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Bio-functionalized silk hydrogel microfluidic systems

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ABSTRACT

Bio-functionalized microfluidic systems were developed based on a silk protein hydrogel elastomeric materials. A facile multilayer fabrication method using gelatin sacrificial molding and layer-by-layer assembly was implemented to construct interconnected, three dimensional (3D) microchannel networks in silk hydrogels at 100 μm minimum feature resolution. Mechanically activated valves were implemented to demonstrate pneumatic control of microflow. The silk hydrogel microfluidics exhibit controllable mechanical properties, long-term stability in various environmental conditions, tunable *in vitro* and *in vivo* degradability in addition to optical transparency, providing unique features for cell/tissue-related applications than conventional polydimethylsiloxane (PDMS) and existing hydrogel-based microfluidic options. As demonstrated in the work here, the all aqueous-based fabrication process at ambient conditions enabled the incorporation of active biological substances in the bulk phase of these new silk microfluidic systems during device fabrication, including enzymes and living cells, which are able to interact with the fluid flow in the microchannels. These silk hydrogel-based microfluidic systems offer new opportunities in engineering active diagnostic devices, tissues and organs that could be integrated *in vivo*, and for on-chip cell sensing systems.

1. Introduction

Microfluidics have enabled precise fluidic manipulation and control at extremely small volumes [1–3] to address challenges in the fields of high throughput biomolecule analysis, diseases diagnosis and integrated cell studies. The introduction of PDMS revolutionized traditional microfluidics, which was previously largely based on silicon and glass microfabrication [4–13]. Due to the optical transparency, low toxicity and biological/chemical inertness, PDMS microfluidics support a broad range of biological and chemical applications, including multiplexed assays, high throughput synthesis, drug screening, and many related applications [14–16]. However, despite these advantages, PDMS presents significant challenges to the expansion in utility of such devices, particularly with respect to biological interfaces and for needs in tissue engineering, regenerative medicine and drug development. PDMS microfluidic devices are fabricated from silicone prepolymer and involves processing at elevated temperatures [14] which negates the incorporation of bio-functional components during device fabrication. Further, PDMS does not support cell attachment unless surface-modified, via physical or chemical means [16–19]. Moreover, mass transfer and functionalization are limited to the surface of microchannels as the bulk of PDMS devices are generally not accessible to aqueous reagents or cell infiltration. Finally, PDMS is not degradable in a biological context, thus, devices for clinical introduction *in vivo* are not remodeled into native tissues over time. These features reduce the utility of PDMS-based devices in a biological context, such as in emulation of tissues or organs, where mass transfer and biochemical activity are carried out both in channels and the bulk and where biodegradation and remodeling are key features in tissue and organ regeneration.

Recently, more biologically relevant and natural hydrogel materials have been utilized in microfluidics fabrication to address the limitations with PDMS, including collagen [20,21], gelatin [17,22,23], gelatin methacrylate (GelMA) [13,24], agarose [25,26], alginate [27,28] and various extracellular matrix proteins [20,29,30]. These hydrogel materials are cell-compatible, enabling better interfaces with two and three dimensional cell cultures. Simple microfluidic channels with cells cultured both

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on the surfaces of microchannels and in the bulk of the device have been demonstrated [19,31]. More complex and interconnected 3D hydrogel microfluidics have also been recently made possible taking advantage of 3D printing [13,29], which requires costly printing instrumentation [31]. However, existing hydrogel microfluidics have not been able to utilize the full capacity of controllable and scalable 3D microfluidic networks and avoid shrinkage or dimensional changes over time. Moreover, mechanical properties, such as stiffness of current microfluidic hydrogel materials only covers a limited range of native tissues, which limits their utility [18,26]. Furthermore, current microfluidic hydrogel materials are either not degradable or are so only over a limited range of time frames, posing challenges for use in tissue regeneration goals.

To address these persistent challenges with current microfluidic devices, and to better adapt microfluidic systems to the rapidly growing needs in the fields of tissue engineering and regenerative medicine, as well as looking towards future needs for implantable and degradable microfluidic devices, microfluidics based on silk fibroin proteins are described. Silk was chosen here because it is a naturally derived protein biomaterial with excellent biocompatibility and controllable degradation rates, thus suitable for tissue engineering and artificial organ development [18,32–37]. Silk is also approved by FDA for some biomedical devices, which indicates its safety for use *in vivo* [38]. Recently, we reported a new enzymatically crosslinked hydrogel format for silk with highly tunable stiffness [39], which we considered here as a suitable alternative to PDMS but with the added benefits of biological functionalization and degradability. A fabrication method based on gelatin sacrificial molding and layer-by-layer assembly was developed to enable facile construction of truly interconnected 3D microchannel networks inside silk hydrogels at 100 μm resolution without the need of costly equipment [19]. Pneumatically actuated valves, common for high-throughput PDMS microfluidics [40], were implemented in the hydrogel microfluidic setups, to provide external control of microflow. Importantly, the unique all-aqueous fabrication method in ambient conditions allowed the incorporation of bulk functionality during device fabrication, including active biomolecules (alkaline phosphatase) and living cells (human fibroblast), which are able to interact with the fluid flow in the microchannels. The silk hydrogel microfluidic devices exhibited tunable mechanical stiffness over 3 orders of magnitude (from 1 kPa to 1 MPa) via control of the crystalline state, which covers a significant portion of various human tissues [33]. Moreover, the silk microfluidic hydrogels also showed optical transparency and stability in various environmental conditions (from non-neutral pH to non-physiological salinity) in addition to inherent biocompatibility and adjustable degradability.

