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Multi-drug delivery system with sequential release using titania nanotube arrays

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Experimental details

Materials: A high purity titanium foil (thicknes 0.25 mm, 99.6 %) was supplied by Alfa Aesar (USA), ethylene glycol, and ammonium fluoride by Sigma Aldrich (Australia). Indomethacin, itraconazole, gentamicin sulphate., d-α-tocopheryl polyethylene glycol 1000 (TPGS) and 1, 2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N [Methoxy(Polyethylene glycol)-2000 (DGP 2000) were obtained from Sigma-Aldrich Co., and used as received.

Preparation of titania nanotube (TNT) arrays: TNTs on Ti foil were prepared using two-step anodisation process. Anodization was performed using ammonium fluoride/ethylene glycol electrolyte (3 % water and 0.3 % NH_4F) at 20 ^oC at a constant voltage of 100 V for 2 h as described previously. [1-2]

Preparation of drug loaded polymer micelles (drug carriers): Two types of polymeric micelles: (i) d-α-tocopheryl polyethylene glycol 1000 (TPGS) (ii) 1, 2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N [Methoxy(Polyethylene glycol)-2000 (DGP 2000) were synthesised. TPGS was prepared as a regular micelle using the solvent evaporation technique.[3-5] 15 mg TPGS were dissolved in 5 ml chloroform. The formulations were prepared using oil-in-water emulsion techniques involving dissolving the regular polymeric micelles in an aqueous system (TPGS in Milli-Q water) and the drug in an organic solvent (indomethacin or itraconazole in ethanol) and vice versa, i.e. inverse micelles (phospholipid micelle DGP 2000 in ethanol) and the drug in an aqueous system (gentamicin in Milli-Q water).The TPGS micelles (5g) were first dissolved in 500 ml dichloromethane (organic solvent), which yields a micelle concentration of 1 w/v %. The micellar solution was mixed with a shear mixer at 10,000 rpm. Organic solvents were removed under vacuum and a thin film was obtained. Upon solvent evaporation in a rotary vacuum evaporator, micelles were dispersed in 20 ml Milli-Q water using gentle magnetic stirring for 15 min. On the other hand, 0.25 g indomethacin drug or mixture indomethacin /itraconazole in 50 ml of chloroform (a total of 5 % drug mass relative to the micelles mass) was prepared. Drug solutions was added into the micelle solution during mixing in a single portion over 15 sec. Excess drug was evaporated by placing the mixed dispersion in a rotary evaporator under reduced pressure at 50 ºC. Samples were dialysed against Milli-Q water for 2 days and produced in triplicate. Direct dissolution approach was used to synthesis the inverted micelles, i.e. 1, 2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy (Polyethylene glycol)-2000] (DGP 2000). Micelles were first dissolved in tetrahydrofuran, a common organic solvent which is also miscible in water. [5-6] This 5 wt% micelle solvent mixture was stirred and subsequently removed by being dialysed against bi-distilled water. During this dialysis process, micelle was formed and the organic solvent was removed through a 0.2 μm Nylon filter. An aliquot of copolymer water stock solution was prepared. An excess amount of drug stock solution (gentamicin sulphate) in acetone was made by adding the aliquot to an empty vial, before dialysed through a

Spectra/Por® Biotech regenerated cellulose membrane (Spectrum Labs, Inc.). Acetone was allowed to evaporate. The pre-prepared micelle/water mixture was added to the vial which contained the drug to be encapsulated. Excess drug (those non-incorporated in micelles) was separated by filtration of micelle suspension through a 0.2 μm filter. Micelles were evaluated in terms of their size by laser dynamic light scattering before and after drug encapsulation using Zetasizer Nano ZS, Malvern Instrument Ltd. Samples were diluted and the hydrodynamic radius of a micelle was determined.

