WHAT IS CLAIMED IS:

1. A liposomal preparation, comprising a suspension of liposomes having a gel-phase lipid bilayer and an active agent entrapped inside the liposomes; said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols, monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the active agent is selected from the group consisting of doxorubicin, bleomycin, dacarbazine, daunorubicin, dactinomycin, fludarabine, gemcitabine, idarubicin, methotrexate, mitomycin, mitoxantrone, vinblastine, vinorelbine, and vincristine, and

wherein the lipid bilayer constituents are provided in a molar ratio of about 80-90:2-8:2-18; and wherein the size of the liposomes in the suspension is between about 50 and about 150 nm.

2. The liposomal preparation of claim 1, wherein the active agent is doxorubicin, and wherein the relative concentration of impurity A after 6 months of storage at less than or equal to 8 °C is less than 0.5%, and wherein impurity A is a peak with a relative retention time approximately 1.4 in a high performance liquid chromatography (HPLC) with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

3. The liposomal preparation of claim 2, wherein the relative concentration of impurity A after 6 months of storage at less than or equal to 8 °C is less than 0.2%.

4. The liposomal preparation of claim 2, wherein the relative concentration of impurity A after about 1 year of storage at less than or equal to 8 °C is less than about 0.5%.

5. The liposomal preparation of claim 2, wherein the relative concentration of impurity A after about 2 years of storage at less than or equal to 8 °C is less than about 0.75%.

6. The liposomal preparation of claim 2, wherein the relative concentration of 8-desacetyl-8-carboxy daunorubicin after 6 months of storage at less than or equal to 8 °C is less than about 0.5%.

7. The liposomal preparation of claim 2, wherein the relative concentration of 8-desacetyl-8-carboxy daunorubicin after about 1 year of storage at less than or equal to 8 °C is less than about 0.5%.

8. The liposomal preparation of claim 2, wherein the relative concentration of 8-desacetyl-8-carboxy daunorubicin after about 2 years of storage at less than or equal to 8 °C is less than about 1.6%.

9. The liposomal preparation of claim 2, wherein the concentration of doxorubicin after about six months of storage at a temperature of about less than or equal to 8 °C is greater than 99% of the initial doxorubicin concentration, as determined by HPLC using a C18 size exclusion column with an acetic acid/methanol solvent gradient elution conditions.

10. The liposomal preparation of claim 2, wherein the concentration of doxorubicin after about one year of storage at a temperature of about less than or equal to 8 °C is greater than 97% of the initial doxorubicin concentration, as determined by HPLC with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

11. The liposomal preparation of claim 2, wherein the concentration of doxorubicin after about two years of storage at a temperature of about less than or equal to 8 °C is greater than 95% of the initial doxorubicin concentration, as determined by HPLC with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

12. The liposomal preparation of claim 2, wherein the formation of total degradation products after about six months of storage at a temperature of about less than or equal to 8 °C is less than 1%.

13. The liposomal preparation of claim 2, wherein the formation of total degradation products after about one year of storage at a temperature of about less than or equal to 8 °C is less than 1%.

14. The liposomal preparation of claim 2, wherein the formation of total degradation products after about two years of storage at a temperature of about less than or equal to 8 °C is less than 2.5%.

15. The liposomal preparation of claim 2, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl glycerol (DSPG), or a combination thereof; the lysolipid is monopalmitoylphosphatidylcholine (MPPC), monolaurylphosphatidylcholine (MLPC), monomyristoylphosphatidylcholine (MMPC), monostearoylphosphatidylcholine (MSPC), or mixtures thereof; and the one or more phospholipids derivatized with a hydrophilic polymer is a PEGylated lipid.

16. The liposomal preparation of claim 2, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine, one or more phospholipids derivatized with a hydrophilic polymer is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000], and the one or more lysolipids is monostearoylphosphatidylcholine.

17. The liposomal preparation of claim 2, wherein the liposomes further comprise an imaging agent or a diagnostic agent.

18. The liposomal preparation of claim 17, wherein the imaging agent or diagnostic agent is entrapped in the liposome.

19. The pharmaceutical composition of claim 18, wherein the imaging agent or diagnostic agent is an X-ray contrast agent, an MRI contrast agent, an ultrasonic imaging agent, a fluorescent agent or a radioactive agent.

20. A method for loading an active agent into temperature sensitive liposomes, comprising:

(a) preparing a suspension of liposomes having a gel-phase lipid bilayer and a greater concentration of ammonium ions inside the liposomes than outside the liposomes, said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols, monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the lipid bilayer constituents are provided in a molar ratio of about 80-90:2-8:2-18; and

where said preparing includes reducing the size of the liposomes in the suspension to an average particle size of between about 50 and about 150 nm;

(b) adding a solution of the active agent to the suspension of liposomes, wherein the active agent is taken up into the liposomes,

wherein the active agent is selected from the group consisting of doxorubicin, bleomycin, dacarbazine, daunorubicin, dactinomycin, fludarabine, gemcitabine, idarubicin, methotrexate, mitomycin, mitoxantrone, vinblastine, vinorelbine, and vincristine.

