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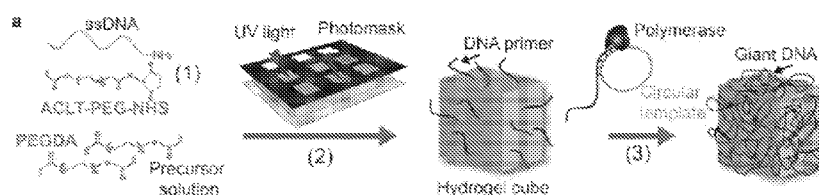
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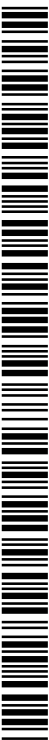
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(54) Title: NUCLEIC ACID HYDROGEL SELF-ASSEMBLY

FIG. 2



(57) Abstract: Provided herein are self-assembling shaped nucleic acid hydrogel subunits and methods of use thereof.



NUCLEIC ACID HYDROGEL SELF-ASSEMBLY

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 61/727,344, filed November 16, 2012, which is incorporated by reference herein in its entirety.

FEDERALLY SPONSORED RESEARCH

This invention was made with government support under EB008392, DE019024, HL099073, AR057837, HL092836 and 1DP2OD007292 awarded by National Institutes of Health; under DMR0847287 and CCF1054898 awarded by National Science Foundation; and under N000141110914 and N000141010827 awarded by U.S. Department of Navy. The government has certain rights in the invention.

FIELD OF THE INVENTION

The invention relates, in some embodiments, to the field of nucleic acid nanotechnology.

BACKGROUND OF INVENTION

“Self-assembly” refers to a process by which a disordered system of pre-existing components forms an organized structure as a consequence of specific local interactions among the individual components without external direction. Self-assembly processes are particularly useful in the field of nucleic acid nanotechnology. These processes take advantage of the molecular recognition properties of nucleic acids to create, for technological purposes, artificial, often intricate, nucleic acid structures.

SUMMARY OF INVENTION

Provided herein are individual self-assembling hydrogel subunits. A single hydrogel subunit is covalently conjugated to at least one nucleic acid (*e.g.*, at least one nucleic acid concatemer). The nucleic acid on each subunit acts as “glue” to bring together (*i.e.*, self-assemble) multiple hydrogel subunits in a controlled fashion. Based on the specific nucleotide sequence of each nucleic acid, a plurality of hydrogel subunits can be directed to self-assemble into single or multiple architecturally distinct, typically predetermined, structures.

Thus, in some aspects of the invention, provided herein are shaped hydrogel subunits surface-modified with at least one nucleic acid (referred to herein as a shaped nucleic acid hydrogel subunit). In some embodiments, the nucleic acid is a concatemer.

5 In other aspects of the invention, provided herein are compositions comprising at least two shaped hydrogel subunits, each surface-modified with at least one nucleic acid, wherein the at least two shaped hydrogel subunits are joined to each other through sequence-specific hybridization between the nucleic acids. In some embodiments, the nucleic acid is a concatemer.

10 In yet other aspects of the invention, provided herein are pluralities of shaped hydrogel subunits each surface-modified with at least one nucleic acid concatemer, wherein the shaped hydrogel subunits are joined to each other through sequence-specific hybridization between the nucleic acids. In some embodiments, the pluralities of shaped hydrogel subunits are specifically arranged to form three-dimensional structures.

15 In still other aspects of the invention, provided herein are methods of producing a three-dimensional hydrogel structure comprising combining at least two shaped hydrogel subunits, each surface-modified with at least one nucleic acid concatemer, in an aqueous assembly system or in an interfacial assembly system, thereby providing for nucleic acid hybridization and shaped hydrogel subunit self-assembly.

20 In additional aspects of the invention, provided herein are methods of producing a shaped hydrogel subunit surface-modified with a nucleic acid comprising conjugating a nucleic acid to a polyethylene glycol (PEG) monomer to form nucleic acid-PEG-acrylate, combining the nucleic acid-PEG-acrylate with PEG-diacrylate and a photoinitiator to form a mixture, and exposing the mixture to ultraviolet light under a photomask to produce the shaped hydrogel subunit modified with at least one nucleic acid. In some embodiments, methods further
25 comprise producing at least one concatemer through rolling circle amplification of the at least one nucleic acid. In some embodiments, the nucleic acid is a concatemer.

In further aspects of the invention, provided herein are methods comprising delivering cells or other biomolecules to a site of interest *in vivo* using a shaped nucleic acid hydrogel subunit of the invention, a composition of the invention, or a plurality of shaped nucleic acid
30 hydrogel subunits of the invention.

In some embodiments, at least one nucleic acid is covalently attached to the subunit. In some embodiments, the at least one nucleic acid is single-stranded.

In some embodiments, a shaped hydrogel subunit is a cube, a tube or a sphere. In some embodiments, a shaped hydrogel subunit is comprised of polyethylene glycol, gelatin methacrylate or alginate. In some embodiments, a shaped hydrogel subunit has a diameter of about 1 μm to about 1 mm.

5 In some embodiments, a nucleic acid concatemer is produced through rolling circle amplification (RCA).

In some embodiments, a shaped hydrogel subunit is surface-modified with at least two nucleic acids. In some embodiments, at least two nucleic acids are different from each other and are present on different surfaces of the shaped hydrogel subunit. In some embodiments, a
10 shaped hydrogel subunit is surface-modified with at least three nucleic acids. The nucleic acids, in some embodiments, are nucleic acid concatemers.

In some embodiments, a shaped hydrogel subunit contains cells. In some embodiments, cells are stem cells, progenitor cells or differentiated cells.

15 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 (steps (1) to (5)) shows a process of a non-limiting example of stencil-based hydrogel fabrication and surface modification with nuclei acid concatemers;

FIG. 2A (steps (1) to (3)) shows schematics of a non-limiting example of a fabrication process of hydrogel cubes uniformly modified with DNA concatemers; FIGs. 2B-2D show a
20 phase contrast image (FIG. 2B), a fluorescent image (FIG. 2C), and a scanning electron microscopy (SEM) image (FIG. 2D) of hydrogels carrying short 56-nucleotide (nt) single-stranded DNA primers; FIGs. 2E-2G show a phase contrast image (FIG. 2E), a fluorescent image (FIG. 2F), and a scanning electron microscopy (SEM) image (FIG. 2G) of amplified single-stranded DNA concatemers (the gels in (FIG. 2C) and (FIG. 2F) were stained with
25 SYBR[®] Gold before imaging);

FIGs. 3A-3K show non-limiting examples of self-assembly of hydrogel cubes with uniform DNA concatemer modification;

FIGs. 4A-4E show non-limiting examples of self-assembly of hydrogel subunits (*e.g.*, cubes) with face-specific DNA concatemer modifications;

30 FIG. 5A shows a schematic of a non-limiting example of hydrogel cube self-assembly at a liquid-liquid interface (hydrogel cubes were floated at the interface formed between aqueous phosphate buffered saline (PBS) liquid (upper) and fluorinert FC-40 liquid (bottom) and agitated with rotary shaking); FIG. 5B shows dimers formed from red and blue hydrogel

cubes (left, schematics; right, stereomicroscopy image); FIGs. 5C-5E show examples of four hydrogel cubes self-assembled into a chain (FIG. 5C), a T-junction (FIG. 5D) or a square (FIG. 5E) based on their surface DNA concatemer modification pattern (left, schematics; right, color stereomicroscopy image);

FIGs. 6A-6E show non-limiting examples of patterned DNA concatemer modification on the surface of shaped hydrogel cubes and DNA concatemer-directed hydrogel cube dimer formation (*e.g.*, self-assembly of two hydrogel cubes);

FIGs. 7A-7F show phase contrast (left) and fluorescent (right) images for non-limiting examples of DNA concatemer-modified cell-free hydrogel cubes (top), cell-encapsulated hydrogel cubes without DNA concatemers (middle), and cell-encapsulated hydrogel cubes modified with DNA concatemers (bottom) (DNA concatemers and cells fluoresce; scale bar, 100 microns);

FIG. 8A shows a single-stranded 84-nt DNA primer before amplification (middle lane) and a post-amplification DNA concatemer (right lane) on 1% agarose gel, stained with SYBR[®] Gold (left lane, 100 bp DNA ladder with the 2072 bp fragment labeled); FIG. 8B shows DNA concatemers amplified from a 84-nt primer tethered on a PEG surface (the DNA concatemers were stained with SYBR[®] gold); FIG. 8C shows a scanning electron microscopy (SEM) image of amplified DNA concatemers on a glass surface; FIG. 8D shows patterned amplified DNA concatemers on a PEG hydrogel surface (the region on the left is the surface of a naked PEG gel; the region on the right is decorated with DNA concatemers);

FIG. 9 shows two copies of T-junctions structures self-assembled from four red, blue, yellow, and violet 1 mm × 1 mm × 0.3 mm PEG hydrogel cuboids carrying patterned DNA concatemers (in the schematic, the parts that contain DNA are dark gray and the sequences are labeled with letters, where x and x* denote two complementary DNA sequences); and

FIG. 10A shows a non-limiting example of a fabrication schematic of cell-encapsulated hydrogel cubes. FIGs. 10B-10D show phase contrast (B, D) and fluorescent (C, E) microscopy images of a cell viability assay. FIGs. 10F and 10G show phase-contrast (F) and fluorescent (G) microscopy images of cell-encapsulated hydrogel dimer assembled from human umbilical vein endothelial cell (HUVEC) (green) and smooth muscle cell (SMC) (red) encapsulated hydrogels.

DETAILED DESCRIPTION OF INVENTION

Tissue engineering holds great promise for developing therapies to treat injured tissue; however, existing technologies face several challenges. First, the lack of vascularization in most engineered tissue scaffolds results in cell death and loss of function, thereby limiting the size of the scaffold. Second, the difficulty in uniformly seeding cells throughout a scaffold prevents high initial cell loading. Lastly, the inability to mimic the complex microarchitecture of tissues limits the application of the scaffold. Provided herein are embodiments that address each of these challenges.

The present invention is premised, in part, on the finding that nucleic acids can be conjugated to biocompatible, shaped hydrogel subunits to form “self-assembling” units, referred to herein as “nucleic acid hydrogel subunits.” The shaped hydrogel subunits (which may be referred to simply as “hydrogel subunits”) provided herein are modified with (*e.g.*, attached to) nucleic acids that can hybridize to each other through complementary (or sequence-specific) nucleic acid hybridization. The nucleic acids attached to the hydrogel subunits function as “glue” to assemble the subunits, which assembly can be directed by introducing specific “address” sequences into the nucleic acids. In this way, it is possible to produce a multitude of specific, stable, three-dimensional hydrogel structures (*e.g.*, linear chains and net-like structures) for use in many biomedical and bioengineering applications.

One example of a basic self-assembly process using three nucleic acid hydrogel subunits is as follows: a first hydrogel subunit in the shape of a cube is conjugated on one side to a concatemer of sequence A, while the opposite side of the cube is conjugated to a concatemer of sequence B. The A sequence of the concatemer of the first subunit will hybridize to another concatemer of sequence A* (complementary to A) conjugated on one side of a second cube. The concatemer of sequence B of the first subunit will hybridize to its complementary sequence on a third shaped nucleic acid hydrogel subunit designed to have a concatemer of sequence complementary to B. In this way, a basic “chain” of three subunits is formed. This process can continue, using multiple hydrogel subunits of various shapes, each conjugated to at least one (one or more) specifically designed nucleic acid concatemers, to create chains, sheets or other intricate architectures, depending on the shapes of the hydrogel subunits and how they are attached to each other.

In some embodiments, the nucleic acid hydrogel subunits can be used in combination with existing tissue and cell (*e.g.*, stem cell) bioengineering technology to create micro-vascularized tissue architectures for use in applications such as, for example, skin grafting to

support cell growth and function. In some embodiments, cells or other biomolecules may be embedded in the nucleic acid hydrogel subunits for delivery *in vivo* to a site of interest such as, for example, an injured tissue to promote tissue cell growth and vascularization. In some embodiments, the nucleic acid hydrogel subunits may be produced using a biocompatible gel such as polyethylene glycol (PEG) and may be seeded with cells such as, but not limited to, stem cells, progenitor cells or differentiated cells, depending on the application, and then used to produce tissue scaffolds (*e.g.*, *in vivo* grafts). Cells may be embedded into the hydrogel subunits during subunit formation. In some instances, however, cells may be embedded into the hydrogel subunits after subunit formation. The hydrogel subunits are conjugated to at least one nucleic acid designed to have a specific address sequence, which will be amplified to produce a concatemer of that sequence. Such addressable subunits can be directed to assemble into a two- or three-dimensional structure, mimicking an *in vivo* tissue structure.

Hydrogel subunits

Hydrogel subunits of the invention may be synthesized by any means known to one of ordinary skill in the art including, without limitation, photolithography, emulsification, microfluidic synthesis and micromolding (*see, e.g.*, Khademhosseini and Lander, *Biomaterials*, 28:5087-5092 (2007); Khademhosseini *et al.*, *Proc. Natl. Acad. Sci. USA*, 103:2480-2487 (2006), each of which is incorporated herein by reference).

