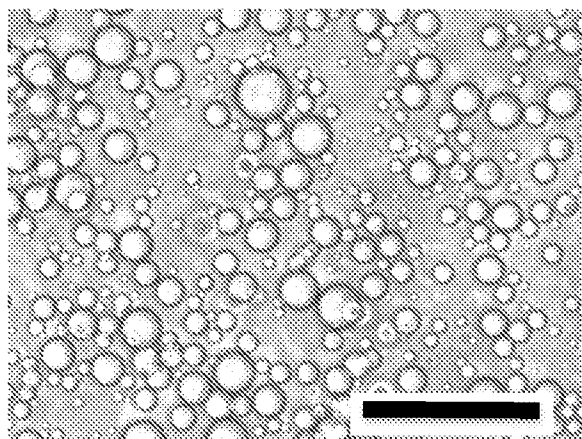
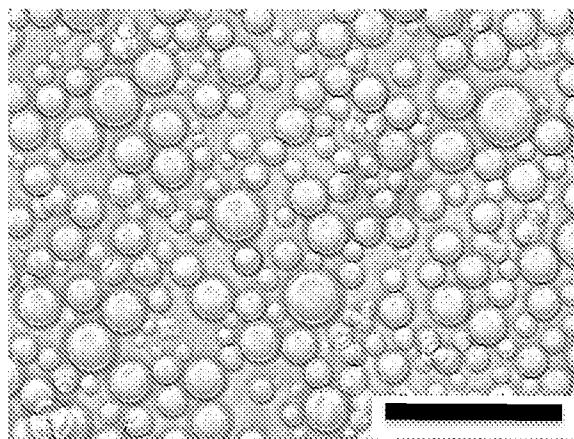
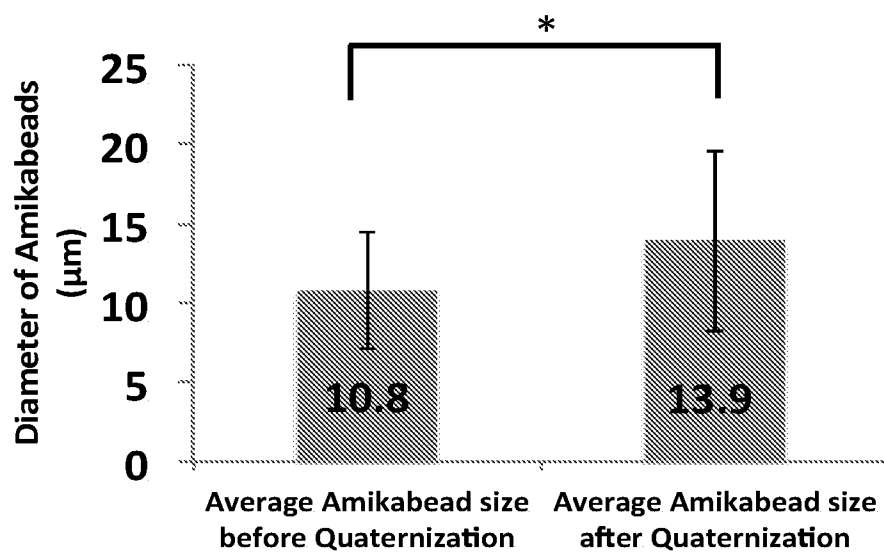
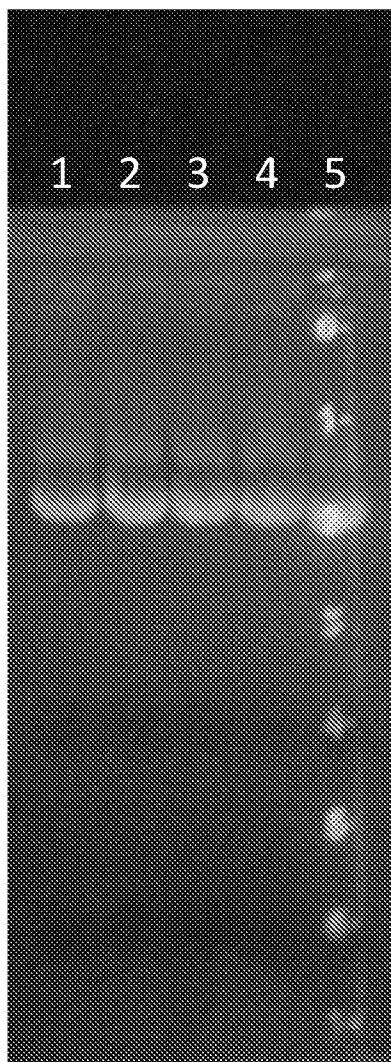
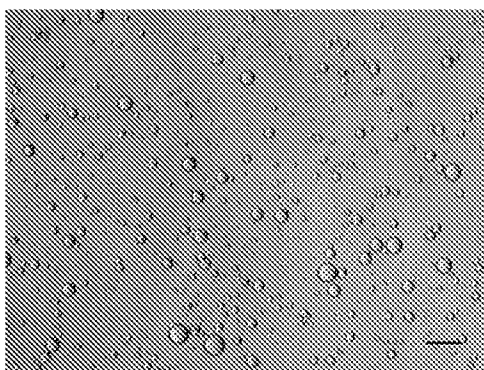
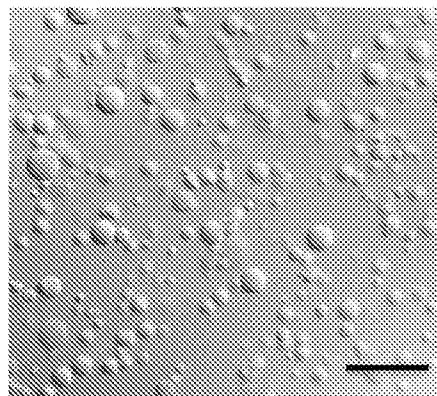