21. The method of claim 20, wherein the active agent is doxorubicin.

22. The method of claim 21, wherein at least 95% of the doxorubicin present in the solution is taken up into the liposomes.

23. The method of claim 21, wherein the concentration of doxorubicin taken up into the liposomes is about 50 mM.

24. The method of claim 21, wherein the concentration of doxorubicin taken up into the liposomes is about 75 mM.

25. The method of claim 21, wherein said preparing comprises preparing the liposomes in the presence of an ammonium salt.

26. The method of claim 25, wherein the ammonium salt is provided as an ammonium sulfate solution.

27. The method of claim 26, wherein the concentration of ammonium sulfate is about 100 mM to about 300 mM.

28. The method of claim 26, further comprising replacing the ammonium ions outside the liposomes with a monosaccharide or disaccharide solution.

29. The method of claim 28, wherein the concentration of the monosaccharide or disaccharide solution is about 5-15%.

30. The method of claim 29, wherein the ammonium ions outside the liposomes are replaced with a monosaccharide solution.

31. The method of claim 30, wherein the monosaccharide solution is a lactose solution.

32. The method of claim 21, further comprising adding a histidine buffer after step (b).

33. The method of claim 32, wherein the concentration of the histidine buffer is about 5 mM to about 15 mM.

34. The method of claim 21, wherein the one or more phospholipids have two same or different $C_{14}$-$C_{20}$ acyl groups.

35. The method of claim 34, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl glycerol (DSPG), or a combination thereof.

36. The method of claim 21, wherein the lysolipid is monopalmitoylphosphatidylcholine (MPPC), monolaurylphosphatidylcholine (MLPC), monomyristoylphosphatidylcholine (MMPC), monostearoylphosphatidylcholine (MSPC), or mixtures thereof.

37. The method of claim 21, wherein the one or more phospholipids derivatized with a hydrophilic polymer is a PEGylated lipid.

38. The method of claim 37, wherein said PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000].

39. The method of claim 21, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine, one or more phospholipids derivatized with a hydrophilic polymer is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000], and the one or more lysolipids is monostearoylphosphatidylcholine.

40. A liposome preparation made by the method of claim 21.

release of the drug to its target, and 4) liposome size that permits its extravasation from the blood stream across leaky tumor micro-vasculature permitting targeting of chemotherapeutic drugs to a tumor site.

**[0011]** An additional important design issue discovered by the inventors with liposomal formulations of doxorubicin (*e.g.* disclosed in U.S. Patent No. 7,901,709) is the stabilizing effect of doxorubicin complex (co-crystal or salt) formation on the stability of the finished drug product. Successful control of degradation rates will result in significant impact on the storage temperature and long term stability of the drug product. Two degradation products of interest are 8-desacetyl-8-carboxy daunorubicin and impurity A.

**[0012]** The present invention solves a persistent problem with drug degradation in doxorubicin liposomal formulation that results in a citrate complex (co-crystal or salt). It has been found that the citrate complex plays a significant role in doxorubicin instability and formation of degradates. More specifically, the formation of 8-desacetyl-8-carboxy daunorubicin and impurity A can be significantly reduced by changing the formation of a citrate complex to a sulfate complex (co-crystal or salt). In addition, the present invention maintains the key design principles listed above for an efficacious hyperthermically activated liposomal formulation containing an active agent.

## BRIEF SUMMARY OF THE INVENTION

**[0012A]** In one aspect, there is provided a liposomal preparation, comprising a suspension of liposomes having a gel-phase lipid bilayer and ~~an active agent~~doxorubicin sulfate entrapped inside the liposomes; said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines,

monoacylphosphatidylglycerols,
monoacylphosphatidylinositols, and
monoacylphosphatidyl-ethanolamines;

~~wherein the active agent is selected from the group consisting of doxorubicin, bleomycin, dacarbazine, daunorubicin, dactinomycin, fludarabine, gemcitabine, idarubicin, methotrexate, mitomycin, mitoxantrone, vinblastine, vinorelbine, and vincristine, and~~ wherein the lipid bilayer constituents are provided in a molar ratio of ~~about~~ 80-90:2-8:2-18; and wherein the size of the liposomes in the suspension is between ~~about~~ 50 and ~~about~~ 150 nm; and wherein the relative concentration of impurity A after 6 months of storage at less than or equal to 8°C is less than 0.5%, and wherein impurity A is a peak with a relative retention time approximately 1.4 in a high performace liquid chromatography (HPLC) with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

**[0013]** Also disclosed is a pharmaceutical composition, comprising a suspension of liposomes having a gel-phase lipid bilayer and doxorubicin entrapped inside the liposomes; said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols,

monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the lipid bilayer constituents are provided in a molar ratio (i):(ii):(iii) of about 80-90:2-8:2-18; wherein the liposomes in the suspension have an average particle size of between about 50 and about 150 nm; and

wherein the relative concentration of impurity A after 6 months of storage at about less than or equal to 8 $^{o}$C is less than 0.5%, and wherein impurity A is a peak with a relative retention time of about 1.4 when separation is achieved using high performance liquid chromatography (HPLC) with a C18 reverse phase column and acetic acid/methanol solvent gradient elution buffer.