In some embodiments, photolithography may be used for a variety of biomedical applications to engineer hydrogel subunits, including microscale subunits. In some embodiments, synthetic or natural photocrosslinkable prepolymers may be crosslinked to form hydrogel subunits (Peppas *et al.*, *Adv. Mater.*, 18:1-17 (2006), incorporated herein by reference). In photolithographic processes, a thin film of a polymer is exposed to ultraviolet (UV) light through a mask. As the light reaches the photosensitive polymer through the transparent regions of the mask, it causes a photoreaction that crosslinks the polymer. In some embodiments, photolithography may be used to create the shaped hydrogel subunits or to immobilize cells within the shaped hydrogel subunits. The photocrosslinkable hydrogel subunits of the invention may be made from various types of synthetic polymers including, without limitation, polyethylene glycol (PEG) (Koh *et al.*, *Langmuir*, 18(7):2459-62 (2002); Liu *et al.*, *Biomed. Microdev.*, 4(4):257-66 (2002), each of which is incorporated herein by reference). In some embodiments, natural photocrosslinkable prepolymers may be used to form the hydrogel subunits (Khademhosseini *et al.*, *J Biomed Mater Res A* (2006),

incorporated herein by reference). In some embodiments, photolithography may be used to conjugate chemical entities (*e.g.*, nucleic acids) to hydrogel subunits with controlled spatial resolution (Hahn, *et al.*, *Adv. Mater.* 18(20):2679-84 (2006); Luo *et al.*, *Nat Mater.*, 3(4):249-53 (2004), each of which is incorporated herein by reference). The dimensions that can be achieved by using photolithography range from submicron to millimeter scale.

In some embodiments, emulsification may be used to fabricate hydrogel subunits of the invention. In this process, a multi-phase mixture is stirred to generate small aqueous droplets of hydrogel precursors within an organic phase. The size of the droplets may be controlled by the degree of mechanical agitation, viscosity of each phase, or the presence of surfactants that can modify the surface tension between the two phases. The resulting droplets may be gelled using a variety of crosslinking mechanisms to generate spherical hydrogels. In some embodiments, cells may be added to the aqueous phase to fabricate cell-encapsulated, or cell-embedded, hydrogel subunits.

In some embodiments, microfluidics may be used to fabricate nucleic acid hydrogel subunits of the invention. In some embodiments, a multi-phase system may be used to generate microparticles. In this system, the viscous and surface tension forces may be used to create homogeneous particles that can be crosslinked to form microscale hydrogel subunits. Such crosslinking may be, without limitation, chemical crosslinking or pH crosslinking. A range of particle sizes and shapes may be created based on the design of the microfluidic channels. For example, by changing the dimensions of the microchannels, the flow rates and the droplet shapes, it is possible to create hydrogels in the form of spheres and rods (Xu *et al.*, *Angew. Chem. Int. Ed. Engl.*, 44(25):3799 (2005), incorporated herein by reference).

In some embodiments, microfluidic fabrication may be used to control the spatial properties of hydrogel subunits. For example, a microfluidic device that creates concentration gradients at two or more inlets may be used to create hydrogels with controlled gradients of signaling molecules or material properties embedded in the hydrogel subunit (Burdick *et al.*, *Langmuir*, 20(13):5153-6 (2004), incorporated herein by reference). In some embodiments, such hydrogel subunits may be used for various tissue-engineering applications in which concentration gradients are desired in the scaffolds. In some embodiments, Janus particles (*i.e.*, particles with two or more distinct sides) may be generated by flowing multiple streams and generating droplets contributing to the two or more sides (Roh *et al.*, *Nat. Mater.*, 4(10):759-63 (2005), incorporated herein by reference).

In some embodiments, micromolding may be used to generate hydrogel subunits. Precursor polymers may be initially molded and subsequently gelled to generate structures of a variety of shapes and sizes (Fukada *et al.*, *Biomaterials*, 27:1479-1486 (2006); Yeh *et al.* *Biomaterials*, 27:5391-5398 (2006); Ling *et al.*, *Lab Chip*, 7:756-762; Franzesi *et al.*, *J Am Chem Soc.*, 128(47):15064-5 (2006), each of which is incorporated herein by reference). In some embodiments, micromolding may be used to generate three-dimensional hydrogel structures or microstructures. This may be accomplished by first using a sacrificial template around which the hydrogel can be formed (Stachowiak *et al.*, *Adv. Mater.*, 17(4):399-403 (2005), incorporated herein by reference).

It is to be understood that the shaped hydrogel subunits of the invention may be made from any biocompatible gel composition. A “biocompatible gel composition” herein refers to any non-toxic, aqueous-based, biodegradable composition including, without limitation, polyethylene glycol (PEG), polyethylene glycol diacrylate (PEGDA), gelatin, agarose, collagen, calcium alginate, hyaluronic acid, hydrophobically modified chitosan, fibrogen and polysaccharides (Becker *et al.*, *Neurosurgery*, 56(4):793-801 (2005); Himeda *et al.*, *Journal of Gynecological Surgery*, 20(2):39-46 (2004); Dennis *et al.*, *Soft Matter*, 7(9):4170-73 (2011), Khademhosseini *et al.*, *Lab Chip*, 4(5):425-30 (2004); U.S. Patent No. 7,709,462, each of which is incorporated herein by reference). Other biocompatible gel compositions may be used in accordance with the invention.

One example of hydrogel subunit formation follows. A 2.0 percent by weight (wt %) sodium alginate prepolymer solution, containing biotin-conjugated alginate and streptavidin-conjugated DNA primers, is applied to a microfabricated stencil that is reversibly sealed to a flat substrate. Alginate gels modified with DNA primers are produced (immersion in 100 mM CaCl_2 , 30-90 min, room temperature) and stabilized (in 0.9 wt % NaCl, 5.0 M sodium triphosphate, 30 minutes, room temperature). Finally, concatemers are amplified from the DNA primers, as described elsewhere herein. Divalent cation (*e.g.*, Ca^{2+}) induced gelation of alginate is reversed by ethylenediaminetetraacetic acid (EDTA) or citric acid treatment, and such controlled dissolution is used to control lumen formation.

The hydrogel subunits described herein may be a range of subunit sizes and shapes including, without limitation, hemi-sphere, cube, cuboidal, tetrahedron, cylinder, cone, octahedron, prism, sphere, pyramid, dodecahedron, tubular, irregular or abstract. Other hydrogel subunit sizes and shapes may be used in accordance with the invention.

The hydrogel subunits may vary in diameter, or edge length, from about 1 micrometer (μm) to about 1 millimeter (mm). In some embodiments, the subunits are about 1 μm to about 10 μm , about 1 μm to about 100 μm , or about 1 μm to about 500 μm in diameter (or edge length). In some embodiments, the hydrogel subunits are about 10 μm , about 20 μm , about 30 μm , about 40 μm , about 50 μm , about 60 μm , about 70 μm , about 80 μm , about 90 μm , about 100 μm , about 150 μm , about 200 μm , about 250 μm , about 300 μm , about 350 μm , about 400 μm , about 450 μm , about 500 μm , about 550 μm , about 600 μm , about 650 μm , about 700 μm , about 750 μm , about 800 μm , about 850 μm , about 900 μm , about 950 μm or about 1000 μm in diameter (or edge length). In some embodiments, the hydrogel subunits are submicron scale, while in other embodiments, the subunits are as large as 1 centimeter (cm). The “edge length” of a hydrogel subunit refers to the length of one edge of the hydrogel subunit such as, for example, a cube (having a total of 12 edges).

Nucleic acid primers and concatemers

The nucleic acid hydrogel subunits of the invention comprise, on at least one of their surfaces, a nucleic acid. The nucleic acids attached to the hydrogel subunits are referred to herein as being surface accessible, to denote their presence at the surface of the subunit and their ability to interact with other nucleic acids, including those on other hydrogel subunits.

The term “nucleic acid,” as used herein, refers to a polymeric form of deoxyribonucleotides or ribonucleotides, or analogs thereof, of any length. A nucleic acid may comprise naturally occurring nucleotides and/or non-naturally occurring nucleotides such as modified nucleotides, for example, methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before, or after, the nucleic acid is conjugated to the hydrogel subunit. Accordingly, nucleic acids include, without limitation, single-stranded or partially single-stranded DNA, double-stranded or partially double-stranded DNA, single-stranded or partially single-stranded RNA, double-stranded or partially double-stranded RNA, cDNA, aptamers, peptide nucleic acids (“PNA”), 2'-5' DNA (a synthetic material with a shortened backbone that has a base-spacing that matches the A conformation of DNA – 2'-5' DNA will not normally hybridize with DNA in the B form, but it will hybridize readily with RNA), and locked nucleic acids (“LNA”). Nucleic acid analogues include known analogues of natural nucleotides that have similar or improved binding, hybridization or base-pairing properties. “Analogous” forms of purines and pyrimidines are well known in the art and include, without limitation, aziridinylcytosine, 4-acetylcytosine, 5-fluorouracil, 5-

bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, inosine, N⁶-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N⁶-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5-methoxyuracil, 2-methylthio-N⁶-isopentenyladenine, uracil-5-oxyacetic acid methylester, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid and 2,6-diaminopurine. DNA backbone analogues include, without limitation, phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, peptide nucleic acids (PNAs), methylphosphonate linkages, and alternating methylphosphonate and phosphodiester linkages.

A nucleic acid of invention may be further modified, such as by conjugation with a labeling component. Other nucleic acid modifications may be used.

A nucleic acid of the invention may be linear or circular.

A nucleic acid of the invention may be recombinant or isolated. The term “recombinant nucleic acid,” as used herein, refers to a nucleic acid of genomic origin, cDNA origin, semi-synthetic origin or synthetic origin, which either does not occur in nature or is linked to another nucleic acid in a non-natural arrangement. The term “isolated nucleic acid,” as used herein, refers to a nucleic acid of natural or synthetic origin, or some combination thereof, which (1) is separated, at least in part, from the environment and/or components with which it exists, and/or (2) is operably linked to a nucleic acid to which it is not linked in nature. The nucleic acids of the invention may be extracted from cells or synthetically prepared according to any means known to those skilled in the art. For example, the nucleic acids may be chemically synthesized, transcribed or reverse transcribed from cDNA, transcribed or reverse transcribed from mRNA, or transcribed or reverse transcribed from other sources.

As used herein, two nucleic acids, or two nucleic acid regions or sequences, are “complementary” to one another if they base-pair with each other to form a double-stranded nucleic acid molecule. Regions of complementarity between nucleic acids may range in length from about 5 to about 100 nucleotides. For example, regions of complementarity between nucleic acids may be about 5, about 10, about 15, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90 or about 100 nucleotides in length. In some embodiments, regions of complementarity between nucleic acids may be more than 100 nucleotides in length.

In some embodiments, a nucleic acid may be about 15 nucleotides to about 1000 nucleotides in length. For example, the nucleic acid may be about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900 or about 1000 nucleotides in length.

5 A nucleic acid “primer,” as used herein, refers to a short nucleic acid (*e.g.*, less than 100 nucleotides in length). In some embodiments, a nucleic acid primer is about 90, about 80, about 70, about 60, about 50, about 40, about 30, about 20 or about 15 nucleotides in length.

Methods of nucleic acid conjugation to hydrogel subunits

Nucleic acids may be conjugated (or linked, as the terms are used interchangeably
10 herein) to the hydrogel subunits using any technique known in the art. In some embodiments, *N*-hydroxysuccinimide (NHS) chemistry is used to conjugate the nucleic acids to the hydrogel subunits (*see* FIG. 2). For example, amine-bearing nucleic acids may be conjugated to PEG-NHS monomers using a standard protocol, as described in further detail in the Examples section. In some embodiments, other chemical crosslinking agents are used. Examples of
15 chemical crosslinking agents for use in accordance with the invention include, without limitation, thiol-thiol, amine-amine, amine-thiol and amine-acrylate, any of which may be used to conjugate DNA with, for example, PEG or other polypeptide hydrogel polymers. In some embodiments, biotin-modified DNA may be linked to a hydrogel subunit modified with tethered streptavidin.

Methods of nucleic acid concatemerization

In some embodiments, a shaped hydrogel subunit is conjugated to a nucleic acid, and then the nucleic acid is amplified to produce a concatemer. A “concatemer,” as used herein, refers to a nucleic acid molecule comprising at least two identical copies of the same
25 nucleotide sequence covalently linked to each other in tandem. In some embodiments, a nucleic acid (*e.g.*, DNA) concatemer is produced through rolling circle amplification (RCA) (Schopf *et al.*, *Anal Biochem* 397: 115-117 (2011), incorporated herein by reference) or a variation thereof (Fire and Xu, *Proc. Natl. Acad. Sci. USA*, 92:4641-4645 (1995); Nilsson *et al.*, *TRENDS in Biotech.*, 24(2):83-88 (2006); Dahl *et al.*, *PNAS*, 101(13):4548-4553 (2004),
30 each of which is incorporated herein by reference). Rolling circle amplification involves two simultaneous processes. DNA polymerase synthesizes sequences complementary to a circular template, and as this replication proceeds, the parental duplex is unwound to allow the polymerase to advance. Continued DNA synthesis produces multiple single-stranded linear

copies of the original DNA in a continuous head-to-tail series, forming a concatemer. Herein, a DNA concatemer may also be referred to as a “giant DNA” or a “DNA nanoball,” as concatemers tend to coil into a large, ball-like shape.

