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Enzyme Prodrug Therapy Engineered into Biomaterials

*Ana C. Mendes and Alexander N. Zelikin**

In this work, enzyme-prodrug therapy (EPT) is engineered into hydrogel biomaterials to achieve localized synthesis of the drugs and their delivery to the adhering cells. The use of EPT in the context of drug delivery mediated by biomaterials significantly empowers the latter in that the same hydrogel is used to successfully synthesize several drugs with dissimilar structures and therapeutic effects. The concentration of the synthesized drugs is conveniently controlled by the concentration of the administered prodrugs. Using prodrugs for two therapeutic agents allows their synthesis and delivery with independent control over the concentration and the time of administration of each of the drugs. Using these tools, sequential delivery of drugs for anti-inflammatory and anti-proliferative activity is accomplished whereby the synthesis of drugs is mediated by the same enzyme-functionalized hydrogel. The use of EPT to perform combination therapy mediated by an implantable biomaterial is also reported. Taken together, these results contribute significantly to the development of flexible and highly powerful tools of substrate-mediated drug delivery with applications in the design of therapeutic implants and tissue engineering.

1. Introduction

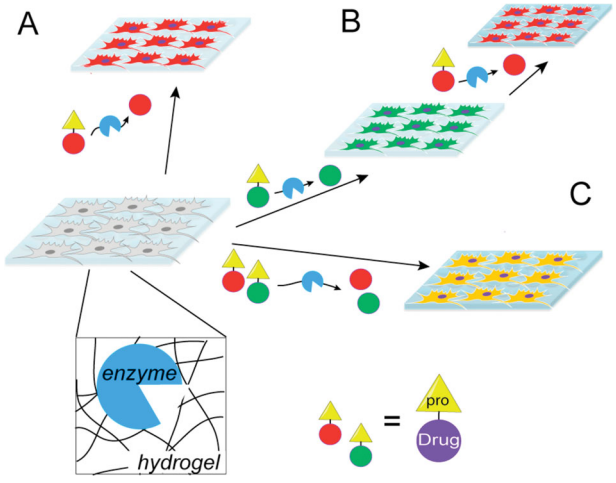
Enzyme prodrug therapies (EPT) comprise a suit of techniques that allow synthesizing the drugs locally, at the site of action, using systemically administered benign prodrugs.[1–5] Compared to conventional drug administration techniques, EPT creates a higher local concentration of the therapeutic thus favouring enhanced response. At the same time, EPT results in a lower systemic distribution of the drug thus minimizing side effects. Key to success of EPT is the choice of the enzyme and highest specificity of treatment is achieved with the use of enzymes to which human analogues do not exist (e.g. bacterial lactamase) or which exhibit a localized distribution (e.g. glucuronidase). Enzyme localization at the site of action can be achieved via a number of ways, such as with the use of an antibody to navigate the conjugated enzyme to the desired site (antibody directed EPT, ADEPT).[1] Another method includes association of the enzyme with a polymer chain in which case accumulation of the conjugate in the tumour tissue is achieved via the enhanced permeation–retention effect (polymer directed EPT, PDEPT).[3] Gene transfer and expression of a non-natural enzyme within a defined tissue (gene directed EPT, GDEPT)[4,6,7] has also proven to be a successful technique attracting significant research attention. Bacteria directed EPT relies on the use of bacteria which were genetically engineered to produce a prodrug-converting enzyme and this tool has also shown promise in anti-cancer therapies.[8]

EPT strategies have proven to be highly successful tools of biomedicine being significantly advantaged over systemic administration of the drugs in achieving localized, site-specific therapeutic effect. Further to this, EPT stands out as unique tools of drug delivery in that each individual enzyme molecule successfully placed at the desired site performs numerous acts of drug synthesis thus achieving a tremendous "amplification of the deliverable payload". However, phenomenological limitation of EPT techniques as described above is a requirement to navigate the enzyme to the desired site, a task that presents itself as highly challenging. Indeed, antibody-assisted targeting of conjugated drugs is highly successful yet only ca. 1% of the payload actually reaches the desired target,[9] and this limitation is inherited by ADEPT. PDEPT strategy faces the same obstacles as its sister-technique, polymer therapeutics, whereby drugs are directed to the tumour via the passive or active targeting.[10,11] GDEPT is as challenging as any gene delivery approach and remains successful in laboratory settings but highly challenging in clinic.

Recently, to overcome the above mentioned obstacles, we hypothesized that an enzyme can be physically placed at the desired site of action, similar to the highly successful techniques employed for the site specific drug delivery using eroding biomaterials or drug-eluting surface coatings. We proposed to engineer the tools of EPT into biomaterials to achieve "substrate mediated enzyme prodrug therapy", SMEPT.[12,13] Towards this end, we used an enzyme with excellent prior characterization in EPT techniques, β-Glucuronidase (β-Glu),[1,12] functionalized hydrogel biomaterials[13] and multilayered polymer coatings[14,15] with this enzyme, and subsequently used the resulting biomaterials as substrates for mammalian cell culture. Biocatalytic hydrogels and coatings successfully accomplished conversion of the externally added prodrugs for potent anti-cancer agents for delivery to adhering cells, thus verifying the proof of concept of SMEPT.[13–15] Both the hydrogel biomaterials and the coatings

Dr. A. C. Mendes, Dr. A. N. Zelikin
Department of Chemistry
Aarhus University
Aarhus 8000, Denmark
E-mail: zelikin@chem.au.dk
Dr. A. N. Zelikin
iNano Interdisciplinary Nanoscience Centre
Aarhus University
Aarhus 8000, Denmark

