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High-throughput screening of PLGA thin films utilizing hydrophobic fluorescent dyes for hydrophobic drug compounds.

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29 approval for submission.

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31

Abstract:

Hydrophobic, anti-restenotic drugs such as paclitaxel and rapamycin are often incorporated into thin film coatings for local delivery using implantable medical devices and polymers, such as drug eluting stents and balloons. Selecting the optimum coating formulation through screening the release profile of these drugs in thin films is time consuming and labour intensive. We describe here a high-throughput assay utilizing three model hydrophobic fluorescent compounds; fluorescein diacetate, coumarin-6, and rhodamine-6g that were incorporated into poly-DL-lactide-co-glycolide (PLGA) and PLGA/polyethylene glycol (PEG) films. Raman Microscopy determined the hydrophobic fluorescent dye distribution within the PLGA thin films in comparison to that of paclitaxel. Their subsequent release was screened in a high-throughput assay and directly compared to HPLC quantification of paclitaxel release. It was observed that paclitaxel controlled release kinetics could be mimicked by a hydrophobic dye that had similar logP values and homogenous dissolution in a PLGA matrix as the drug. In particular, fluorescein diacetate was found to be the optimal hydrophobic dye at modelling the burst release as well as the total amount of paclitaxel released over a period of 30 days.

48 **Keywords:** PLGA, paclitaxel, drug delivery, screening, raman, fluorescein

49

51 **1.0 Introduction**

52 Paclitaxel is well described as an important treatment in various cancer pathologies including
53 ovarian, breast, and lung cancers. A lesser known application for this compound is in treating
54 cardiovascular disease, in particular limiting the growth of scar tissue, or restenosis. Drug
55 eluting stents have incorporated paclitaxel into the slow release thin films, which confers reduced
56 restenosis over several weeks¹. The Taxus® Stent, by Boston-Scientific, is a commercial stent
57 with a non-biodegradable thin film coating on a stainless steel stent. It was initially well
58 received, but was later associated with higher thrombosis rates compared to bare metal stents^{2,3}.
59 Due to this detrimental effect, it has been proposed that localized delivery of paclitaxel
60 immediately following percutaneous transluminal (coronary) angioplasty treatment, without
61 stenting, should improve the long term outcome of the procedure; favorable results in early
62 clinical trials have justified this notion^{4,5}. Various studies focused on producing paclitaxel
63 incorporating thin films are presently under way. Films with release profiles that control the
64 burst and extended dosages from weeks to months are being developed⁶⁻⁸. However, to date,
65 there are no high-throughput methods to screen thin films for the release of paclitaxel and other
66 hydrophobic drugs such as, rapamycin, or everolimus.

67
68 The gold standard technique of paclitaxel quantification has been high performance liquid
69 chromatography (HPLC) with C18 column separation and detection with UV absorbance (λ_{max} :
70 227 nm, $\epsilon = 2.8 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). On optimized isocratic systems, individual analyses run under
71 10 min with limit of quantification at 0.05 $\mu\text{g/mL}$ ⁹. This is only 7 times lower than the
72 maximum solubility of paclitaxel in aqueous solutions¹⁰. To increase the solubility of paclitaxel,
73 addition of organic solvents or surfactants was needed, thus complicating HPLC analyses^{11,12}.
74 The inefficient rate of sample throughput using HPLC is also a major flaw, since the
75 quantification of drug release by one HPLC apparatus is limited to a maximum rate of 10 thin
76 film formulations per day. The extensive time required to complete HPLC analysis, including
77 sample preparation, for a time course experiment with replicates restricts the number of films a
78 worker can realistically study in a single session. It is therefore of great use to develop an

approach towards quick turnover, bulk screening, allowing film optimization in substantially shorter time frame.

An alternative approach would be to use fluorescence spectroscopy, as this would avoid the most laborious preparation steps and thus speed up the quantification by orders of magnitude. Indeed, quantification by fluorescence in a 96-well plate takes seconds per sample compared to 10 mins per sample in a HPLC. However, hydrophobic compounds such as paclitaxel or rapamycin are not fluorescent. Fluorescent analogues of these compounds do exist, for use in biochemistry mechanism investigations, where microgram quantities are required¹³. Currently these fluorescent compounds are prohibitively expensive for use in the milligram to gram amounts needed in thin film-extended release preparations.

The next best option would be to acquire more economical hydrophobic fluorescent compounds that are suitable for screening the thin film formulations. These could be used to select best candidate film formulations from a larger panel, for further assessment using the more laborious HPLC hydrophobic drug quantification. The use of hydrophobic fluorescent compounds in place of hydrophobic drugs for visualization and tracking has been previously documented. For example, fluorescent Nile red was used in place of paclitaxel in PLGA nanoparticles to observe the nanoparticle cellular uptake and subsequent Nile red release¹⁴. Similar experiments with PLGA/Montmorillonite nanoparticles used coumarin-6 (structurally similar to Nile red) in place of paclitaxel for cellular uptake observations¹⁵.