Concatemers of the invention may comprise about 2 to about 1000 copies of the same nucleotides sequence. For example, concatemers may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 copies of the same nucleotides sequence. In some embodiments, concatemers may comprise more than 1000 copies of the same nucleotide sequence.

Exemplary synthesis of a hydrogel subunit having surface accessible nucleic acids

The following illustrates synthesis of a hydrogel subunit having nucleic acid concatemers in accordance with the invention and is intended for exemplary purposes only and not to limit the scope of the invention.

A stencil-based photolithography is used to pattern three distinct concatemer species on specific surfaces of polyethylene glycol (PEG) and gelatin gels, as illustrated in FIG. 1. In step (1), a stencil with 100 μm size cubic holes is produced using standard techniques. In step (2), a hydrogel precursor solution (PEG-diacrylate (DA) or gelatin methacrylate), including photoinitiator and PEG-acrylate conjugated DNA primer 1, is applied to the stencil, which is sandwiched between two hydrophobic glass slides (pre-treated with octadecyltrichlorosilane). The shaped hydrogels form upon UV-crosslinking, and subsequently the glass slides are detached. In step (3), a prepolymer hydrogel solution containing gelatin methacrylate, or PEG-DA and PEG-acrylate, conjugated to DNA primer 2 is applied on the top surface, which is then covered with one glass slide. A photomask is aligned with the shaped hydrogel subunit in stencil over the glass slide for secondary ultra violet (UV)-crosslinking of DNA primer 2 to the top surface of the hydrogel subunits. In the same way, the bottom surface is modified with DNA primer 3. In step (4), the stencil is peeled off, and hydrogel subunits with primer 2 on top, primer 3 on bottom, and primer 1 on the sides are collected. In step (5), the primers are converted into concatemers (DNA concatemers) by using rolling circle amplification (RCA) in aqueous solution with circular DNA templates complementary to primers 1, 2, and 3, respectively. This procedure produces three distinct DNA concatemer species on the top, bottom, and the sides of each hydrogel subunit.

To verify the correct surface DNA primer modification, the gel is treated with three DNA probes, respectively complementary to primers 1, 2 and 3. Each probe is labeled with a

distinct fluorophore. Three-color multiplexed imaging is used to quantify each primer density. Similarly, multiplexed imaging with complementary fluorescent DNA probes is used to quantify concatemer density. Additionally, concatemers are selectively released from the gel through endonuclease treatment and quantified by gel electrophoresis.

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Directed assembly of nucleic acid hydrogel subunits

There are several existing self-assembly approaches to building three-dimensional hydrogel structures; however, such approaches lack control of the resulting hydrogel structure and orientation (McGuigan *et al.*, *Proc. Natl. Acad. Sci. USA*, 103:11461-11466 (2006); Liu Tsang *et al.*, *FASEB J.*, 21:790-801 (2007); Yeh *et al.*, *Biomaterials*, 27:5391-5398 (2006)) and others involve cytotoxic materials and processes (Bowden *et al.*, *Science*, 276:233-235 (1997); Breen *et al.*, *Science*, 284:948-951 (1999); Choi *et al.*, *Angew. Chem. Int. Ed. Engl.*, 38:3078-3081 (1999)).

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Provided herein are biocompatible shaped hydrogels (*e.g.*, resembling a natural extracellular matrix) that can assemble into intricate patterns through specific, addressable nucleic acid interactions. The shaped hydrogel subunits of the invention may be linked to form a single chain or a sheet of multiple chains linked together (*e.g.*, similar to a net or net-like structure). The term “linked” refers to the joining of at least two shaped hydrogel subunits through their nucleic acid-modified surfaces. For example, subunit A is considered to be linked to subunit B if a nucleic acid conjugated to subunit A hybridizes, in a sequence-specific manner, to a nucleic acid conjugated to subunit B.

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In some embodiments, the shaped subunits are directed to form simple chains, sheets, or more complex branched structures (*e.g.*, T-junctions). To achieve such patterned structures, each shaped hydrogel subunit may be designed to have particular nucleic acids conjugated to specific surfaces, or specific surface regions, such that nucleic acid hybridization of complementary sequences between neighboring nucleic acids brings together the hydrogel subunits in a predictable manner. In some embodiments, the shaped hydrogel subunits are linked such that the individual subunits form a larger, more intricate shape, for example, mimicking a particular tissue architecture such as, for example, branched vasculature.

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In some embodiments, nucleic acid hydrogel subunits (*e.g.*, within a mixed population of complementary nucleic acid hydrogel subunits) self-assemble under conditions that allow hybridization of sequences having at least 65% complementarity. For example, in some embodiments, the nucleic acid hydrogel subunits self-assemble with a sequence specificity of

at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98%. In some embodiments, non-specific binding between nucleic acid hydrogel subunits is less than 20%. For example, in some embodiments, non-specific binding between nucleic acid hydrogel subunits is less than 18%, less than 17%, less than 16%, less than 15%,
5 less than 14%, less than 13%, less than 12%, less than 11%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5%.

Nucleic acid hydrogel subunits of the invention can be assembled in an aqueous assembly system or an interfacial assembly system. An aqueous assembly system refers to a system in which hydrogels are suspended in solution and when rotated can freely move in all
10 directions. For example, an aqueous assembly system may be comprised of a microtube filled with phosphate buffered saline (PBS) supplemented with 0.5 M NaCl, 0.5mM EDTA and 0.05% TWEEN[®] 20. An interfacial assembly system refers to a system in which hydrogels are floated on an interface formed between aqueous buffer and hydrophobic liquid (*e.g.*, aqueous PBS and liquid fluor-inert FC-40) and under mild shaking conditions can only move along the
15 liquid/liquid interface.

Applications

The nucleic acid hydrogel subunits of the invention may be used to generate structures (*e.g.*, microstructures) of defined and predetermined shape and complexity. Such structures
20 may be used as tissue grafts *in vivo* or *in vitro*. For example, in some embodiments, the hydrogel subunits may be seeded with (*e.g.*, encapsulated with) living cells such as stem cells, progenitor cells and/or differentiated cells. The stem cells may be embryonic stem cells or adult stem cells. Examples of stem cells for use in accordance with the invention include, without limitation, endothelial stem cells, mesenchymal stem cells and hematopoietic stem
25 cells. Examples of progenitor cells for use in accordance with the invention include, without limitation, angioblasts and endothelial progenitor cells. Examples of differentiated cells for use in accordance with the invention include, without limitation, red blood cells, white blood cells, platelets, stromal cells, fat cells, bone cells, skin cells and muscle cells. In some
30 embodiments, the cells are fibroblasts. In some embodiments, the cells are induced pluripotent cells.

In some embodiments, the hydrogel subunits encapsulate biomolecules such as, for example, proteins, lipids, polysaccharides, metabolites, nucleic acids or any combination of two or more of the foregoing.

In some embodiments, nucleic acid hydrogel structures of the invention may be used as, or as part of, a tissue graft. Examples of tissues that may be grafted using the nucleic acid hydrogel structures of the invention include, without limitation, skin, bone, nerves, tendons, neurons, blood vessels, fat and cornea. A hydrogel subunit-based skin graft may be used, for example, to treat skin loss due to a wound, burn, infection or surgery. A hydrogel subunit-based vascular graft may be used, for example, as prosthetic blood vessels in surgical procedures.

Kits

The invention provides kits comprising the shaped hydrogel subunits of the invention with attached non-amplified primers or attached concatemers.

The invention also provides kits for the synthesis of the shaped hydrogel subunits, including subunits with non-amplified primers or subunits with the amplified concatemers.

Examples

Example 1: Self-assembly of hydrogel cubes with uniform surface DNA modifications

In this example, a strategy was developed to use complementary DNA molecules as “glue” to direct the self-assembly of hydrogel cubes (also referred to herein as subunits) with edge lengths of 250 μm . An initial attempt to assemble such hydrogel cubes carrying short complementary DNA strands (36 nucleotide (nt) poly-T linker followed by 20 nt complementary sequences) failed to induce hydrogel cube assembly (FIG. 3C). This failure may be attributed to the rugged surface morphology of the hydrogel, as revealed by scanning electron microscopy (SEM, FIG. 2D), and the relative small size and weak binding interactions between short complementary DNA strands. To accommodate for this rugged hydrogel surface, a strategy was developed to decorate the hydrogel surface with single-stranded DNA concatemers (FIG. 2A). Specifically, in step (1), amine-bearing short DNA strands (brown, 56 nt) were conjugated to PEG-NHS monomers (MW 3500 Da) using a standard protocol (Schlingman *et al.*, *B: Biointerfaces* 83: 91-95 (2011), incorporated herein by reference). In step (2), the DNA-PEG-acrylate was mixed with photocrosslinkable poly(ethylene glycol)-diacrylate (PEG-DA, 4000 MW) and 0.5 wt % photoinitiator, and exposed to UV under a photomask with 250 μm $\times$ 250 μm square holes. The height of the cubes was controlled by using microscope cover glass slides (No. 2; 250 μm in thickness) as spacers. Upon UV exposure, 250 μm $\times$ 250 μm $\times$ 250 μm hydrogel cubes, uniformly modified with short DNA

primers, were produced. In step (3), the DNA primers hybridized with complementary, circular DNA templates (produced by circularization of short linear DNA using CIRCLIGASE™). Through rolling circle amplification (RCA), the DNA primers were amplified to produce long strands with repeated sequences complementary to the circular template, referred to herein as concatemers, DNA concatemers or “giant” DNAs.

The successful production of DNA concatemers through a RCA reaction in free solution and on glass slides was first verified. Agarose gel electrophoresis showed the synthesis of a high molecular weight product, fluorescent microscopy showed the formation of large membrane-like DNA concatemers, and scanning electron microscopy (SEM) showed the fiber-like DNA concatemers on glass slides. Hydrogel cubes carrying DNA concatemers, as described above, were then fabricated and characterized using fluorescent DNA staining and SEM. In comparison with hydrogels carrying short 56-nt DNA primers (FIG. 2: (B) phase contrast imaging; (C) fluorescent imaging, DNA was stained by SYBR® Gold; and (D) SEM imaging), three hours of RCA amplification at 37 °C resulted in significant DNA staining by SYBR® Gold (FIG. 2: (E) phase contrast imaging; and (F) fluorescent imaging), and the hydrogel surface was covered by fiber-like structures (SEM image, FIG. 2G). These experiments suggested that the surfaces of the hydrogel cubes were decorated with DNA concatemers produced by RCA, as designed.

DNA concatemer-directed assembly of hydrogel cubes was next demonstrated. Using the procedure described above, 250 µm × 250 µm × 250 µm hydrogel cubes carrying DNA concatemers containing tandem repeated complementary 48-nucleotide sequences (FIG. 3A) were fabricated. The DNA concatemers were uniformly amplified on the surface of hydrogel cubes, with one cube having on its surface an “a” sequence and the other cube having on its surface an “a*” complementary sequence (such sequences are shown in FIG. 3A as SEQ ID NO:1 (top) and SEQ ID NO:42 (bottom)). Hydrogel cubes carrying complementary DNA “a” or “a*” were labeled with red or blue fluorescent microbeads, respectively, and stained with SYBR® Gold. Hybridization between the complementary DNA sequences resulted in assembly of hydrogel cubes. Self-assembly was performed by mixing these hydrogel cubes in a 0.5 ml microtube filled with assembly buffer under mild rotation, using a tube rotator with a fixed speed of 18 rpm (FIG. 3B, see details under *Method* heading below).

To visualize the assembled hydrogel structures, the two species of hydrogel cubes carrying complementary DNA sequences “a” and “a*” were labeled with red (FLUORESBRITE® microspheres with excitation at 512 nm and emission at 554 nm) or blue

(FLUORESBRITE[®] microspheres with excitation 360 nm and emission 407 nm) fluorescent microbeads, respectively. After the assembly reaction in the tube, the hydrogel cubes were transferred to a 1.6 cm diameter Petri dish for imaging (FIG. 3B). Large aggregates were observed for hydrogel cubes carrying complementary DNA concatemers (FIG. 3D, left, phase contrast imaging; right, fluorescent imaging). By contrast, hydrogel cubes carrying only 56-nt short DNA primers, without RCA amplification, failed to produce aggregates under the same assembly conditions (FIG. 3C, left, phase contrast imaging; right, fluorescent imaging). Including DNA-free yellow hydrogel cubes (*i.e.*, cubes that contain yellow microbeads) in the reaction system did not change the assembly outcome for either the hydrogel cubes carrying 56-nt short DNA primers (FIG. 3E, left) or hydrogel cubes carrying DNA concatemers (FIG. 3E, right). Moreover, yellow hydrogel cubes were not observed in the assembled structure, confirming that the assembly was directed by DNA concatemers on the hydrogel cube surface. This DNA concatemer-dependent nature of the assembly was further verified in a DNA degradation experiment: assembled hydrogel cubes carrying complementary DNA concatemers became dispersed after a 1-hour treatment with Baseline-ZERO[™] DNase, which was expected to degrade the DNA concatemers on the hydrogel cube surface (FIG. 3F: left- before DNase treatment; right- after DNase treatment).