Loading of drug loaded polymer micelles (drug carriers) on TNTs: Prepared TNT/Ti platforms were loaded with drug encapsulated polymer micelles using a simplified lyophilisation method where different arrangement of polymer micelles, their proportion and number of encapsulated drugs were explored.[68] The TNT substrates (12 cm X 12 cm) were cleaned with deionized Milli-Q water prior to the loading of the polymeric micelles by repeated drop-wise loading. In general micelle dispersion (1 μL) was pipetted drop wise onto the TNT surface each time and gently spread to ensure an even coverage. After every loading, at least 30 min was allowed for micelle solution to be loaded into the nanotube structures. The TNTs was placed in vacuum desiccators for 1 h under vacuum at room temperature for 2 h to evaporate solvent. After drying, the surface of the TNT was wiped gently using a soft tissue to ensure no excess drug was present and accumulated on the top layer. The loading of second polymer micelles was performed after the completion of loading for the first following a similar procedure. In total 24 or 25 loading steps were applied, which was found in our previous work enough to have good loading capacity of 12-16 %. In this work the first loading of invereted micelle (DGP 2000-gent) followed by regular micelle (TPGS-ind or TPGS-ind-itr) was performed. Their ratio was varied from 25% to 75% and controlled by number of loading steps. As an example, for a ratio of 50 : 50 loading for TPGS-ind : DGP 2000-gent, the loading and drying steps were performed in a total of 12 times (cycle) for DGP 2000-gent, before another 12 times for TPGS-ind for complete dual-drug loading in TNT. For a ratio of 25 : 75 ratio, the loading and drying steps were performed in a total of 6 times (cycle) for DGP 2000-gent, followed by 18 times for TPGS-ind. Control experiment using only single micelle loading (100 %) was also performed.

Characterizations:

Scanning electron microscopy (SEM): High resolution images were taken using Philips XL 30 SEM. The samples were cut into small pieces, mounted on a holder with double sided conductive tape and coated with platinum layer (3-5 nm). Images with a range of scan sizes at normal incidence and at a 30o angle were acquired from the top and cross-section.

Dinamic light scattering: Hydrodynamic diameter of the micelles were evaluated in terms of their size by laser dynamic light scattering before and after drug encapsulation using Zetasizer Nano ZS, Malvern Instrument Ltd. Samples were diluted and the hydrodynamic radius of a micelle was determined.

Thermograviemtric analysis (TGA): Drug loading efficiency was expressed as the percentage of drug loaded in micelles with respect to the initially added drug. Drug-micelle loaded in TNT was determined by the thermogravimetric (TGA) method (Hi-Res Modulated 2950, Q SeriesTM Thermal Analysis). 15 mg Titania nanotubes was loaded on the platinum pan then placed inside the burning furnace, heated from 20 ºC to 800 oC at a scanning rate of 10 ºC/min under nitrogen gas flow of 50 ml/min. The obtained thermograms was analysed using the software Universal Analysis 2000 (TA Instruments).

Drug release characterization: In order to release drug-loaded micelles from the nanopores, AAO substrates were immersed in 5 mL phosphate buffer (pH = 7.4) at room temperature for in

vitro release studies. Aliquots of 1 mL each were taken more frequently in the first 6 h of the first day during the initial burst, i.e. at pre-determined 5-15 min time intervals. Repeated volumes from the samples were withdrawn and their intensities measured periodically for up to 2 weeks whereby release reaches completion, with a frequency of two measurements per day. The extracted solution was replaced with 1 mL of fresh phosphate buffer every time an equal volume of the samples was taken. The samples were analyzed by Cary 1E UV-Vis spectroscopy (Varian Ltd, Inc.) in order to determine drug release amount using pre-constructed calibration curve of respective drug in micelle solution immersed in phosphate buffer. UV detection wavelength was set at λ=280 nm, 300 and 340 nm for itracanazole, gentamicin and indomethacin. Each experiment was carried out in triplicate. To prove the presence of both indomethacin and itraconazole inside TPGS micelles, UV-VIS spectrum (Cary 5, Varian Inc.) was carried out to obtain the absorbance peak of each of the drug in the micelle solution. Their individual existence, was then confirmed using their wavelength peak observed for each drug.