In this investigation, a novel method of high-throughput quantification of the thin film drug release was optimized, in the form of a 96-well plate assay. Three commercially available fluorescent compounds were chosen with similar hydrophobic properties to paclitaxel (octanol/water partition coefficient, logP, of 4.0-4.4^{16,17}) and compared against the release of paclitaxel in PLGA and PLGA/PEG films by HPLC methods. Table 1 lists the three hydrophobic fluorescent compounds: rhodamine 6G, fluorescein diacetate, and coumarin-6, and with logP values of 4.02, 4.2, and 5.43, respectively¹⁸⁻²⁰. The fluorescent compounds were chosen due to their similar logP values to the drug, as earlier reports list the solubility in the PLGA matrix (dry or wet) as the predominant factor that influences the rate of delivery²¹.

109 Molecular weight, molecular volume, and topological surface areas²² are also listed for
110 comparison of paclitaxel to the hydrophobic dyes.
111

2.0 Materials and Methods

2.1 Materials

Poly (DL-lactide-co-glycolide) (PLGA) 53/47 (PLGA with a 53/47 lactide/glycolide ratio) with intrinsic viscosity (i.v.) of 0.2 (~ 10 kDa MW) and 1.03 dL/g (~100 kDa MW) was purchased from Purac, Netherlands. Paclitaxel was purchased from Yunnan Hande Bio-Tech, China. HPLC-grade dichloromethane (DCM) and acetonitrile was purchased from Tedia, USA. Deuterated chloroform (CDCl_3 + 0.03 % v/v TMS D99.8% + silver foil) was purchased from Cambridge Isotope Laboratories, Andover, USA. Polyethylene glycol (PEG) of molecular weight of 8000 and 35,000, and polysorbate 80 (Tween 80) were purchased from Sigma-Aldrich, Singapore. Rhodamine 6g, coumarin-6, and fluorescein diacetate (FDAC) were purchased from TCI Japan, Singapore. All other polar solvents used were of high performance liquid chromatography (HPLC) grade and were supplied by Sigma-Aldrich, Singapore. All chemicals and materials were used as received.

2.2 Film Formulation

Solutions of PLGA/PEG prepared at a concentration of 20 % w/v (in DCM) were used to incorporate 10 % w/w paclitaxel/fluorescent dye (paclitaxel/fluorescent dye in PLGA/PEG) in DCM. Hence, a typical film formulation consisted of 60 mg of paclitaxel/fluorescent dye and 600 mg of polymer (PLGA + 0-50 % PEG) in 3 mL of DCM. Such a preparation was carried out as follows: a 15 % 8k PEG/PLGA 53/47 solution was dissolved in 3 mL DCM overnight with 60 mg paclitaxel, 510 mg of PLGA 53/47 i.v. 1.03 and 90 mg of 8k PEG.

Film casting was performed with the film applicator height set at 300 μm and the viscous solution was cast onto 50 μm polyethylene terephthalate substrate at 50 mm/s, room temperature (RT), in a fume extractor hood. DCM was evaporated at RT for 24 h followed by 55 $^{\circ}\text{C}$ for 2 d in a vacuum oven. Punch-outs of 6 mm diameter were prepared for release studies, with the PLGA films still mounted on the polyethylene terephthalate substrate.

2.3 PEG Quantification by ^1H NMR

One cm square films were dissolved in $1050 \pm 10 \mu\text{g}$ (700 μL) of CDCl_3 , mixed by vortexing, and centrifuged at 10,000 rpm for 5 min, the subsequent supernatant was transferred into NMR

tubes. ^1H NMR spectra were recorded on Bruker Advance Spectrometer at 400 MHz using the signal of tetramethylsilane (TMS) present in deuterated chloroform at 0.03 % as an internal standard. ^1H NMR (400 MHz, CDCl_3 , δ) 1.5-1.7 (bs, PLGA 3H, $-\text{C}(=\text{O})-\text{CH}(\text{CH}_3)-\text{O}-\text{C}(=\text{O})-\text{CH}_2-\text{O}-$), 3.45-3.85 (bs, PEG 4H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), 4.6-5.0 (bs, PLGA 2H, $-\text{C}(=\text{O})-\text{CH}(\text{CH}_3)-\text{O}-\text{C}(=\text{O})-\text{CH}_2-\text{O}-$), 5.0-5.3 (bs, PLGA 1H, $-\text{C}(=\text{O})-\text{CH}(\text{CH}_3)-\text{O}-\text{C}(=\text{O})-\text{CH}_2-\text{O}-$).

2.4 Film Surface and Film Cross-Section Topography by Scanning Electron Microscopy

Surface and cross-sectioned PEG incorporated PCTX-PLGA films were coated with platinum for 50 s under a chamber pressure of less than 5 Pa at 20 mA using JEOL JFC-1600 Auto Fine Coater, Japan. Secondary electron images were acquired at 5.0 kV, 12 μA , at a working distance of 8 mm under the Field Emission Scanning Electron Microscopy (FESEM) (JEOL JSM-6340F, Japan). Film cross-sections were prepared by flash freezing the films in Tissue-Tek O.C.T. Compound at -80°C . Embedded film blocks were sliced while frozen at 10 μm and subsequently lyophilized under vacuum.

2.5 Raman Microscopy

Raman microscopy analysis has been performed as described earlier²³. Briefly, the thin films were placed under the microscope objective and Raman point-by-point mapping measurements were performed on the sample. The sample was irradiated with a 785 nm near infrared diode laser and a 50 $\times$ or 100 $\times$ objective lens was used to collect the backscattered light. The collected Raman mapping spectra were then preprocessed (spike removal and baseline correction) before the data was further analysed using the band-target entropy minimisation (BTEM) algorithm. The BTEM algorithm²⁴ was used to reconstruct the pure component spectra of underlying constituents from a set of mixture spectra without recourse to any a prior known spectral libraries. This has been proven to be effective in reconstructing the pure component spectra of minor components²⁵⁻²⁷. When all normalised pure component spectra of all the underlying constituents have been reconstructed, the relative contributions of each constituent can be calculated by projecting them back onto the baseline-corrected and normalised data set. The spatial distribution of each underlying constituents are then generated.