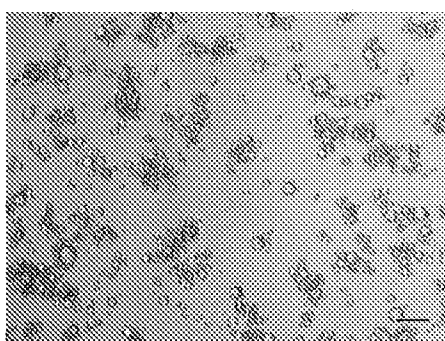
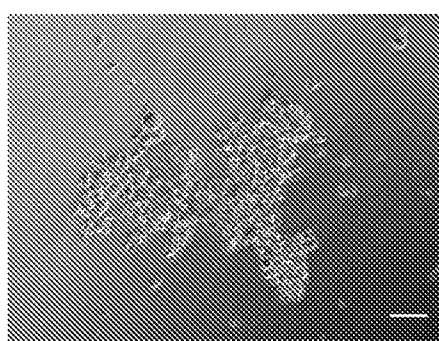
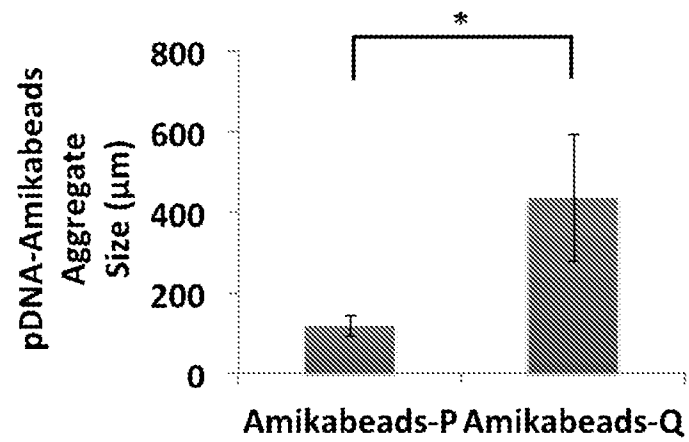
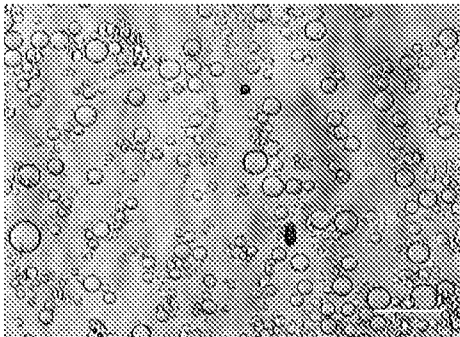
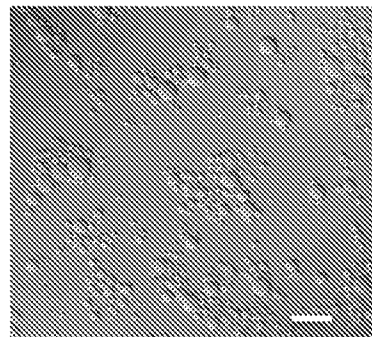


