Enzyme Prodrug Therapy Engineered into Electrospun Fibers with Embedded Liposomes for Controlled, Localized Synthesis of Therapeutics

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Enzyme prodrug therapy (EPT) enables localized conversion of inert prodrugs to active drugs by enzymes. Performance of EPT necessitates that the enzyme remains active throughout the time frame of the envisioned therapeutic application. β-glucuronidase is an enzyme with historically validated performance in EPT, however it retains its activity in biomaterials for an insufficiently long period of time, typically not exceeding 7 d. Herein, the encapsulation of β-glucuronidase in liposomal subcompartments within poly(vinyl alcohol) electrospun fibers is reported, leading to the assembly of biocatalytically active materials with activity of the enzyme sustained over at least seven weeks. It is further shown that liposomes provide the highly beneficial stabilization of the enzyme when incubated in cell culture media. The assembled biocatalytic materials successfully produce antiproliferative drugs (SN-38) using externally administered prodrugs (SN-38-glucuronide) and effectively suppress cell proliferation, with envisioned utility in the design of cardiovascular grafts.

Implantable biomaterials designed for controlled drug release have entered the mainstream of medicinal and pharmaceutical sciences. Indeed, delivery of hormonal contraceptives can be sustained for years using implantable matrices comprised of organic biodegradable polymers.[1,2] Hydrogel biomaterials with embedded growth factors that aid in wound healing are highly warranted in the treatment of diabetic ulcers.[3,4] Specific delivery of antiproliferative drugs from cardiovascular stents can prevent vascular smooth muscle cell proliferation and reduce the incidence of thrombosis and restenosis.[5–7] These designs are highly successful in their own right but are not without limitations. Specifically, using implantable biomaterials, it is still challenging to control drug dosing after implantation, to stop drug elution on demand, or to control the release of multiple drugs from the same implant. These opportunities are highly warranted in drug delivery, and designs of biomaterials enabling such drug dosing would open up broader opportunities in biomedicine.

Recently, we introduced an approach to accomplish advanced drug delivery design through engineering biomaterials equipped with the tools of enzyme prodrug therapy (EPT) to enable localized conversion of inert inactive prodrugs by enzymes.[8–11] Key to success of EPT is localization of the enzyme at the nominated target, which can be accomplished using antibody-enzyme conjugates (antibody-directed enzyme prodrug therapy, ADEPT).[12] Alternatively, enzymes can be expressed by cells upon their transfection, specifically using viral vectors. This mode of EPT is often termed gene-directed enzyme prodrug therapy (GDEPT) or “suicide gene therapy.”[13] The cells are transfected to produce enzymes that are unnatural to the mammalian cells, such as herpes simplex virus kinase. This kinase is particularly active in phosphorylating acyclovir, ganciclovir, and their close analogues leading to pronounced toxicity of the otherwise benign nucleosides to the cells expressing these genes. An elegant strategy to suicide gene therapy was developed by Kasahara and co-workers, whereby viral vectors were not only used to transfect the cells but also to achieve viral proliferation and infection of neighbor cells—leading to enhanced distribution of the viral vector, enhanced expression of the transgene, and thereby increased therapeutic benefits.[14]
In our hands, substrate-mediated enzyme prodrug therapy (SMEPT) proved to be highly enabling in the design of functional biomaterials. Specifically, we designed hydrogel biomaterials that contained the tools of biocatalysis, that is, the β-glucuronidase enzyme (β-glu). The same enzyme was capable of converting multiple prodrugs, as long as each of these was based on the same protecting trigger, glucuronic acid. Taking advantage of this, we achieved conversion of two or more fluorogenic (imaging) molecules and drugs. The same biomaterial was capable of generating two or more drugs—simply instructed by administration of the proper prodrug. When administered in sequence, biomaterials successfully produced anti-inflammatory drugs followed by cytotoxic drugs at the nominated time points—with relevance to the treatment of atherosclerosis.[9] When prodrugs were administered simultaneously, biomaterials were equipped with EPT-mediated combination therapy—a highly prized therapeutic opportunity for treatments of cancer and infectious diseases.[9] We have subsequently adapted SMEPT for applications within the multilayered polyelectrolyte surface coatings.[16] Assembly of these thin films is solution-based and can therefore be performed on biomaterials with no restriction on the surface geometry, topography, or materials porosity, such as wire meshes used in the production of balloons for cardiovascular angioplasty. Equipped with the tools of EPT, the multilayered coatings successfully mediated localized production of therapeutic drugs in static and under flow conditions. Most importantly, the therapeutic effect (cell killing) was the highest on the biocatalytic coatings and was significantly lower “downstream” on coatings with no immobilized enzyme, that is, cell killing through the bystander effect. Tools of EPT were also engineered into implantable biomaterials by Wang and Yang, specifically toward cardiovascular applications. Enzymes were immobilized on the surface of vascular stents, and local release of nitric oxide (NO), a key signaling molecule in the cardiovascular physiology, was achieved upon administration of NO prodrugs. Taken together, the prior reports presented above illustrate that tools of EPT engineered into implantable biomaterials significantly enable implants in terms of their functionality, specifically with regards to the broad opportunities associated with the localized synthesis of therapeutic agents for delivery to the cells and tissues in the immediate vicinity.