2.6 High-Throughput Screening of Fluorescent Dyes

The 6 mm diameter discs consisting of fluorescent dye incorporated into PLGA were immersed in 200 μ L of PBS/2% Tween 80 solution, within a 96 well Costar flat black polystyrene flat bottom plate. Samples were assayed in pentaplicates and stored in a 37°C incubator in between sampling events. For rhodamine 6G (ex/em: 480/560) and coumarin-6 (ex/em: 465/505), 100 μ L aliquot was drawn out of the release plate and placed into a separate black read plate. The read plate was diluted with another 100 μ L of PBS 2% Tween 80 and assayed using the Infinite M200 Tecan Microplate spectrofluorometer. For fluorescein diacetate, 20 of the aliquot was drawn out of the release plate and diluted with 180 μ L of 100 mM NaOH in the read plate, immediately yielding fluorescein (ex/em: 490/520). The amount of hydrophobic dye released was quantitated using three calibration curves (with separate gain settings) spanning three orders of magnitude; 0.01 – 10 μ g/mL. The release plate had the remaining solution carefully drawn out, and replaced with another 200 μ L worth of PBS/2% Tween 80. Rhodamine 6G, coumarin-6, and FDAC all had solubilities of >100 μ g/mL in PBS/2% Tween 80. Each well was flashed 25 times, with a 20 μ sec integration. The entire 96-well plate was measured in < 5 min for three gain settings. Instrument gain settings were experimentally determined by calibrating 90% of the maximum fluorescent signal (50,000 Fluorescent units) at the highest standard concentration for the high gain, (0.01 – 0.1 μ g/mL), medium gain (0.01 – 0.10 μ g/mL), and low gain (1.00 – 10.0 μ g/mL) standard concentration ranges. The gain settings were then subsequently used for all controlled release-fluorescent dye quantitation.

2.7 In-Vitro Paclitaxel Release

The in-vitro release of paclitaxel was conducted in 2 mL of PBS/2% Tween 80 release buffer (pH 7.4) at 37 °C, using 15 mm punch-outs in triplicate. At predetermine time-points the buffer was removed and replaced with another 2 mL release buffer, thus maintaining sink conditions throughout the release. The withdrawn aliquots (or standards/dissolutions samples) were flushed through a 0.2 μ m syringe filter, directly into HPLC vials and immediately capped. The paclitaxel content was quantified with an Agilent Series 1100 HPLC (Santa Clara, CA, USA) equipped with UV/Vis detector, autosampler, and column heater set to 35°C. A ZORBAX Eclipse XDB-C18 (5 μ m) column of acetonitrile/water 60/40 (v/v) served as the mobile buffer,

eluting the paclitaxel peak at ~ 5.4 mins with a flow rate 1.0 mL/min and the UV/Vis detector recording at 227 nm. At the final endpoint, a total dissolution study of the 15 mm discs in triplicate was conducted by dissolving the films in acetone and diluting in release buffer to determine the surface concentration of paclitaxel ($\mu\text{g}/\text{mm}^2$). The solubility limit of paclitaxel in PBS/2% Tween-80 release buffer was determined to be 20 $\mu\text{g}/\text{mL}$, ~50x that of PBS buffer.

3.0 Results and Discussion

3.1 Thin Film Physical Properties

The method (viscous polymer solution knife-casted on polyethylene terephthalate substrates) employed for film fabrication containing the paclitaxel and that for the hydrophobic dyes was identical. The analysis was optimised for using both HPLC paclitaxel quantitation and fluorescence measurements of the hydrophobic dyes. Figure 1 summarizes the film fabrication and analysis steps employed.

Initial analysis on the hydrophobic dyes and paclitaxel revealed all four compounds were incorporated into two PLGA matrices of intrinsic viscosity (i.v., dL/g) 0.2 and 1.03. Additionally, the four compounds were incorporated into PLGA i.v. 1.03 with two types of PEG MW at two w/w ratios: 8k and 35k PEG at 15 and 50% w/w ratios each (Table 1). Table 2 displays the thin film values of thickness, surface drug concentration, and the percentage of PEG. Representative films from FDA and paclitaxel-containing films are shown, but similar values were seen for the fluorescent dyes of coumarin-6 and rhodamine 6G. The addition of large amounts of PEG (i.e. 50% formulations) yielded thinner viscosities in the dichloromethane (DCM) solutions, which subsequently generated thinner films and lower drug concentrations when dried. Paclitaxel caused thicker films in the PLGA i.v. 0.2 films versus fluorescein diacetate, although this was likely attributed to slight differences in film fabrication.

