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Controlled and Sequential Delivery of Fluorophores from 3D Printed Alginate-PLGA Tubes

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Abstract

Controlled drug delivery systems, that include sequential and/or sustained drug delivery, have been utilized to enhance the therapeutic effects of many current drugs by effectively delivering drugs in a time-dependent and repeatable manner. In this study, with the aid of 3D printing technology, a novel drug delivery device was fabricated and tested to evaluate sequential delivery functionality. With an alginate shell and a poly(lactic-co-glycolic acid) (PLGA) core, the fabricated tubes displayed sequential release of distinct fluorescent dyes and showed no cytotoxicity when incubated with the human embryonic kidney (HEK293) cell line or bone marrow stromal stem cells (BMSC). The controlled differential release of drugs or proteins through such a delivery system has the potential to be used in a wide variety of biomedical applications from treating cancer to regenerative medicine.

Keywords

Extrusion printing; PLGA; Alginate; Controlled Delivery; Differential Delivery; Composite Materials

1. INTRODUCTION

The ability to control the means by which drugs are delivered, whether through pulsatile^{8, 14, 30}, sequential^{1, 26}, or on-demand release¹⁵, has the potential to provide more effective dosage regimens and enhanced therapeutic effects for various diseases and injuries.

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Not only do these systems have the potential to increase the efficacy of current drugs, they could also help to address many patient compliance and adherence issues caused by forgetfulness⁹, complicated dosage schedules⁷, and inability to physically handle the drugs²⁷. In the research presented here, we utilize two distinct fluorophores as model drug molecules to demonstrate a “proof of concept” 3D printed PLGA filled alginate tube capable of controlled sequential drug release. Sequential release involves a differential temporal release of two or more agents. Sequential release has been demonstrated using titania (TiO₂) nanotubes and polymer micelles to sequentially deliver both hydrophobic (indomethacin and itraconazole) and hydrophilic (gentamicin) drugs¹. Sequential drug release has also provided a potential cancer treatment system where the sequential release of ibandronate and tamoxifen has been reported to act synergistically in preventing the proliferation of an estrogen receptor-positive breast cancer cell line, MCF7¹¹. Sequential drug release may also be beneficial for tissue engineering. For example, a silica calcium phosphate nanocomposite scaffold has been developed that is capable of controlled drug delivery, initially providing protection from infection through the release of an antibiotic, followed by the release of a bone morphogenetic protein²¹.

The impact of three-dimensional (3D) printing in a range of industries is already evident and this technology is expected to have increasing applications in many biomedical applications with advancements in printer performance and resolution^{23, 28}, and the emergence of new bioprinting technologies²⁰. 3D printing of tablets has demonstrated the potential for controlled release of drugs^{6, 10, 12, 22, 25}. Here, we aimed to build on these previous tablet-based technological innovations and demonstrate for the *first* time the controlled and sequential release of distinct fluorophores from 3D printed PLGA and alginate hybrid tubes. Three-dimensional bioprinting of alginate tubes using a coaxial extrusion-based system was recently carried out to mimic natural vascular networks with the ultimate aim of generating blood vessels during scale-up tissue fabrication^{2, 3, 19}. Here, we have 3D printed a drug delivery device capable of sequential drug delivery by utilizing a double layer system. The delivery system comprises a 3D-printed (through a coaxial extrusion system) alginate tube housing a poly (lactic-co-glycolic acid) (PLGA) core. The PLGA core was added to fortify the structural integrity of the alginate tube as well as to provide versatility with respect to controlled drug delivery applications¹⁸. In addition, PLGA is a Food and Drug Administration (FDA) approved biocompatible and biodegradable polymer that has been used in many drug delivery systems¹⁶. Thus, in the study presented here, tubes comprising 4% w/v alginate (shell) and 1% (w/v) PLGA (core) (alginate-PLGA tubes) were fabricated, and tested for controlled sequential delivery of different fluorophores. Biocompatibility of the alginate-PLGA tubes used in our studies was assessed through cytotoxicity assays using a human embryonic kidney cell line, HEK293, and bone marrow stromal cells (BMSCs). Mechanical analysis was performed to test compressive strengths of various alginate versus alginate-PLGA tubes $\pm$ fluorophores in order to assess the contribution of the PLGA core to the mechanical strength of the tubes as well as testing if the integrity of the tubes was load-dependent. The development of these alginate-PLGA tubes through 3D coaxial-extrusion-based printing provides a unique drug delivery system capable of sequential release. Such devices have the potential to be used in a multitude of applications, including but not limited to, scaffold fabrication for bone regeneration and cancer vaccine/therapy implants.

