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<p>Please submit a plain text version of your cover letter here.</p> <p>If you are submitting a revision of your manuscript, please do not overwrite your original cover letter. There is an opportunity for you to provide your responses to the reviewers later; please do not add them here.</p>	<p>5 April 2016</p> <p>Dr José Oliveira Editor-in-Chief, Small</p> <p>Dr Joachim Loo Associate Professor Nanyang Technological University School of Materials Science and Engineering 50 Nanyang Avenue, N4.1 #02-09, Singapore 639798</p> <p>Re: Submission of Revised manuscript to SMALL</p> <p>Dear Dr Oliveira,</p> <p>My co-authors and I would like to thank you and the reviewers for taking time to review our submitted manuscript entitled " Multi-drug-loaded Microcapsules with Controlled Release for Management of Parkinson's Disease" (201600067R1). In this re-submission, we have carefully considered the comments by the reviewers and incorporated the amendments into the manuscript (see attached "Response to Reviewers").</p> <p>We hereby state that this submission has not been published previously, that it is not under consideration for publication elsewhere, and that if accepted it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher.</p> <p>Any correspondences with regards to this submitted manuscript can be referred directly to me through email at joachimloo@ntu.edu.sg. Alternatively, I can also be contacted through telephone (+65 67904603) or fax (+65 67909081). My co-authors and I would greatly appreciate if you could kindly re-review this article for publication. Thank you.</p> <p>Yours Sincerely, Joachim Loo</p>
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Multi-drug-loaded Microcapsules with Controlled Release for Management of Parkinson's Disease

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Keywords: Parkinson's disease, controlled release, spray-coating, floating drug delivery system

Parkinson's disease (PD) is a progressive disease of the nervous system, and is currently managed through commercial tablets that do not sufficient enable controlled, sustained release capabilities. It is hypothesized that a drug delivery system that provides controlled and sustained release of PD drugs would afford better management of PD. Hollow microcapsules composed of poly-L-lactide (PLLA) and poly (caprolactone) (PCL) were prepared through a modified double-emulsion technique. They were loaded with three PD drugs, i.e. levodopa (LD), carbidopa (CD) and entacapone (ENT), at a ratio of 4:1:8, similar to commercial PD tablets. LD and CD were localized in both the hollow cavity and PLLA/PCL shell, while ENT was localized in the PLLA/PCL shell. Release kinetics of hydrophobic ENT was observed to be relatively slow as compared to the other hydrophilic drugs. It was further hypothesized that encapsulating ENT into PCL as a surface coating onto these microcapsules can aid in accelerating its release. Now, these spray-coated hollow microcapsules exhibited similar release kinetics, according to Higuchi's rate, for all three drugs. The results suggest that multiple drug encapsulation of LD, CD and ENT in gastric floating microcapsules could be further developed for *in vivo* evaluation for the management of PD.

1. Introduction

Parkinson's disease (PD) is well-known as a progressive and degenerative disease of the nervous system. It is more common in the elderly, with most cases occurring after the age of 50. The degeneration of dopaminergic neurons in the substantia nigra, and a reduction in the amount of the neurotransmitter dopamine available in the striatum relate symptoms of this disease.^[1-5] PD is currently treated through, (i) dopamine substitution via administration of its metabolic precursor levodopa (LD), (ii) administration of drugs that inhibit the metabolism of dopamine, and (iii) activation of striatal dopamine receptors by dopamine agonists.^[6,7]

Dopamine substitution via levodopa is currently the “gold standard”, as unlike dopamine, levodopa is able to cross the blood brain barrier. In a case of oral administration, however, levodopa undesirably undergoes extensive decarboxylation during its transport through the liver – a problem that requires it to be administered in combination with other drugs to improve its efficacy.

LD is well-known as the most effective therapeutic agent for the treatment of PD.^[8-10] LD is absorbed mainly in the proximal small intestine and then quickly metabolized to dopamine by the aromatic L-amino acid decarboxylase (AADC) enzyme. Its elimination half-life is approximately 90 minutes, and only a small amount (~1 %) of LD eventually reaches the brain after a single oral dose.^[11] In addition, chronic administration of LD leads to LD-induced dyskinesia (LID). Dyskinesia occurs most commonly at the time of peak LD plasma concentrations during intermittent or pulsatile LD stimulation, and is referred to as peak-dose dyskinesia.^[11-13] Achieving a continuous and sustained delivery of LD could nonetheless reduce the emergence of LID.

Carbidopa (CD) is in a class of medications called decarboxylase inhibitors. It works by preventing LD from being broken down before it reaches the brain. Hence, LD products that are formulated with CD as AADC inhibitor inhibit metabolism of LD at periphery and increase the number of fractions delivered to the brain for dopamine conversion.^[14,15] In

addition, LD can also be administered with a dopa-decarboxylase inhibitor (DDCI) that shifts the peripheral metabolism of LD to an alternative pathway of catabolism-methylation [catechol-O-methyltransferase (COMT)].^[16,17] COMT inhibitors are able to block peripheral LD metabolism, prolong the half-life of LD and increase its delivery to the brain.^[18] Entacapone (ENT) is one peripheral COMT inhibitor that extends the half-life of LD elimination by up to 85 %.^[19] The addition of ENT to LD in patients with moderate/severe PD achieved sustained plasma levels of LD, and therefore provides a more constant dopaminergic stimulation in the brain. This reduces the required dosage and frequency of LD administration, thus decreasing the “off” time and increasing the “on” time, which essentially results in an improved and prolonged clinical response to LD.^[20-22]