Whilst rarely reported for assessing PLGA films, we found that ^1H NMR analysis was a robust technique to analyze the matrix chemical composition. The percentage of PEG was verified and measured through the ^1H NMR PEG integrals present from 3.45-3.85 ppm, as seen in Figure 2. FdAc was an ideal candidate for ^1H NMR quantification as the 2 methyl esters (with 6 protons) had a strong singlet at 2.33 ppm. The dichloromethane (DCM) dissolved-knife cast films required 24 h of drying at atmospheric pressure before 55°C vacuum oven exposure. Without the

24h of air drying, unpredictable foam matrices occurred (data not shown). Higher boiling point solvents were avoided in this study as longer incubations at atmospheric pressures would be needed. The optimized drying procedure employed a vacuum oven at 55°C after RT drying, which yielded <500 ppm and <3 µg residual DCM, for an assumed 5 mg PLGA film (Figure 1 Inset displays the ¹H NMR DCM peaks at 2 drying conditions). It may be noted that this level falls under FDA guidelines of <600 ppm and <6 mg/d residual DCM ²⁸.

The cross section and surface topology of the PLGA films was analyzed using Field Emission Scanning Electron Microscopy (FESEM) at x700 magnification. Figure 3 displays a representative photograph of PLGA (53/47 with i.v. 1.03) with 10 % drug encapsulation (no PEG additives). At day 0, a solid matrix was seen with no pores present at the surface. After 10 days in release buffer, pore formation on the surface and within the matrix cross-section displayed a porous film that had swelled to almost twice its original thickness (~25 µm). The uniform degradation from the top to the bottom surface suggests bulk degradation with no autocatalytic processes occurring. Autocatalytic degradation tends to display a more degraded core than film surface, and is usually noted only in films thicker than 200 µm²⁹. The effect of combining PEG with PLGA (53/47 with i.v. 1.03) and subsequent degradation has previously been discussed ²³.

3.2 PLGA film characterization by Raman Microscopy: Partitioning of the Hydrophobic dyes and Paclitaxel

In earlier work we demonstrated with the use of Raman Microscopy, how the presence of co-localized paclitaxel in crystalline PEG (vs amorphous PEG) can affect the overall drug release from PLGA films²³. Furthermore, Raman peaks of crystalline PEG at 844 and 860 cm⁻¹ have been previously used to differentiate the PEG crystalline phase from its amorphous phase³⁰. The Raman peak assignments for all the compounds tested are found in Table 3. When paclitaxel was distributed homogeneously (see Figure 4A and 4B), the controlled release was slow (< 2 µg/cm²-day). Depending on the MW, increasing the amount of PEG caused localized phase separations of crystalline PEG, as seen in Figure 4C with 15% 35k PEG in PLGA 53/47. Paclitaxel preferentially co-localizes in these phase-separated crystalline PEG regions, and was subsequently released rapidly with the dissolved PEG. Hydrophobic dyes would require similar partitioning behavior to mimic the paclitaxel release. Coumarin-6 and rhodamine 6G were found

to be heterogeneous even in PLGA 53/47 films without PEG. Upon drying the films, crystalline regions of coumarin-6 and rhodamine 6G were visualized with the naked eye and confirmed with Raman Microscopy.

Figure 4D displays an example of coumarin-6 analyzed by Raman microscopy. Phase separation of the crystalline coumarin-6 within the PLGA matrix region (bottom and top of color map, respectively in Figure 3D) was readily detectable. FDAc films exhibited homogenous distribution for PLGA 53/47 (data not shown), and with 15% PEG (both 8k and 35k MW). A representative image of the FDAc distribution within 15% 35k MW PEG is represented in Figure 4E. FDAc in these three films was assumed to be dissolved completely in the PLGA 53/47 matrix, as the Raman signals were weak. At the highest concentration of PEG (50% of 8k and 35k MW) a slight amount of fluorescein diacetate/crystalline PEG co-localization occurred. However, a more pronounced co-localization of paclitaxel/crystalline PEG was present at 15% PEG than the FDAc/crystalline PEG at 50% (Figure 4C vs. 4E). In general, the FDAc was distributed evenly within the film, regardless of crystalline/amorphous PEG and PLGA 53/47 phase separations.

3.3 Assay sensitivity using 96-well plate/M200 Tecan Spectrofluorometer

The most convincing arguments for switching from the HPLC to a 96-well plate/fluorescence assay was the savings on labour, consumables cost, and increased method sensitivity. The specifications of the M200 spectrofluorometer allowed for the sensitivity of fluorescein detection/quantification in 96-well plates to be within the picomolar range. The instrument has the capability 20 sec/plate read throughput³¹, although in practice, we found it ranged from 1-2 min/plate at a single gain and excitation/emission setting. Linear curves down to 1 ng/mL (~4 nM) fluorescein were easy to reproduce. Quantification in this range was important for experimental use *in vivo* formulations and for *ex vivo* bioreactors, where thin films often release encapsulated drugs into liters of medium.

Fluorescein was best read at high pH values, as high pH instantly cleaves the esters present in non-fluorescent FDAc to the fluorescent form. This allowed it to be utilized in the cells and vesicles as a 'pH-meter'³². Indeed, we found that small changes in physiological pH (+/- 0.04 pH units) did cause an increase in fluorescence unit standard deviation. At basic pH of ~12-13,

fluorescence was at its brightest, and did not show an increase in standard deviation (data not shown). Fluorescent dyes coumarin-6 and rhodamine 6G did not show this pH-dependent fluorescence fluctuation, and were measured at pH 7.4. Average plate read time was ~4.5 min was for all three fluorescent dyes, using three gain settings; High gain: 0.01-0.10 $\mu\text{g/mL}$, Medium gain: 0.10-1.0 $\mu\text{g/mL}$, and Low gain: 1.0-10.0 $\mu\text{g/mL}$.