2. MATERIALS AND METHODS

2.1. Alginate-PLGA Tube Fabrication

Sodium alginate (Sigma, St. Louis, MO) was used as the tube sheath material and calcium chloride (CaCl_2) powder (Sigma) was used as a crosslinking agent. Sodium alginate (4% w/v) and CaCl_2 (4% w/v) were each dissolved in sterile deionized water. The fabrication system consisted of a single-arm robotic printer (EFD[®] Nordson, East Providence, RI) controlled by a proprietary computer system and a homemade coaxial nozzle unit connected to a pneumatic air dispenser (EFD[®] Nordson) and a mechanical pump (New Era Pump System Inc., Farmingdale, NY) for alginate and CaCl_2 extrusion, respectively (Figure 1A). The coaxial nozzle comprised a 14-gauge outer needle and a 22-gauge inner needle (Figure 1B). Alginate precursor solution was dispensed through the sheath section of the coaxial-nozzle unit while CaCl_2 solution was dispensed through the core section. The alginate dispensing pressure was set at 82.7 kPa and the CaCl_2 dispensing rate was set at 16 mL/min. The 3D printed alginate tubes were then soaked overnight in 4% CaCl_2 solution to allow complete crosslinking. Poly (lactic-co-glycolic acid) 50:50 (RG503, Evonik, Darmstadt, Germany) was dissolved in chloroform (Sigma) at a concentration of 1% (w/v) and was then injected into the alginate tubes using a custom syringe unit (Hamilton Company, Reno, NV, USA). These PLGA-loaded alginate tubes, or alginate-PLGA tubes, were then clamped together by surgical micro-vessel clips (30 g) (World Precision Instruments, Sarasota, FL). The alginate-PLGA tubes were soaked for 48 h in deionized (DI) water to achieve maximum crosslinking and to prevent leakage. This soaking process also performed the function of potentially washing away any residual chloroform or chemical impurities.

2.2. Morphology Imaging of Alginate-PLGA Tube

Alginate shell and PLGA core layers were visualized and imaged using an optical microscope. From these images, the layer diameters were measured using the open source ImageJ software. The ultra-morphology of alginate-PLGA tubes was examined using Scanning Electron Microscopy (SEM). Samples were placed on aluminum stubs and left to dry overnight in ambient air for 24 h prior to being coated with gold-palladium using an argon beam K550 sputter coater (Emitech Ltd., Kent, England). Once coated, samples were imaged using a Hitachi S-4800 SEM (Hitachi High-Technologies, Tokyo, Japan).

2.3. Fluorophore Release Study

Release studies were performed with tubes comprising 4% (w/v) alginate (shell) and 1% (w/v) PLGA (core) where fluorescein (Molecular Probes, Eugene, OR) was mixed with alginate and rhodamine B (Sigma, St. Louis, MO) was mixed with PLGA. These fluorophores were chosen due to their distinct (non-overlapping) excitation and emission wavelengths. Alginate-PLGA tubes containing both dyes were made with one concentration of fluorescein (0.025 mg/ml) and either of two concentrations of rhodamine B (0.80 mg/ml (**R1**) or 0.40 mg/ml (**R2**)). These concentrations were chosen due to the solubility and the detection limits of the fluorophores. The two concentrations of rhodamine B were also selected to observe any concentration dependent effects on alginate-PLGA tube stability and fluorophore release. Alginate-PLGA tubes of 5 cm length were created by cutting with a surgical blade, ensuring that each section did not contain air bubbles. The cutting process

also provided a means to close off the ends of the tubes because the pressure applied by the incision created a seal at each end. These alginate-PLGA tubes were added singly to scintillation vials containing 3 mL of phosphate-buffered saline (PBS) and then placed in a shaker incubator set at 300 rpm and 37°C. In order to mitigate photodegradation of fluorophores, the vials were covered with aluminum foil. Samples were collected after 1, 3, 6, 12, 24, 48, 72, 120, and 168 h. Sampling involved removing 200 µl from the vial (being cautious to avoid sampling remnants of the alginate-PLGA tubes) and then 200 µl of fresh PBS was added back to the vials. The samples were measured for fluorescein ($\lambda_{\text{ex}}494$, $\lambda_{\text{em}}521$) and rhodamine B ($\lambda_{\text{ex}}540$, $\lambda_{\text{em}}625$) fluorescence using a SPECTRAmax M5 Microplate Spectrofluorometer (Molecular Devices, San Diego, CA). These readings were then compared to a standard curve to determine the amount of each fluorescence dye released. In addition, degradation (or loss of fluorescence) of the fluorophores was taken into account by monitoring (at $t = 0, 1, 3, 6, 12, 24, 49, 120, 144$, and 168 h) the concentrations of both fluorophore solutions in PBS in parallel samples at starting concentrations of 0.025 mg/ml fluorescein and 0.8 mg/ml rhodamine. The degradation of the fluorophores yielded a degradation rate equation of $y = 7.7 \ln(x) + 100$ for fluorescein and $y = -2.1 \ln(x) + 98$ for rhodamine B (Figure 2). Using these equations, the release study data were adjusted to account for any fluorophore degradation.