Despite the synergism of these drugs in managing PD, their short elimination half-lives ($T_{1/2}$ -LD = 50 to 90 min; $T_{1/2}$ -CD = 1-2h; $T_{1/2}$ -ENT = 0.4-0.7h)^[23] pose other challenges. For example, in one pharmacokinetic study, LD/CD (LC) and LD/CD/ENT (LCE) tablets administered four times daily at 3.5-hour intervals to mice still showed insufficiency in achieving a continuous delivery of LD.^[24] In order to maintain adequate plasma drug levels over prolonged periods, frequent dosing is therefore required. This would inevitably result in sharp fluctuations in plasma drug level, giving rise to end-of-dose "wearing-off" symptoms. Compounding to this problem, a large number of these patients are elderly and probably already taking multiple pills for other ailments. These elderly patients are also more likely to overlook or “miss” their dose. High pill burden therefore decreases compliance to drug therapy, arising from the need to take a large quantity of these pills on a regular basis. All these factors, in combination, increase the possibility of side effects and medication errors for PD patients. These challenges therefore necessitate for controlled and sustained delivery of PD drugs in order to reduce dosing frequency, while improving therapeutic efficacy and patient compliance.

At present, there are only a few medications that are commercially available to patients, and these include Sinemet[®], Stalevo[®], and Rytary[®]. Unfortunately, Sinemet[®] and Stalevo[®] are non-sustained releasing medications, and do not address to the above issues. Although Rytary[®] has sustained release capabilities, it contains only two of the three PD drugs, i.e. LC/CD. Our group previously reported on a multi-drug-loaded microcapsule delivery system that showed great buoyancy in simulated gastric fluid, while providing controlled release of multiple cardiovascular drugs.^[25] The floatability of the designed microcapsules enabled for a prolonged gastric residence time, to provide sustained release while improving drug bioavailability. It is hypothesized that this microcapsule delivery system would provide controlled, and a more sustained, release of PD drugs as compared to commercially available PD tablets, thus potentially improving treatment outcomes and patient compliance. The aim of this study was therefore to develop a PD-specific drug delivery system that provides complete and simultaneous release of three PD drugs, i.e. LD, CD (both hydrophilic) and ENT (hydrophobic), towards better management of PD. Here, FDA-approved, biocompatible and biodegradable polymers, such as poly(L-lactide) (PLLA) and poly(caprolactone) (PCL), were used in the design of the delivery system.^[26,27] Other design parameters, such as PLLA/PCL ratio, spray-coating and the amount and localization of drugs, to modulate drug release profiles were also investigated. Drug localization was examined through scanning electron microscopy, Raman mapping, and confocal laser microscopy. Drug release profiles from various formulations were evaluated in simulated gastric and intestinal fluid for up to 24 h. Subsequently, buoyancy and *in vitro* hydrolytic degradation of these microcapsules were also investigated to lend support to the drug release profiles and kinetics observed.

2. Results and Discussion

2.1. Uncoated microcapsules

Microcapsules co-loaded with LD, CD and ENT were fabricated through a double emulsion ($W_1/O/W_2$) solvent evaporation method. In order to attain prolonged gastric residence time of these microcapsules, they were designed to be hollow, i.e. of lower density, to attain better floating properties. Modifications were therefore made to our previously reported fabrication process to produce hollow microcapsules through a single-step process. With the use of a rotary evaporator under reduced pressure, microcapsules with hollow cavities were obtained because of the fast solvent extraction rate under **this** condition (**Figure 1**). A fast solvent extraction rate shortens the time for any coalescence of the inner aqueous phase (W_1) with the outer aqueous phase (W_2), such that hollow microcapsules can be generated.^[26] To achieve different drug release kinetics, several hollow microcapsule samples (i.e. F1 to F3) were fabricated (details are summarized in **Table 1**). While all samples were composed of PLLA/PCL blends, they differed in their polymer blend ratios. The same amount of PD drugs were also encapsulated in these samples at a ratio of 4:1:8 (LD:CD:ENT), so as to replicate the same drug ratio as commercially available PD tablets (i.e. Stalevo[®]-100) for comparison purposes.