3.4 Release in fast (i.v. 0.2) and slow (i.v. 1.03) degrading PLGA thin films

To mimic the in vivo solubility conditions for paclitaxel, in vitro release conditions were modulated using 2% Tween 80 in PBS buffer at 37°C. The addition of 2% Tween 80 allows soluble paclitaxel concentrations of 20 $\mu\text{g/mL}$, 50 times that of PBS alone and equivalent to albumin-bound blood concentrations³³. Tween 80 has commonly been used to dissolve paclitaxel for this purpose^{34,35}, and allowed solutions of >100 $\mu\text{g/mL}$ concentrations of the fluorescent compounds to be prepared (data not shown).

The drug release behavior of the hydrophobic fluorescent molecules: FDAc, coumarin-6, rhodamine 6g and paclitaxel revealed diffusion controlled release in all of the pure PLGA films of intrinsic viscosity (i.v.) 0.2 and 1.03 dL/g. The FDAc and paclitaxel revealed a similar drug release profile with no burst release. With similar logP values and homogenous (aka amorphous) PLGA matrix distribution, this was to be expected. Rhodamine 6G and coumarin-6 displayed higher burst release, likely due to the phase separated, crystalline state of the two dyes within the matrix. At the 10% w/w concentration, rhodamine 6G and coumarin-6 were not completely soluble within the PLGA matrix

The structures of rhodamine 6G and coumarin-6 (See Table 1) may explain why they were insoluble at 10 % w/w in PLGA. Even though these molecules have a hydrophobic logP similar to paclitaxel, their amine substituents provide a local polar behaviour that decreased their solubility in the hydrophobic PLGA matrix. This effect was unexpected, as coumarin-6 substitution for paclitaxel was common for previous cell uptake and imaging studies, although these used smaller w/w concentrations^{15,36}.

The controlled release of the four compounds was then compared across two MW neat PLGAs of i.v. 0.2 and 1.03 dL/g in Figure 5A and 5B. The 0.2 i.v. PLGA matrices revealed a faster dye and paclitaxel release than i.v. of 1.03 in PLGA. At the higher i.v. (and MW), the longer and less

soluble polymer chains slowed the diffusion rate of the drugs. Larger PLGA chains tend to retain more of the encapsulated dye and for longer³⁷. Coumarin-6, FDAc, and paclitaxel had roughly twice the release kinetics at i.v. 0.2 as that of i.v. 1.03 PLGA. Rhodamine 6G exhibited a gradual release in i.v. 0.2 PLGA, whereas the i.v. 1.03 PLGA gave an abrupt release from 20-25 days.

Overall, FDAc was found to mimic the paclitaxel release kinetics the most. Based on the equations of the semi-empirical Power Law, rate K and the diffusion coefficients were calculated and listed in Table 1, allowing a comparison between paclitaxel and FDAc³⁸. Despite paclitaxel almost having twice the molecular volume of FDAc (see Table 1) the diffusion coefficients were comparable for both i.v. 0.2 and 1.03 PLGA matrices.

3.5 Release in low and high % PLGA additives using 8000 and 35000 MW PEG

The drug release behavior of paclitaxel and hydrophobic dye FDAc, coumarin-6, and rhodamine 6G also showed diffusion controlled release with the addition of PEG additives at PLGA i.v. of 1.03 dL/g. Distinct observation can be made from the release profiles seen in Figure 6A and 6B, whereby, from the drug panel, only rhodamine 6G exhibited a burst release from PLGA with either 15% 8K or 35K PEG, of 20% and 60% respectively. Hence an increased burst release occurs with larger PEG MW additive. In addition, an increase in the percentage of PEG additive also causes greater burst release. These two effects are confirmed by the highest burst release being detected in PLGA incorporated with PEG 35000 at 50% w/w concentration; i.e. the highest MW and percentage additive tested (Figure 7). Such a large burst release could be partly attributed to the following; lower LogP value in rhodamine 6G, ionic molecule formation in solution, crystalline phase separation, or combination there-of. In the case of coumarin-6, it is assumed the crystalline phase separation accounts for the higher rate of release observed as well.

The subsequent controlled releases of the four dyes were then further compared across two MW PEG additives of 8000 and 35000 at two percentages; 15% and 50% (Figures 6 and 7). The larger 35000 MW PEG generally gave a smaller percentage of burst release as compared to 8000 MW PEG at 50% concentration (Figure 7). An increase in co-localization of the larger MW PEG (analyzed for FDAc and paclitaxel) resulted in a sustained release. Paclitaxel is known to co-localize with PEG chains via intercalating chemistry due to its aromatic rings³⁹. Such an

occurrence may also be expected for the three fluorescent dyes. A higher concentration of PEG at 50% gave an increased rate of release compared to 15% PEG, as deduced from the steeper release curve over the initial days of the release profile. The steeper curve results from the PEG dissolution and leaching²³. Since the greater co-localization was likely associated with higher PEG concentrations, an increased release was therefore expected to occur for all drug molecules at high PEG content.

