**Claims**

1. A method of forming different covalently modified surfaces in a regioselective manner within a microfluidic device, wherein the microfluidic device comprises an enclosure having a base, a cover, and a microfluidic circuit material defining a microfluidic circuit therein, wherein the microfluidic circuit comprises a flow region and a sequestration pen, and wherein the sequestration pen comprises an isolation region and a connection region, the connection region comprising a proximal opening to the flow region and fluidically connecting the isolation region to the flow region, the method comprising:

flowing a first modifying reagent through the flow region under conditions such that the first modifying reagent does not enter the isolation region of the sequestration pen;

reacting the first modifying reagent with reactive moieties on at least one surface of the flow region, thereby forming a first modified surface within the flow region, wherein the first modified surface does not extend into the isolation region of the sequestration pen;

flowing a second modifying reagent through the flow region under conditions such that the second modifying reagent enters into the isolation region of the sequestration pen; and

reacting the second modifying reagent with reactive moieties on at least one surface of the isolation region of the sequestration pen, thereby forming a second modified surface within the isolation region of the sequestration pen,

wherein the first modifying reagent does not have the same structure as the second modifying reagent.

2. The method of claim 1, wherein the conditions for flowing the first modifying reagent through the flow region comprise applying a negative pressure to the flow region.

3. The method of claim 1, wherein the conditions for flowing the second modifying reagent through the flow region comprise applying a positive pressure to the flow region.

4. The method of any one of claims 1 to 3, wherein the second modifying reagent does not substantially react with moieties on the surfaces of the flow region.

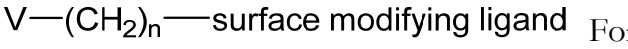
5. The method of any one of claims 1 to 4, wherein:

the first modifying reagent comprises a first connecting moiety and a first modifying moiety, the first modifying moiety comprising a first surface contact moiety; and

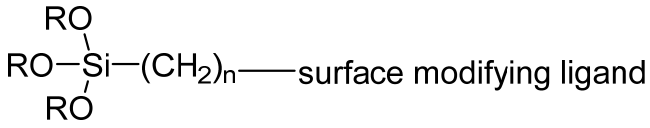
the second modifying reagent comprises a second connecting moiety and a second modifying moiety, the second modifying moiety comprising a second surface contact moiety,

wherein the first modifying moiety is different from the second modifying moiety.

6. The method of any one of claims 1 to 5, wherein the first modifying reagent has a structure of one of the following formulae:



Formula I;



Formula III;

V-$\underline{L_{sm}}$-surface modifying ligand Formula XXXII;

wherein:

V is $-P(O)(OH)_2$ or $-Si(T)_2W$;

W is –T, -SH, or $-NH_2$ and is the moiety configured to form a covalent bond with the at least one surface of the flow region;

T is independently OH, $OC_{1-6}$ alkyl, or halo;

R is $C_{1-6}$ alkyl;

n is an integer of 3 to 21; and

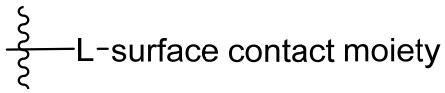
$L_{sm}$ is a linker comprising 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms, and further comprises 0, 1, 2, or 3 coupling groups CG.

7. The method of claim 6, wherein W is $OC_{1-6}$ alkyl or halo.

8. The method of claim 6 or 7, wherein n is 7 to 21.

9. The method of any one of claims 6 to 8, wherein T is $OC_{1-3}$ alkyl or halo and/or R is $C_{1-3}$ alkyl.

10. The method of any one of claims 6 to 9, wherein the surface modifying ligand of the first modifying reagent has a structure of Formula X or Formula XI:




Formula X




Formula XI

wherein:

CG is a coupling group;

L is a linker comprising a bond or 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms;

the sum of $L_{sm}$ and L is 1 to 200 non-hydrogen atoms, not including atoms of the CG if present; and

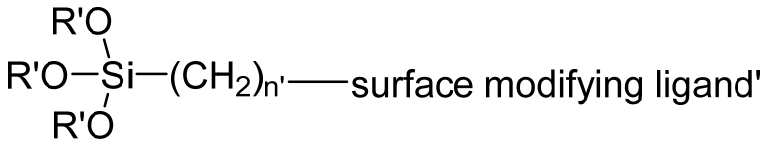
the surface contact moiety is a moiety configured to support cell growth, viability, portability, or any combination thereof in the microfluidic device.

11. The method of claim 10, wherein the surface contact moiety comprises one or more of an alkyl, fluoroalkyl, monosaccharide, polysaccharide, alcohol, polyalcohol, alkylene ether, polyelectrolytes, amino, carboxylic acid, phosphonic acid, sulfonate anion, carboxybetaines, sulfobetaine, sulfamic acid, amino acid moiety, or cleavable moiety.