DOI: 10.1002/adfm.201304312



Scheme 1. Enzyme-prodrug therapy (EPT) is a strategy applied to achieve site specific synthesis and localized delivery of the drugs. In this work, EPT is engineered into hydrogel biomaterials and this affords several advanced opportunities in drug delivery mediated by implantable biomaterials. Specifically, EPT allows facile adjustment of the drug dosage in monotherapy (A), synthesizing two drugs at nominated timepoints for sequential, dissimilar therapeutic responses (B) and combination therapy (C).

retained their enzymatic activity over as long as 7 days.[14,16] Furthermore, in the case of polymer coatings, we demonstrated that EPT allows an on-demand synthesis of the product at the desired time, for which purpose we used flow chambers and illustrated substrate conversion under flow conditions.[15] This experimental setup also revealed that a more pronounced therapeutic effect was achieved locally, at the site of drug synthesis, compared to that observed on cells proliferating "downstream" from the location of drug release. In this work, we build on the success of our initial reports and investigate utility of enzyme-functionalized, biocatalytic biomaterials in several substrate mediated drug delivery settings (**Scheme 1**).

Flexibility of drug dosage is a feature characteristic of conventional, pill-based drug administration. Indeed, treating a common head-ache is highly flexible in that patients have a choice of drugs (aspirin, paracetamol, ibuprofen, etc), choice of the drug dosage, and choice of time of administration. However, oral administration is highly non-specific and only a minor fraction of the active ingredient actually reaches the site of action. In contrast, drug delivery using implantable biomaterials is site specific, that is, release of the embedded therapeutic is achieved locally.[17,18] However, typical examples of substrate- or surface- mediated drug delivery are completely devoid of the flexibility of dosage as defined above. Individual implants or coatings are designed to release a particular drug at an engineered dose in unit time and it is practically impossible to e.g. change the drug, double the dose, or stop drug release altogether. By design, EPT strategies are in a unique position to combine the benefits of the two drug delivery approaches discussed above. Thus, localized synthesis of the drug affords the benefits that are well advertised in the context of site-specific drug delivery. In turn, external administration of the prodrugs shares similarities with the pill-based drug administration with regard to the flexibility of drug dosage. The latter opportunity is made possible by that the same enzyme is poised to convert a panel of prodrugs, taken individually or in a combination, and achieve their delivery to the surrounding cells, organs and tissues in a site-specific manner. However, these opportunities remain un-fulfilled and potential of EPT strategies to achieve these highly promising possibilities is to be unravelled. In this work, we specifically address this issue and on the example of SMEPT investigate advanced opportunities in drug delivery. In particular, we investigate the time-controlled synthesis of the desired therapeutics as well as combination therapy mediated by the enzyme-functionalized biomaterials.

As biomaterial hosts for SMEPT, we chose hydrogels based on poly(vinyl alcohol), PVA, well-characterized matrices based on a polymer with decades of utility in biomedicine and clinic.[19–21] PVA-based materials are formulated as drug eluting beads which are then used as embolic bodies in trans-arterial catheter embolization for localized delivery of chemotherapeutic agents to treat hepatocellular carcinoma.[21,22] Vascular grafts based on PVA are investigated due to a suit of favourable characteristics such as good tissue compatibility and tuneable mechanical characteristics.[23] This hydrogel-making polymer is also popular in production of electrospun fibres for applications in tissue engineering.[24] Unlike fibres prepared using organic, water-insoluble polymers, PVA is readily dissolved in water and affords well-defined fibres with incorporated cargo such as proteins[25–28] and liposomes.[29] We believe that biomedical applications using these materials would become significantly empowered once functionalized with tools of biocatalysis for localized synthesis and site-specific delivery of drugs.

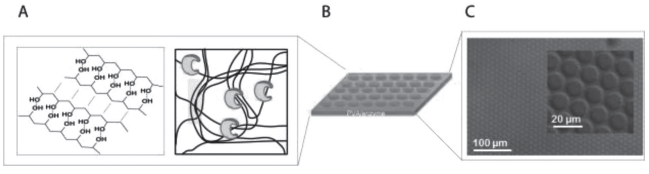
As candidate drugs, we focused on anti-inflammatory therapeutics as well as cytotoxic, anticancer drugs. Scope and utility of the latter in chemotherapy significantly depends on the opportunities in targeted drug delivery.[9,11,21,30] Tools of EPT are particularly important in this context[31] and, together with success of SMEPT in achieving localized synthesis and delivery of anti-proliferative drugs,[15] this justifies broader investigation of SMEPT for these applications. In turn, anti-inflammatory drugs are less toxic and are generally regarded as safe medication agents. However, the Centre for Drug Control in the USA reports that up to 18,000 deaths are registered annually in the US alone as a result of overdose using non-steroidal anti-inflammatory drugs (NSAID). From a different perspective, NSAIDs and other anti-inflammatory agents are gaining recognition as tools in anti-cancer[32–34] and anti-viral[35] therapy further highlighting the need for development of tools for localized delivery of these drugs.