3.6 FDAc as a model hydrophobic drug for screening thin film preparations

The release profile of FDAc was found to have a good correlation with that of paclitaxel when encapsulated within pure PLGA films. This was found to be independent of the two molecular weight PLGA matrices examined in Figure 5. The release of FDAc only began to differ from paclitaxel upon addition of PEG, an additive that was known to phase separate within PLGA (see Figures 6 and 7). Within these heterogenous films, our hypothesis of mimicking the paclitaxel logP with a similar hydrophobic fluorescent compound was no longer valid. LogP only predicts preference towards hydrophobic solvents (or PLGA matrices) in contact with in an aqueous phase. It cannot provide any information toward tertiary amphiphilic compounds like PEG that phase separate within the PLGA. Fortunately, these phase separations were typically easy to visualize by the naked eye, light microscopy, Raman microscopy or combination thereof. A quick assessment of the homogenous structure by these techniques will decide if this high-throughput method may be of use.

The differences in FDAc and paclitaxel distribution among PEG and PLGA described here may be explained with another solubility descriptor; topological polar surface area (TPSA, listed in Table 1.) TPSA represents the sum of polar surfaces of nitrogen, oxygen, and their bound hydrogen atoms. It has proved to be a useful parameter for drug transport analysis in intestinal and blood-brain barrier absorption²². Within the context of our experiments, higher TPSA was associated with PEG partitioning. The lowest TPSA values were observed with coumarin-6 and rhodamine 6G; no PEG partitioning was detected in the PLGA films with either of these two molecules. The second highest TPSA value was for FDAc with 88 Å², which had a minor partitioning at the highest 50% PEG concentration. Paclitaxel was present with the most amount of crystalline PEG partitioning, had greater than two fold TPSA of FDAc with 221 Å².

4.0 Conclusions

Three fluorescent dyes with logP's similar to paclitaxel have been evaluated in PLGA films with Raman Microscopy for molecular distribution and compared against paclitaxel release of < 30 days. Two of the dyes, rhodamine 6g and coumarin-6, were found to be inadequate, as they were not soluble within the PLGA matrix, and tended to phase separate and crystallize. One hydrophobic dye, FDAc (converted to fluorescein when treated with base) was found to be a model drug towards estimating the paclitaxel release profile. It displayed similar diffusion coefficients in homogenous PLGA films (intrinsic viscosity of 0.2 and 1.03 dL/g). When PEG additives were present, FDAc was still found to mimic the paclitaxel release, when phase separation was not substantial. As the MW and concentration of PEG increased, more crystalline PEG phase separation was present. Under these conditions, paclitaxel preferentially co-localized into the crystalline PEG more than the FDAc, with varying release profiles. If phase separating additives are introduced, alternative drug models will have to be considered.

This report demonstrates that FDAc incorporation into PLGA can be controlled to model that of paclitaxal inclusion. Thus, a method for high-throughput screening of PLGA thin film formulations has been presented. With the use of FDAc in place of paclitaxel, fluorescent 96-well or possibly 384-well plate readers can be used while reducing the amount of consumables and decreasing the dependency for HPLC quantitation. We recommend the following guidelines for those who are considering this method:

1. FDAc, paclitaxel, additives, and polymer matrix (i.e. PLGA or other polyester matrices) will need to have similar hydrophobic properties to make homogenous films.
2. Visualization by light microscopy or raman microscopy to asses microstructure for additive or drug phase separated regions.
3. Polymer films thicknesses should be similar and less than 200 microns, to avoid autocatalytic degradation effects.
4. This method should be considered when multiple formulations ($10 \geq$ thins films) need to be simultaneously screened for paclitaxel delivery. When testing less than 10 films overall, the time saved by this fluorescence protocol does not justify the need for preparation of separate FDAc containing-films.
5. Applicable to studies when the drug release period is less than 30 days.

