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Development of a custom biological scaffold for investigating ultrasound-mediated intracellular delivery

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ABSTRACT

In vitro investigations of ultrasound mediated, intracellular drug and gene delivery (*i.e.* sonoporation) are typically carried out in cells cultured in standard plastic well plates. This creates conditions that poorly resemble *in vivo* conditions, as well as generating unwanted ultrasound phenomena that may confound the interpretation of results. Here, we present our results in the development of a biological scaffold for sonoporation studies. The scaffolds were comprised of cellulose fibers coated with chitosan and gelatin. Scaffold formulation was optimized for adherence and proliferation of mouse fibroblasts in terms of the ratio and relative concentration of the two constituents. The scaffolds were also shown to significantly reduce ultrasound reflections compared to the plastic well plates. A custom treatment chamber was designed and built, and the occurrence of acoustic cavitation in the chamber during the ultrasound treatments was detected; a requirement for the process of sonoporation. Finally, experiments were carried out to optimize the ultrasound exposures to minimize cellular damage. Ultrasound exposure was then shown to enable the uptake of 100 nm fluorescently labeled polystyrene nanoparticles in suspension into the cells seeded on scaffolds, compared to incubation of cell-seeded scaffolds with nanoparticles alone. These preliminary results set the basis for further development of this platform. They also provide motivation for the development of similar platforms for the controlled investigation of other ultrasound mediated cell and tissue therapies.

1. Introduction

Sufficient and uniform delivery of drugs and genes is crucial for an effective therapeutic response. Obtaining safe and efficient drug and gene delivery, however, is still a major challenge. In gene delivery, viral vectors have been used in about 70% of clinical trials [1]. Despite the efficiency of viral vectors for gene delivery, they possess several drawbacks including immunogenicity, cytotoxicity, oncogenic effects, and potentially generating an inflammatory response. These vectors are also costly for large scale production [2,3]. Non-viral vectors on the other hand typically lack many of these undesirable characteristics. However, they are generally less efficient in terms of delivery [4,5]. Various methodologies and approaches exist for enhancing intracellular drug and gene delivery. These include pharmaceutical and biomaterial approaches such as liposomes and cell penetrating proteins and peptides [6]. Device-based approaches also exist, such as electroporation. This procedure, which can be very effective, requires the use of electrodes, and hence is considered invasive [7–9] when compared to extracorperal ultrasound-based methods.

Sonoporation is the process by which transient pores are created in the outer membranes of cells with the application of ultrasound waves. Through this process, therapeutic agents such as drugs and genes can be delivered to individual cells, which normally would not be able to cross an intact cellular membrane [10]. Sonoporation occurs when a gas containing bubble is activated adjacent to a cell surface and set into oscillation by the varying pressure field of an ultrasound wave. When the bubble collapses, constraints in fluid flow surrounding the bubble may cause the outer surface to collapse faster, towards the cell, generating what has been termed a 'wall-direct-reentrant jet'. These jets, which pass through the inner surface of the bubble, can impact cellular membranes, producing small pores that may increase the cells' permeability. Whereas this is the most commonly considered mechanism responsible for the sonoporation process, other possible mechanisms have also been proposed. These include shock waves

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generated when a bubble collapses more distant to the cell, as well as shear forces occurring as the result of microstreaming, generated in proximity to a stably oscillating bubble [11]. The formation of bilayer sonophores has more recently also been proposed [12]. Of all of the existing physical approaches for enhancing intracellular delivery, sonoporation is the only technique that is non-invasive. It can also be carried out deep inside the body under image guidance in a non-destructive manner [10]. Ultrasound devices are typically inexpensive and involve the application of non-ionizing mechanical waves. Despite these many advantages, sonoporation still has some drawbacks. The manner by which cell membranes are affected is not a perfectly controllable process, and outcomes have been shown to vary among the different reported studies [13,14].

A variety of experimental setups have been used to investigate sonoporation *in vitro*. Whereas some of these setups involve the treatment of cells in suspension, the vast majority are comprised of cells in monolayer in plastic cell culture dishes, treated either from above or below the cell layer [15,16]. Despite their widespread use, these setups are considered to be problematic. Cells in suspension are not representative of conditions *in vivo*. A single layer of cells in culture wells, backed on one side by incompressible plastic and a relatively large volume (compared to the cells) of unconfined fluid on the other side does not represent *in vivo* conditions either. In addition, the transmission of ultrasound through culture wells is inefficient, and can result in heat generation, mode conversion and the potential formation of standing waves. Combined, these factors can lead to uncertainties of up to 700% in the actual ultrasound exposure being experienced by the treated cells [17,18]. As a result it can be difficult to carry out systematic investigations on the true relationship between the physical nature of the ultrasound mechanisms and the bioeffects that they produce.