Specific aim of this project was to investigate flexibility of EPT strategies with regard to the choice of drugs, facile drug dosing, and the potential to achieve substrate-mediated combination therapy. Novelty of this presentation lies in that to the best of our knowledge, we provide the first example of drug eluting biomaterials with a facile external control over the nature of the drug to be released (anti-inflammatory, cytotoxic), drug dosage, and time of release. Importance of this is that these possibilities are demonstrated using hydrogel biomaterials that are already used in biotechnology and biomedicine, including clinical applications.[19–21,23] In doing so, our results significantly extend functionality of these and other

biomaterials and open up novel opportunities in design and applications of functional matrices for drug delivery and tissue engineering.

2. Results and Discussion

Hydrogels are among the most widely used biomaterials and are truly unique due to a set of characteristics not observed in any other material.[36–40] Specifically, high degree of hydration of these three-dimensional matrices renders hydrogels human tissue-like and makes these materials indispensable in applications such as tissue engineering.[39,41,42] Historically, characterization of the hydrogel samples was typically conducted using macroscopic, bulk specimen. Advent of nano/microtechnology enabled detailed investigation of biointerfaces and associated techniques have become imperative in the characterization of biomaterials.[43–45] In our previous reports, we developed a method to produce PVA hydrogels as micrometer-thin, surface adhered materials and used the arsenal of surface characterization and visualization techniques for an in-depth investigation of these matrices.[46–48] Specifically, we used micro-transfer molding (μTM) to replicate engineered topography of elastomeric stamps with dimensions of individual features varied across the length scales, from centimetres to (sub)micrometers. Surface adhered format of hydrogels enabled facile investigation and optimization of these matrices with regard to kinetics of their biodegradation,[16] opportunities in bioconjugations,[47] and substrate-mediated drug delivery.[13] Specifically for the latter, surface-adhered hydrogels were used as substrates for mammalian cell culture and this allowed quantifying intracellular processes using relatively high throughput, multi-well plate assays. This format also afforded facile visualization of the cells and interfaces using readily available tools of microscopy.[46,48] These opportunities make surface-adhered hydrogels convenient tools to investigate drug delivery achieved through the EPT and hydrogel biomaterials. In this work, micro-structured (μS) PVA hydrogels were prepared via μTM by casting solutions of mixtures of PVA with β-glucuronidase between a patterned elastomeric stamp and a glass cover-slip in a custom made clamps at finger-tight pressure for 24 h. Separation of the coverslip form the stamp resulted in glass-adhered PVA-based materials. Stabilization of the hydrogels was achieved using a kosmotropic electrolyte, sodium sulfate, and subsequent rehydration in PBS afforded the target preparation, μS PVA hydrogels (**Scheme 2**).



Scheme 2. Schematic illustration of the structure of the enzyme-functionalized physical hydrogels based on PVA (A) and micro-structured topography of the hydrogel biomaterials used in this study (B) as well as a differential interference contrast (DIC) image of the hydrogels in their hydrated state in PBS (C).

2.1. Choice of the Prodrug Defines the Active Ingredient

Degradable and/or erodible matrices as well as surface coatings have been developed to accommodate sustained delivery of diverse cargo, from small organic molecules to proteins and nucleic acids, with fine-tuned kinetics and duration of drug delivery.[17,18] Significant limitation of these therapeutic implants, powerful in their own right, is that their design does not allow for a change in drug prescription. As a result, the same mass-produced biomaterial is expected to cater successfully for each case – disregarding patient-to-patient variation, differences in susceptibility to treatment etc. In contrast, EPT strategies are poised to overcome this shortcoming and should allow nominating the drug of choice through a selection of an appropriate candidate prodrug. To investigate this for biocatalytic biomaterials, and with broader interest in hepatic drug delivery[49–51] and specifically in the treatment of hepatocellular carcinoma (HCC), we used a hepatocyte cell line (HepG2). Identical β-Glu functionalized hydrogels were used as substrates for cell culture and three different prodrugs were used to elicit the same therapeutic response, i.e. cell killing, with relevance to anticancer treatment. SN-38 belongs to the class of topoisomerase I inhibitors and elicits anti-cancer effect through the interruption of DNA replication.[52] 5-fluorouracil (5-FU) is an antimetabolite drug acting through inhibition of thymidylate synthase.[53] Curcumin has the capability to modulate activity of the growth factors, functioning of receptors, cytokines, enzymes, and modulate transcription of genes regulating cell proliferation and apoptosis.[54] For SMEPT, hepatocytes were seeded onto biocatalytic hydrogels and allowed to adhere to the substrate for 24 h, following which the (pro)drugs were administered and cell culture was conducted for additional 48 h. After this time, metabolic activity of the cells was quantified using a commercial cell viability assay. In control experiments, pristine drugs (10 μM SN-38, 100 μM curcumin, 100 μM 5-FU) afforded a statistically significant decrease in cell viability, **Figure 1**. Magnitude of this effect was different and viability of the cells dropped to <20% for SN-38 and curcumin but was at 60% for 5-FU reflecting poor suitability of the latter anticancer agent for the anti-proliferative treatment of this hepatocyte cell line. Corresponding prodrugs in the absence of β-Glu have a negligible effect on the cell viability revealing that hepatocytes do not possess inherent glucuronidase activity and conversion of the prodrugs necessitates the presence of the enzyme in the biomaterial (Supporting Information, Figure S1). In turn, administration of the prodrugs under SMEPT conditions resulted in therapeutic responses matching well that of the corresponding drugs. In each case, cell viability was marginally higher in the case of the prodrugs possibly reflecting an incomplete substrate conversion. However, loss of activity in the case of prodrugs is expected and this shortcoming is typically over-compensated by pharmacokinetic gains.[55,56] Results in Figure 1 demonstrate that with SMEPT, very much alike traditional "pill based" drug administration, it is possible to nominate the desired drug, find an optimized agent to elicit the required therapeutic effect, and achieve this using the same biomaterial. This possibility may prove particularly important for the treatment of HCC for which case drug eluting hydrogel beads have shown significant promise as tools for localized drug delivery.[57] We envision that