Fig. 13C

**Fig. 14A****Fig. 14B****Fig. 14C**

**Fig. 15**

**Fig. 16A****Fig. 16B****Fig. 16C****Fig. 16D**

**Fig. 16E****Fig. 16F****Fig. 16G**

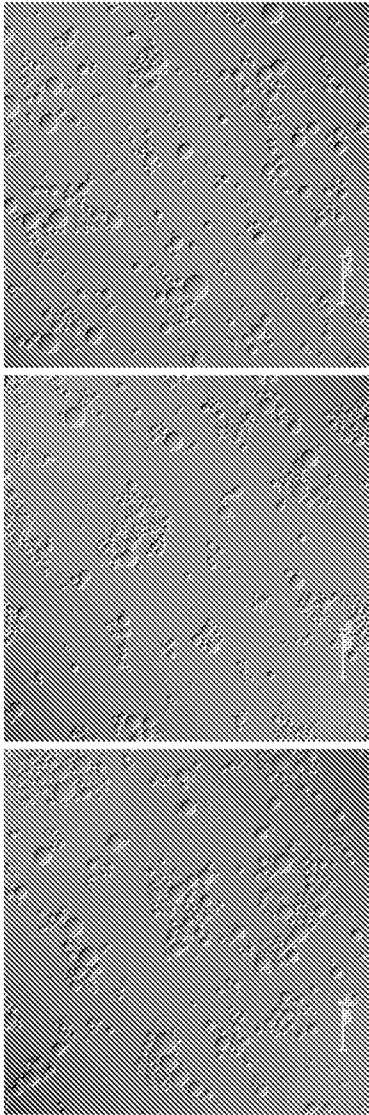


FIG. 17A

FIG. 17B

FIG. 17C

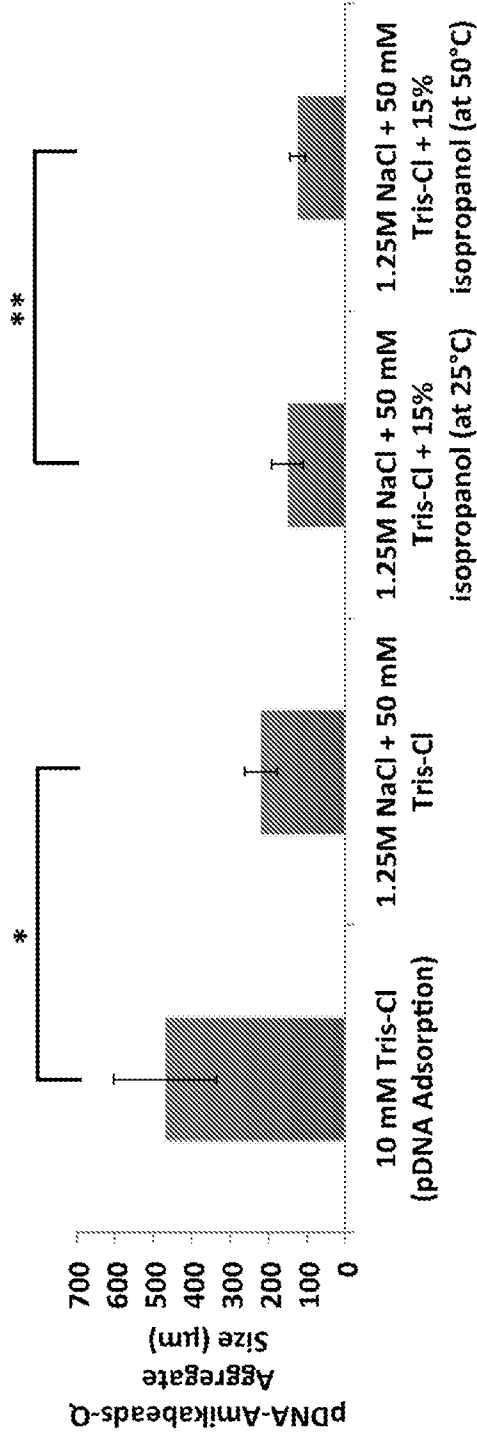


FIG. 17D

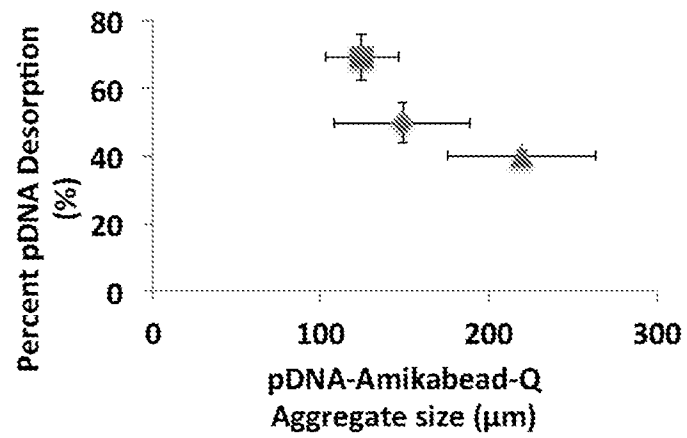


FIG. 17E