12. The method of any one of claims 6 to 11, wherein the second modifying reagent has a structure of one of the following formulae:




Formula I'




Formula III'




Formula XXXII';

wherein:

V' is $-P(O)(OH)_2$ or $-Si(T')_2W'$;

W' is –T', -SH, or $–NH_2$ and is the moiety configured to form a covalent bond with the at least one inner surface;

T' is independently OH, $OC_{1-6}$ alkyl, or halo;

R' is $C_{1-6}$ alkyl;

n' is an integer of 3 to 21, and

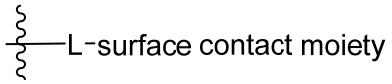
$L'_{sm}$ is a linker comprising 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms and further comprises 0, 1, 2, or 3 coupling groups CG.

13. The method of claim 12, wherein W' is $OC_{1-6}$ alkyl or halo.

14. The method of claim 12 or 13, wherein n' is 7 to 21.

15. The method of any one of claims 12 to 14, wherein T' is $OC_{1-3}$ alkyl or halo and/or R' is $C_{1-3}$ alkyl.

16. The method of any one of claims 12 to 15, wherein the surface modifying ligand of the second modifying reagent has a structure of Formula X or Formula XI:




Formula X




Formula XI

wherein:

CG is a coupling group;

L is a linker comprising a bond or 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms;

the sum of $L'_{sm}$ and L is 1 to 200 non-hydrogen atoms, not including atoms of the CG if present; and

the surface contact moiety is a moiety configured to support cell growth, viability, portability, or any combination thereof in the microfluidic device.

17. The method of claim 16, wherein the surface contact moiety comprises one or more of an alkyl, fluoroalkyl, monosaccharide, polysaccharide, alcohol, polyalcohol, alkylene ether,

polyelectrolytes, amino, carboxylic acid, phosphonic acid, sulfonate anion, carboxybetaines, sulfobetaine, sulfamic acid, amino acid moiety, or cleavable moiety.

18. The method of claim 16, wherein the surface contact moiety comprises a polyethylene glycol, a dextran moiety, a proteinaceous moiety, a poly carboxylic acid, or a poly lysine moiety.

19. The method of any one of claims 1 to 18, wherein the conditions such that the second modifying reagent enters into the isolation region of the sequestration pen comprises priming the microfluidic device with carbon dioxide prior to flowing a second modifying reagent through the flow region but after reacting the first modifying reagent with nucleophilic moieties on at least one surface of the flow region.

20. A microfluidic device comprising:

an enclosure comprising a base, a cover, and microfluidic circuit material defining a fluidic circuit therein,

wherein the fluidic circuit comprises a flow region and sequestration pens,

wherein at least one surface of the flow region is modified with a plurality of first covalently bound surface modifications, each comprising

a first linking group, and

a first moiety, wherein the first moiety is a first surface contact moiety covalently linked to the first linking group, and the first surface contact moiety is configured to inhibit self-propelled movement of cells from one sequestration pen to another sequestration pen;

wherein at least one surface of the sequestration pens is modified with a plurality of second covalently bound surface modifications, each comprising

a second linking group, and

a second moiety, wherein the second moiety is a second surface contact moiety or a reactive moiety, the reactive moiety configured to react with a surface modifying reagent to form the second surface contact moiety, and

wherein the first moiety is different from the second moiety.

21. The microfluidic device of claim 20, wherein the first moiety and the second moiety are each covalently bound to the surface via a linking group LG independently selected from –W-Si$(OZ)_2$O- and –O$P(O)_2$O-, wherein W is O, S, or N, and wherein Z is a bond to a silicon atom in an adjacent linking group LG or is a bond to the surface.

22. The microfluidic device of claim 20 or 21, wherein the first surface contact moiety comprises one or more of an alkyl, fluoroalkyl, monosaccharide, polysaccharide, alcohol, polyalcohol, alkylene ether, polyelectrolytes, amino, carboxylic acid, phosphonic acid, sulfonate anion, carboxybetaines, sulfobetaine, sulfamic acid, amino acid moiety, or cleavable moiety; and/or

wherein the second surface contact moiety comprises one or more of an alkyl, fluoroalkyl, monosaccharide, polysaccharide, alcohol, polyalcohol, alkylene ether, polyelectrolytes, amino, carboxylic acid, phosphonic acid, sulfonate anion, carboxybetaines, sulfobetaine, sulfamic acid, amino acid moiety, or cleavable moiety.

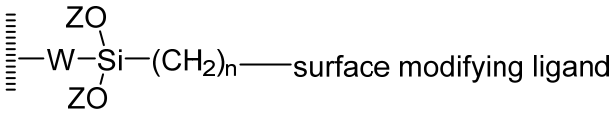
23. The microfluidic device of claim 20 or 22, wherein the first surface contact moiety comprises a polyethylene glycol moiety, a dextran moiety, a proteinaceous moiety, a poly carboxylic acid, a polylysine moiety, or any combination thereof; and/or

wherein the second surface contact moiety comprises a polyethylene glycol moiety, a dextran moiety, a proteinaceous moiety, a poly carboxylic acid, a polylysine moiety, or any combination thereof.