2.4 Individual Release of Fluorophore from Polymers

Release studies were performed with polymer and fluorophore combinations comprising either 4% (w/v) alginate (shell) or 1% (w/v) PLGA (core) with either fluorescein or rhodamine B. Alginate hydrogel was combined with rhodamine solution to yield a 0.80 mg/ml solution, while PLGA was solubilized and mixed with fluorescein or rhodamine B to yield a polymer-fluorophore mixture of 0.025 mg/ml and 0.80 mg/ml, respectively. Samples in one mL volumes of each desired polymer-fluorophore combinations were made, to which 3 mL of PBS was added and then placed in a shaker incubator set at 300 rpm and 37°C and were covered with aluminum foil. Samples were then collected after 1, 3, 6, 12, 24, 48, 72, 120, and 168 h. Sampling involved removing 300 µl out of each vial and replacing with 300 µl of PBS. The samples were measured for fluorescein ($\lambda_{\text{ex}}494$, $\lambda_{\text{em}}521$) and rhodamine B ($\lambda_{\text{ex}}540$, $\lambda_{\text{em}}625$) fluorescence using a SPECTRAmax M5 Microplate Spectrofluorometer (Molecular Devices). The readings were compared to a standard curve and photodegradation equations applied to normalize the results.

2.5. Evaluating Cell Viability and Cytotoxicity

Cell Lines and Cell Culture—Human embryonic kidney 293 (HEK293) cells and bone marrow stromal stem cells (BMSCs) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco®, Life Technologies Corporations, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10 mM HEPES (Gibco®, 50 µg/mL gentamycin sulfate (Cellgro, Manassas, VA), 1 mM sodium pyruvate (Gibco®), and 1 mM Glutamax (Gibco®). Both cell types were incubated at 37°C and 5% CO₂ in a humidified atmosphere.

Evaluation of Alginate-PLGA Tubes on Cell Viability—Cell viability studies were performed using an MTS assay (CellTiter 96®, Promega, Madison, WI). The MTS assay was performed following manufacturer's instructions. In brief, cells were plated in a 96-well plate at a density of 1×10^4 cells/well in 100 μ l of DMEM (+ supplements) for 24 h prior to treatments. The media was then aspirated and fresh media was added along with alginate-PLGA tubes of different lengths. The cultures were then incubated for 24 h, after which the alginate-PLGA tubes were removed, the media was aspirated and then replaced with fresh media. It was also crucial to ensure during media removal, no remnants of the alginate-PLGA tubes remained in the well because this could affect absorbance readings. Then, 20 μ l of MTS reagent was added and cells were incubated for a further 2 h. The absorbance was determined at 490 nm using a SpectraMax plus 384 Microplate spectrometer (Molecular Devices, Sunnyvale, CA). Relative cell viability was analyzed using untreated cells as the control group. The resultant absorbance of the soluble formazan at 490 nm is directly proportional to the cellular metabolic activity of living cells in each well.

2.6. Mechanical Analysis

Mechanical properties were measured using a dynamic mechanical analyzer (DMA Q800 V7.0 Build 113, TA Instruments, New Castle, DE) equipped with a submersion compression clamp in static mode. Prior to the measurements, the drive shaft position, clamp mass, clamp offset, and clamp compliance were calibrated according to the protocols suggested by the manufacturer. The samples were cut with a razor blade to be ~10 mm in length and the exact length and diameter of each sample was measured using calipers. Each sample was carefully transferred to the basin of the DMA clamp using forceps and the top portion of the clamp was gently lowered onto the sample. A preload force of 0.0001 N was initially applied and the force was then increased to a final value of 0.2 N at a rate of 0.02 N/min. Data were collected every 2 seconds as each sample was compressed. The stress was plotted against strain data with the slope of the line representing the compressive modulus of the sample.

2.7. Data Analysis

Data were analyzed using GraphPad Prism 6. All graphs were generated using GraphPad Prism 6. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test to compare the experimental group with a single control group. All error bars represent standard deviation.