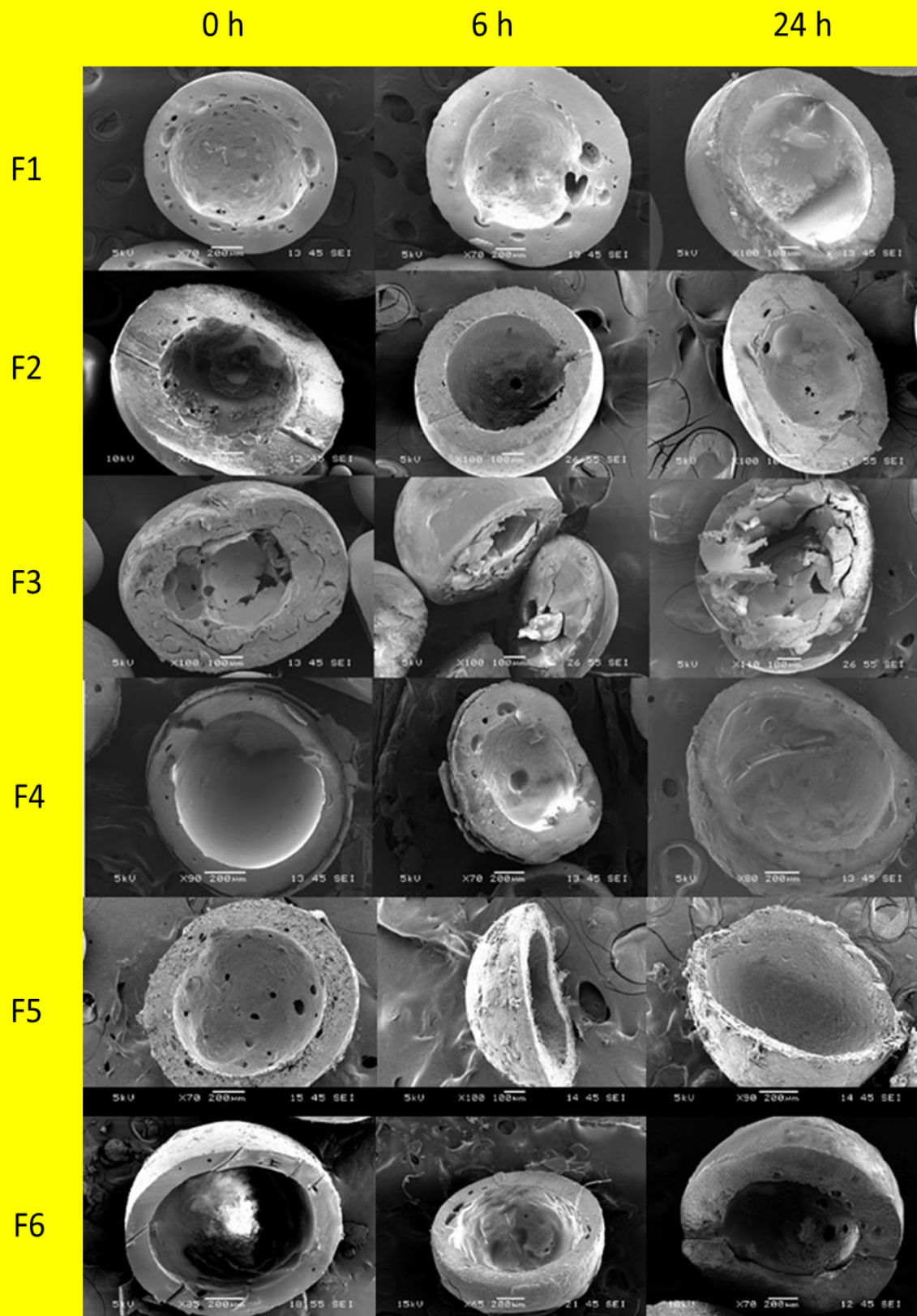


Figure 1. SEM image of a cross-sectioned uncoated and spray-coated microcapsules loaded LD, CD and ENT in SGF after 0 (Left column), 6 (Middle column), and 24 h (Right column). Row 1: F1; Row 2: F2; Row 3: F3; Row 4: F4; Row 5: F5, Row 6: F6.

Table 1. Compositions used to achieve different release profiles of the microparticles.

Samples	Organic Phase	Coating Layer 1		Coating Layer 2		Coating Layer 3	
	Solution	Solution	Flow Rate (mL/min)	Solution	Flow Rate (mL/min)	Solution	Flow Rate (mL/min)
F1 - PLLA/PCL 3:1	ENT (0.08 g)	-	-	-	-	-	-
F2 - PLLA/PCL 2:1	ENT (0.08 g)	-	-	-	-	-	-
F3 - PLLA/PCL 1:1	ENT (0.08 g)	-	-	-	-	-	-
F4 - PLLA/PCL 3:1 +PCL	ENT (0.04 g)	DCM (0.5 mL)	0.5	PCL (0.1 g) ENT (0.02 g)	0.3	PCL (0.1 g) ENT (0.02 g)	0.3
F5 - PLLA/PCL 2:1 +PCL	ENT (0.04 g)	DCM (0.5 mL)	0.5	PCL (0.1 g) ENT (0.02 g)	0.3	PCL (0.1 g) ENT (0.02 g)	0.3
F6 - PLLA/PCL 2:1 +PCL B	ENT (0.02 g)	DCM (0.5 mL)	0.5	PCL (0.1 g) ENT (0.03 g)	0.3	PCL (0.1 g) ENT (0.03 g)	0.3

Figure 1 shows the scanning electron microscopy (SEM) images of the fabricated microcapsules, and their corresponding images (from top to bottom) with increasing drug release time (left to right column). Regardless of the polymer blend ratios, all microcapsules were spherical in shape, have a mean diameter of ~600 μm , and possess a smooth surface finishing, uniform layer thickness and a hollow cavity. Unlike samples F1 and F2, sample F3 was, however, observed to have distinctively larger PCL particulates dispersed within the PLLA shell.^[28] This was due to the phase separation of immiscible PCL within PLLA that resulted in such a structural morphology. With the exception of particle morphology, there were no other significant observable differences, across all three samples. The drug loading (%) of LD, CD and ENT in all these microcapsules were also comparable (Table S1, Supporting Information).