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408 **6.0 References**

- 409 1. Heldman AW, Cheng L, Jenkins GM, Heller PF, Kim DW, Ware M, Jr., Nater C, Hruban RH, Rezai
410 B, Abella BS, Bunge KE, Kinsella JL, Sollott SJ, Lakatta EG, Brinker JA, Hunter WL, Froehlich JP 2001.
411 Paclitaxel stent coating inhibits neointimal hyperplasia at 4 weeks in a porcine model of coronary
412 restenosis. *Circulation* 103(18):2289-2295.
- 413 2. Lagerqvist B, James SK, Stenestrand U, Lindback J, Nilsson T, Wallentin L 2007. Long-term
414 outcomes with drug-eluting stents versus bare-metal stents in Sweden. *N Engl J Med* 356(10):1009-
415 1019.
- 416 3. Galloe AM, Thuesen L, Kelbaek H, Thayssen P, Rasmussen K, Hansen PR, Bligaard N, Saunamaki
417 K, Junker A, Aaroe J, Abildgaard U, Ravkilde J, Engstrom T, Jensen JS, Andersen HR, Botker HE, Galatius S,
418 Kristensen SD, Madsen JK, Krusell LR, Abildstrom SZ, Stephansen GB, Lassen JF 2008. Comparison of
419 paclitaxel- and sirolimus-eluting stents in everyday clinical practice: the SORT OUT II randomized trial.
420 *JAMA* 299(4):409-416.
- 421 4. Tepe G, Schmitmeier S, Speck U, Schnorr B, Kelsch B, Scheller B 2010. Advances on drug-coated
422 balloons. *J Cardiovasc Surg (Torino)* 51(1):125-143.
- 423 5. Unverdorben M, Kleber FX, Heuer H, Figulla HR, Vallbracht C, Leschke M, Cremers B, Hardt S,
424 Buerke M, Ackermann H, Boxberger M, Degenhardt R, Scheller B 2010. Treatment of small coronary
425 arteries with a paclitaxel-coated balloon catheter. *Clin Res Cardiol* 99(3):165-174.
- 426 6. Chia NK, Venkatraman SS, Boey FY, Cadart S, Loo JS 2008. Controlled degradation of
427 multilayered poly(lactide-co-glycolide) films using electron beam irradiation. *J Biomed Mater Res A*
428 84(4):980-987.
- 429 7. Wang XT, Venkatraman S, Boey F, Loo SC, Tan LP 2007. Effects of controlled-released sirolimus
430 from polymer matrices on human coronary artery smooth muscle cells. *J Biomater Sci Polym Ed*
431 18(11):1401-1414.
- 432 8. Loo SC, Tan ZY, Chow YJ, Lin SL Drug release from irradiated PLGA and PLLA multi-layered films. *J*
433 *Pharm Sci* 99(7):3060-3071.
- 434 9. Mohammadi A, Esmaeili F, Dinarvand R, Atyabi F, Walker RB 2009. Development and validation
435 of a stability-indicating method for the quantitation of Paclitaxel in pharmaceutical dosage forms. *J*
436 *Chromatogr Sci* 47(7):599-604.
- 437 10. Hamada H, Ishihara K, Masuoka N, Mikuni K, Nakajima N 2006. Enhancement of water-solubility
438 and bioactivity of paclitaxel using modified cyclodextrins. *J Biosci Bioeng* 102(4):369-371.
- 439 11. Alexis F, Venkatraman SS, Rath SK, Boey F 2004. In vitro study of release mechanisms of
440 paclitaxel and rapamycin from drug-incorporated biodegradable stent matrices. *J Control Release*
441 98(1):67-74.
- 442 12. Yang T, Cui FD, Choi MK, Cho JW, Chung SJ, Shim CK, Kim DD 2007. Enhanced solubility and
443 stability of PEGylated liposomal paclitaxel: in vitro and in vivo evaluation. *Int J Pharm* 338(1-2):317-326.
- 444 13. Han Y, Chaudhary AG, Chordia MD, Sackett DL, Perez-Ramirez B, Kingston DG, Bane S 1996.
445 Interaction of a fluorescent derivative of paclitaxel (Taxol) with microtubules and tubulin-colchicine.
446 *Biochemistry* 35(45):14173-14183.
- 447 14. Xu P, Gullotti E, Tong L, Highley CB, Errabelli DR, Hasan T, Cheng JX, Kohane DS, Yeo Y 2009.
448 Intracellular drug delivery by poly(lactic-co-glycolic acid) nanoparticles, revisited. *Mol Pharm* 6(1):190-
449 201.
- 450 15. Dong Y, Feng SS 2005. Poly(d,l-lactide-co-glycolide)/montmorillonite nanoparticles for oral
451 delivery of anticancer drugs. *Biomaterials* 26(30):6068-6076.

16. Forrest ML, Yanez JA, Remsberg CM, Ohgami Y, Kwon GS, Davies NM 2008. Paclitaxel prodrugs with sustained release and high solubility in poly(ethylene glycol)-b-poly(epsilon-caprolactone) micelle nanocarriers: pharmacokinetic disposition, tolerability, and cytotoxicity. *Pharm Res* 25(1):194-206.
17. Liu J, Lee H, Huesca M, Young A, Allen C 2006. Liposome formulation of a novel hydrophobic aryl-imidazole compound for anti-cancer therapy. *Cancer Chemother Pharmacol* 58(3):306-318.
18. Cheruvu NP, Kompella UB 2006. Bovine and porcine transscleral solute transport: influence of lipophilicity and the Choroid-Bruch's layer. *Invest Ophthalmol Vis Sci* 47(10):4513-4522.
19. Lombry C, Bosquillon C, Preat V, Vanbever R 2002. Confocal imaging of rat lungs following intratracheal delivery of dry powders or solutions of fluorescent probes. *J Control Release* 83(3):331-341.
20. Wright KM, Horobin RW, Oparka KJ 1996. Phloem mobility of fluorescent xenobiotics in *Arabidopsis* in relation to their physicochemical properties. *J Exp Bot* 47(11):1779-1787.
21. Makoto Miyajima* AK, Jun'ichi Okada, Akira Kusai and Masaru Ikeda 1998. The effects of drug physico-chemical properties on release from copoly (lactic/glycolic acid) matrix *International Journal of Pharmaceutics* 169(2):255-263.
22. Cheminformatics M. 2010. Molinspiration, <http://www.molinspiration.com/>. ed.
23. Steele T, Huang C, Widjaja E, Loo J, Venkatraman S 2011. The effect of polyethylene glycol structure on paclitaxel drug release and mechanical properties of PLGA thin films. *Acta Biomaterialia* Feb. 17(doi:10.1016/j.actbio.2011.02.002).
24. Widjaja E, Li C, Chew W, Garland M 2003. Band-target entropy minimization. A robust algorithm for pure component spectral recovery. Application to complex randomized mixtures of six components. *Anal Chem* 75(17):4499-4507.
25. Widjaja E, Seah RK 2008. Application of Raman microscopy and band-target entropy minimization to identify minor components in model pharmaceutical tablets. *J Pharm Biomed Anal* 46(2):274-281.
26. Widjaja E, Garland M 2008. Use of Raman microscopy and band-target entropy minimization analysis to identify dyes in a commercial stamp. Implications for authentication and counterfeit detection. *Anal Chem* 80(3):729-733.
27. Seah RK, Garland M, Loo JS, Widjaja E 2009. Use of Raman microscopy and multivariate data analysis to observe the biomimetic growth of carbonated hydroxyapatite on bioactive glass. *Anal Chem* 81(4):1442-1449.
28. FDA. 2003. Guidance for Industry: Q3C--Tables and List. <http://www.fda.gov/>. ed.
29. Lu L, Garcia CA, Mikos AG 1999. In vitro degradation of thin poly(DL-lactic-co-glycolic acid) films. *J Biomed Mater Res* 46(2):236-244.
30. Maxfield J, Shepherd I 1975. Conformation of poly(ethylene oxide) in the solid state, melt, and solution measured by Raman scattering. *Polymer* 16:505-509.
31. Tecan. 2009. Tecan M200 Multimode Manual. ed.
32. Chen Y, Arriaga EA 2006. Individual acidic organelle pH measurements by capillary electrophoresis. *Anal Chem* 78(3):820-826.
33. Lovich MA, Creel C, Hong K, Hwang CW, Edelman ER 2001. Carrier proteins determine local pharmacokinetics and arterial distribution of paclitaxel. *J Pharm Sci* 90(9):1324-1335.
34. Kang Y, Yin G, Ouyang P, Huang Z, Yao Y, Liao X, Chen A, Pu X 2008. Preparation of PLLA/PLGA microparticles using solution enhanced dispersion by supercritical fluids (SEDS). *J Colloid Interface Sci* 322(1):87-94.
35. Jauhari S, Dash AK 2006. A mucoadhesive in situ gel delivery system for paclitaxel. *AAPS PharmSciTech* 7(2):E53.