3. RESULTS

3.1 Visualization and dimensions of alginate-PLGA tubes

Alginate tubes were printed through coaxial extrusion and then manually injected with PLGA. The fabricated device can be seen in Figure 3. The two layers were visualized using light microscopy (Figure 3B). With the addition of rhodamine B mixed into PLGA, it could be seen that the dye was homogenously mixed and dispersed throughout the core of the alginate-PLGA tube (Figure 3C). The diameters of the alginate-PLGA tubes ranged from 1.5 - 1.7 mm; however, tubes could be extruded to any length. The diameters of the alginate sheath layers and the PLGA cores were $140 \pm 14 \mu\text{m}$ and $1,200 \pm 120 \mu\text{m}$, respectively. Scanning electron microscopy images were also captured to visualize the surface

morphology of the alginate-PLGA tubes (Figure 3D). The small variation in diameter (1.5 – 1.7 mm) of the alginate-PLGA tubes may have been caused by the inherent variability associated with the manual injection of the PLGA solution that can result in a non-uniform dispersal of PLGA within the alginate tubes. This process of injection of the core solution will be automated in the future in an attempt to reduce diameter variability.

3.2 Release kinetics of fluorophores from alginate-PLGA tubes

Release studies were performed with alginate-PLGA tubes where fluorescein (0.025 mg/ml) was loaded into the alginate sheath whilst rhodamine B (0.8 mg/ml (R1) or 0.4 mg/ml (R2)) was loaded into the PLGA core. Sequential release was observed where fluorescein began to be released immediately (burst release) from the alginate sheath and the majority of this fluorophore was released within 24 hours (Figure 4). In contrast, no detectable rhodamine B was released for the first 24 hours followed by a steady release over the next 3 – 4 days.

In order to eliminate the possibility that chemical interactions between polymer and fluorophore impacted on release kinetics, the release rates of different polymer and fluorophore combinations were assessed. All combinations yielded similar release kinetics demonstrating a burst release for each fluorophore/polymer matrix combination (Figure 5). Of particular importance here, was the observation that rhodamine B was released from the PLGA polymer much faster (Figure 5B) than was observed for the alginate-PLGA tube (Figure 4) highlighting the influence of the alginate sheath in slowing release of this fluorophore.

3.3 Cytotoxicity of alginate-PLGA tube

In order to evaluate the effect of alginate-PLGA tubes on cell viability, HEK293 cells or BMSCs were cultured for 24 h in the presence of alginate-PLGA tubes of different lengths (ranging from 1 to 5 mm). After 24 h the alginate-PLGA tubes were removed and MTS reagent was added, as described in the methods section, to assess the viability of each of the differently treated cultures. As shown in Figure 6, the alginate-PLGA tubes were found to have no detectable detrimental effect on viability of HEK293 cells or BMSCs.

3.4 Compression moduli of alginate-PLGA tubes

The compression moduli were determined using dynamic mechanical analysis as described in the methods section. Comparisons of the compression moduli data obtained for empty alginate tubes versus alginate-PLGA tubes (with or without fluorophore) were made and the results are shown in Figure 7. It was found that alginate-PLGA (without fluorophore) had a four-fold higher compression modulus compared to empty alginate tubes. The loading of fluorophore into PLGA resulted in a concentration dependent reduction in the compression modulus.

4. DISCUSSION

The alginate-PLGA tubes manufactured in these studies comprised a 3D printed alginate (4% w/v) sheath and an injected PLGA core. The rationale for using 4% alginate solution was based on previous studies showing that 3 - 4% (w/v) alginate solutions were optimal for

generating structurally intact tubular structures^{3, 29}. The properties of the two-layered system used here were investigated, in vitro, for sequential drug delivery, biocompatibility and mechanical strength. The fluorophore release study performed with alginate-PLGA tubes loaded with fluorescein (in the alginate sheath) and rhodamine B (in the PLGA core) demonstrated the ability of the device to release two distinct molecules in a discrete and sequential fashion. The initial release of fluorescein followed by the delayed release of rhodamine B is consistent with the nature of the layered system, where the fluorescein diffusion through the alginate sheath occurred before the release of rhodamine B from the PLGA core. We showed that rhodamine B in combination with PLGA is released very quickly into solution when it is not incorporated into the core of the alginate tube (Figure 5B) thus demonstrating the ability of the alginate-PLGA tube to achieve sequential fluorophore release.