In order to increase drug bioavailability, these microcapsules should possess good buoyancy, so as to prolong their gastric retention time and allow for drugs to be released in the upper gastrointestinal tract. For example, it was reported that floating tablets exhibited higher bioavailability (162.7%) of Norfloxacin in healthy male human volunteers as compared to conventional non-floatable tablet forms.^[29] In addition, it was also reported that propranolol HCl loaded into a floating gum showed a significant improvement in its bioavailability (132%) as compared with commercial propranolol tablets (Ciplar LA 80).^[30] Buoyancy of the microcapsules was subsequently evaluated to examine their floatability in SGF over 24 h (**Figure 2**). It was observed that microcapsule samples F1 to F3 possess floating capabilities, but to varying degree of success. The ability to stay afloat was attributed to their low density aided by an oil-filled hollow cavity, together with the hydrophobic nature of a PCL/PLLA blend that restricted rapid water uptake into these hollow microcapsules.^[31-33] Sample F1 had 94 % of the microcapsules remaining afloat at 24 h, whereas samples F2 and F3 fared poorer in this regards. Specifically, sample F3 only had ~60 % of the microcapsules afloat at 24 h. The floating tendency of these microcapsules was observed to be affected by its PCL/PLLA blend ratio, whereby floatability decreased with increasing PCL content. PCL, as an amorphous polymer with low glass transition temperature (below 0 °C), promoted the influx of water into the microcapsules (**Figure 3a**) The more open and mobile polymer chains in PCL resulted in higher water uptake,^[3] thereby decreasing their buoyancy over time. This further resulted in the formation of pores that further promote water ingress, thus translating to inferior buoyancy.^[34]

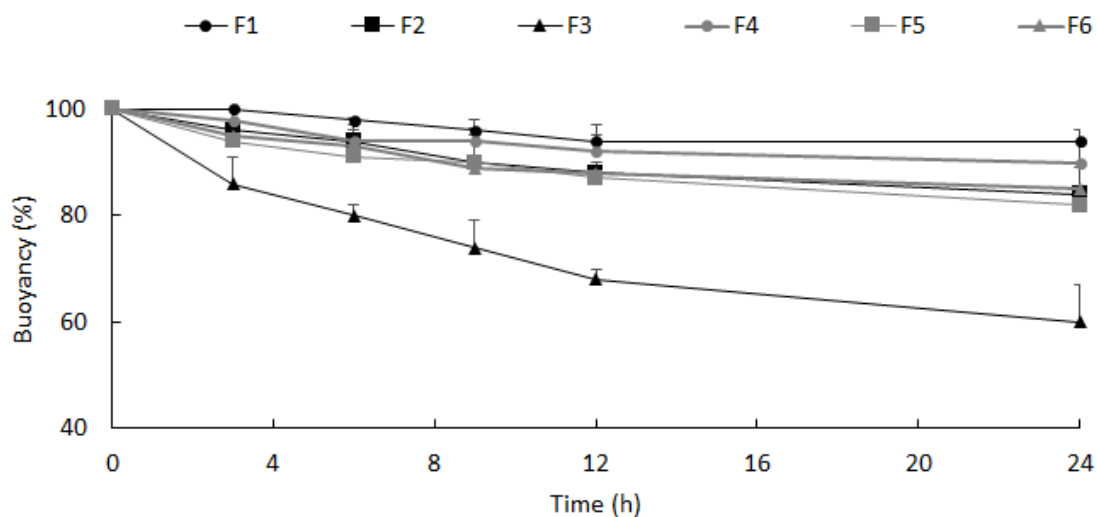


Figure 2. Buoyancy (%) of the various microcapsules encapsulating three drugs in SGF at 37 °C for 24 h (n=3).