Biomaterials based on poly(vinyl alcohol) (PVA) have a long and successful history of biomedical applications. PVA hydrogels are biocompatible and are marketed as postoperative tissue sealants and as drug eluting beads for hepatic embolization. They can be formed through chemical or physical stabilization (crosslinking). In the latter case, hydrogelation is nondamaging and accommodates incorporation of proteins and live cells into the biomaterial. In our previous work, we observed that β-glu, being a relatively large-sized protein, can be incorporated into PVA hydrogels solely through size exclusion effects and does not necessitate chemical immobilization within the hydrogel as is the case for a number of other enzymes incorporated into PVA matrices, for example, for biomass conversion. However, we also observed that β-glu retained its activity for an insufficienly long period of time, typically not exceeding 7 d. In this work, we address significant limitations of SMEPT that were not considered in prior works but are critical to the performance of this platform in biomedical applications. Specifically, performance of EPT necessitates that the enzyme remains active throughout the time frame of the envisioned therapeutic application. For cardiovascular stenting, re-endothelialization typically takes at least six weeks—providing an indication of the desired duration of activity of the enzyme for SMEPT, and therefore leaving significant room for improvement in terms of achieving extended lifetime of the enzyme within the biomaterials.

We hypothesize that liposomes, which are vesicular structures formed by the self-assembly of amphiphilic lipids, can provide the sought-after stabilization of the enzyme during manufacturing, storage, and active use of the biomaterial in EPT. Motivated by this, in this work, we use electrospinning to engineer biomaterials based on PVA, which can be conveniently performed from aqueous polymer solutions, that is, conditions which are nondenaturing to the enzymatic cargo and facilitate the incorporation of liposomes. PVA fibers have been used as wound dressings and, in tubular form, as cardiovascular grafts. For the first time, we achieve immobilization of β-glu within the spun fibers through encapsulation in liposomal subcompartments, and illustrate that this strategy provides the highly sought-after stabilization of the protein against proteolytic degradation and loss of activity. In particular, we: (i) verify the integrity of the liposomes in electrospun fibers, (ii) demonstrate biocatalytic activity of β-glu that is loaded into the liposomes, (iii) investigate the long-term stability and functional activity of the enzyme, and (iv) demonstrate the local synthesis of antiproliferative drugs and their therapeutic effects on an in vitro model system. Here, we show that the assembled biomaterials performed well as biocatalytic matrices and mediated the synthesis of antiproliferative drugs—as warranted in the design of cardiovascular grafts and stents.

β-glu (0.1 mg mL⁻¹) was incorporated into liposomes composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmityl-sn-glycero-3-phosphocholine (DPPC) (DMPC:DPPC = 2:3). Encapsulated enzyme was then mixed into a PVA solution (12 wt% in water), which was processed into nonwoven fibers by electrospinning (Scheme 1). We stabilized our liposome-containing fibers using a kosmotropic electrolyte, sodium sulfate (Na₂SO₄), and their morphology before and after stabilization was assessed using scanning electron microscopy (SEM). Figure 1a shows morphology of a nonwoven mesh as is typical for electrospun fibers and reveals that incorporation of liposomes into the feed polymer solution has minimal detrimental effects on the suitability of PVA solution for electrospinning. Fiber diameter and porosity were characterized by ImageJ analysis and determined to be 307 ± 71 nm and 77 ± 5%, and 350 ± 74 nm and 77 ± 4%, for fibers before and after stabilization, respectively (Table 1).