Table ST1.
The size of polymer micelles used as drug cariers before, after loading and after release. The individual loading characteristics of TNTs for drugs encaspulated in two layers of regular (TPGS) and inverted micelles (DGP)

Polymer Micelles	*Size (nm)* [a]	*Weight loss (%)* [b] *Ratio: 25*	*50*	*75*	*Drug/micelle loading in TNT (wt %)* [b]
Before drug loading					
TPGS	15.0±5.0				
DGP	20.0±3.5	-	-	-	-
After drug loading					
TPGS-ind	22.3±2.4	4.1	9.3	12.2	16.6
TPGS-ind-itr	29±6.0	4.4	9.2	12.0	15.8
DGP -gen	28.1±2.3	3.2	6.5	9.4	12.3
After drug release					
TPGS-ind	21.1±5.2	-	-	-	-
DGP-gen	25.1±3.8	-	-	-	-

[a] SD was calculated as the mean of 10 separate measurements on 10 different batches
[b] Determined by thermogravimetric (TGA) analysis. TNT (pore size = 110 nm).

Individual drug loading in TNT (wt%) = single drug loaded in TNT/total weight of TNT sample X 100%
Individual drug loading efficiency (wt%) = actual single drug loaded in TNT/theoretical amount of drug loaded in TNT X 100%

Table ST2.
Loading quantification of drugs (indomethacin and gentamicin) encapsulated in polymer micelles (regular, TPGS and inverted, DGP-2000) and loaded as two independent layers in TNT with different proportions (0 to 100%). The mass loss was determined by TGA measurements.

Ratio % (wt) TPGS-ind: DGP-gen	*Theoretical mass loss (mg)*	*Actual mass loss (mg)*	*Drug loading (%)*	*Encapsulation efficiency (%)*
0 : 100	0.0 : 2.5	0 : 2.4	0 : 14.2	0.0 99.1
25: 75	0.64: 1.9	0.63: 1.6	4.2 : 11	98.4 84.4
50: 50	1.3 : 1.3	1.2 : 0.92	8.1 : 6.2	95.4 71.6
75: 25	1.7 : 0.57	1.7 : 0.54	11: 3.5	98.7 94.0
100 : 0	2.86: 0.0	2.8 : 0.0	0 : 16.1	98.9 0.0

Drug loading (%) = drug amount loaded in TNT/total sample weight X 100%

Encapsulation efficiency = drug amount loaded micelles successfully entrapped inside TNT/total sample weight X 100%

Table ST3.
Release characterization of drugs (indomethacin and gentamicin) encapsulated in regular (TPGS) and inverted micelles (DGP-2000) loaded as two layers in TNTs with different proportions (25%, 50% and 75%)

Ratio % (wt) TPGS-ind: DGP-gen	*Time Release (hours or days)*								
	$t_{B,indo}$	$t_{B,gen}$	$t_{B,total}$ *(h)*	$t_{50\%,indo}$	$t_{50\%,gen}$	$t_{50\%,total}$ *(h)*	$t_{c,indo}$	$t_{c,gen}$	$t_{c,total}$ *(d)*
25: 75	8	-	8	11	44	102	2.2	7.6	9.8
50: 50	6	-	6	3	21	109	4.0	5.3	9.3
75: 25	6	-	6	8	16	8	6.1	2.7	8.6

t_B is the time for burst release; $t_{50\%}$ is the time for 50 % overall drug-micelle release; t_c is the time for complete release of drugs or micelles.

Table ST4:
Release efficiency of the two-drugs loaded in regular and inverted micelles with respect to the overall total release from TNT arrays (indomethacin and gentamicin)

Ratio % (wt) *TPGS-ind: DGP-gen*	*Release efficiency (%)*								
	6 h_{indo}	*6 h_{gen}*	*6 h_{total}*	*1 d_{indo}*	*1 d_{gen}*	*1 d_{total}*	*7 d_{indo}*	*7 d_{gen}*	*7 d_{total}*
0: 100	-	63	63	-	71	71	-	95	95
25: 75	16	1 9	25	36	15	51	47	49	96
50: 50	19	12	31	37	26	63	21	21	42
100 : 0	59	-	59	66	-	66	91	-	91

Release efficiency is defined as the wt % of drug release (mg)/wt of total drug release (mg) X 100%