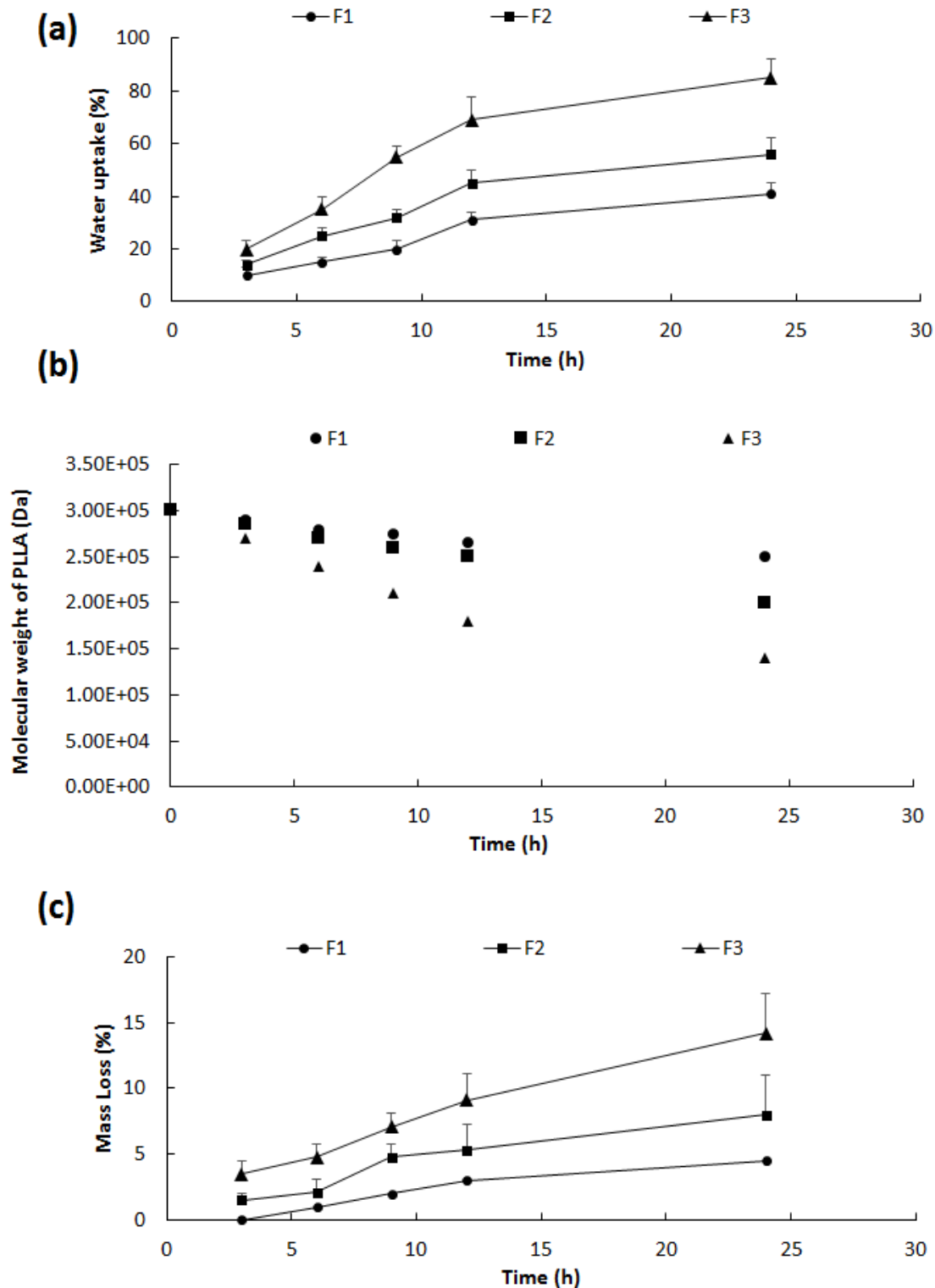


Figure 3. (a) Water uptake, (b) change in molecular weight of PLLA and (c) mass loss of the degrading uncoated microcapsules as a function of incubation time ($n=3$).

To test the hypothesis that this drug delivery system can better provide sustained and controlled release of triple PD drugs, as opposed to commercially available tablets, the release profiles of all three PD drugs from the samples were subsequently investigated. As observed from **Figure 4a**, commercial PD tablets (Stalevo[®]) in SGF gave a complete release of all three drugs within a short span of 2 h, as these tablets were not controlled-release formulations. Figure 4 further plots the drug release profiles for all three samples for each of the three PD drugs. A correlation between drug release kinetics and PCL content can be drawn from these plots, whereby all three drugs had faster release rates in samples with higher PCL content (i.e. F3>F2>F1). This, again, **was** attributed to the higher water uptake arising from the higher PCL content in the polymer blends.

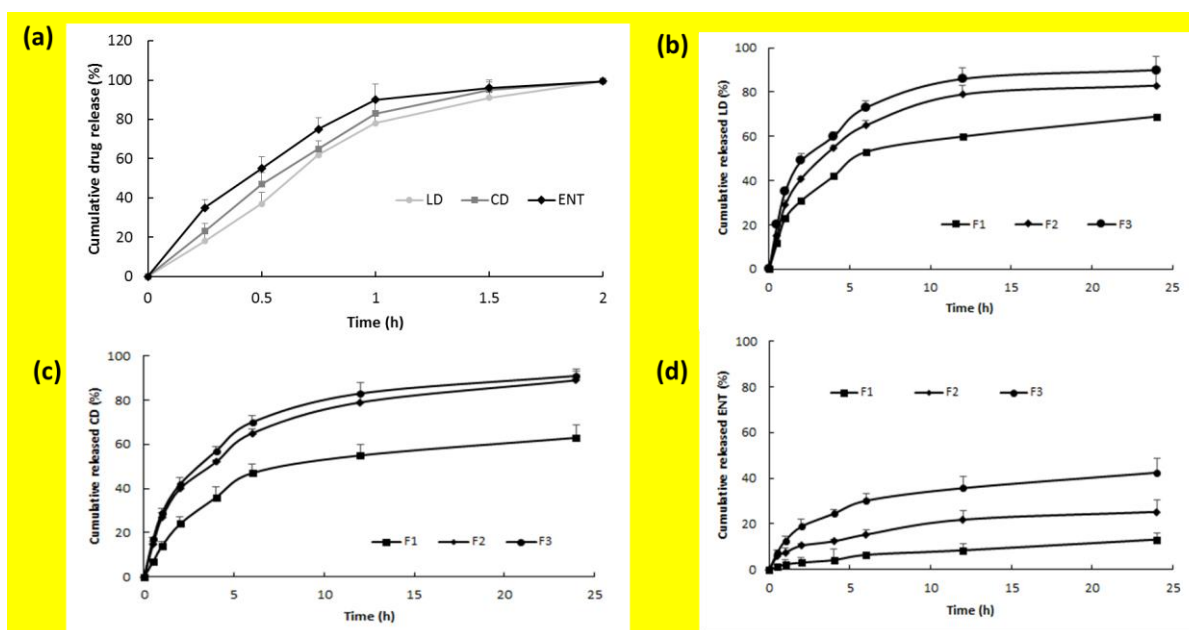


Figure 4. Release profiles of (a) commercial tablets (Stalevo[®]), (b) LD, (c) CD and (d) ENT from uncoated microcapsules in SGF at 37 °C for 24 h (n=3).