To confirm the incorporation of liposomes, we visualized the fibers using fluorescence microscopy. For this purpose, liposomes composed of DMPC and DPPC were prepared to contain 1.5 mol% of 1,2-dipalmityl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE), and fibers were produced using PVA solutions with two different concentrations of fluorescently labeled liposomes: 1% v/v (Figure 1bi and Figure S1, Supporting Information) and 5% v/v (Figure 1bii). Images show punctuated fluorescent signals (best
viewed at low content of liposomes in the fibers, Figure 1bi and Figure S1, Supporting Information) as would be expected from labeled liposomes. Increasing the content of liposomes in the polymer solution afforded an expected increase in the abundance of fluorescent subcompartments. Even at the higher concentration of liposomes (5% v/v), fibers reveal nonmerged fluorescent signals, suggesting the existence of individual liposomes within the fibers (Figure 1bii).

To verify the integrity of liposomes in the electrospun fibers, we encapsulated a fluorescent probe, carboxyfluorescein (CF, \(50 \times 10^{-11} \text{m}\)), in liposomes, and produced PVA fibers containing CF-loaded liposomes. We incubated stabilized electrospun fibers in phosphate buffered saline (PBS, pH 7.4) for 1 h at 24 or 37 °C and analyzed the fluorescence intensity of supernatants. Liposomes composed of a DMPC:DPPC (2:3) mixture have a phase transition temperature (\(T_m\)) of 34 °C, above which the lipids are in a disordered liquid-like phase and the liposomes are expected to have enhanced permeability to solutes. In full accord with this, we detected a two-fold higher CF released in supernatants at 37 °C as compared to the 24 °C incubation (Figure S2, Supporting Information). On the other hand, when CF was not encapsulated in liposomes and was instead directly mixed with PVA for the electrospinning process, the fluorescent molecules were readily detected in the supernatant and the behavior was not dependent on temperature (within the range from 24 to 37 °C). This provides evidence that the liposomes remained intact within the structure of the electrospun fibers and that cargo release can be mediated via phase transition temperature of the liposomes.

To further validate the integrity of liposomes and to illustrate the functional activity of \(\beta\)-glu within liposomes, we investigated enzymatic conversion of a model fluorogenic substrate, fluorescein di-\(\beta\)-\(D\)-glucuronide (FDGlcU), to fluorescein (Figure 2a). This probe is virtually nonfluorescent and exhibits a drastic increase in the quantum yield of fluorescence upon cleavage of the glucuronide bonds by \(\beta\)-glu. PVA fibers containing \(\beta\)-glu-loaded liposomes were incubated with FDGlcU (5 \(\mu\)g mL\(^{-1}\)) in PBS for 30 min at 24 or 37 °C, and the fluorescence intensity of supernatants was analyzed. Incubation of the fibers with FDGlcU at 24 °C afforded a minor increase in fluorescence, likely due to a restricted access of the substrate into the liposomes, and indicated activity of the enzyme in the fibers (Figure 2b). Increasing the temperature to 37 °C resulted in a four-fold higher fluorescence signal, indicating significantly increased conversion of the fluorogenic substrate into its product by \(\beta\)-glu due to the enhanced permeability of FDGlcU through
the lipid bilayer above the phase transition temperature of the lipids. The presented results further confirmed that the liposomes remained intact within the fibers. By varying the concentration of the administered substrate (0.05, 0.5, or 5 µg mL$^{-1}$ of FDGlcU), the amount of produced fluorescein can be tuned, providing a convenient tool to externally program the catalytic output of the enzyme containing fibers (Figure 2c).