It was shown that relatively large cuboid-shaped particles (250 mm edge width) needed to assemble in a strong agitation (to disrupt non-specific interactions) solution-based system, and thus much stronger “glue-like” interactions were likely required to enable the assembly. Over 70% specific binding was observed by analyzing the binding event between two hydrogel cubes uniformly carrying the same or complementary DNA concatemers. The DNA concatemer-dependent hydrogel cube binding was also quantified under different temperatures (4 °C, 25 °C and 37 °C, respectively). The specific assembly yield increased as the temperature decreased (data not shown). However, non-specific binding also increased significantly at 4 °C (data not shown).

It was next demonstrated that the interaction between complementary DNA concatemers was capable of directing assembly of hydrogel cubes with a wide range of edge lengths. Cubes carrying complementary DNA “a” or “a*” were labeled with red or blue color microbeads respectively. The assembly reaction was performed as described above. Aggregates were observed from red and blue hydrogel cubes with edge lengths of 30 μm (FIG. 3G(i)), 200 μm (FIG. 3G(ii)), 500 μm (FIG. 3G(iii)) and 1000 μm (FIG. 3G(iv)). DNA concatemers were uniformly amplified on the hydrogel surface. These experiments indicated

that the hybridization between complementary DNA concatemers was sufficiently strong to induce the assembly of hydrogel cubes across scales.

As complementary DNA molecules hybridize with each other in a sequence-dependent fashion, it is possible to generate a large number of specific interactions using DNA

concatemers with orthogonal sequences. To test whether such DNA sequence dependent specificity can be applied in the hydrogel cube self-assembly system provided herein, fifty 24-nt DNA sequences were designed to produce 25 pairs of orthogonal specific interactions (see *Methods* below). To visually differentiate distinct cube species under stereomicroscope, colored microbeads were trapped in the core and periphery parts of the cubic hydrogel, respectively (FIG. 3J, top left corner schematic) and pairwise combinations of five colors (red, blue, yellow, black, and violet) generated 25 distinct signatures. Following fabrication, DNA concatemers containing tandem repeated sequence were amplified using RCA reaction on the hydrogel surface, as described above. The 25 structures were self-assembled in an aqueous assembly system. FIG. 3H shows a schematic for multiplexed self-assembly of 25 orthogonal pairs of dimers in five independent experiments. To avoid forming large aggregates that can trap microgels inside and hinder the quantification of specific assembly yields, only one copy for each of the 50 hydrogel species was included in one of five independent experiments (FIG. 3H). The assembly process was conducted using agitation of repeated mild rotation at a fixed speed of 18 rpm and strong hand shaking every 30 minutes to disrupt non-specific binding.

The final assembled nucleic acid hydrogel structures from the five independent experiments were pooled together into a single Petri dish for imaging and quantification. Each of the 25 expected specific hydrogel dimers were all identified (FIGs. 3I, 3J; the assembled structures are highlighted by white triangles; scale bar: 1 mm). FIG. 3J shows a schematic (top left corner) depicting the double-component structure of the hydrogel cube used in the multiplexed self-assembly of 25 hydrogel cube dimers. The core “pad” cube was $100\ \mu\text{m} \times 100\ \mu\text{m} \times 100\ \mu\text{m}$, and the periphery “body” cube was $300\ \mu\text{m} \times 300\ \mu\text{m} \times 300\ \mu\text{m}$. The core and periphery hydrogel cubes were labeled with distinct, colored microbeads, and pairwise combinations of five colored cubes (red, blue, yellow, black and violet) generated 25 distinct signatures.

Images of the 25 corresponding hydrogel cube dimers are shown in FIG. 3J. Hydrogel cube dimers were identified under microscopy and quantified as specific ($66.4 \pm 4.5\%$) and non-specific ($18.4 \pm 7.2\%$) binding events, $n = 5$, $*P < 0.05$ (FIG. 3K). With the exception of 15.2% of non-assembled single hydrogel cubes, and hydrogel cubes missed in the operation process putatively due to the nonspecific binding to microtube surface, about 66.4% specific

binding and 18.4% nonspecific binding was obtained (FIG. 3K). These experiments demonstrated that the interaction between DNA concatemers is sequence-specific and that highly multiplexed assembly can be achieved using the system provided herein. This system includes more specific interactions than reported mesoscale self-assembly systems.

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Example 2: Self-assembly of hydrogel cubes with face-specific DNA modifications.

To assemble structures with controlled architecture, rather than aggregates, hydrogel cubes were fabricated with face-specific DNA concatemer modification. The procedure is illustrated in FIG. 4. In steps 1-4, a procedure is described for making a two-component cube composite structure where a larger “body-cube” displays smaller DNA-modified “pad-cubes” on its designated sides; in steps 5 and 6, the agitation system for their assembly is described.

Step 1. The $150\text{ }\mu\text{m} \times 150\text{ }\mu\text{m} \times 150\text{ }\mu\text{m}$ hydrogel pad cubes were made from a precursor solution that contained 20 wt % PEGDA (4 KDa) and PEG (3.5 KDa) acrylate single-stranded DNA primers using photolithography. A photomask with $150\text{ }\mu\text{m} \times 150\text{ }\mu\text{m}$ square holes was used to control the cross-section shape of the pad cube. Microscope cover glass slides (No. 1; $150\text{ }\mu\text{m}$ in thickness) were used as spacers to control the height of the pad cube.

Step 2. The un-polymerized reagent was washed away, and DNA concatemers were produced through a RCA reaction, as described above. FIG. 4A shows arrays of $150\text{ }\mu\text{m}$ pad cubes (dark gray) with uniform DNA concatemer modification.

Step 3. To make the larger body cube, a second solution containing only 20 wt % polyethylene glycol diacrylate (PEGDA) (4 KDa) was added, and the cube was covered with a second photomask with $250\text{ }\mu\text{m} \times 250\text{ }\mu\text{m}$ square holes. This photomask was aligned carefully with the pad cubes made in step 2 such that this photomask covered half of the cross-section area of each pad cubes (to protect them from subsequent UV exposure). Microscope cover glass slides (No. 2; $250\text{ }\mu\text{m}$ in thickness) were used as spacers to control the height of the body cubes.

Step 4. Subsequent ultraviolet UV treatment resulted in the polymerization of the second $250\text{ }\mu\text{m} \times 250\text{ }\mu\text{m} \times 250\text{ }\mu\text{m}$ body cube. Un-polymerized reagent was washed away. At the end of step 4, an array of hydrogel cubes was produced: the $250\text{ }\mu\text{m}$ body cube covered half of the $150\text{ }\mu\text{m}$ pad cubes—only the $150\text{ }\mu\text{m}$ pad cubes were modified with DNA concatemers. As a consequence, the hydrogel cube composite had only DNA concatemer

modification on designated faces that display the pads. This composite structure may be referred to herein as a hydrogel cube with surface-specific DNA modifications.

Step 5. The hydrogel cubes were collected into a 0.5 ml microtube filled with the assembly buffer.

Step 6. Assembly was performed by rotating the tube.

Step 7. The solution was transferred to a Petri dish and imaged under a microscope.

Using the above strategy, the multiplexed assembly of three hydrogel cube dimer species was demonstrated (FIGs. 4B and 4C). In this experiment, six hydrogel cube species were manufactured (FIG. 4B, left). The first species was a red hydrogel cube (*i.e.* the body cube contained red microbeads) that displayed DNA concatemers with tandem repeating sequence “a.” This cube is referred to as “red cube a.” The other five species are referred to as “red cube a*” (where sequence a* is complementary to sequence a), “blue cube b,” “blue cube b*,” “yellow cube c” and “yellow cube c*.” FIG. 4B depicts hydrogel cubes displaying face-specific DNA concatemers with tandem repeats and assembled cubes based on “a/a*,” “b/b*,” or “c/c*” complementarity. The smaller pad cube carrying the DNA concatemer is depicted in dark gray. Letters “n” (*e.g.*, “a,” “b” and “c”) and “n*” (*e.g.*, “a*,” “b*” and “c*”) denote concatemers with complementary DNA sequences. Multiple copies of each of the six cube species were made separately and then mixed in the same tube for assembly (see *Methods* for details). After 6 hours of rotation, the solution was imaged (FIG. 4B, right). Three populations of structures were observed and quantified (FIG. 4C, *P < 0.05): 46.1% of the structures were the expected, specific hydrogel dimers that formed between two same-color hydrogel cubes that carried complementary sequences; 10.4% of the structures were the hydrogel dimers that formed between two different-color hydrogel cubes that carried non-complementary sequences; and 43.5% of the structures were the un-assembled, single hydrogel cubes. The specificity of dimer formation was quantified as 82% by dividing the number of specific dimers over the total number of dimers.

Using hydrogel cubes that display DNA concatemers on multiple designated faces, linear chain structures and net-like structures were next constructed. To make the chain structures, two hydrogel cube species were made: a red cube that displays DNA concatemer “a” on two opposite faces, and a blue cube that displays DNA concatemer “a*” on two opposite faces (FIG. 4D, left, schematic). The assembly of these two species produced chain structures that contained alternating red and blue hydrogel cubes, as expected (FIG. 4D, right, microscopy image). The longest chain observed contained seven cubes (FIG. 4D, top-right

corner). The following hydrogel cubes were then made: (i) a red cube species that displays DNA concatemer “a” on two opposite faces and DNA concatemer “b” on another two opposite faces, and (ii) a blue cube species that displays pairs of “a*” and “b*” on opposite faces (FIG. 4E, left). The assembly of these two species resulted in the formation of net-like structures with alternating red and blue hydrogel cubes that were connected through DNA modified sides (FIG. 4E, right). The linear and net-like structures were each assembled in three independent experiments, where each experiment used 40 red and 40 blue complementary cubes. In this experimental system, 39% of the gel cubes remained as unassembled, whereas 58% were assembled into a linear structure (due to their small size, 3% of cubes were lost during the assembly and quantification process). Among the 58% assembled cubes, 42% were only connected to a different color cube and, thus, referred to specifically assembled cubes; the remaining 16% were connected non-specifically to at least one same color cube and were referred to as non-specifically assembled cubes. For the net-like structures, the specifically assembled cubes, non-specifically assembled cubes and unassembled cube monomers were, respectively, 56%, 30% and 14%.

Example 3: Interfacial self-assembly of hydrogel cuboids into complex structures.

The self-assembly of hydrogel cuboids were next directed to assemble into prescribed, finite structures. To avoid the rotation of the hydrogel cuboids in the vertical direction during assembly, hydrogel cuboids were fabricated and floated on a liquid/liquid interface between aqueous phosphate buffered saline (PBS) and fluor-inert FC-40 liquid, and horizontal shaking was applied to facilitated assembly (FIG. 5A). The assembled structures were directly imaged in this interfacial system. Using a similar fabrication strategy as in FIG. 4A, a two-component hydrogel composite was made: the body cuboids were 1 mm (length) $\times$ 1 mm (width) $\times$ 0.3 mm (height), and the pads were DNA-modified 250 μ m $\times$ 250 μ m $\times$ 250 μ m cubes. Note that the length ratio between the body cuboid and the pad cubes was increased to 4:1 (compare to the 2.5:1 in FIG. 4). Using the interfacial system and the cuboids that carry relatively smaller pad cubes, it was possible to assemble dimers (FIG. 5B), linear chains with finite length (FIG. 5C), a T-junction (FIG. 5D) and a square structure (FIG. 5E). The linear chain, the T-junction and the square were all composed of four distinct cuboid species. By simply changing the pad cube (and hence surface DNA concatemer) modification pattern, the assembled hydrogel structure was changed from a chain to a T-junction to a square. The self-assembly of two T-junctions in the same reaction system was also demonstrated.

For the dimer experiments (FIG. 5B), two copies of each component were included; for the linear (FIG. 5C), T-junction (FIG. 5D) and square (FIG. 5E) structures, only one copy of each component was included. The assembly of each system was tested three times or more, and, in total, more than 20 experiments were performed for these structures. Out of these more than 20 experiments, the structure always formed as intended. Such systems (involving only one copy of each component) are simpler than systems that involve multiple copies of the same components. Additional experiments have been performed to form two copies of the T-junction. These experiments (data not shown) started with 14 hydrogel cubes: two copies of the center hydrogel cube and four copies of each of the three side hydrogel cubes. The experiments were conducted three times, and two copies of T-junctions were successfully assembled in one of the three experiments.

Example 4: Patterned DNA modification with specific design

FIGs. 6A and 6B illustrate another example of assembling the shaped nucleic acid hydrogels. Two hydrogel cubes were first aligned with respect to the center of each cube. A small area on top surface of a hydrogel cube was modified with DNA. This was achieved by aligning a secondary mask having a specific design of 150 μm in diameter with the center of the hydrogel cube having a diameter of about 250 μm , followed by concatemerization of the DNA. A dimeric structure was assembled from the hydrogel cubes with patterned DNA on the top surface. The alignment of assembled hydrogel cubes was controlled through patterning DNA on different areas of the hydrogel surface (*e.g.*, at the corner or at the center of the cube) (FIGs. 6D and 6E), and the orientation of hydrogel in the final assembled structure was controlled through patterning the DNA on the hydrogel surface with a cross design (FIGs. 6B and 6C).