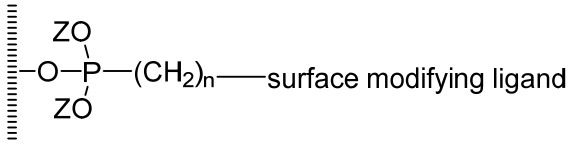
24. The microfluidic device of any one of claims 20 to 23, wherein each first covalently bound surface modification comprises a linker, wherein the linker comprises 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms; and/or

wherein each second covalently bound surface modification comprises a linker, wherein the linker comprises 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms.

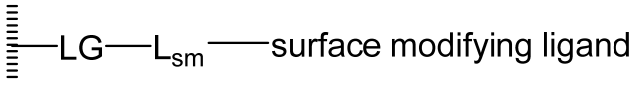
25. The microfluidic device of claim 20, wherein the first covalently bound surface modifications have a structure selected from Formula XXXI, Formula VIII, and Formula IX:




Formula VIII;




Formula IX;




Formula XXXI;

wherein:

LG is -W-Si$(OZ)_2$O- or -O$P(O)_2$O-;

W is O, S, or N;

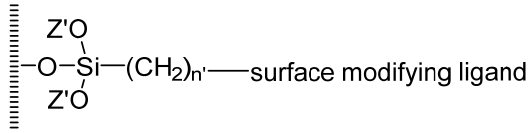
Z is a bond to an adjacent silicon atom or is a bond to the surface;

n is an integer of 3 to 21;

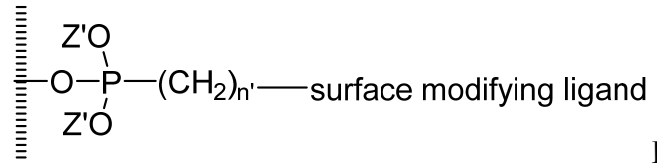
$L_{sm}$ is a linker comprising 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms and further comprises 0, 1, 2, or 3 coupling groups CG; and

 is the surface.

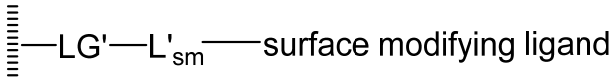
26. The microfluidic device of claim 25, wherein the second covalently bound surface modifications have a structure selected from Formula XXXI', Formula VIII', and Formula IX':




Formula VIII';




Formula IX';




Formula XXXI';

wherein:

LG' is –W'-Si(OZ')$_2$O- or –OP(O)$_2$O-;

W' is O, S, or N;

Z' is a bond to an adjacent silicon atom or is a bond to the surface;

n' is an integer of 3 to 21;

L'$_{sm}$ is a linker comprising 1 to 200 non-hydrogen atoms selected from any combination of silicon, carbon, nitrogen, oxygen, sulfur and phosphorus atoms and further comprises 0, 1, 2, or 3 coupling groups CG; and

 is the surface.

27. The microfluidic device of claim 26, wherein LG is –W-Si(OZ)2O- and W is O and/or wherein LG' is –W'-Si(OZ')$_2$O- and W' is O.

28. The microfluidic device of claim 26 or 27, wherein n is 7 to 21 and/or n' is 7 to 21.

29. The microfluidic device of any one of claims 26 to 28, wherein the first covalently bound surface modifications and the second covalently bound surface modifications are on a common inner surface of the base, the cover, and/or the microfluidic circuit material.

30. The microfluidic device of claim 29, wherein the common inner surface comprises a first region comprising the first covalently bound surface modifications and a second region comprising the second covalently bound surface modifications, and wherein the first region is adjacent to the second region.

31. The microfluidic device of any one of claims 20 to 30, wherein the second covalently bound surface modifications comprises a surface contact moiety configured to anchor adherent cells.

32. The microfluidic device of any one of claims 20 to 31 wherein the first covalently bound surface modifications comprises a surface contact moiety configured to inhibit migration of motile cells out of the sequestration pen.

33. The microfluidic device of any one of claims 20 to 32, wherein the fluidic circuit further comprises a plurality of sequestration pens each having at least one inner surface modified with the second covalently bound surface modifications.

34. The microfluidic device of any one of claims 20 to 33, wherein substantially all of the inner surfaces of the enclosure are covalently modified.

35. The method of any one of claims 1 to 19 and the microfluidic device of any one of claims 20 to 34, wherein reacting the first modifying reagent with nucleophilic moieties on at least one surface of the flow region comprises reacting the first modifying reagent with nucleophilic moieties on all surfaces of the flow region, and wherein reacting the second modifying reagent with nucleophilic moieties on at least one surface of the isolation region of the sequestration pen comprises reacting the second modifying reagent with nucleophilic moieties on all surfaces of the isolation region of the sequestration pen.