To demonstrate the potential biocompatibility of alginate-PLGA tubes, cytotoxicity assays were performed with HEK293 and BMSC cells, and no detrimental effects on cell viability for either cell type were observed. HEK293 cells were tested because they are routinely used in drug and gene delivery studies. Bone marrow stromal cells were tested due to the fact that they are widely used in tissue engineering, specifically for bone regeneration^{4, 5}. Mechanical testing revealed that insertion of a PLGA core enhanced the mechanical strength of the alginate tubes by up to 4-fold. The presence of rhodamine B in the PLGA core reduced the contribution of the PLGA core to the mechanical strength of the alginate-PLGA tube and we speculate that loading of any agent may have the same effect. This effect is possibly due to the dye (or any prospective drug) reducing the overall density of the PLGA polymer core^{13, 24}. Thus, if that is the case, the core formulation will require minor adjustments to improve its strength if such drug-loaded alginate-PLGA tubes are to be used in applications where compression moduli of > 2 MPa are required. Situations where a compression modulus of > 2 MPa would be important include the development and design of scaffolds for tissue engineering, where the scaffolds/implants are required to tolerate compression forces caused by cellular processes in order to maintain their structural integrity¹⁷.

The main purpose of this study was to design and test a proof of concept 3D printed controlled drug or molecule delivery system capable of sequential release. This was accomplished through the fabrication of a delivery device, comprising a 3D-printed outer alginate layer (or sheath) and an inner PLGA core, that was capable of differential release of fluorescent dyes. The sequential release observed was due to the delayed release of dye from the PLGA core with the surrounding alginate layer facilitating its retention, whilst the dye in the alginate layer was released more rapidly. It was also established that alginate-PLGA tubes were non-toxic in vitro for two cell types tested, suggesting their potential biocompatibility in a range of in vivo applications. The mechanical strength of alginate-PLGA tubes were greatly increased with the addition of PLGA. Further optimization is ongoing to further improve design features and mechanical strength of the alginate-PLGA tubes. This includes switching from manual to automated injection of PLGA solution, as well as manipulating layer thickness to optimize release profiles and mechanical strength. These alginate-PLGA tubes have rapid and practical processability, and have the potential to provide more efficacious drug treatments.

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Figure 1.

A) Schematic of the coaxial extrusion printing system utilizing a mechanical pump to extrude sodium alginate (blue fluid) and a dispensing unit to introduce CaCl₂ (yellow fluid). **B)** Illustration of the coaxial nozzle used to print alginate conduits tubes, where the hydrogel flow contains the sodium alginate and CaCl₂ solutions in the sheath and core sections, respectively.

Figure 2. Degradation curves for: **A)** fluorescein ($\lambda_{\text{ex}}494$, $\lambda_{\text{em}}521$) and **B)** rhodamine B ($\lambda_{\text{ex}}540$, $\lambda_{\text{em}}625$).

Figure 3.

Images of 3D printed alginate-PLGA tubes. **A)** A photograph of an alginate-PLGA tube. **B)** A light microscope image of an alginate-PLGA tube with the alginate sheath and PLGA core indicated with arrows. **C)** A light microscope image (clear shell showing alginate, light-purple core showing PLGA containing rhodamine) of an alginate-PLGA tube **B)**. **D)** A SEM image of alginate-PLGA tube.

Figure 4.

Cumulative release from: **A)** alginate-PLGA tubes containing 0.8 mg/ml of rhodamine B in the PLGA core and 0.025 mg/ml of fluorescein in the alginate sheath ($n = 3$) and **B)** alginate-PLGA tubes containing 0.4 mg/ml of rhodamine B in the PLGA core and 0.025 mg/ml of fluorescein in the alginate sheath ($n = 3$).

Figure 5. Cumulative release of fluorophores from: **A)** PLGA loaded with fluorescein (0.025 mg/ml), **B)** PLGA loaded with rhodamine B (0.8 mg/ml), **C)** alginate loaded with fluorescein (0.025 mg/ml), and **D)** alginate loaded with rhodamine B (0.8 mg/ml) ($n = 4$ for A, B and C).

Figure 6.

Cell viability assays: **A)** HEK293 or **B)** BMSCs were incubated with alginate-PLGA tubes of indicated varying lengths. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test at $n = 4$ (* signifies $p < 0.05$, ** $p < 0.01$).

Figure 7.

Compression moduli of blank alginate-PLGA tubes, alginate-PLGA tubes loaded with 0.025 mg/ml fluorescein (in alginate) and 0.80 mg/ml (R1) or 0.40 mg/ml (R2) rhodamine B (in PLGA), and alginate tubes alone. Statistical analysis was carried out using one-way ANOVA with Dunnett's multiple comparison test at $n=4$ (* signifies $p<0.05$, ** $p<0.01$, and *** $p<0.0001$).