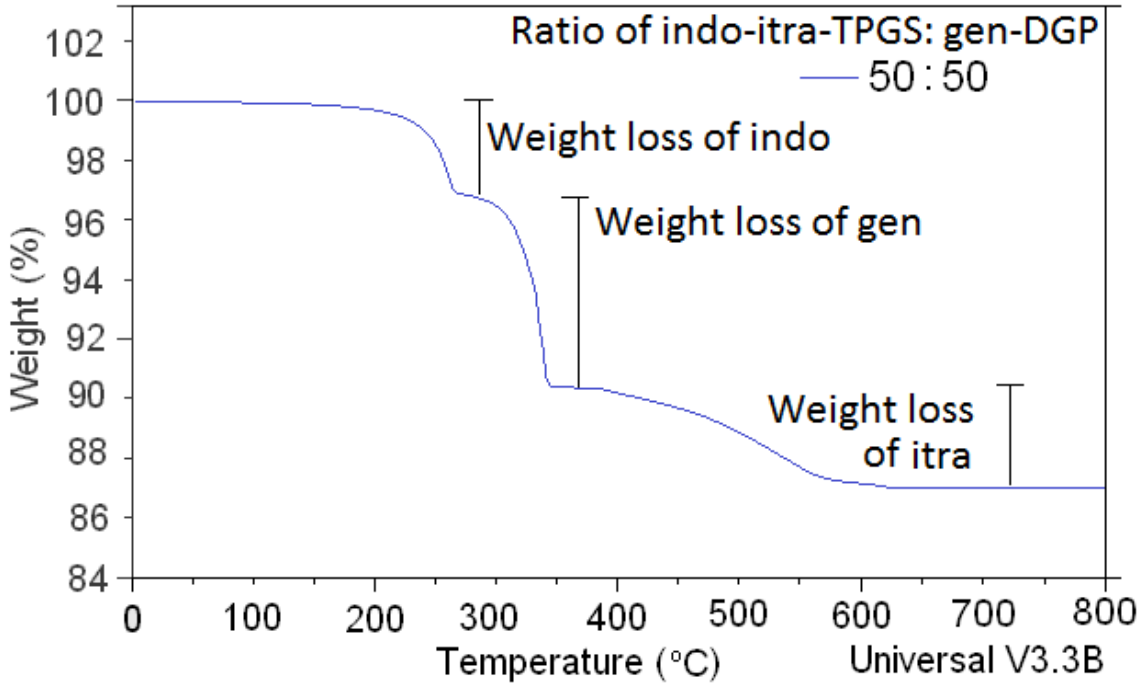

Figure S1.
Thermogravimetric analysis of the weight loss of TNTs loaded with two layers of polymer micelles (TPGS and DGP 2000) with 50% :50% proportion encapsulated with three drugs (two hydrophobic drugs indomethacin and itraconazole in regular micelle TPGS and hydrophilic drug gentamicin in reversed micelle DGP 2000).