Table 1. Diameter and porosity of PVA fibers with embedded liposomes before and after stabilization through a sodium sulfate (Na$_2$SO$_4$) treatment.

|                  | Diameter [nm] | Porosity [%] |
|------------------|---------------|--------------|
| Before stabilization | 307.7 ± 71.1  | 77.3 ± 4.6   |
| After stabilization | 350.2 ± 74.1  | 77.0 ± 4.0   |

To evaluate the stability of the encapsulated enzyme within electrospun fibers and to illustrate the long-term performance of the designed biocatalytic materials, we repeated the enzymatic catalysis over several cycles by periodically removing fluorescent products (fluorescein) and adding fresh FDGlcU (5 µg mL$^{-1}$) to the same PVA fibers containing β-glu-loaded liposomes. Altogether, ten rounds of readout were made over a period of seven weeks (Figure 2d). Fluorescence of the supernatant, as achieved through enzymatic conversion of FDGlcU by the encapsulated enzyme, was only marginally lower at the reading taken on week 7 as compared to the readings during

Figure 2. Biocatalytic activity of electrospun PVA fibers containing β-glucuronidase-loaded liposomes. a) Enzymatic activity of β-glucuronidase results in the release of fluorescein (products) from fluorescein di-β-d-glucuronic acid (FDAcU, substrates). Liposomes have enhanced permeability to FDGlcU above the phase transition temperature ($T_m$) of the lipids. b) Enzymatic conversion of FDGlcU (5 µg mL$^{-1}$) to fluorescein by PVA fibers containing β-glucuronidase-loaded liposomes at 24 or 37 °C (below or above the $T_m$ of the lipids, respectively). c) By varying the concentration of the administered substrate (0.05, 0.5, or 5 µg mL$^{-1}$ of FDGlcU), the amount of fluorescent product produced by PVA fibers containing β-glucuronidase-loaded liposomes can be controlled. d) The enzymatic catalysis of FDGlcU to fluorescein was repeated over ten cycles by periodically removing hydrolyzed substrates and adding fresh FDGlcU (5 µg mL$^{-1}$) to the same PVA fibers containing β-glucuronidase-loaded liposomes. e) Fluorescein was produced at a similar rate and the enzymatic activity was preserved at a nearly constant level over seven weeks.
the first days of incubation (Figure 2e), thus illustrating that the enzymatic activity was preserved at a nearly constant level. In other words, encapsulation of β-glu within liposomes as subcompartments within electrospun fibers affords a highly stable preparation of biocatalytically active materials for SMEPT and other biomedical applications with activity of the enzyme sustained over at least seven weeks. This time frame exceeds which is quoted to be the typical time required for re-endothelialization of vascular grafts,[26] and the fibers were stable over 14 weeks at physiological conditions (Figure S3, Supporting Information).

Further tests into stability of the enzyme within liposomal subcompartments in PBS were performed and compared to those in cell culture media. β-glu-loaded liposomes or non-encapsulated β-glu were incubated in PBS or in cell culture media, and the enzymatic activity was monitored over time through conversion of FDGlcU to fluorescein. In PBS, β-glu (encapsulated and nonencapsulated) exhibited only a minor loss of activity over 48 h of incubation (Figure 3a). In contrast, in cell culture media, this enzyme underwent drastic deactivation and over 48 h of incubation, catalytic output of the β-glu in the absence of liposomes decreased by over 100-fold (Figure 3b). Liposomes provided stabilization of the enzyme, which exhibited only a minor degree of deactivation in cell culture media. These results further confirm the benefit and necessity of liposomal encapsulation in protecting enzymes and maintaining their functional activity. This result is highly encouraging and together with the data in Figure 2, presents a highly active, durable biomaterial with sustained enzymatic activity.

Finally, to illustrate the therapeutic utility of electrospun PVA fibers containing β-glu-loaded liposomes, we engineered these biocatalytic materials to produce an antiproliferative drug, SN-38, using its benign, externally administered prodrug (SN-38-glucuronide). Human cancer-derived cell lines are fundamental models to test the therapeutic efficacy of antiproliferative drugs, and in this study, we employed human cervical cancer cells (HeLa) as an in vitro model system. Stabilized PVA fibers containing β-glu-loaded liposomes were immersed and positioned immediately over HeLa cells cultured in 96-well plates—in a setting to investigate drug delivery to surrounding cells and tissues. SN-38-glucuronide (1 × 10^{-6} m) was added into the cell culture media and cell metabolic activity was monitored after 48 h treatment. SN-38 (1 × 10^{-6} m) was used as a positive control. The presence of spun fibers did not alter metabolic activity of the cells and had no significant effect on the antiproliferative activity of SN-38 (Figure 4 and Figure S4, Supporting Information). With or without the fibers, the presence of SN-38 led to a decrease of metabolic activity of the cells to ~40%. We found that IC_{50} for SN-38 corresponded to 47 × 10^{-9} m (Figure S5, Supporting Information). Addition of SN-38-glucuronide in the absence of fibers produced a minor change in the cell viability, most likely due to minor impurities of SN-38 in the sample of β-glu-glucuronidase-loaded liposomes or nonencapsulated β-glu in a) PBS or b) cell culture media for 0, 24, and 48 h through monitoring the conversion of fluorescein di-β-o-glucuronide (FDGlcU) to fluorescein. Liposomes provided stabilization of the enzyme with only a minor degree of deactivation when incubated in cell culture media. *p < 0.05, **p < 0.01.