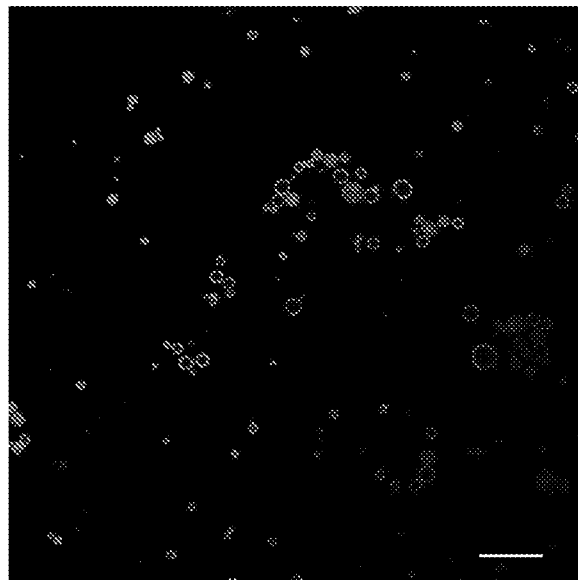


FIG. 17F

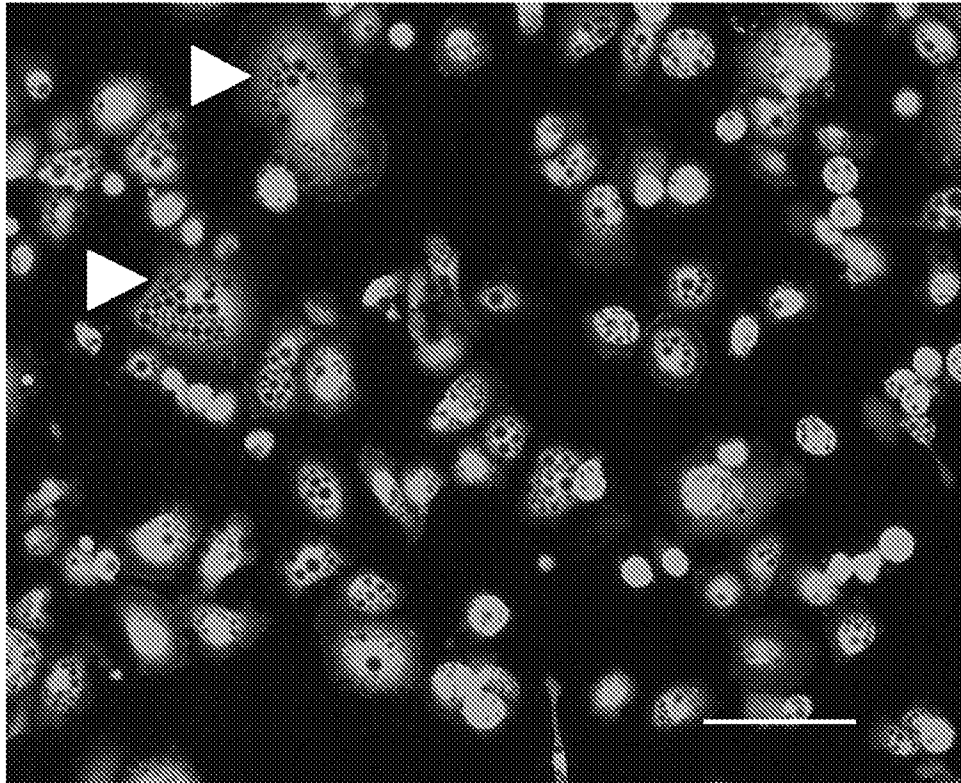


FIG. 18

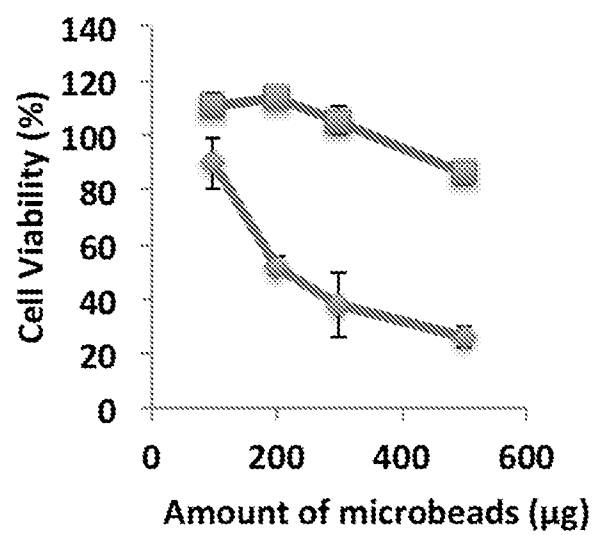


FIG. 19