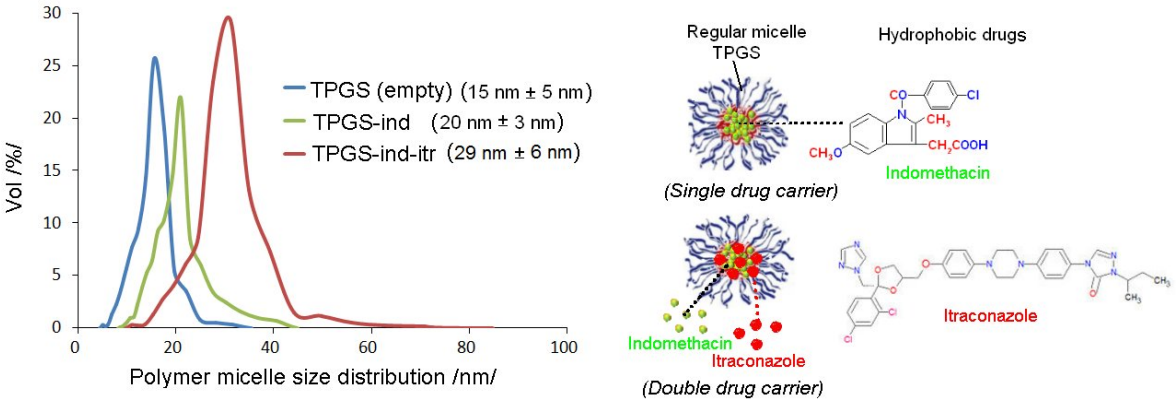

Figure S2.
Size distribution curves of regular polymer micelle (TPGS) showing changes in size after encapsulation with single hydrophobic drug (Indomethacin) and two hydrophobic drugs (indomethacin and itraconazole). The scheme of micelles with loaded drug is shown on the right.

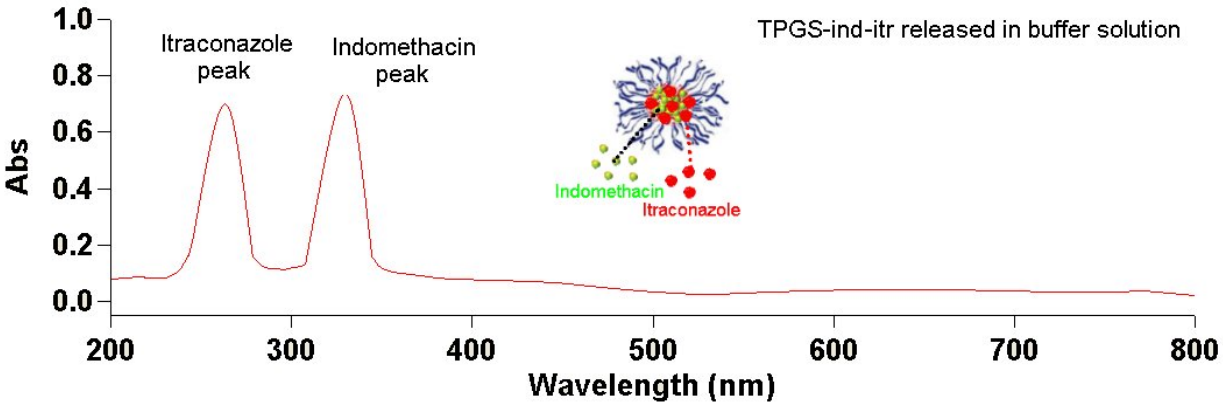

Figure S3.
The UV-Vis spectrum of TPGS micelle encapsulated with 2 hydrophobic drugs (indomethacin and itraconazole) and relased in PBS solution (pH = 7.4) from TNTs. Two prominent absorbance peaks which corespond to indomethacin and itraconazole were obsreved. The specrum was obtained by scanning in Cary 5 UV-Vis spectrophotometer.