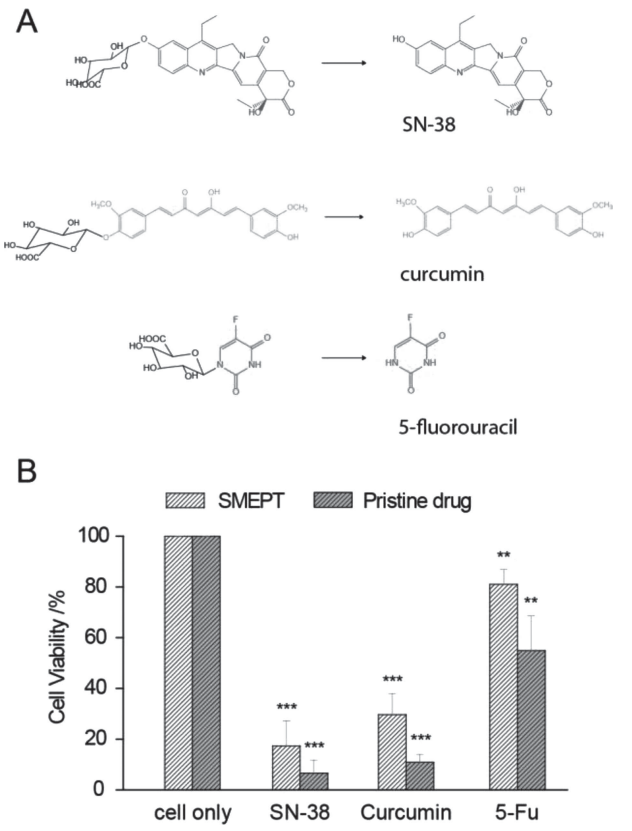


Figure 1. (A) Chemical formulae of the glucuronide derivatives and schematic illustration of glucuronide conversion into respective pristine drugs for the nominated anti-proliferative therapeutics, SN-38, curcumin, and 5-fluorouracil. (B) Viability of hepatocyte cells cultured in the presence of 10 μM SN-38, 100 μM curcumin, 100 μM 5-FU of the same concentration of corresponding glucuronide derivatives under SMEPT conditions, i.e. cell culture on enzyme-functionalized hydrogel biomaterials. Presented results are average of at least three independent experiments and are presented as mean ± standard deviation.

coupled with EPT, implantable hydrogel beads will become significantly empowered tools for anticancer treatment.

2.2. SMEPT Provides Facile Adjustment of Drug Dosage

Another shortcoming of surface mediated drug delivery, such as performed by implantable devices, lies in a lack of opportunities to control the drug dosage. Once designed and engineered using materials properties, the amount of the drug released in unit time cannot be changed externally. In contrast, traditional systemic drug administration allows doing this via simple adjustment of the administered dose. EPT strategies and SMEPT in particular inherit this feature from systemic administration techniques and are designed to afford facile adjustment of the amount of the drug released by a biomaterial through the choice of prodrug concentration administered externally. To verify this, we used a macrophage cell line (RAW 264.7) and lipopolysaccharide (LPS) stimulation of the cells towards inflammatory condition. Using this tool, we investigated opportunities in adjusting the dosage of anti-inflammatory therapeutics released by a hydrogel biomaterial using concentration of the corresponding prodrug.

Inflammatory processes are largely mediated by two enzymes, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).[58] Improper up-regulation of these enzymes can result in pathophysiology of certain types of human cancers as well as inflammatory disorders. Curcumin plays a major role in anti-inflammatory activities via the inhibition of COX-2 and iNOS.[54,58] However, this drug has significant associated problems such as limited stability and a low solubility in aqueous solutions. In contrast, glucuronide derivative of curcumin shows significantly enhanced solubility in physiological buffers thus making EPT strategy for delivery of this drug further attractive. For both, pristine drug and its glucuronide derivative, therapeutic response and associated cytotoxicity of treatment was quantified in LPS-stimulated macrophages using concurrent administration of the (pro)drug and LPS and 24 h duration of cell culture. Relative levels of nitric oxide and cell viability were determined through the Griess assay[59] and PrestoBlue reagent, respectively. Addition of LPS resulted in a pronounced increase in the levels of nitric oxide produced by macrophages (from those below the detection limit of the Griess assay to > 20 μM). Upon pro-inflammatory stimulation, macrophages also exhibited a ca. 20% decrease in the rate of their proliferation compared to the non-treated cells (**Figure 2**). Administration of curcumin glucuronide in concentrations ranging from 0.1 to 100 μM onto cells cultured on the enzyme-functionalized hydrogels inhibited the production of NO in a concentration-dependent manner. Anti-inflammatory activity of the drug became apparent at 0.1 μM dose of the therapeutic. At 10 μM concentration of curcumin, therapeutic effect was statistically significant and levels of NO were suppressed to under 40% with minor expense to the cytotoxicity. Further increase in the prodrug content to 100 μM resulted in a pronounced cytotoxic effect and low levels of NO were likely a result of the overall cell mortality rather than anti-inflammatory activity of the drug. We note that these data are qualitatively similar to those obtained using pristine curcumin