The different release kinetics of the samples could be further explained by the hydrolytic degradation of these microcapsules (Figure 3). The faster release rate also **correlated** to the

1 faster degradation of the PLLA in the PCL/PLLA shell capsule arising from the higher water
 2 uptake (Figure 3a). As PLLA has a semi-crystalline morphology and relatively more
 3 hydrophobic nature, PLLA retards any appreciable water influx, [38] which can explain for a
 4 slower release rate from sample F1. Microcapsule shells with a higher PCL content were more
 5 rubbery, due to the presence of larger phase-separated PCL particulates (Figure 1). At 24 h,
 6 samples F3 were observed to have a more porous shell that would inevitably further
 7 accelerate drug release (Figure 4). Faster release rates were therefore attributed to larger PCL
 8 particulates that promote water ingress that increases the rate of PLLA degradation (Figure 3b
 9 and 3c). Taken in totality, these factors allowed for a faster diffusion of drugs through a more
 10 porous shell, translating to a faster release rate.

11 While the release rates for hydrophilic LD and CD were observed to be reasonable, and
 12 somewhat related to a diffusion-controlled releasing mechanism, the release kinetics for the
 13 more hydrophobic ENT was observed to be relatively much slower. In order to replicate the
 14 drug content from commercial tablets at a ratio of 4:1:8 (LD:CD:ENT), it was essential to
 15 deliver all three drugs at the same rate. From Figure 4d, the release of hydrophobic ENT from
 16 F1, F2 and F3 was found to be highly retarded at only ~13.2, 25.3 and 42.5 % within 24 h. As
 17 the partition coefficient of ENT for [octanol]/[water] is 2.0, [39] the slow release of
 18 hydrophobic ENT is expected. At the same time, a more hydrophobic ENT would interact
 19 strongly with PLLA through hydrophobic-hydrophobic affinity, further retarding its
 20 release. [40]

2.2. Spray-Coated Microcapsules

21 Although the hollow microcapsules showed some promise, i.e. good buoyancy and the ability
 22 to sustain the release of LD and CD, the release of hydrophobic ENT was too slow and did
 23 not match up to the release kinetics of the other two hydrophilic drugs. To address to this
 24 issue, it was further hypothesized that the encapsulation of ENT into PCL as an additional

surface coating onto the hollow microcapsules can aid in accelerating its release, by reducing the drug diffusion distance to the release environment. To test this hypothesis, samples F1 and F2 were chosen for further development, by coating with PCL-ENT; but with differing ENT amounts (Table 1). In order to keep to the same drug ratio of 4:1:8, half of the original amount of ENT was loaded into the hollow microcapsule, while the other half was coated onto the surface of the microcapsule, i.e. PLLA/PCL 3:1 (F4) and PLLA/PCL 2:1 (F5). A third sample, F6, had a quarter of the original ENT amount loaded in the hollow microcapsules, while the remaining ENT was surface coated. The rationale of choosing PCL as the coating polymer was because of its amorphous nature that allows for a faster water uptake which is expected to translate to a more rapid release rate.

Figure 1 displays the SEM images of samples F4 to F6. It was observed that freshly spray-coated microcapsules ($t = 0$ h) were likewise spherical in shape, but had an additional coating layer on the surface. With the additional coating, the size of the microcapsules was observed to increase by about 100 μm . To determine the drug and polymer localization within the microcapsules, Raman spectroscopy and mapping (**Figure 5**) was conducted for sample F5, as a representative sample. Figure 5a shows the Raman spectrum of each drug and polymer in the microcapsule, from the inner hollow cavity to the particle shell and finally to the outer coating layer. Raman mapping was further employed to distinguish the distribution of the polymers and drugs. Raman mapping shows that while the hydrophobic ENT had preferential interactions with the hydrophobic polymer shell, the hydrophilic LD and CD drugs were uniformly distributed both in the hollow cavity and capsule shell. As ENT itself possess yellow fluorescence, confocal laser scanning microscopy (CLSM) was utilized to determine its distribution. CLSM images obtained for F5 are shown in **Figure 6**. The images further confirmed the localization of ENT both within the microcapsules and on the surface of the microcapsule, as a uniform coating layer. From the z-stack images, the surface of the particle exhibited the strongest fluorescence from ENT, confirming the effectiveness of the spray

coating process. The largest amount of ENT was localized in the outer layer, i.e. coating/shell, while the hollow core exhibited a much weaker intensity.