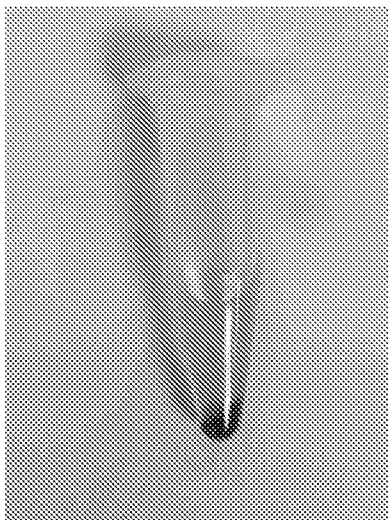


FIG. 20



FIG. 21A



FIG. 21B



FIG. 21C

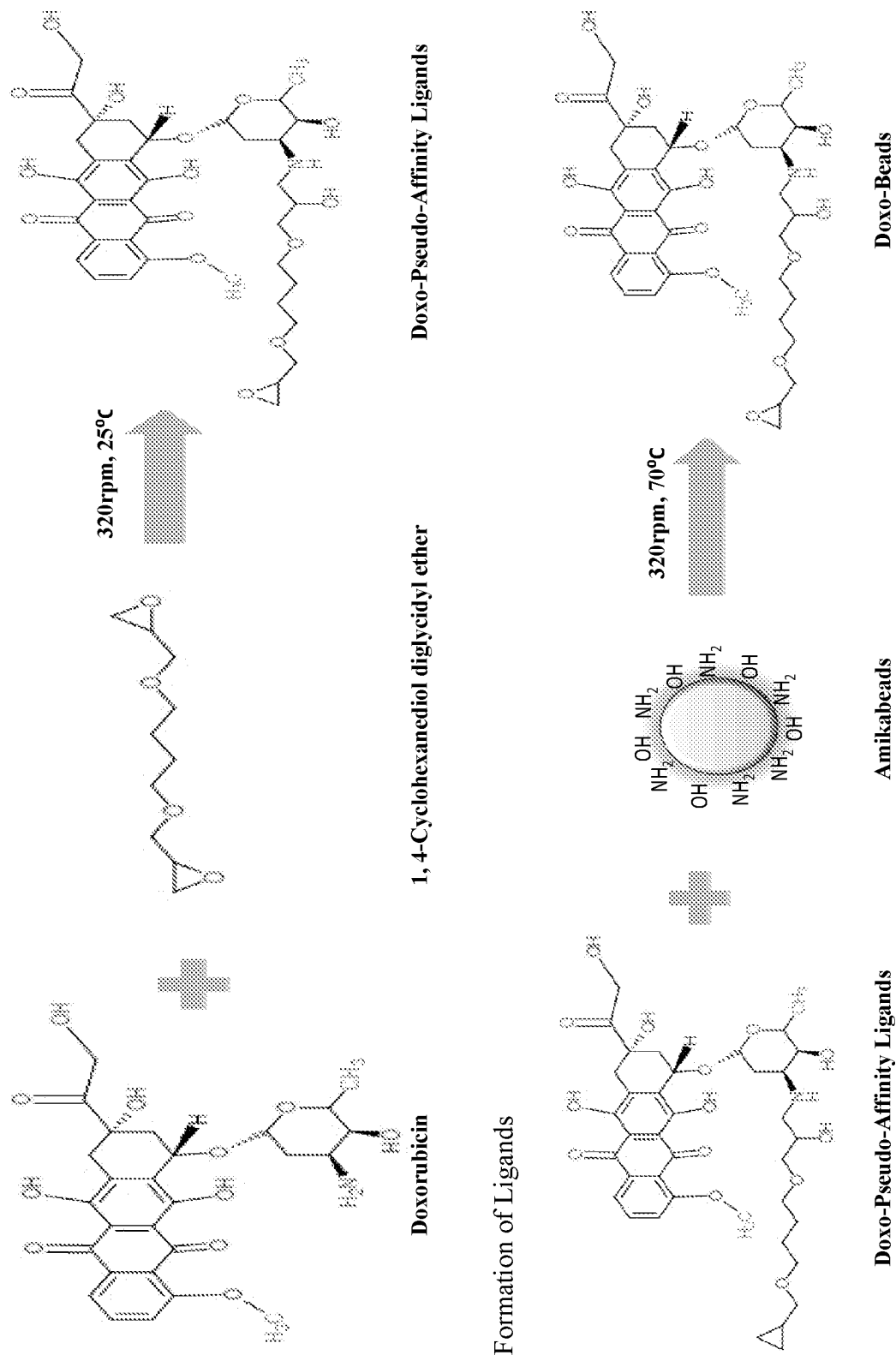
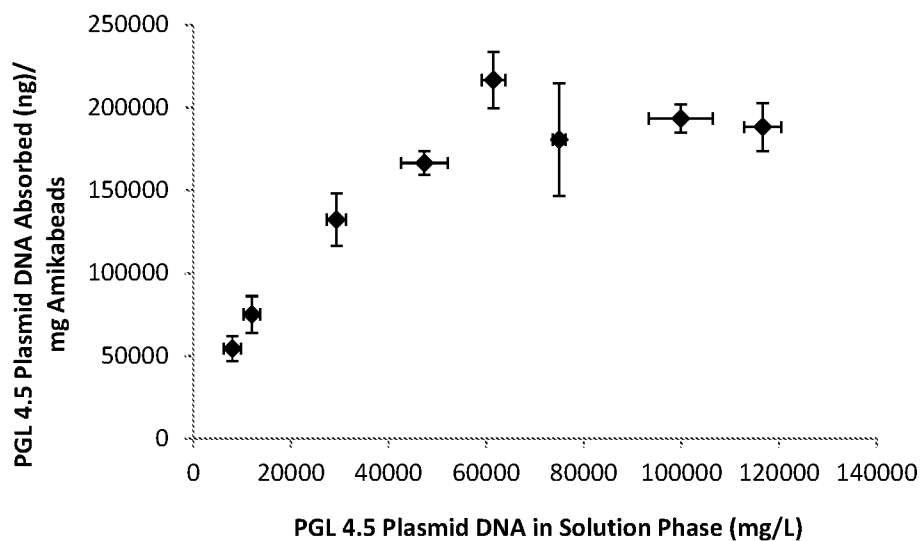