Example 5: Cell encapsulated hydrogel fabrication

NIH 3T3 cells were encapsulated inside PEG hydrogel cubes, and DNA amplification was performed in a cell medium-based reaction solution. As showed in FIG. 7, amplified DNA was detected on hydrogel cubes with cells encapsulated therein. Fluorescent imaging of SYBR[®] Green stained concatemers in cell-encapsulated and cell-free hydrogel cubes (control) were compared to quantify the effects of the cell cultures on RCA efficiency. The efficiency was subsequently optimized by varying RCA reaction conditions as well as the density and size of DNA concatemers. Similarly, imaging of the change of fluorescence after various

incubation time (~1-24 hours) after RCA was used to study the stability/degradation of DNA concatemers in cell-encapsulated hydrogels. The hydrogels were imaged after saline (*e.g.*, PBS) washing to remove potential DNA degradation products.

FIG. 10A shows a fabrication schematic of cell-encapsulated hydrogel cubes in accordance with the invention. In step (1), a DNA pad-cube was fabricated as described in FIG. 2A. In step (2), a DNA concatemer was amplified from the tethered ssDNA primer using rolling circle amplification (RCA). In step (3), a cell-encapsulated body-cube was fabricated from a polymer precursor solution containing living cells. The final microstructure was controlled by aligning the second photomask with the first small pad-cube as described in FIG. 4A and in the Examples. FIGs. 10B-10D show phase contrast (FIGs. 10B, 10D) and fluorescent (FIGs. 10C, 10E) microscopy images of a cell viability assay. The cell viability of NIH 3T3 cells encapsulated in 20% PEG hydrogel cubes with edge lengths of 250 μm was measured by calcein AM and ethidium homodimer-1 (LIVE/DEAD Reagent from Invitrogen). FIGs. 10F and 10G show phase-contrast (FIG. 10F) and fluorescent (FIG. 10G) microscopy images of cell-encapsulated hydrogel dimer assembled from human umbilical vein endothelial cell (HUVEC) and smooth muscle cell (SMC) encapsulated hydrogels.

Summary

The invention provides DNA-directed self-assembly of shape-controlled hydrogel subunits to build complex structures in a programmable fashion. Acting like sequence specific glue and tethered onto a hydrogel surface, single-stranded DNA concatemers exhibit a significant capability for binding objects across scales, with sizes ranging from 30 micrometers to a millimeter (or more). Additionally, DNA concatemers offer significant diversity over current mesoscale self-assembly systems: 50 DNA sequences were designed to generate 25 orthogonal pairs of specific interactions. The designable DNA “glues” thus provide improved methods for programming (*e.g.*, mesoscale) self-assembly.

For self-assembling complex hydrogel structures, the hydrogel subunit fabrication is important. Provided herein is a precisely controlled fabrication technique by which specific DNA concatemers are decorated on a prescribed face of a hydrogel subunit (*e.g.*, cube). By changing the position of each DNA concatemer, various structures including dimers, linear chains and open networks were assembled. It was further demonstrated (*e.g.*, in an interfacial system) that hydrogel cuboids can be fabricated with four different DNA concatemers on four designated faces, and by simply changing the surface DNA decoration pattern, discrete

hydrogel structures were assembled, including dimers, T-junctions, linear chains with fixed length and squares. Thus, the invention provides for successful introduction of programmability into self-assembling (e.g., mesoscale) structures.

Another aspect of the invention is biocompatibility. Preliminary experiments suggest that mammalian cells encapsulated inside hydrogel subunits maintained high viability through the fabrication and assembly process (FIGs. 10A-G). The DNA-directed hydrogel self-assembly system provided herein has promising potential in bottom-up tissue engineering applications. Cell-laden hydrogel units may be engineered to self-assemble into structures that mimic the microarchitecture of native tissues.

By coupling novel *in situ* DNA amplification methods and microfabrication techniques, the diversity and specificity of biomolecular interactions were successfully introduced to mesoscale assembly. DNA-directed self-assembly of shape-controlled hydrogel subunits proved to be highly programmable and controllable and will open new doors to address the challenge of building complex self-assembled 3D structures for diverse applications.

Methods

Materials. The prepolymer solution of PEG-DA with average molecular weight 4000 Da was prepared by diluting PEG (Monomer- Polymer&Dajac Labs) in DPBS (Gibco) to a final concentration of 20 wt % with 1 wt % photo-initiator, 1-(4-(2-Hydroxyethoxy)- phenyl)-2-hydroxy-2-methyl-1-propane-1-one (IRGACURE 2959 Ciba) for hydrogel modules fabrication. Circular DNA template was produced by ligating 5' and 3' terminal of oligonucleotides (DNA) (Invitrogen) with 5'-terminal phosphate modification using a CIRCLIGASE[™] single-stranded DNA (ssDNA) ligase (EPICENTRE Biotechnology) under a standard reaction condition according to manufacturer's instructions. DNA sequence for DNA glue synthesis was designed using software of nucleic acid package (NUPACK) with minimized mis-hybridization between each two non-complementary sequences and ordered from Invitrogen. Baseline-ZERO[™] DNase was obtained from Epicentre Biotechnology and used with final concentration of 1 U/ml.

Sequence design. Twenty-five orthogonal sequence pairs were designated using a modified version of the Domain Design (DD) software described by Zhang *et al.* (Zhang *et al.*, *Lecture Notes in Computer Science* 6518:162-175 (2011), incorporated herein by reference). Twenty-five domains of 24 bases each were first designed, and then these domains were concatenated together into 25 domains of 48 bases each. Sequences were designed using a

three-letter alphabet to reduce spurious hybridization. In order to reduce long regions of repeated bases (*e.g.*, poly-A, poly-G, *etc.*), sequences, which had a higher Shannon entropy, were rewarded. The NUPACK thermodynamic analysis package was used to calculate that unintended interactions between the concatenated products would be ~108 times less favorable than the intended interactions (Dirks *et al.*, *SIAM Rev* 49:65-88 (2007), incorporated herein by reference).

Fabrication of PEG hydrogels carrying DNA glues. A standard protocol was followed to synthesize acrylate-PEG-DNA by adopting NHS chemistry to conjugate the oligonucleotides DNA primer with a amine modification at the 5'-terminal of Poly(T-36) linker to the acrylate-PEG-NHS (Jenkem Technology). Shape-controlled PEG hydrogel was fabricated by following a general photolithograph process, as described (Du *et al.*, *Proc. Natl. Acad. Sci. USA* 105: 9522-9527 (2008), incorporated herein by reference), in which photomask was designed using AutoCAD software with 20,000 dpi resolution (CAD/Art Services; Bandon, OR). Prior to DNA amplification, the hydrogel subunits were washed with PBS and DNA amplification buffer thoroughly (40 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 5 mM (NH₄)₂SO₄, and 4 mM DTT). DNA concatemers were amplified by soaking the hydrogel subunits in reaction solution including 50 μM circular DNA template and 5 U/μl of Phi29 DNA polymerase (EPICENTRE Biotechnology) in 1× DNA amplification buffer at 37 °C overnight, according the manufacturer's instructions.

Hydrogel subunits carrying patterned DNA concatemers were fabricated in a two-step fabrication process. (1) DNA concatemers were amplified on a small hydrogel cube with size of 150- 300 μm, fabricated by photolithography, as described above. (2) Following DNA amplification, the DNA modified hydrogel subunits were washed thoroughly with 1× assembly buffer (0.5 M NaCl, 0.5 mM EDTA, and 0.05% Tween-20 in 1× general PBS buffer), and then a 20 wt % prepolymer PEG solution, including 1 v/v% color microbeads, was added. The final shape of the hydrogel subunits was controlled by a secondary photomask being aligned with the DNA hydrogel subunits in accordance with the designed patterning of DNA under microscope.

Self-assembly of hydrogels in aqueous solution. Hydrogel subunits carrying specific DNA concatemers were collected in a 0.5 ml microtube fulfilled with assembly buffer containing 0.5 M NaCl, 0.5 mM EDTA, and 0.05% Tween-20 in 1× general PBS buffer. To prevent non-specific binding between hydrogel and microtube, the inside surface of microtube was treated with a corona treater (BD-20AC from Electro-Technic Products Inc.) and coated

with 10% PEGDA (MW 1000) beforehand. To achieve DNA-directed hydrogel subunit assembly, the microtube was subjected to agitation of continuous 360-degree upright rotation on a VWR Multimix tube rotator with a fixed speed of 18 rpm and intermittent soft vortex or hand shaking every 30-60 minutes to disrupt non-specific binding or aggregates. After assembly, the hydrogel subunits were transferred to a petri dish filled with solution of 20 wt % PEG (MW 3350) in 1× assembly buffer and subject to further horizontal shaking with speed of about 60 rpm on a VWR standard orbital shaker. Assembled hydrogel structure were identified, quantified and imaged using a Stereo Microscope. Cell-laden hydrogel subunits were assembled in a general culture medium, Dulbecco's Modified Eagle's medium (DMEM, Invitrogen), without serum supplement.

Liquid/liquid interface self-assembly. Liquid/liquid interface was generated between Fluorinert electronic liquid FC-40 (bottom liquid, 3MTM Chemicals) and aqueous assembly buffer (top liquid) in a Petri dish. Hydrogel subunits were floated on the interface and subjected to agitation of continuous horizontal shaking at low speed of 60 rpm on a VWR standard orbital shaker (Model 1000, VWR) and intermittent 120 rpm shaking or strong hand shaking every 30-60 minutes to break undesired, spurious aggregates. The assembly process was recorded using an image recording software, HyperCam Version 2, under a Stereo Microscope.

Quantification of DNA directed hydrogel assembly. To quantify the specificity of hydrogel subunit dimer formation, hydrogel cubes with size of $250\ \mu\text{m} \times 250\ \mu\text{m} \times 250\ \mu\text{m}$, carrying three different complementary pairs of DNA (a/a*, red; b/b*, blue; c/c*, yellow) at single surface, were fabricated. Ten hydrogels of each pair were placed in an Eppendorf tube (n=6) filled assembly buffer and rotated for over 3 hours. Rotating the hydrogels generated dimers with all different combinations of the three colors. Self-assembly was performed in aqueous liquid system, as described above, and the total specific binding among the pairs was identified under a Stereo Microscope with 81.5% of specific binding, while non-specific binding was 18.50%, which confirmed the specificity of DNA interactions.

DNA analysis. RCA DNA product was amplified and analyzed on a 1% agarose gel. Surface of hydrogel carrying giant DNA glue was analyzed using a scanning electron microscopy (Zeiss EVO SEM). Hydrogel carrying ssDNA probe was fabricated on a glass slide surface as described earlier and giant single-strand DNA was amplified by soaking hydrogel in RCA reaction solution including $50\ \mu\text{M}$ circular DNA template and 5 U/ μl of Phi29 DNA polymerase (EPICENTRE Biotechnology) in 1× DNA amplification buffer at 37

°C overnight according to the manufacturer's instructions. After amplification, the hydrogel was rinsed thoroughly with general PBS (GIBCO, DPBS), and liquid around the gel was dried carefully using Kimwipes. Following washing, the hydrogel was frozen in -80 °C freezer for 3 hours and transferred to a freeze drier for 2 days for scanning electron microscopy imaging.

5 *Twenty-five structures assembly.* DNA oligos, D001, D001*, D002, D002* D025, D025* and poly(T-36) with a 5'-terminal amine modification were ordered from Integrated DNA Technology (IDT) and dissolved in water upon arrival. One phosphate group was added to the 3'- terminal of DNA oligos by T4 Polynucleotide kinase (PNK, EPICENTRE Biotechnology) for ligation with poly(T- 36) or circularization. Modification master reaction
10 comprising of 33 mM Tris-HCl (pH 7.5), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 5 mM ATP, 100 µM DNA oligo and 10 U/µl PNK enzyme, was incubate in 37 °C for 3 hours and then PNK enzyme was inactivated by incubation at 70 °C for 30min. Circular DNA template was prepared by circulating phosphate modified DNA oligo using Cirligase II ssDNA ligase (EPICENTRE Biotechnology). Circularization reaction was
15 performed with supplementing 2.5 mM manganese chloride, 1 M Betaine, and 5 U/µl ligase in phosphate modified DNA oligo solution and incubate at 60 °C for 6 hours, and ligase was inactivated at 80 °C for 10 minutes. Following this, exonuclease I (EPICENTRE Biotechnology) was added to remove linear DNA oligo at 37 °C for 1 hour and inactivated at 80 °C for 30 minutes. DNA primer was prepared by ligating modified DNA oligos to amine-
20 poly(T-36). Ligation reaction was performed with supplementing 20 U/µl T4 RNA ligase (New England Biolab) and 100 µM amine-poly(T-36) in phosphate modified DNA oligo solution and incubated at room temperature (25 °C) overnight.