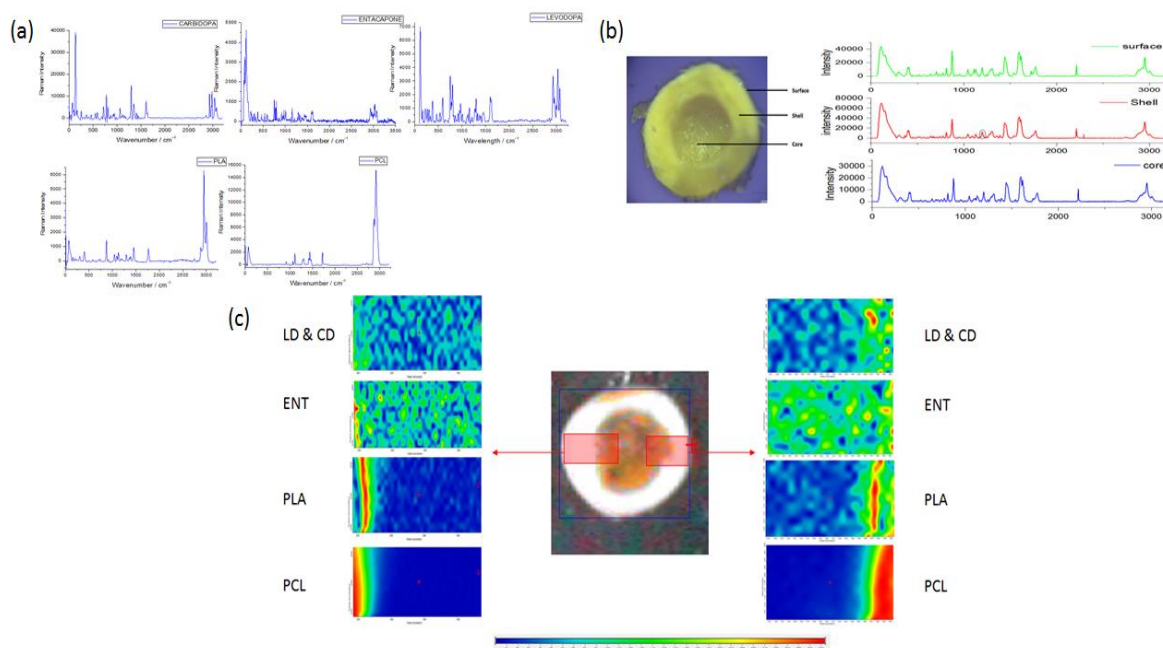


Figure 5. (a) Raman spectra of pure component, (b) spectra from hollow core, shell and coating layer of F5, and (c) Raman mapping images of F5.

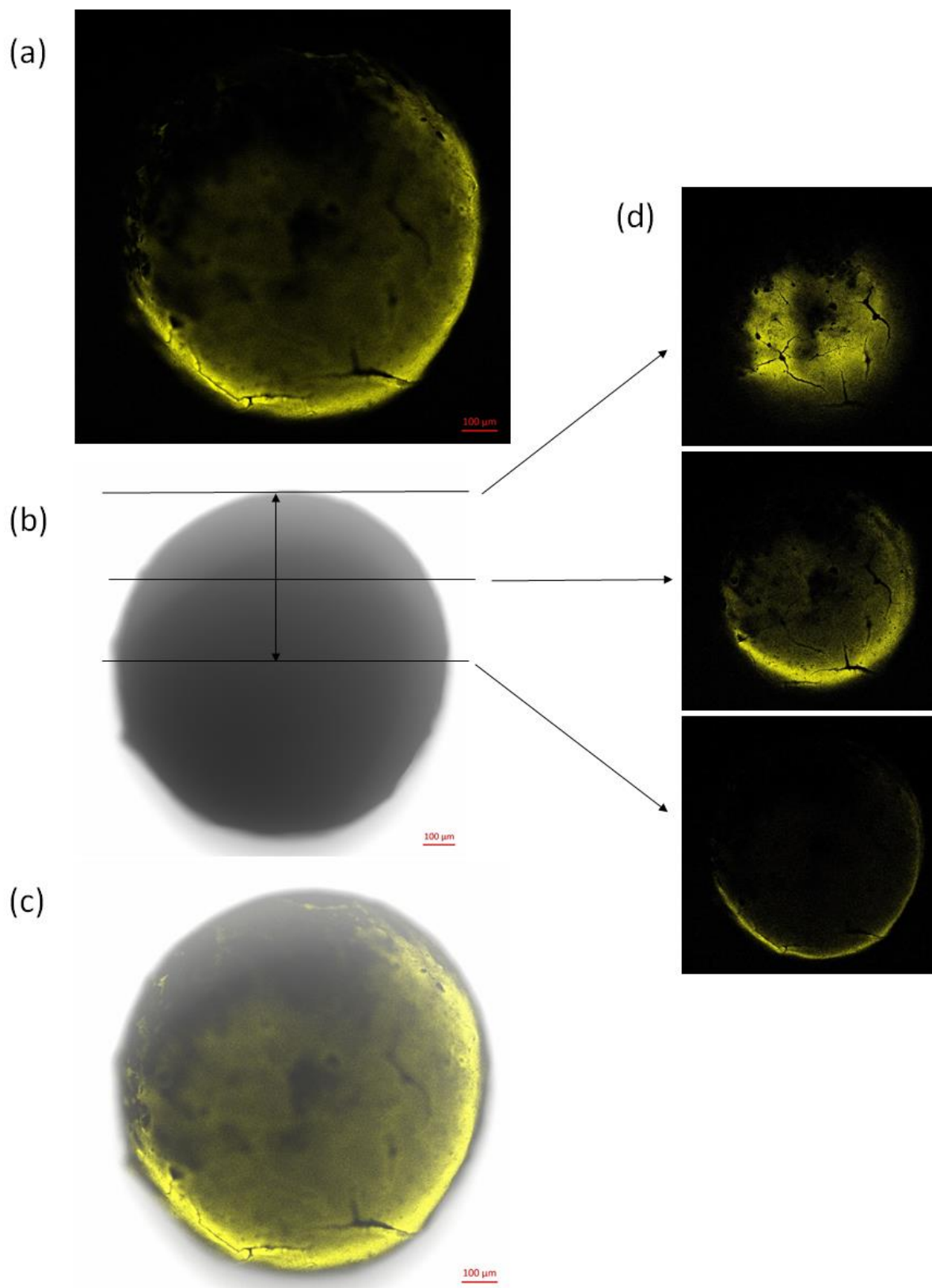


Figure 6. CLSM images of a cross-sectional microcapsule (F5). (a) emission of the ENT, (b) no laser, (c) emission of the ENT, and (d) z-stack images from center to top of microcapsule.