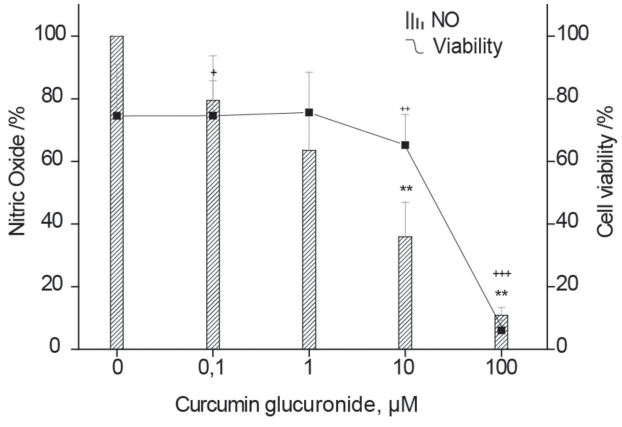


Figure 2. Level of nitric oxide produced by the RAW 264.7 macrophages and corresponding viability of cells as determined in cell culture conducted on the enzyme-functionalized hydrogel biomaterials in the presence of varied concentration of curcumin glucuronide. Presented results are average of at least three independent experiments and are presented as mean ± standard deviation.

and solution-based administration of the drug (see Supporting Information, Figure S2). Thus, prodrug conversion does not appear to be a rate-limiting step and as such, SMEPT strategy does not lead to noticeable detrimental effects such as loss in activity or dramatic shift in the effective concentration of the drug. Furthermore, our data illustrate that EPT approach to drug delivery engineered into a hydrogel biomaterial allows fine-tuning the concentration of the drug synthesized by the cell-adhesive material. The same biomaterial allows achieving mild anti-inflammatory effect (1 μM prodrug), pronounced anti-inflammatory activity (10 μM prodrug), or toxicity-associated treatment. In each of these samples, identical preparations of biocatalytic hydrogels were used and desired therapeutic effect was achieved using external means of control, specifically the choice of prodrug concentration.

2.3. Sequential Therapeutic Responses Mediated by a Biocatalytic Biomaterial

Time-controlled drug release is one of the main challenges for drug delivery engineered into matrices for tissue engineering. Techniques for a gradual, sustained release of the embedded drug are well established and widely implemented.[17] In contrast, advanced possibilities such as pulsatile release or release on demand at specified time points are significantly harder to accomplish and successes in these undertaking are few.[60] On-demand drug release is typically achieved using external triggers to initiate drug release and materials exhibiting stimuli responsive behaviour. On-demand release of two and more drugs with their respective and independently nominated times of release would require two orthogonal mechanisms of stimuli response to be engineered into the same biomaterial, and to the best of our knowledge, such design has never been accomplished. To investigate if tools of EPT allow circumnavigating this biomedical challenge, we aimed to perform the synthesis of two drugs using the same biomaterial and register two distinctly different therapeutic effects in the same cells, at the desired time. Towards this goal, we used a macrophage cell line, curcumin and SN-38 as drug agents, and anti-inflammatory and cytotoxic effects as model therapeutic responses. First, macrophage lineage cells were allowed to adhere to the enzyme-functionalized hydrogel biomaterial and stimulated towards an inflammatory response using LPS. At a nominated time-point (24 h), curcumin glucuronide was administered onto the cultured cells to a concentration of 10 μM and following further 24 h of cell culture, production of nitric oxide and cell viability were independently quantified. At this concentration of curcumin, experimental data reveal a drastic inhibition in production of nitric oxide and a minor decrease in cell viability, **Figure 3**. Following this, media covering the cells was changed to fresh and charged with a prodrug of SN-38 towards eliciting a cytotoxic effect. Cell viability was quantified after a 24 h of cell culture and the data reveal a decrease in cell viability to a level ~ 10%. Thus, the same biomaterial was successfully used to sequentially elicit two types of cell responses in the same population of adhering cells. This was achieved through administration of respective prodrugs at their desired dose and time of administration and conversion of the prodrugs into respective active therapeutic by the enzyme-functionalized biomaterial. Biomedical relevance

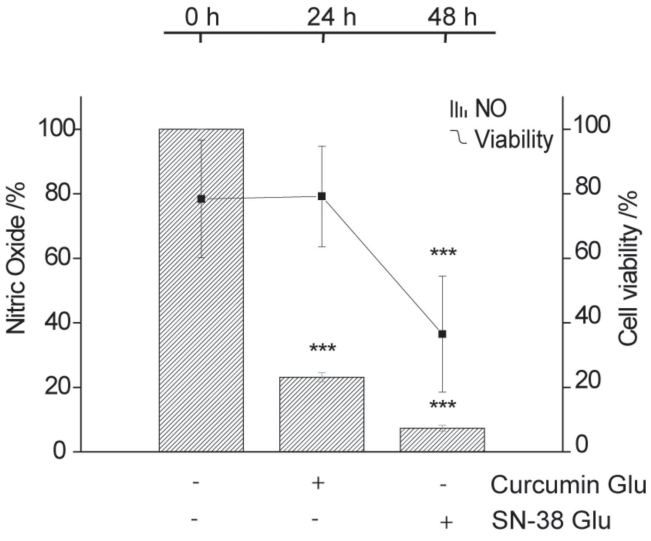


Figure 3. Sequential administration of the two prodrugs at their nominated timepoints affords corresponding therapeutic responses. Macrophage cells were first treated under SMEPT conditions with a glucuronide derivative of curcumin, a therapeutic with anti-inflammatory activity and no accompanying cytotoxic effect. Subsequently, the cells were treated with a glucuronide of SN 38 to achieve a pronounced anti-proliferative effect. Presented results are average of at least three independent experiments and are presented as mean ± standard deviation.