Cubic PEG hydrogel subunits with a size of 250 µm × 250 µm × 250 µm carrying DNA primer was fabricated and giant DNA was amplified as described earlier. With labeling core
25 and periphery part separately, pairwise combination of 5 color microbeads including polybead Red Dyed 1.0 µm microspheres, polybead Blue Dyed 0.5 µm microspheres, Polybead Yellow Dyed 3.0 µm microspheres, polybead Violet Dyed 1.0 µm microsphere and polybead Black Dyed 10.0 µm microspheres (Polysciences) generated 25 distinct labeling. The fabrication process is similar to that of hydrogel for dimer assembly, as described herein, but with minor
30 modification. During the secondary photolithography, the second hydrogel (periphery part) was fabricated to completely warp the first hydrogel (core part) forming the final structure. To decrease assembly time and avoid aggregation, a single copy of each hydrogel uniformly carrying DNA D001, D001*, D002, D002* D025, D025* was collected in 1.5 ml

Eppendorf microtubes fulfilled with 1× assembly buffer and self-assembly was performed in aqueous liquid as described above. The assembled structure was identified under a Stereo Microscope and specific assembly was quantified with a Student's t-Test (n=6).

Cell Culture. NIH-3T3 mouse fibroblast cells, smooth muscle cells (SMCs), and GFP-transfected human umbilical vein endothelial cells (HUVECs) expressing green fluorescence protein were cultured using an approved protocol. SMC's were cultured in SMC basal medium (RPIM 1640; Invitrogen; Carlsbad, CA).

Cell Viability. NIH-3T3 fibroblast cells were encapsulated inside hydrogels and cell viability was analyzed after assembly. Cells were trypsinized, counted, and re-suspended inside the prepolymer solution at concentration of 1x10⁷ cells/ml. After fabrication, cells containing hydrogels were placed in PCR Eppendorf tubes (0.5 ml; Hamburg, Germany) filled with cell culture medium and rotated for 3 hours. Cell-encapsulated hydrogels were transferred into a Petri dish and the viability of the cells was determined using LIVE/DEAD Viability/Cytotoxicity Kit (2 µl of Calcein AM and 0.5 µl of Ethidium homodimer-1 in DPBS; Invitrogen; Carlsbad, CA).

Cell-laden Dimer. HUVECs and SMCs were trypsinized, harvested, and labeled with PKH26 Red Florescent Cell Linker Kit (Sigma Aldrich; St. Louise, MO) and CellTracker™ Green CMFDA (Invitrogen; Carlsbad, CA) accordingly. In addition, the nuclei of both groups of cells were stained with DAPI Nucleic Acid Stain (Invitrogen; Carlsbad, CA) to facilitate imaging. Labeled cells were re-suspended in prepolymer solution (PEG 20 % wt) at a density of 1x10⁷ cells/ml. Single side-modified hydrogels were made out of the prepolymer-cell solution using the same method mentioned earlier. Hydrogels were collected and placed in an Eppendorf tube containing a mixture of HUVEC and SMC cell culture medium (Dulbecco's Modified Eagle's medium, (DEME), Invitrogen) without serum and were rotated for 3 hours before being imaged.

SEQUENCES

Name	Sequence
(5'---3')	
a	CTCTACTACCTTCTCCCTCCCACAAACGCAAACCCACTACCACCAAAC (SEQ ID NO:1)
a*	GTTTGGTGGTAGTGGGTTTGCGTTTGTGGGAGGGAGAAGGTAGTAGAG (SEQ ID NO:2)
b	TCAATGTAAGTGCAGATAAAGTACTCGCGCACTACTATGTTTTAGCTA (SEQ ID NO:3)

	b*	TAGCTAAAACATAGTAGTGCGCGAGTACTTTATCTGCACTTACATTGA (SEQ ID NO:4)
	c	GACTATATCGTATCGACTCATCCATGATAGTAATCAATTCAGGCCATC (SEQ ID NO:5)
5	c*	GATGGCCTGAATTGATTACTATCATGGATGAGTCGATACGATATAGTC (SEQ ID NO:6)
	Primer a	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGAGGGAGAAGGTAGTAGAG (SEQ ID NO:7)
	Primer a*	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTCTACTACCTTCTCCCTCC (SEQ ID NO:8)
10	Primer b	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGCGAGTACTTTATCTGC (SEQ ID NO:9)
	Primer b*	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGCACTACTATGTTTTAGC (SEQ ID NO:10)
15	Primer c	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTATCATGGATGAGTCG (SEQ ID NO:11)
	Primer c*	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATCGACTCATCCATGATAG (SEQ ID NO:12)
	PolyT	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT (SEQ ID NO:13)
20	D001	AAAAAACTAACATTTAACTAATA (SEQ ID NO:14)
	D001S	TATTAGTTAAATGTTAGTTTTTTT (SEQ ID NO:15)
	D002	AAAACTTATTATCAAATTACTAT (SEQ ID NO:16)
	D002S	ATAGTAATTTGATAATAAGTTTTT (SEQ ID NO:17)
	D003	ATTACATAATTCAATTTTTCATA (SEQ ID NO:18)
25	D003S	TATGAAAAATTGAATTATGTAAAT (SEQ ID NO:19)
	D004	AAATTACATTACTTAACTTTATA (SEQ ID NO:20)
	D004S	TATAAAGTTTAAGTAATGTAATTT (SEQ ID NO:21)
	D005	ATTATTCTAAATTCTAAAACAATA (SEQ ID NO:22)
	D005S	TATTGTTTTAGAAATTTAGAATAAT (SEQ ID NO:23)
30	D006	AAATATACTATATACTAACTAAA (SEQ ID NO:24)
	D006S	TTTAGTTTAGTATATAGTATATTT (SEQ ID NO:25)
	D007	TTATCATTATACAATTTACTTTAA (SEQ ID NO:26)
	D007S	TTAAAGTAAATTGTATAATGATAA (SEQ ID NO:27)
	D008	TAAATTCTTTTTCATTATCATAAA (SEQ ID NO:28)
35	D008S	TTTATGATAATGAAAAAGAATTTA (SEQ ID NO:29)
	D009	AAAAACAAATACTATACTATAAAT (SEQ ID NO:30)
	D009S	ATTTATAGTATAGTATTTGTTTTT (SEQ ID NO:31)
	D010	AATACTTATCAAATACATATAAAT (SEQ ID NO:32)
	D010S	ATTTATATGTATTTGATAAGTATT (SEQ ID NO:33)
40	D011	TTATTCAAAAATACTATTCTTAAA (SEQ ID NO:34)

D011S TTTAAGAATAGTATTTTTGAATAA (SEQ ID NO:35)
D012 TAAATTCATATATCTTACAATAAA (SEQ ID NO:36)
D012S TTTATTGTAAGATATATGAATTTA (SEQ ID NO:37)
D013 AAAATTACTTTATACAAACATTTA (SEQ ID NO:38)
5 D013S TAAATGTTTGTATAAAGTAATTTT (SEQ ID NO:39)
D014 TTATCTTAATATTCATTTCTAAAA (SEQ ID NO:40)
D014S TTTTAGAAATGAATATTAAGATAA (SEQ ID NO:41)

EQUIVALENTS

10 While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art
15 will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments
20 described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition,
25 any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

 All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary
30 meanings of the defined terms.

 All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

 The indefinite articles “a” and “an,” as used herein in the specification and in the
35 claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, *i.e.*, “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); *etc.*

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, *i.e.*, the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (*i.e.* “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment,

to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); *etc.*

5 It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

10 In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

15

CLAIMS

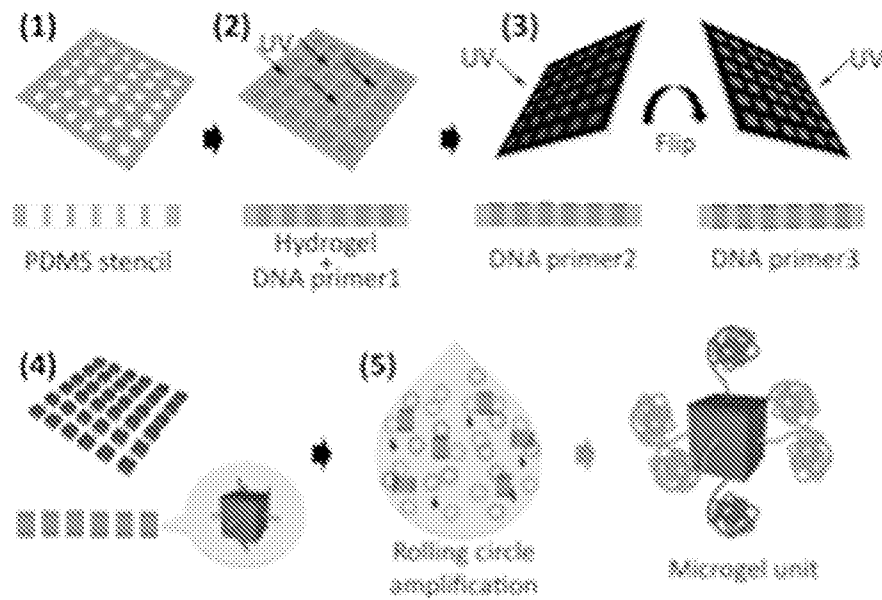
What is claimed is:

1. A shaped hydrogel subunit surface-modified with at least one nucleic acid.
2. The shaped hydrogel subunit of claim 1, wherein the at least one nucleic acid is covalently attached to the shaped hydrogel subunit.
3. The shaped hydrogel subunit of claim 2, wherein the at least one nucleic acid is single-stranded.
4. The shaped hydrogel subunit of any one of the foregoing claims, wherein the nucleic acid is a concatemer.
5. The shaped hydrogel subunit of claim 4, wherein the nucleic acid concatemer is produced through rolling circle amplification (RCA).
6. The shaped hydrogel subunit of any one of the foregoing claims, wherein the shaped hydrogel subunit is a cube, a tube or a sphere.
7. The shaped hydrogel subunit of any one of the foregoing claims, wherein the shaped hydrogel subunit is comprised of polyethylene glycol, gelatin methacrylate or alginate.
8. The shaped hydrogel subunit of any one of the foregoing claims, wherein the shaped hydrogel subunit has a diameter of about 1 μm to about 1 mm.
9. The shaped hydrogel subunit of any one of the foregoing claims, where the shaped hydrogel subunit is surface-modified with at least two nucleic acids.
10. The shaped hydrogel subunit of claim 9, where the at least two nucleic acids are different from each other and are present on different surfaces of the shaped hydrogel subunit.
11. The shaped hydrogel subunit of any one of the foregoing claims, where the shaped hydrogel subunit is surface-modified with at least three nucleic acids.
12. The shaped hydrogel subunit of any one of the foregoing claims, where the shaped hydrogel subunit contains cells.

13. The shaped hydrogel subunit of claim 12, wherein the cells are stem cells, progenitor cells or differentiated cells.
14. A composition comprising at least two shaped hydrogel subunits, each surface-modified with at least one nucleic acid, wherein the at least two shaped hydrogel subunits are joined to each other through sequence-specific hybridization between the nucleic acids.
15. A plurality of shaped hydrogel subunits each surface-modified with at least one nucleic acid concatemer, wherein the shaped hydrogel subunits are joined to each other through sequence-specific hybridization between the nucleic acids.
16. The plurality of shaped hydrogel subunits of claim 15 specifically arranged to form a three-dimensional structure.
17. A method of producing a three-dimensional hydrogel structure comprising combining at least two shaped hydrogel subunits, each surface-modified with at least one nucleic acid concatemer, in an aqueous assembly system or in an interfacial assembly system, thereby providing for nucleic acid hybridization and hydrogel subunit self-assembly.
18. A method of producing a shaped hydrogel subunit surface-modified with a nucleic acid comprising
 - conjugating a nucleic acid to a polyethylene glycol (PEG) monomer to form nucleic acid-PEG-acrylate;
 - combining the nucleic acid-PEG-acrylate with PEG-diacrylate and a photoinitiator to form a mixture; and
 - exposing the mixture to ultraviolet light under a photomask to produce the shaped hydrogel subunit modified with at least one nucleic acid.
19. The method of 18, further comprising producing at least one concatemer through rolling circle amplification of the at least one nucleic acid.
20. A method comprising delivering cells or other biomolecules to a site of interest *in vivo* using the nucleic acid hydrogel subunits of any one of the foregoing claims, the composition of any one of the foregoing claims, or the plurality of shaped hydrogel subunits of any one of the foregoing claims.