- 498 36. Liu Y, Pan J, Feng SS Nanoparticles of lipid monolayer shell and biodegradable polymer core for
499 controlled release of paclitaxel: effects of surfactants on particles size, characteristics and in vitro
500 performance. *Int J Pharm* 395(1-2):243-250.
- 501 37. Korber M 2010. PLGA Erosion: Solubility- or Diffusion-Controlled? *Pharm Res*.
- 502 38. Baker R. 1987. *Diffusion-controlled systems*. ed., New York: Wiley.
- 503 39. Kang E, Robinson J, Park K, Cheng JX 2007. Paclitaxel distribution in poly(ethylene
504 glycol)/poly(lactide-co-glycolic acid) blends and its release visualized by coherent anti-Stokes Raman
505 scattering microscopy. *J Control Release* 122(3):261-268.
- 506
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Table and Figure Captions

Table 1. Properties of hydrophobic fluorescent dyes and paclitaxel.

Table 2. Physical properties of selected paclitaxel and fluorescein diacetate thin films.

Table 3. Raman Microscopy peaks of analyzed compounds.

Figure 1. Scheme of PLGA thin film fabrication and analysis by HPLC or high throughput 96-well plate spectrofluorometry.

Figure 2. ^1H NMR of 15% 35K PEG/PLGA (53/47 with i.v. 1.03) containing 10% fluorescein diacetate (FDAC). Tetramethylsilane was used as the internal standard for ppm and ^1H quantification. Inset: Dichloromethane (DCM) peak present after 48h vacuum oven drying at 25°C , but not at 55°C .

Figure 3. Cross-section (CS) and surface topology of PLGA (53/47 with i.v. 1.03) containing 10% FDAC films at day 0 (top) and day 10 (bottom) incubation in release buffer. Images were taken at x700 magnification on a Field Emission Scanning Electron Microscopy (JEOL JSM-6340F, Japan).

Figure 4. Raman Microscopy of selected PLGA 53/47 (neat) and PLGA 53/47 with PEG films containing 10 % (w/w) hydrophobic dyes and paclitaxel. A) PLGA 53/47 with 10% paclitaxel, B) PLGA 53/47, 15% 8k PEG, and 10% paclitaxel, C) PLGA 53/47, 15% 35k PEG, and 10% paclitaxel, D) PLGA 53/47 with 10% coumarin- 6, E) PLGA 53/47, 15% 35k PEG, and 10% fluorescein diacetate, F) PLGA 53/47, 50% 35k PEG, and 10% fluorescein diacetate.

Figure 5. Slow and fast release of hydrophobic fluorescent dyes and in 0.2 (A) and 1.03 (B) i.v. PLGA's.

Figure 6. Low percentage additive release using A) 15% 8000 MW PEG and B) 15% 35000 MW PEG

Figure 7. High percentage additive release using A) 50% 8000 MW PEG and B) 50% 35000 MW PEG. Some error bars have been removed for clarity.

Homogenous PLGA polymer blends

Micrometer dial set
to wet thickness

Film Applicator

Film applicator spreads
polymer blend solution at
speed of 50 mm/s

Macro-view of films after drying in vacuum oven for 48 h @ 55°C.

15 mm punchout

6 mm punchout

PCTX-PEG8K-PLGA

24-well plate

FDAC-PEG8K-PLGA

96-well plate

HPLC Analysis

Time per sample: 10 min

Plate Spectrofluorometer
Time per sample: 2 s

CS

Surface, day 10

CS

Surface, day 0

A **B**

A **B**

A **B**