1. **[0013A]** In another aspect, there is provided a method for loading ~~an active agent~~doxorubicin sulfate into temperature sensitive liposomes, comprising:

(a) preparing a suspension of liposomes having a gel-phase lipid bilayer and a greater concentration of ammonium ions inside the liposomes than outside the liposomes, said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols, monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the lipid bilayer constituents are provided in a molar ratio of about 80-90:2-8:2-18; and

where said preparing includes reducing the size of the liposomes in the suspension to an average particle size of between about 50 and about 150 nm;

(b) adding a doxorubicin solution ~~of the active agent~~ to the suspension of liposomes, wherein the ~~active agent~~doxorubicin is taken up into the liposomes in the form of doxorubicin sulfate~~,~~

~~wherein the active agent is selected from the group consisting of doxorubicin, bleomycin, dacarbazine, daunorubicin, dactinomycin, fludarabine, gemcitabine, idarubicin, methotrexate, mitomycin, mitoxantrone, vinblastine, vinorelbine, and vincristine~~.

**[0014]** Also disclosed is a method for loading doxorubicin into temperature sensitive liposomes, comprising:

(a) preparing a suspension of liposomes having a gel-phase lipid bilayer and a greater concentration of ammonium ions inside the liposomes than outside the liposomes, said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols, monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the lipid bilayer constituents are provided in a molar ratio (i):(ii):(iii) of about 80-90:2-8:2-18; and

where said preparing includes reducing the size of the liposomes in the suspension to an average particle size of between about 50 and about 150 nm;

(b) adding a doxorubicin solution to the suspension of liposomes, wherein the doxorubicin is taken up into the liposomes.

WHAT IS CLAIMED IS:

1. A liposomal preparation, comprising a suspension of liposomes having a gel-phase lipid bilayer and doxorubicin sulfate entrapped inside the liposomes; said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols, monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the lipid bilayer constituents are provided in a molar ratio of 80-90:2-8:2-18; wherein the size of the liposomes in the suspension is between 50 and 150 nm; and wherein the relative concentration of impurity A after 6 months of storage at less than or equal to 8°C is less than 0.5%, and wherein impurity A is a peak with a relative retention time approximately 1.4 in a high performace liquid chromatography (HPLC) with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

2. The liposomal preparation of claim 1, wherein the relative concentration of impurity A after 6 months of storage at less than or equal to 8 °C is less than 0.2%.

3. The liposomal preparation of claim 1, wherein the relative concentration of impurity A after about 1 year of storage at less than or equal to 8 °C is less than about 0.5%.

4. The liposomal preparation of claim 1, wherein the relative concentration of impurity A after about 2 years of storage at less than or equal to 8 °C is less than about 0.75%.

5. The liposomal preparation of claim 1, wherein the relative concentration of 8-desacetyl-8-carboxy daunorubicin after 6 months of storage at less than or equal to 8 °C is less than about 0.5%.

6. The liposomal preparation of claim 1, wherein the relative concentration of 8-desacetyl-8-carboxy daunorubicin after about 1 year of storage at less than or equal to 8 °C is less than about 0.5%.

7. The liposomal preparation of claim 1, wherein the relative concentration of 8-desacetyl-8-carboxy daunorubicin after about 2 years of storage at less than or equal to 8 °C is less than about 1.6%.

8. The liposomal preparation of claim 1, wherein the concentration of doxorubicin after about six months of storage at a temperature of less than or equal to 8 °C is greater than 99% of the initial doxorubicin concentration, as determined by HPLC using a C18 size exclusion column with an acetic acid/methanol solvent gradient elution conditions.

9. The liposomal preparation of claim 1, wherein the concentration of doxorubicin after about one year of storage at a temperature of less than or equal to 8 °C is greater than 97% of the initial doxorubicin concentration, as determined by HPLC with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

10. The liposomal preparation of claim 1, wherein the concentration of doxorubicin after about two years of storage at a temperature of less than or equal to 8 °C is greater than 95% of the initial doxorubicin concentration, as determined by HPLC with a C18 reverse phase column with an acetic acid/methanol solvent gradient elution conditions.