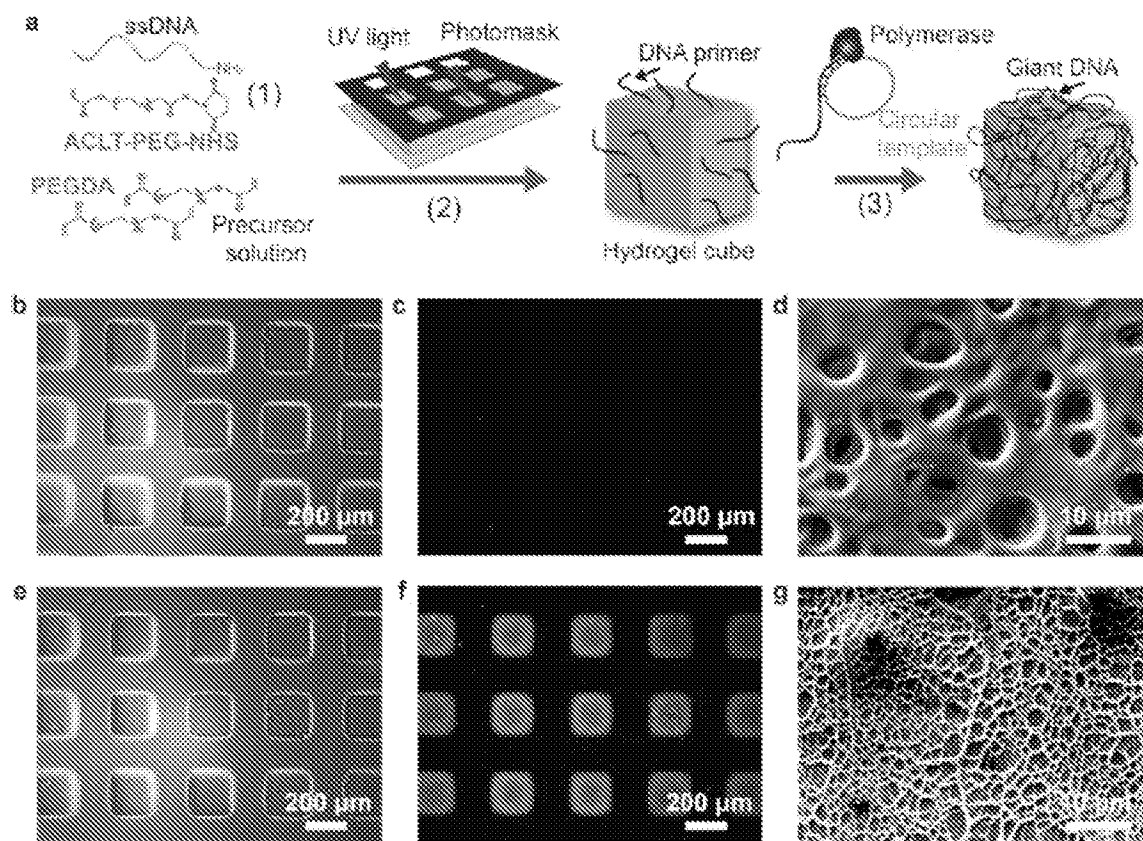
1/8

FIG. 1



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FIG. 2



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FIG. 3

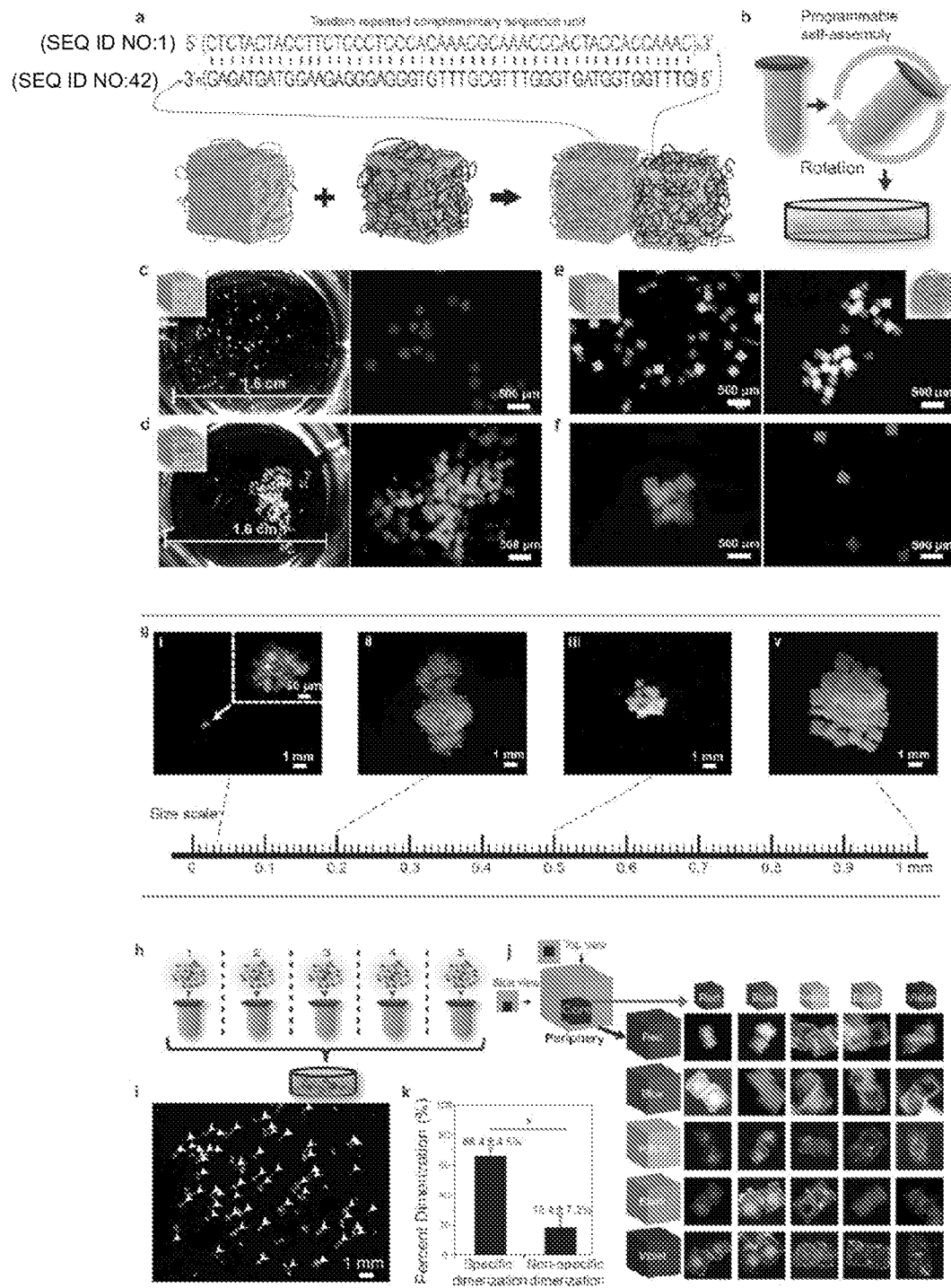


FIG. 4

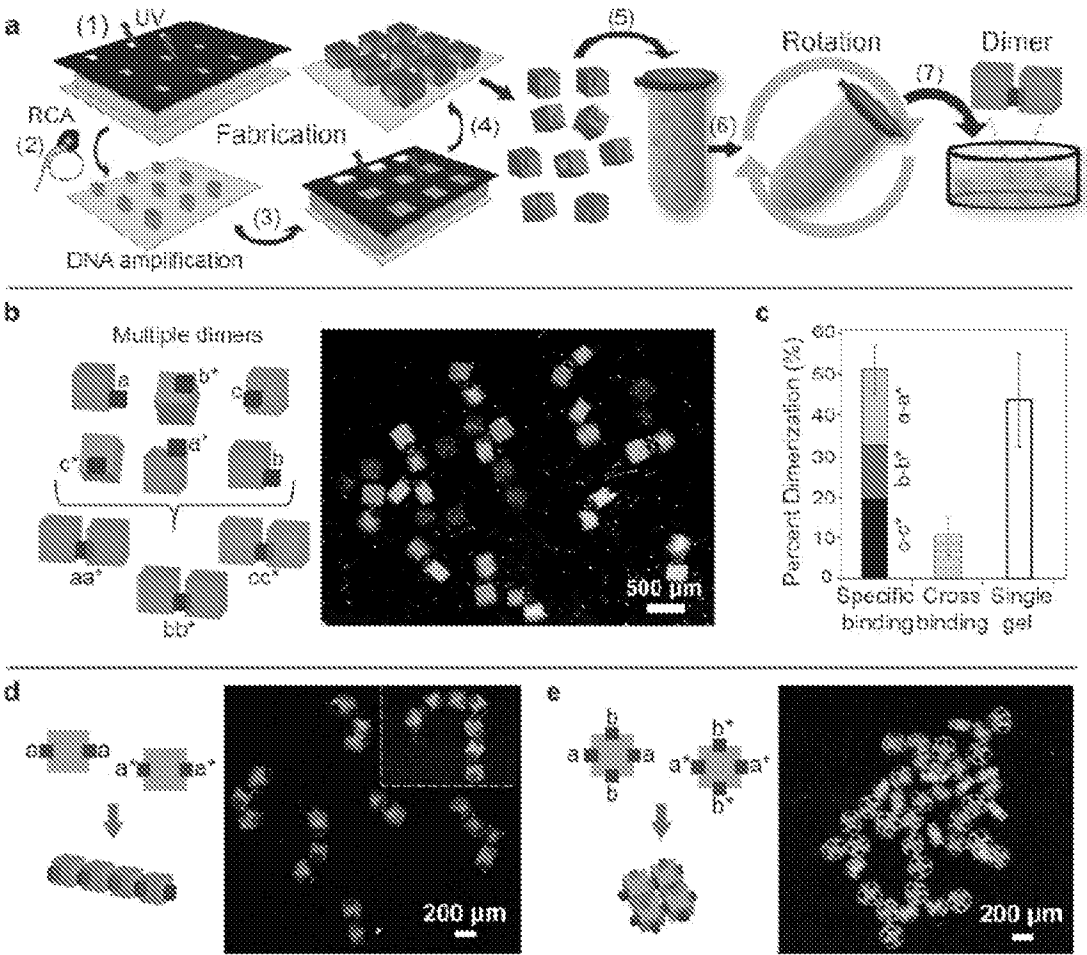
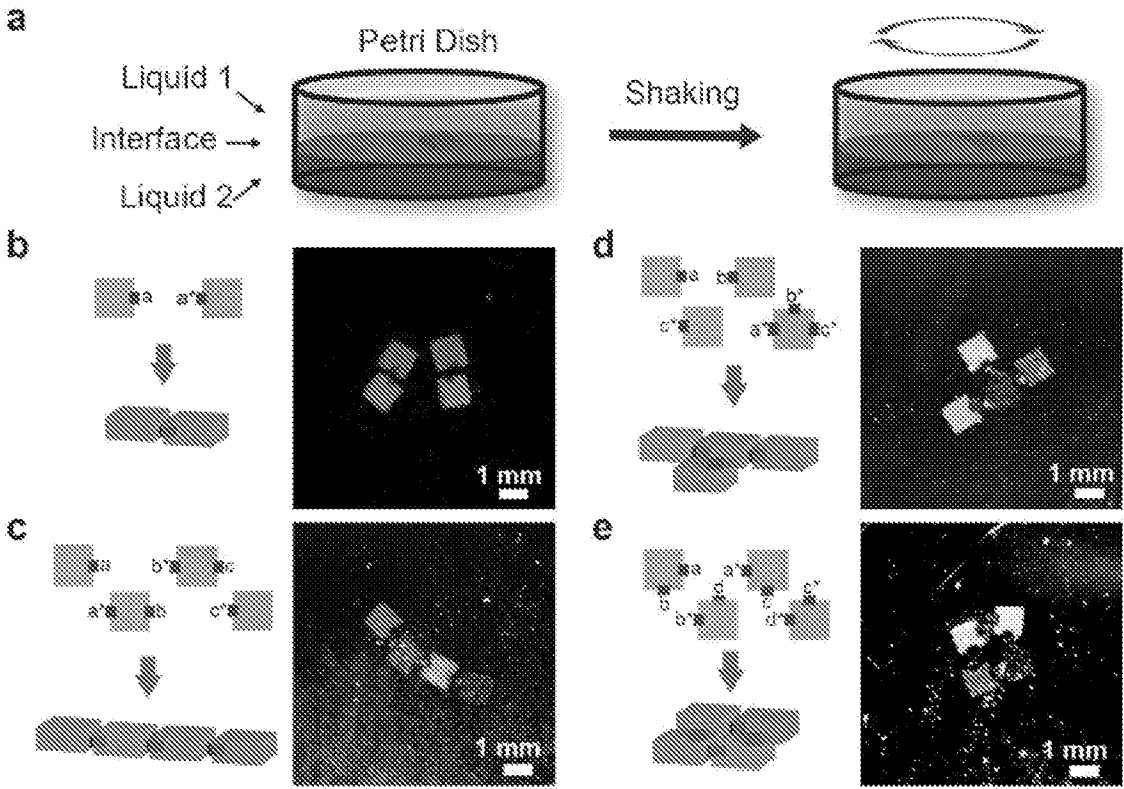