Biological scaffolds are artificial structures that support different cell processes such as proliferation and differentiation. Today, scaffolds are being used for regenerative purposes [19,20], as well as investigative platforms [21,22]. In scaffolds, cells are attached to an extra cellular matrix-like material compared to a flat, rigid and artificial surface in plastic cell culture plates. In addition, scaffolds can be made from naturally derived materials that mimic *in vivo* environments [23,24]. Scaffold fabricated from biomimetic biopolymers (*i.e.* polymers inspired by the ECM to mimic some of its roles) are superior to synthetic polymers in terms of cell adhesion and maintenance of cell differentiation processes [25]. Chitosan and gelatin scaffolds are widely used in biological applications [26]. Gelatin, which is derived from collagen, acts as a substrate for cell adhesion, proliferation and differentiation [26,27], while chitosan provides beneficial structural and support characteristics [28,29].

In this paper, we present early results in the development of a biological scaffold to be used for sonoporation studies. The scaffolds were comprised of layers of cellulose fibers coated with both chitosan and gelatin. Initial experiments were carried out to optimize the formulation of the scaffolds in terms of cells adhesion and proliferation. Additional investigations involved evaluating the ultrasound reflections that they generated compared to the well plates. A custom treatment chamber was designed and built specifically for the sonoporation experiments. And lastly, proof of concept experiments on sonoporation were carried out using the scaffolds and compared to standard well plates.

2. Materials and methods

2.1. Scaffold preparation

Solutions of 1%, 2% and 3% (*w*/*v*) of chitosan were prepared in 2% acetic acid. The same concentrations were prepared for gelatin in deionized (DI) water. Similar (1, 2 or 3%) concentrations of the chitosan and gelatin (CG) solutions were mixed at various ratios of the two constituents (see Section 2.5.1.). Sections (10 × 10 cm) of common laboratory wipes (Kimwipe® Kimberly-Clark, Irving, Texas, USA) were pulled tight over plastic dishes (11.4 × 11.4 cm). Each sheet was saturated with 10 ml of the prepared CG solutions and the excess liquid was removed. After 30 min at room temperature, they were transferred to a freezer at −20 °C for 72 h, and then placed in a freeze/dry chamber for 24 h.

Two separate cross-linking solutions were prepared for chitosan and gelatin, respectively, and mixed together at the same ratio of their respective substrates 2.5% pentasodium tripolyphosphate hexahydrate (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) was used to cross link the chitosan; 0.05% formaldehyde-glutaraldehyde was used to cross link the gelatin. The cross-linking mixture was poured over the scaffolds. 5 min later, after the scaffolds began to turn yellow, they were washed three times with PBS. The scaffolds were then left to dry overnight at room temperature. The following day, the scaffolds were cut into round, 12 mm diameter sections, and placed individually into wells of a 12-well plate. The scaffolds were sterilized by immersing them in 70% ethanol and exposing them to UV light for 20 min. The ethanol was then removed, the scaffolds rinsed in sterile PBS, and left in the PBS overnight with the UV exposure.

2.2. Scaffold cellularization

The scaffolds were cellularized in their individual wells with mouse fibroblast L929 cells. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with10% fetal bovine serum (FBS), and antibiotics penicillin/streptomycin (100 IU/ml and 100 mg/ml), maintained at 37 °C at 5% CO_2. Prior to adding cells, the scaffolds were moistened with 1 ml of media. Scaffolds were cellularized by adding 1 ml of a cell suspension at varying concentrations (see Section 2.5.3.). The scaffolds were then returned to the incubator.

2.3. Scaffold staining

Scaffolds were fixed with 3.3% formalin in PBS, and incubated for 30 min. The fixative was then removed and 0.5% Toluidine blue solution in PBS was added to the scaffolds for 30 min under gentle rotation. The scaffolds were then rinsed with deionized water (DI). Images of the scaffolds were captured by brightfield microscopy.

2.4. Quantification of cell viability

In order to count viable cells, the scaffolds were rinsed in PBS and incubated in 1 ml of trypsin for 15 min. The detached cells were aspirated and transferred to 1.5 ml tubes and stained with 0.4% Trypan blue for cell viability. Cells were counted using a hemocytometer, where four counts (15 μl per count) were performed for each sample, and the averages determined.

2.5. Scaffold optimization

Three separate experiments were carried out to optimize the scaffolds in terms of cell adhesion and proliferation. Two of these involved the formulation of the scaffolds. The third looked at the concentration of cells that was added to the scaffolds.

2.5.1. Chitosan - gelatin ratio

The effect of varying the ratio of chitosan to gelatin on cell adhesion was investigated. Three different ratios (C:G) were evaluated (C_1G_5; C_2G_5; C_4G_5) in addition to chitosan alone (C) and gelatin alone (G). Both chitosan and gelatin concentrations were 1%. The scaffolds were cellularized with 1 ml of cells at 2×10^5 cells per ml. They were then decellularized at 24 h post-cellularization and cell counts were carried out. Four scaffolds were employed for each treatment, where 5 individual counts were carried out as described in Section 2.4 for each scaffold.