Fig. 22

a



b

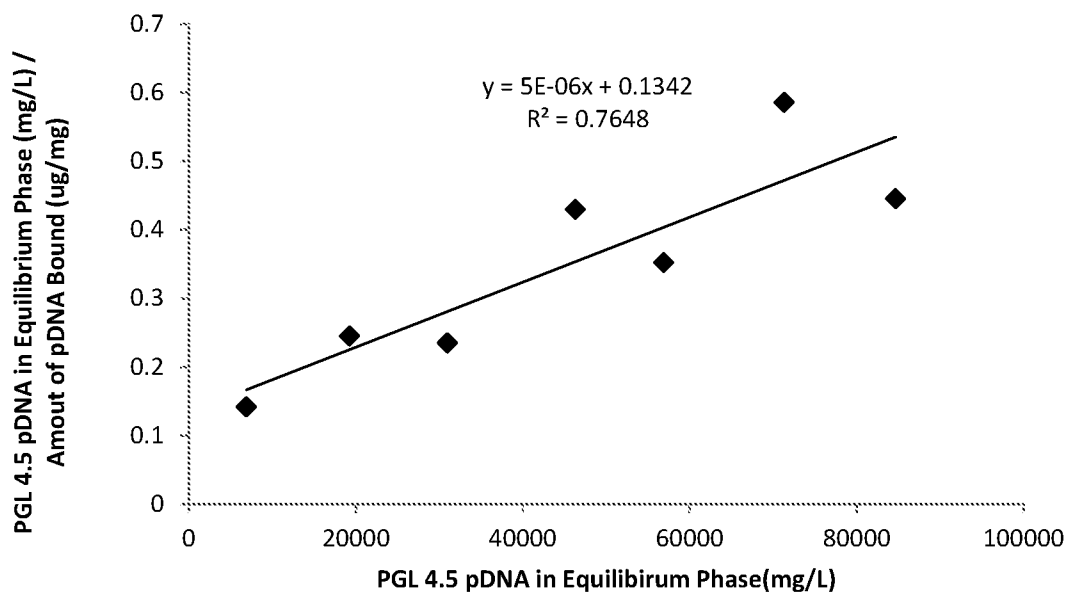
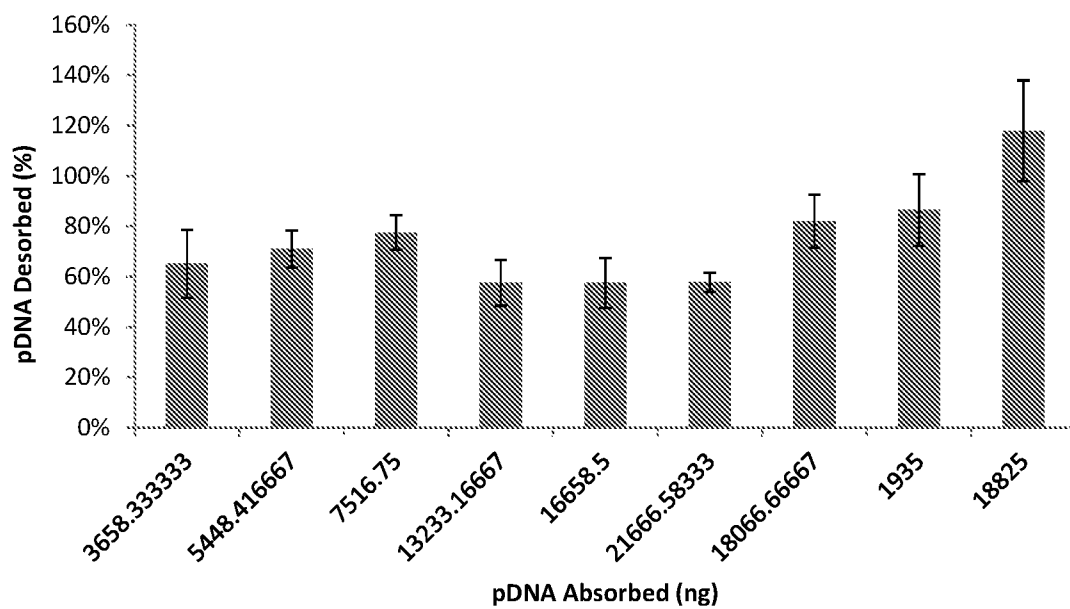
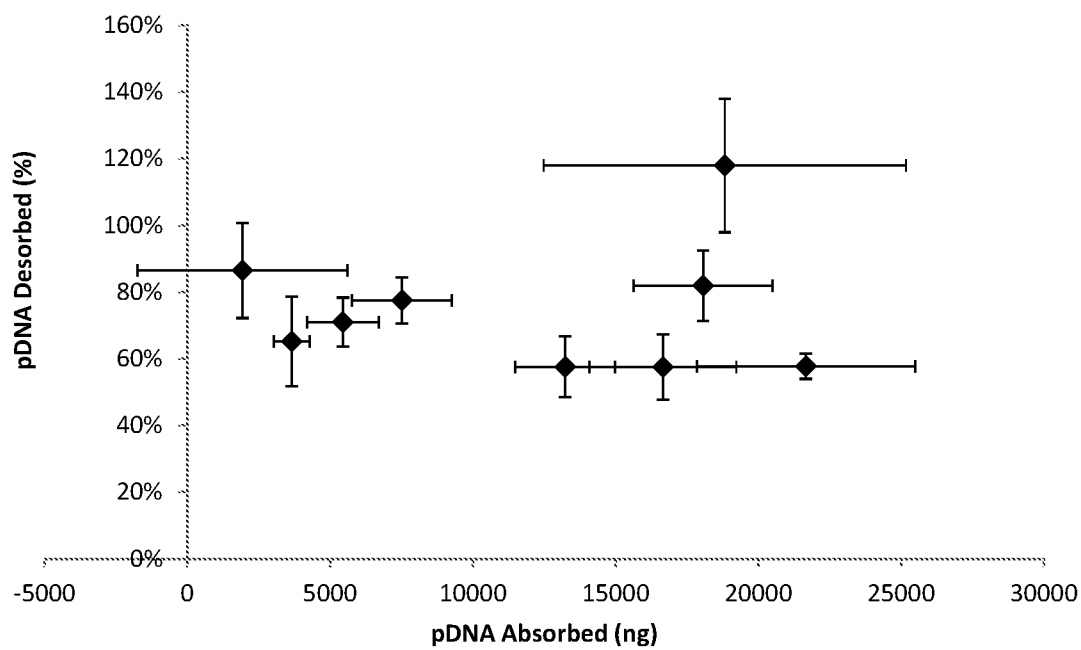


Fig. 23



A.