FIG. 5



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FIG. 6

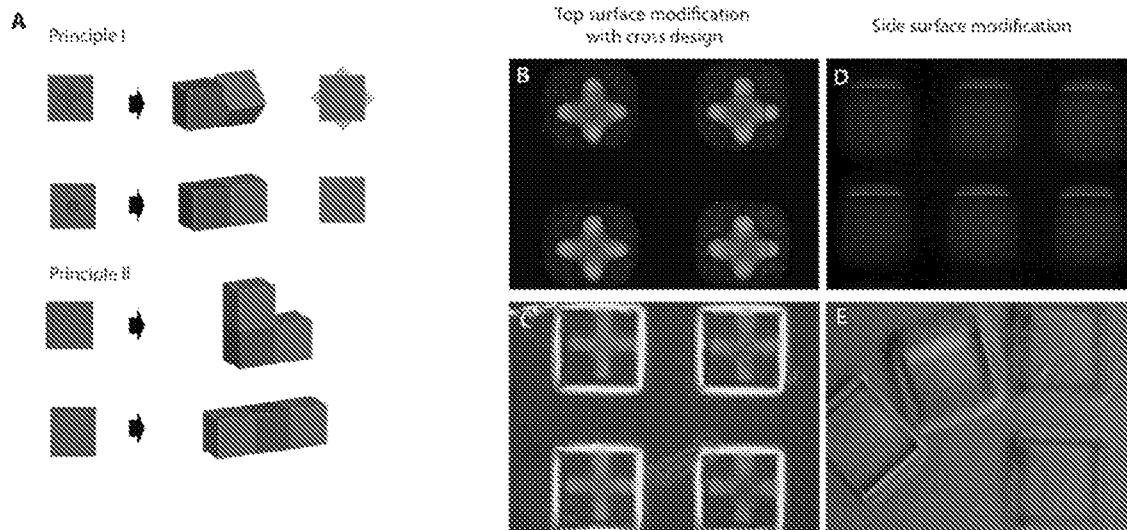


FIG. 7

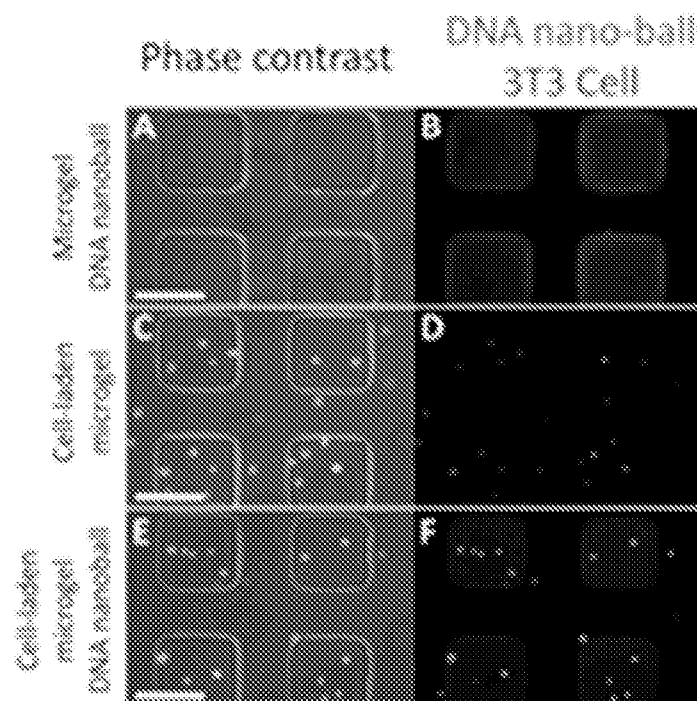


FIG. 8

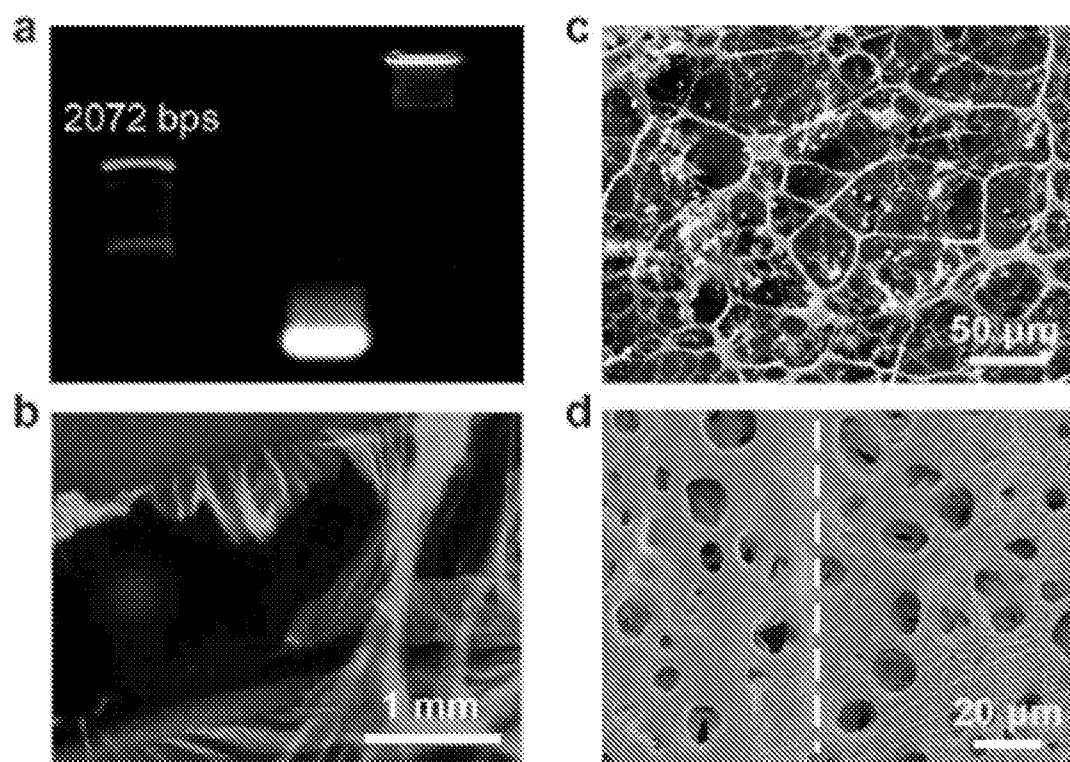


FIG. 9

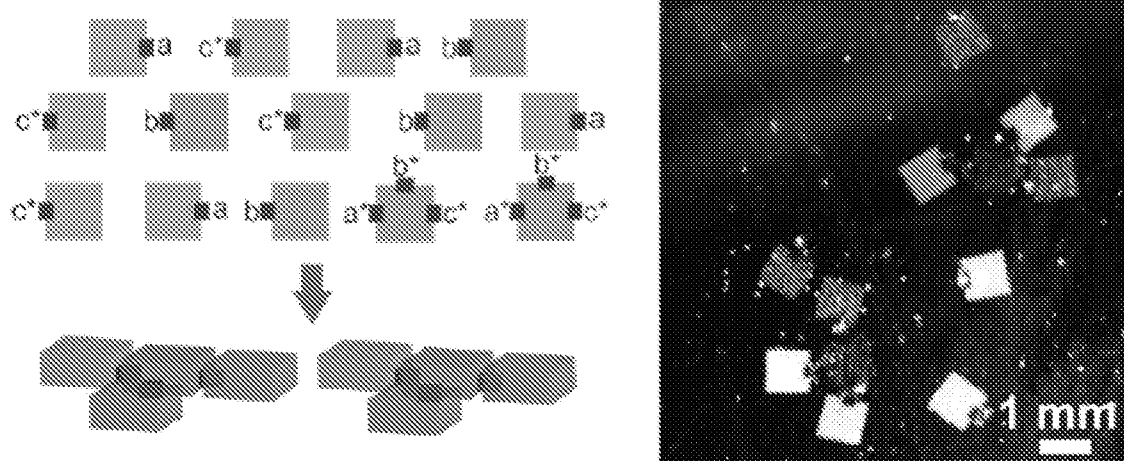
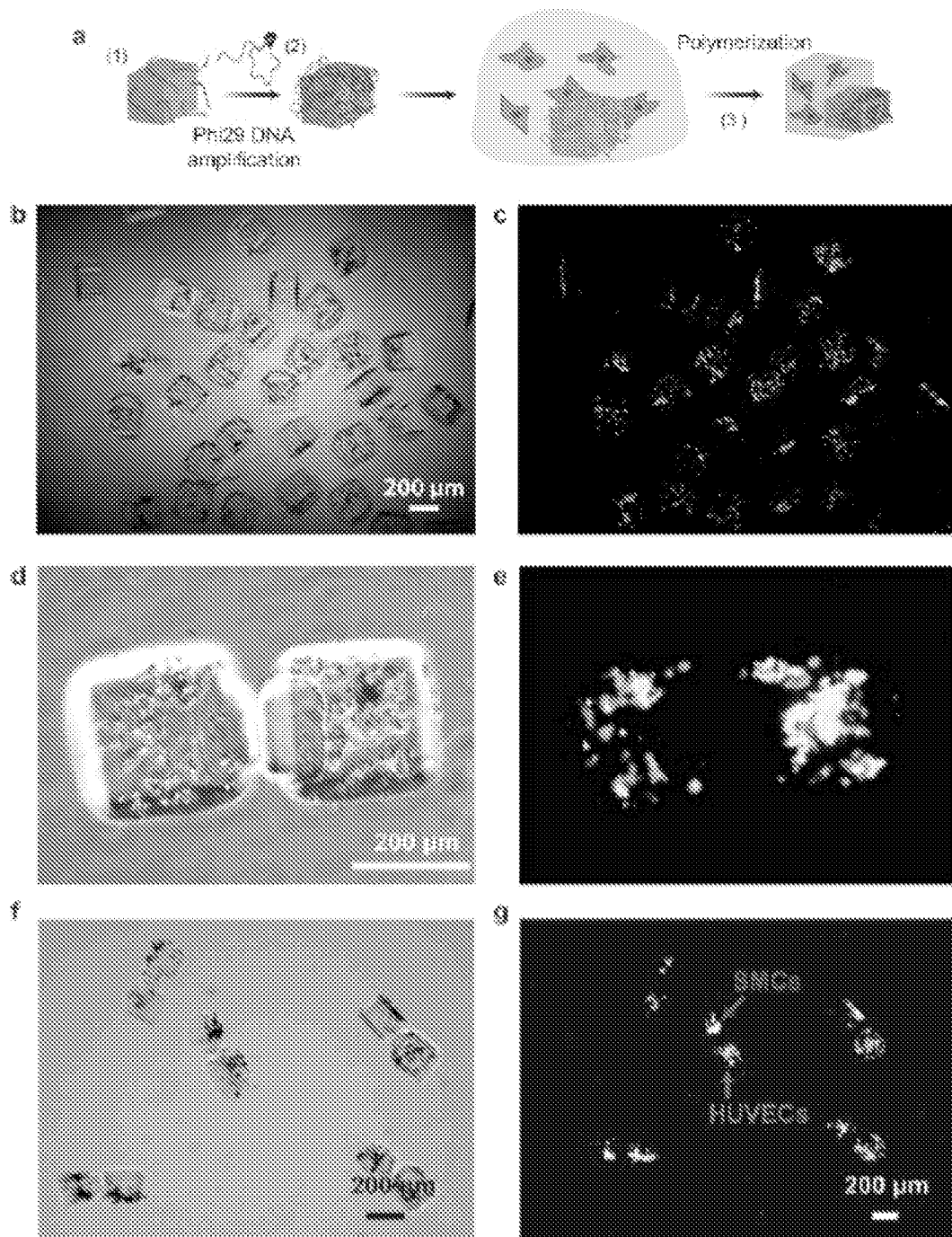


FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/70260

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07H 21/02, 21/04; C08B 37/04 (2013.01)

USPC - 536/23.1; 536/3

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)

USPC: 536/23.1; 536/3

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

USPC: 536/23.1; 536/3; 536/25.3 (text search)

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

Electronic data bases: PatBase, Google Patents, Google Scholar

Search terms: self-assembly*, hydrogel, DNA, polynucleotide, DNA-functionalized, PEG acrylate, photomask, gelatin, metacrylate, alginate, rolling circle amplification (RCA), complementary, concatamer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2008/0193536 A1 (KHADEMHOSEINI et al.) 14 August 2008 (14.08.2008). Especially para [0019], [0131], [0134], [0139]	1-3, 14 ----- 4, 5, 15-19
Y	US 2010/0189794 A1 (LUO et al.) 29 July 2010 (29.07.2010). Especially para [0019], [0093].	4, 5, 15-17, 19
Y	SCHLINGMAN et al. A new method for the covalent attachment of DNA to a surface for single-molecule studies. Colloids Surf B Biointerfaces. March 2011 Vol 83 No 1 Pages 91-95. Especially abstract.	18, 19
A,P	Qi et al. DNA-directed self-assembly of shape-controlled hydrogels. Nat Commun 9 September 2013 Vol 4 No 2275 Pages 1-10. Especially entire article.	1-5, 14-19
A	US 2012/0141551 A1 (BLOEMBERGEN et al.) 7 June 2012 (07.06.2012). Especially para [0019]	1-3
A	VENKATESH et al. Nucleic acid therapeutic carriers with on-demand triggered release. Bioconjug Chem September 2009 Vol 20 No 9 Pages 1773-1782. Especially pg 1775 fig 1, pg 1	1-3

☐ 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

21 December 2013 (21.12.2013)

Date of mailing of the international search report

14 JAN 2014

Name and mailing address of the ISA/US

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

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/70260

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.: 6-13, 20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

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