of this opportunity lies in providing significantly enhanced flexibility in the design of implantable biomaterials. Indeed, site-specific drug release for treatment of e.g. atherosclerosis may benefit from opportunities in independent control over administration of anti-inflammatory drugs, cytotoxic agents, as well as anti-platelet adhesion drugs (e.g. clopidogrel). While engineering on demand delivery of two or more preloaded drugs remains a significant challenge, data in Figure 3 reveal that SMEPT presents a facile approach to accomplish this goal.

2.4. SMEPT for Combination Therapy

Drug synergy and corresponding opportunities in combination therapies have contributed tremendously to the development of safer and more efficient treatments.[61] For example, successful anti-HIV treatment relies on the use of multiple drugs comprising the combination anti-retroviral therapy and this significantly decreases the chances of the virus to escape the treatment and develop resistance.[62,63] In pain management, two drugs administered to intervene at distinctly different points in a biochemical cascade leading to the same overall output signal is known to be a highly effective approach to treatment.[64] Multiple drugs used in anti-cancer therapy also decrease the chances of development of drug resistance.[65] By design, most therapeutic implants may be loaded with multiple active ingredients and be used in delivering combinations of drug. However, independent control over each of the therapeutics is a tremendous challenge and combination therapy mediated by implantable devices remains largely un-explored. EPT strategies appear to be unique in that the same enzyme is capable of converting multiple prodrugs into their respective products.

Furthermore, rational choice of concentration for each prodrug should provide opportunities to fine-tune the kinetics of synthesis of each drug independently. Surprisingly, while EPT strategies are well developed, opportunities in combination therapies using EPT have not been explored.

As a proof-of-concept for the synthesis of combination of drugs, we used fluorogenic glucuronides corresponding to the fluorescent dyes with distinctly different emission spectra, namely glucuronide derivatives of fluorescein and resorufin. Biocatalytic hydrogel matrices were assembled as described above and then immersed in PBS solutions containing the two substrates taken in binary combinations ranging from 100-fold excess of fluorescein di-glucuronide to 100-fold greater concentration of resorufin glucuronide (0.05, 0.5 and 5 μg/mL concentrations for each prodrug). Following 30 min of enzymatic reaction, fluorescence of aspirated supernatants was quantified on a multi-label plate reader. Fluorescence intensities were then used to calculate concentrations of the in situ synthesized products, fluorescein and resorufin, using fluorescence vs concentration calibration curves. At each combination of the concentrations, both substrates were successfully converted into their respective products and even with 100-fold excess of one of the prodrugs, there was a significant level of production of the second product, **Figure 4**. Production of fluorescein was evident to a lower degree, plausibly due to its bi-substituted structure, i.e. two glucuronic acid moieties need to cleaved to produce a fluorescent molecule. Nevertheless, data in Figure 4 demonstrate that the same biomaterial is capable of concurrent conversion of two substrates into their respective products with independent control over the amount of each product synthesized in unit time.

To quantify the potential therapeutic effect achieved via SMEPT through a combination treatment, we employed glucuronide prodrugs of two anti-inflammatory agents, curcumin and dexamethasone (curcumin Glu and dexamethasone Glu, **Figure 5**). Curcumin plays a major role in anti-inflammatory

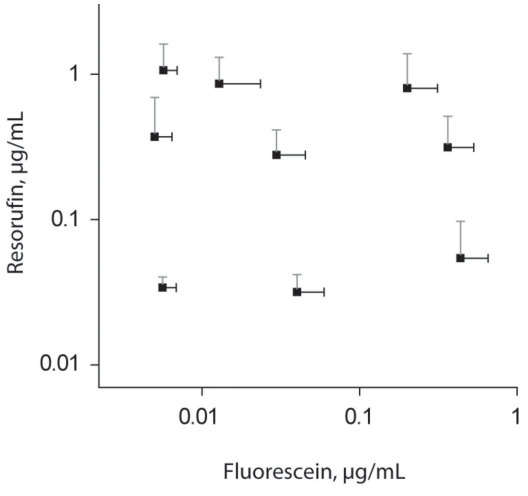


Figure 4. Concentrations of fluorescein and resorufin afforded through enzymatic conversion of the respective glucuronide derivatives in binary mixtures of the two fluoregenic substrates. Feed concentrations for each substrate were 0.05, 0.5 and 5 μg/mL; 30 min of enzymatic conversion. Presented results are average of at least three independent experiments and are presented as mean ± standard deviation.