B.

Fig. 24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/17771

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - B01D 15/08 (2016.01)

CPC - B01D 15/08; G01N 30/56; B01J 2220/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

CPC: B01D15/08; G01N30/56; B01J2220/54; A61L15/32; A61L15/425; A61L15/44; A61L2300/254; C08G18/7621; A61L2300/404
IPC(8): B01D 15/08 (2016.01)Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 210/656; 536/348; 536/13.6; 536/13.7; 536/16.8; 536/13.3; 536/13.2; 426/807; 426/648; 536/13.9; 536/13.8Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Pat Base (AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO), Google Patent, Google Scholar; Search terms: Aminoglycoside organic solvent chromatographic column cross-linker packed salt resin water porous remove macroporous Amikacin Neomycin Streptomycin Tobramycin Sisomicin pore extract foam Apramycin Arbekacin Beckanamycin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/066674 A1 (KCI LICENSING, INC.) 01 May 2014 (01.05.2014) para [0006], [0010], [0023], [0066]	1-8
A	WO 2005/027873 A2 (ALNIS BIOSCIENCES, INC.) 31 March 2005 (31.03.2005) pg 4, ln 20-23; pg 9, ln 10-23; pg 25, ln 1-22;	1-8
A	WO 2014/022749 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 06 February 2014 (06.02.2014) para [0007], [0010], [0020], [0065], [0081]	1-8
A	US 4,347,354 A (CRON et al.) 31 August 1982 (31.08.1982); the entire document	1-8
A	US 3,984,393 A (MAGERLEIN) 05 October 1976 (05.10.1976); the entire document	1-8
A	US 3,860,574 A (NAITO et al.) 14 January 1975 (14.01.1975); the entire document	1-8



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

02 June 2016

Date of mailing of the international search report

01 JUL 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

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Authorized officer:

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/17771

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-8, directed to preparing a macroporous, cross-linked resin.

Group II: Claims 9-22, directed to forming microbeads comprising an aminoglycoside moiety.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

****please see the continuation at the end of this form****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-8

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

****continuation of Box III (Lack of Unity)****

Special Technical Feature:

Group I does not require microbeads comprising an aminoglycoside moiety; polymerizing a reaction mixture comprising an aminoglycoside and a crosslinker; prior to gellation, disposing said reaction mixture into heated mineral oil; collecting said microbeads by centrifugation, as required by Group II.

Group II does not require providing a chromatographic column packed with salt; mixing an aminoglycoside and a cross-linker in an organic solvent; disposing said aminoglycoside/cross-linker/ organic solvent in said chromatographic column; after said aminoglycoside reacts with said cross-linker to form a macroporous porous, cross-linked resin, adding water to said column to dissolve said salt; draining said water from said chromatographic column, as required by group I.

Common technical features:

Group I and II share the technical feature of an aminoglycoside and a crosslinker, and react/reaction. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by WO 2014/066674 A1 to KCI Licensing, Inc. (hereinafter KCI). KCI teaches an aminoglycoside and a crosslinker (para [0006], [0010], [0011] Amine-functionalized foam may comprise aminoglycosides. Examples of aminoglycosides include neomycin. The functionalized material or foam may also be covalently attached with a crosslinker), and react/reaction (para [0072], [00168]; table 1; The table shows 51 potential crosslinker chemistries that react with primary amines. Further, neomycin, which is an aminoglycoside, contains primary amines accessible for chemical reaction. These accessible amines are important because it will enable the crosslinker chemistries depicted in Table 1).

As the shared technical features were known in the art at the time of the invention, they cannot be considered common technical features that would otherwise unify the groups. Therefore, Groups I-II lack unity under PCT Rule 13.

Note:

Claim 13 is improperly depending from claim 8. For the purpose of completing this opinion, claim 13 will be assumed as it is depending from claim 9.

Claim 14 is improperly depending from claim 5. For the purpose of completing this opinion, claim 14 will be assumed as it is depending from claim 9.

Claim 15 is improperly depending from claim 6. For the purpose of completing this opinion, claim 15 will be assumed as it is depending from claim 9.