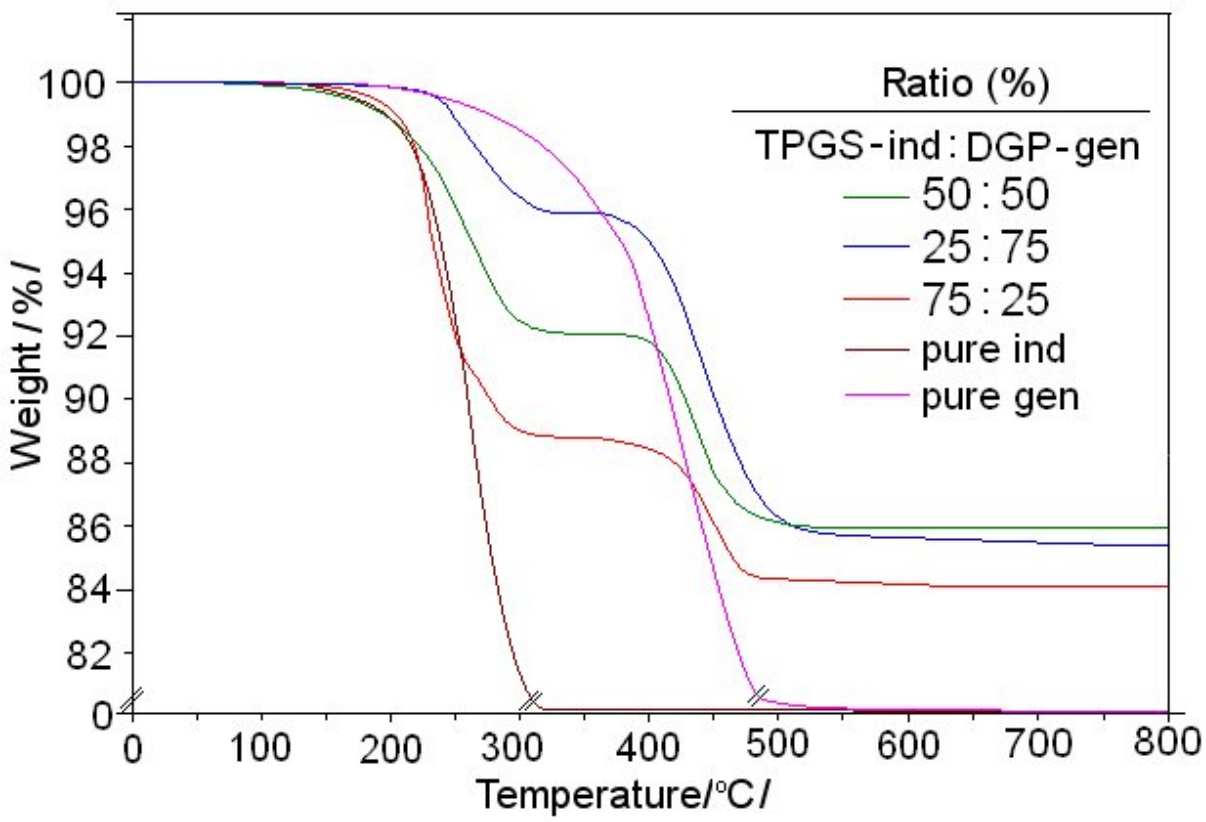

Figure S4.
Comparative TGA thermograms showing weight loss of TNTs loaded with pure drugs (indomethcain and gentamicin) and drug loaded micelles (TPGS-ind and DGP-gen) with different ratios (25%, 50% and 75%).

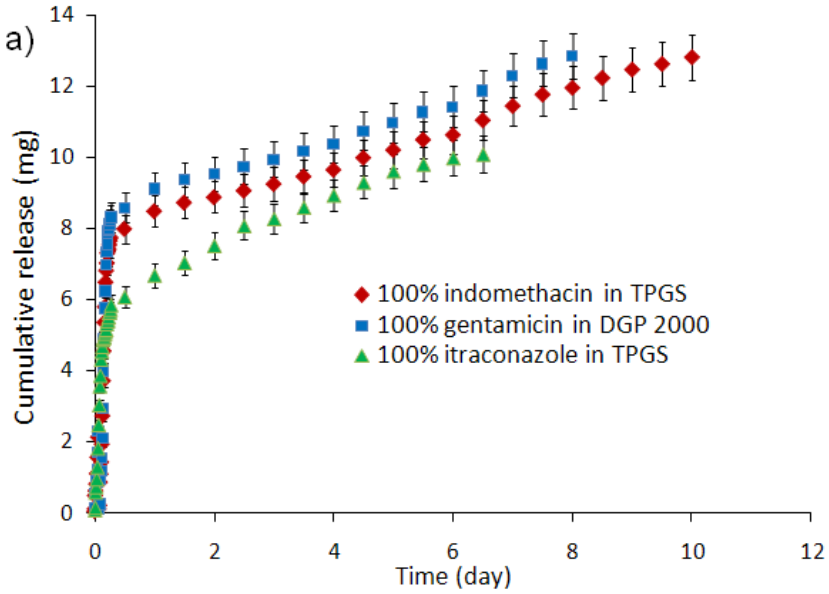

Figure S5.
Control drug release from TNTs loaded with single polymer micelles encapsulated with single drug including TPGS-ind, TPGS-itr and DPG-gen.

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