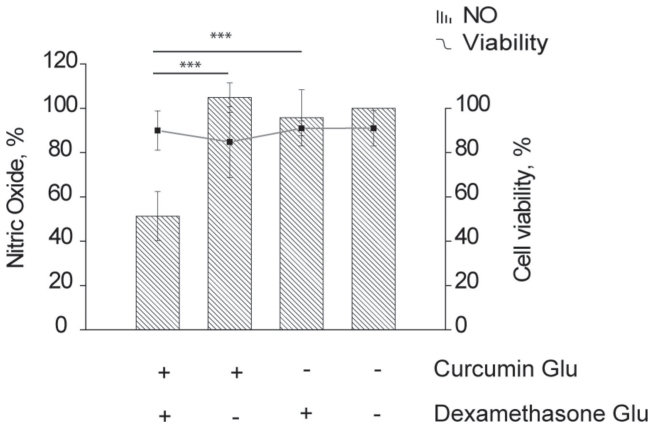


Figure 5. Levels of nitric oxide produced by macrophages and corresponding viabilities for cells as cultured on the biocatalytic hydrogel biomaterials under SMEPT conditions in the presence of glucuronides of curcumin and dexamethasone (0.1 μM each). Combination therapy was accomplished through a concurrent administration of the two prodrugs. Presented results are average of at least three independent experiments and are presented as mean ± standard deviation. Statistical significance is shown for data on production of nitric oxide.

activities via in the inhibition of production of NO through the degradation of iNOS,[66] whereas dexamethasone reduces the translation of iNOS mRNA.[67] This combination of drugs has previously been reported to be effective in decreasing nitric oxide levels in a murine model of asthma and oropharyngeal candidiasis.[68] The anti-inflammatory activity resulted from the concomitant administration of curcumin Glu with dexamethasone Glu under SMEPT conditions was determined in LPS-stimulated murine macrophages after 24h of incubation with the prodrugs. Administration of either of two prodrugs individually at a 0.1 μM concentration resulted in negligible therapeutic effects and levels of NO identical to the cells receiving no drug, Figure 5. In contrast, co-administration of the two prodrugs resulted in a pronounced anti-inflammatory response and a decrease in the registered levels of NO to ~ 50% and this effect was accompanied by no cytotoxicity (Figure 5). Qualitatively, these data were similar to those obtained using a combination of pristine drugs (Figure SI 3). Data in Figure 5 were used to determine combination index (CI)[69,70] for the chosen pair of (pro)drugs at their nominated concentrations. Established CI value was 0.02 and being much lower than unity it indicates a strong synergistic interaction between curcumin and dexamethasone. We note that (pro)drug concentrations were chosen based on an initial screen of combinations of curcumin and dexamethasone and subsequently combination of their glucuronides under SMEPT conditions (data not shown). Data in Figure 5 fully illustrate the competence of SMEPT to perform combination therapy and fine-tune the administered concentrations of the prodrugs to achieve synergistic effect.

3. Conclusion

We have investigated advanced opportunities in drug delivery mediated by the hydrogel biomaterials upon their

functionalization with the tools of enzyme-prodrug therapy. Specifically, we revealed that the same enzyme can convert the prodrugs for multiple therapeutic agents, with the same therapeutic effect or dissimilar mode of action. Prodrugs could be administered individually, in sequence, or in combination, and in each case conversion of the prodrugs resulted in a pronounced therapeutic response. In doing so, we illustrate that the same model implant can sustain substrate-mediated drug delivery for multiple therapeutic agents to elicit the required response at the time it is needed. Furthermore, judicious choice of the prodrugs and their concentrations allowed us to accomplish a biomaterial-mediated combination treatment which was characterized by a pronounced synergistic effect of the therapy. We strongly believe that these results significantly broaden biomedical opportunities associated with biomaterials, specifically drug delivery mediated by therapeutic implants as well as matrices for tissue engineering.

4. Experimental Section

Materials and Reagents: All chemicals were obtained from Sigma/Aldrich (U.S.A.) unless otherwise indicated and used as received. Fluorescein di-β-D-glucuronide (FdG), and PrestoBlue reagents were obtained from Invitrogen (USA). SN-38 glucuronide and curcumin glucuronide were purchased from Toronto Research Chemicals (Canada); dexamethasone glucuronide was acquired from Santa Cruz Biotechnology (USA).

Assembly of μS PVA Hydrogels: The assembly of μS PVA hydrogels was performed as described elsewhere.[12,16] In brief, stock solutions of commercial PVA (Mw 89–98 kDa) at a 12 wt% concentration in distilled water were prepared through heating to 90 °C at least overnight. On the day of experiment, PVA solution was pre-heated to 60 °C for 5 min to homogenize the mixture and subsequently cooled to 37 °C. The PVA solution (at 37 °C) was mixed with β-Glucuronidase reaching the final enzyme concentration of 1 g/L. For cell studies, PVA solutions were supplemented with poly-L-lysine (PLL, 30–70 kDa) to a final concentration of 1 g L^{-1}.

Aliquots of the PVA/enzyme solution (1,5 μL) were casted on the top of the patterned poly-dimethylsiloxane (PDMS) molds, followed by the deposition of a 9 mm glass coverslip and clamping the assembly in a custom made dye set at finger tight pressure (overnight). The patterned PDMS surfaces were composed of pits 10 μm wide, 11.5 μm in separation. Disassembly of the clamps was performed and cover slip-adhered polymer thin films were stabilized via "salting out" effect through 1h of incubation in 0.5 M sodium sulfate (Na_2SO_4) at 37 °C with subsequent 1h of incubation in phosphate buffered saline (PBS) solution. For cellular studies, sterilization was done with UV light for 10–15 min.