**Figure 3.** Effect of liposome encapsulation on the stability of β-glucuronidase. Enzymatic activity was evaluated after incubation of β-glucuronidase-loaded liposomes or nonencapsulated β-glu in a) PBS or b) cell culture media for 0, 24, and 48 h through monitoring the conversion of fluorescein di-β-o-glucuronide (FDGlcU) to fluorescein. Liposomes provided stabilization of the enzyme with only a minor degree of deactivation when incubated in cell culture media. *p < 0.05, **p < 0.01.

administration of SN-38-glucuronide led to local synthesis of antiproliferative drugs by the assembled biocatalytic biomaterials. With a supply of administered prodrugs, SN-38 can be delivered, and EPT enables the opportunities to control the drug dosage within the lifetime of the enzyme. Liposome properties can be tuned by varying lipid composition or by adding components to the lipid mixture during liposome preparation.[34,35] Electrospun fibers can be collected onto a rotating mandrel to assemble tubular conduits with utility as vascular grafts, and PVA is among the materials investigated toward these applications.[31,32,36] Commercially successful vascular stents on the market are engineered to continuously release antiproliferative drugs (e.g., paclitaxel for Vascular Wrap[6,37]) to minimize thrombosis and restenosis. This knowledge served as an inspiration to engineer PVA-based biomaterials to synthesize antiproliferative drugs via EPT for vascular applications.
Experimental Section

PVA Fibers Containing β-Glucuronidase-Loaded Liposomes: PVA (Mw = 85 000–124 000 Da, Sigma-Aldrich) was dissolved in distilled water under constant stirring at 30 °C to generate a 12 wt% solution. Prior to spinning, 10 mL of PVA solution was supplemented with 500 µL of β-glu-loaded liposomes (0.1 mg mL⁻¹ β-glu, 5 mg mL⁻¹ lipid). Polymer solutions were processed by electrosprinning on a custom-built device as previously established.²⁴ Briefly, the device consisted of two high-voltage sources (ETPS Ltd.), one syringe pump (NE-1010 High Pressure Syringe Pump, World Precision Instruments), providing a constant polymer flow through a needle (blunt needle, 18G Terumo), and a planar stainless steel collector. All equipment was placed in an electrically grounded Faraday cage (Phoenix Mechno Ltd.). The positive voltage was applied to the needle, whereas negative voltage was supplied to the collector. High voltage was controlled by a custom-made software based on LabVIEW (Swiss Federal Laboratories for Materials Science and Technology, Switzerland) via a LabJack interface (RS Components).

Fibers were spun at 10/–5 kV, 20 µL min⁻¹ flow rate, and a distance of 15 cm.

Biocatalytic Activity of PVA Fibers Containing β-Glucuronidase-Loaded Liposomes: Electrospun PVA fibers containing β-glu-loaded liposomes were incubated in 0.5 w Na₂SO₄ for 1 h, followed by washing via immersion in PBS for 1 h. Stabilized fibers were incubated with 0.05, 0.5, or 5 µg mL⁻¹ of FDGlcU (Molecular Probes) in PBS for 30 min at 24 or 37 °C, and fluorescence intensity of products (fluorescein) was monitored using a SpectraMax M5 microplate reader (Molecular Devices) (λex = 495 nm; λem = 520 nm). The enzymatic conversion of FDGlcU to fluorescein was repeated over several cycles by periodically removing fluorescent products (fluorescein) and adding fresh FDGlcU (5 µg mL⁻¹) to the same PVA fibers containing β-glu-loaded liposomes.

Detailed methods are available in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

β-glucuronidase, electrosprinning, enzyme prodrug therapy, liposomes, polymer fibers

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