2. Materials and methods

2.1. Materials

Raw silk cocoons produced by *Bombyx mori* silkworms were obtained from Tajima Shoji Co (Yokohama, Japan). Sodium carbonate, lithium bromide (LiBr), gelatin, horseradish peroxidase (HRP), hydrogen peroxide (H_2O_2), polystyrene, FITC labeled bovine serum albumin, alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) liquid substrate and *para*-nitrophenylphosphate (pNPP) liquid substrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Live/dead stain kit and AlamarBlue stain were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Sylgard 184 silicone elastomer kit (PDMS) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Liquid powder dye (Rit liquid dye) was purchased from local Walmart (Saugus, MA, USA). Dialysis cassettes were purchased from Fisher Scientific (Pittsburgh, PA, USA). Human umbilical vein endothelial cells (HUVEC) and media were purchased from Lonza (Walkersville, MD, USA). Human foreskin fibroblast (HFF) cells were a generous gift from Garlick lab at Tufts University. HUVECs and HFF cells were cultured in HUVEC medium and DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, respectively at 37 °C, 5% CO_2.

2.2. Preparation of silk solution

The aqueous silk solution was prepared using our previously published protocol [41]. Briefly, 10 g of silk cocoons were cut into dime-sized pieces and extracted by boiling in 4 L of 0.2% sodium carbonate solution for 10, 30 or 60 min to remove the sericin, which are referred to as 10 min, 30 min and 60 min in the paper. After washing the degummed silk in copious amount of water and drying for 12 h, the silk was dissolved in 9.3M LiBr solution at 60 °C for 4 h. The silk solution was dialyzed against deionized (DI) water in dialysis cassettes with a 3500 molecular weight cutoff for three days to remove the LiBr. After centrifugation at 9000 rpm at 4 °C for 20 min twice to remove any undissolved particles, the resulting solution was used for hydrogel microfluidics fabrication.

2.3. Multilayer microfluidics fabrication

A gelatin sacrificial mold method was utilized for the fabrication of silk hydrogel microfluidics, which prevents deformation of the microchannels during fabrication. The fabrication of silk hydrogel microfluidics started with preparation of the bottom gel layer (Fig. 1A). HRP and H_2O_2 were mixed with silk solution at 20 unit/ml and 0.01% w/w, respectively and the mixture was cast in a PDMS stencil and briefly heated on a 37 °C hotplate for 2 min to half cure the bottom gel layer. A negative PDMS stamp was used to form a gelatin (10% w/v in deionized water) microchannel mold with interlayer connecting pillars. The PDMS stamp together with the gelatin mold was subsequently placed on the half cured bottom gel layer. The silk was allowed to fully cure at room temperature for 10 min and the PDMS stamp was removed, leaving only the gelatin mold on the bottom gel. To facilitate the demolding process, the surface of the PDMS stamp was coated with 5% Pluronic F127 solution to render it non-sticky. It is important to transfer the gelatin when the silk was only half crosslinked to achieve good bonding between the gelatin and silk hydrogels. After the transfer of the gelatin mold, the silk/HRP/H_2O_2 mixture was poured onto the mold and allowed to cure at room temperature to form the channel gel layer. The thickness of the gel layer can be controlled by the volume of the silk solution utilized in the process. By repeating this two-step process of gelatin transfer and silk gel formation, multilayers could be fabricated. Liquid gelatin was used as a glue to connect the gelatin molds of adjacent layers at the joint pillars to ensure good interlayer channel connection. The alignment of different layers was ensured using a stereomicroscope (Cole Parmer, Vernon Hills, IL). To achieve higher tolerance on misalignment, the size of the joint pillars was intentionally designed to be 3 to 4 times bigger than the minimum channel dimension so that slight misalignment would not cause disconnects between adjacent layers. After the assembly was complete, the device was incubated in a 37 °C oven for 5–10 min to completely melt the gelatin, which was subsequently removed by flushing the channel with deionized water.