Cell Culture: RAW 264.7 cells (murine monocyte macrophage cell line) were cultured in basic medium consisting of Dulbeco's Modified Eagle's Medium (DMEM) with phenol red, supplemented with 10% fetal bovine serum (FBS), and 1% of antibiotic–antimycotic mixture. HepG2 cells (human liver carcinoma cell line) were cultured in Minimum Essential Medium Eagle (MEME) supplemented with 10% FBS, 1% of antibiotic–antimycotic mixture, 1% of 2 mM of L-glutamine and 1% of non-essential amino acids (NEA). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and the medium was replaced every two days. RAW cells were detached with scraping while HepG2 cells were detached with trypsin/EDTA 0.05% and further counted in a hemacytometer. For SMEPT, cells were seeded on top of the hydrogel samples adhered to the underlying 9 mm glass cover slips places into the wells of standard 48-well plates.

Cell Viability: Cell viability was quantified using PrestoBlue assay according to the manufacturer's instructions. Reagent (10% of the volume of media in the well) was added to each well containing cells and the plate was incubated at 37 °C for 30 min protected from light. After incubation, 100 μL of solution was transferred to an opaque 96-well plate and fluorescence was measured in a EnSpire Multimode Plate Reader using an excitation wavelength of 560 nm and emission of 590 nm. Each experiment was normalized against a negative control where no (pro)drug has been added to the cells. Controls and samples were prepared in triplicate.

The Potential of SMEPT to Convert Distinct Prodrugs: HepG2 cells (75 000 cells/well) were seeded over μS PVA hydrogels (300 μL MEME) in 48-well plates and allowed to adhere overnight. After this, cell culture media was exchanged to media containing 10 μM of SN-38 glucuronide, 100 μM of curcumin glucuronide, or 100 μM 5-fluoracil glucuronide and incubated for 48 h. After 48 h, cell viability was determined as described above.

SMEPT and iNOS Control: RAW 264.7 cells were seeded over μS PVA hydrogels at an initial cell seeding density of 60 000 cells/well (300 μL DMEM) in 48-well plates and allowed to adhere overnight. Further, cells were stimulated through the addition of 1 μg/mL lipopolysaccharide (LPS, E. coli 026:B6) in DMEM phenol red free for 1 h. Subsequently DMEM/LPS was exchanged by fresh DMEM/LPS containing 100, 10, 1, 0.1 and 0 μM of curcumin glucuronide and incubated for 24 h. L-N^G-nitroarginine methyl ester (L-NAME, 1 mM), dimethyl sulfoxide (DMSO, 20%) were used as controls.

After 24 h, nitric oxide levels were determined by measuring nitrite levels through the Griess assay. In brief, 50 μL media was transferred to a new 96-well plate and 50 μL sulfanilic acid (10 g/L, 5% phosphoric acid) were added. After 5 min of incubation, 50 μL *N*-1-napthylethylenediamine dihydrochloride (1 g/L) was added and absorbance was measured using EnSpire Multimode Plate Reader (548 nm). The nitrite levels were quantified against a freshly-prepared sodium nitrite standard curve and normalized within each experiment against the negative control consisting of LPS stimulated cells without the addition of (pro)drug. Controls and samples were prepared in triplicate. Simultaneously to the quantification of NO levels, cell viability was measured as described above. Similar experiment was done using pristine drug, curcumin, as controls in a 96-well plate, cell density of 20 000 cells/well (100 μL DMEM).

Sequential Synthesis of Two Drugs: Culturing of RAW 264.7 cells, pro-inflammatory stimulation using LPS, treatment with 10 μM curcumin glucuronide, assessment of the levels of nitric oxide and corresponding cell viability were performed as described above. At the end of 24 h of incubation with 10 μM curcumin Glu, the cells were washed with PBS and subsequently incubated in fresh media containing 10 μM SN-38 glucuronide for additional 24 h. Cell viability and NO levels were determined as described above.

SMEPT for Combination Treatment: Fluorescein di-β-D-glucuronide (FdG) and Resorufin β-D-glucuronide (RG) were dissolved in PBS yielding prodrug concentrations of 0.05, 0.5 and 5 μg/mL each. Each concentration of FdG substrate was combined with each concentration of RG and incubated with μS PVA hydrogels at 37 °C during 30 min and protected from light. Solution fluorescence was then quantified using a multi-label plate reader using an excitation wavelength of 500 nm and emission of 600 nm for fluorescein and an excitation wavelength of 550 nm and emission of 585 nm for resorufin.

Culture of RAW 264.7 macrophages was performed as described above. The cells were stimulated through the addition of 1 μg/mL LPS for 1h prior the incubation with curcumin Glu and dexamethasone Glu. Solutions of prodrugs were prepared in DMEM phenol red free/LPS (1 μg/mL) and added to each well to a final concentration of 0.1 μM. Relative levels of NO and cell viability were determined as described above.

The combination index (CI) considering the NO levels was determined according the following equation:

$$I = \frac{D1}{Dm1} + \frac{D2}{Dm2}$$

D1 and D2 are the doses of drug 1 and 2 that in combination produce a specified effect and Dm1, Dm2 are the doses of the drugs at

which the drugs have the same effect when administered individually. The CI values lower than, equal to and higher than 1 denote synergism, additivity and antagonism, respectively.[69]

Data Analysis: Student's T-test was applied to determine statistical significance using Excel software. One-tailed unpaired t-test with 95% confidence interval was considered statistical significant if $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Acknowledgments

This study was supported by a grant from the Novo Nordisk Foundation. Authors thank Benjamin M. Wohl (Aarhus University) for expert advice on cell culture and quantification of nitric oxide.

Received: December 31, 2013
Revised: April 7, 2014
Published online: June 16, 2014

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