11. The liposomal preparation of claim 1, wherein the formation of total degradation products after about six months of storage at a temperature of less than or equal to 8 °C is less than 1%.

12. The liposomal preparation of claim 1, wherein the formation of total degradation products after about one year of storage at a temperature of less than or equal to 8 °C is less than 1%.

13. The liposomal preparation of claim 1, wherein the formation of total degradation products after about two years of storage at a temperature of less than or equal to 8 $^{o}$C is less than 2.5%.

14. The liposomal preparation of claim 1, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl glycerol (DSPG), or a combination thereof; the lysolipid is monopalmitoylphosphatidylcholine (MPPC), monolaurylphosphatidylcholine (MLPC), monomyristoylphosphatidylcholine (MMPC), monostearoylphosphatidylcholine (MSPC), or mixtures thereof; and the one or more phospholipids derivatized with a hydrophilic polymer is a PEGylated lipid.

15. The liposomal preparation of claim 1, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine, one or more phospholipids derivatized with a hydrophilic polymer is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000], and the one or more lysolipids is monostearoylphosphatidylcholine.

16. The liposomal preparation of claim 1, wherein the liposomes further comprise an imaging agent or a diagnostic agent.

17. The liposomal preparation of claim 16, wherein the imaging agent or diagnostic agent is entrapped in the liposome.

18. The pharmaceutical composition of claim 17, wherein the imaging agent or diagnostic agent is an X-ray contrast agent, an MRI contrast agent, an ultrasonic imaging agent, a fluorescent agent or a radioactive agent.

19. A method for loading doxorubicin sulfate into temperature sensitive liposomes, comprising:

(a) preparing a suspension of liposomes having a gel-phase lipid bilayer and a greater concentration of ammonium ions inside the liposomes than outside the liposomes, said lipid bilayer comprising:

(i) one or more phospholipids selected from the group consisting of phosphatidyl cholines, phosphatidyl glycerols, phosphatidyl inositols, and phosphatidyl ethanolamines;

(ii) one or more phospholipids derivatized with a hydrophilic polymer; and

(iii) one or more lysolipids selected from the group consisting of monoacylphosphatidyl cholines, monoacylphosphatidylglycerols, monoacylphosphatidylinositols, and monoacylphosphatidyl-ethanolamines;

wherein the lipid bilayer constituents are provided in a molar ratio of about 80-90:2-8:2-18; and

where said preparing includes reducing the size of the liposomes in the suspension to an average particle size of between about 50 and about 150 nm;

(b) adding a doxorubicin solution to the suspension of liposomes, wherein the doxorubicin is taken up into the liposomes in the form of doxorubicin sulfate.

20. The method of claim 19, wherein the active agent is doxorubicin.

21. The method of claim 20, wherein at least 95% of the doxorubicin present in the solution is taken up into the liposomes.

22. The method of claim 20, wherein the concentration of doxorubicin taken up into the liposomes is about 50 mM.

23. The method of claim 20, wherein the concentration of doxorubicin taken up into the liposomes is about 75 mM.

24. The method of claim 20, wherein said preparing comprises preparing the liposomes in the presence of an ammonium salt.

25. The method of claim 24, wherein the ammonium salt is provided as an ammonium sulfate solution.

26. The method of claim 25, wherein the concentration of ammonium sulfate is about 100 mM to about 300 mM.

27. The method of claim 25, further comprising replacing the ammonium ions outside the liposomes with a monosaccharide or disaccharide solution.

28. The method of claim 27, wherein the concentration of the monosaccharide or disaccharide solution is about 5-15%.

29. The method of claim 28, wherein the ammonium ions outside the liposomes are replaced with a monosaccharide solution.

30. The method of claim 29, wherein the monosaccharide solution is a lactose solution.

31. The method of claim 20, further comprising adding a histidine buffer after step (b).

32. The method of claim 31, wherein the concentration of the histidine buffer is about 5 mM to about 15 mM.

33. The method of claim 20, wherein the one or more phospholipids have two same or different $C_{14}$-$C_{20}$ acyl groups.

34. The method of claim 33, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl glycerol (DSPG), or a combination thereof.

35. The method of claim 20, wherein the lysolipid is monopalmitoylphosphatidylcholine (MPPC), monolaurylphosphatidylcholine (MLPC), monomyristoylphosphatidylcholine (MMPC), monostearoylphosphatidylcholine (MSPC), or mixtures thereof.

36. The method of claim 20, wherein the one or more phospholipids derivatized with a hydrophilic polymer is a PEGylated lipid.

37. The method of claim 36, wherein said PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000].

38. The method of claim 20, wherein the one or more phospholipids is dipalmitoylphosphatidylcholine, one or more phospholipids derivatized with a hydrophilic polymer is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol) 2000], and the one or more lysolipids is monostearoylphosphatidylcholine.

39. A liposome preparation made by the method of claim 20.