As seen in Figure 5c, it was also confirmed that the spray-coated microcapsule had a hollow cavity, a shell and a coating layer. Both Raman mapping showed strong intensity of PLLA and PCL in the shell and coating layer, respectively. A relatively weaker intensity of PCL was observed in the capsule shell as compared to PLLA, as the larger phase would naturally engulf the minor phase (i.e. PCL). Interestingly, no significant intensity for PLLA and PCL was obtained in the hollow cavity of the microcapsule. On the contrary, a uniform distribution of the hydrophilic drugs was observed here. This phenomenon could be explained as during rapid evaporation of organic solvent (DCM) using rotary evaporator, the hydrophilic LD and CD in the water phase remained in the hollow cavity, likely as an internal wall coating, while the more hydrophobic PLLA/PCL shell solidified with ENT-embedded in this shell. Notably, the spray-coated PCL layer on uncoated microcapsules exhibited no affect to their buoyancy.^[25] The F4, F5, and F6 exhibited 90 ± 6 , 85 ± 6 and 82 ± 7 % of buoyancy after 24 h.

The release profiles of each drug from PLLA/PCL microcapsules with PCL coating layer in SGF are shown in **Figure 7**. PCL coated microcapsules (F4 – F6) exhibited significantly faster release of ENT compared to that from uncoated microcapsules (F1 – F3). Furthermore, when a higher amount of ENT was encapsulated in PCL coating layer (F6), the release rate and cumulative release of ENT were faster and higher in quantity, respectively (Figure 7c). As it **had** been reported that floating microspheres are able to provide prolonged GRT of 5 h in the human body,^[41,42] release study was further conducted but, this time, in an environment that would simulate drug release in the stomach, based on typical gastric emptying time, followed by release in the intestinal region.^[35,42] As such, the release profiles of these microcapsules (F4, F5 and F6) were conducted in SGF, followed by SIF (**Figure 8**). Since the major absorption site of all three drugs was reported to be the stomach and upper intestine,^[44] ^{46]} having a 70% release of all three drugs in this regions within 5-7 h would definitely be advantageous. From Figure 8, all the samples now exhibited relatively similar release rates for

all three drugs in SGF. Here, the ENT-loaded PCL coating layer and the ENT-loaded capsule shell provided an initial and sustained release of ENT, respectively. The ENT-loaded rubbery PCL coating layer onto microcapsule not only shortens drug diffusion distance, but also **accelerated** drug dissolution and diffusion. With PCL as a coating layer, the degradation of PLLA shell was observed to be retarded as compared to the non-coated microcapsules (Figure S1, Supporting Information).

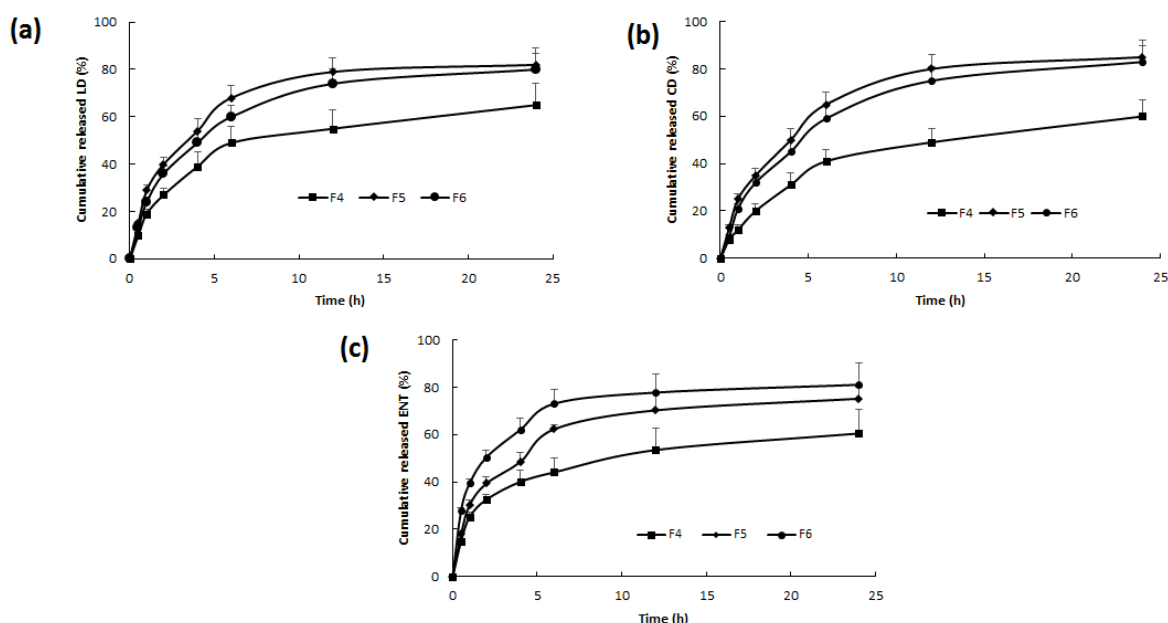


Figure 7. *In vitro* release profiles of (a) LD, (b) CD and (c) ENT from coated microcapsules in SGF for 24 h at 37 °C (n=3).

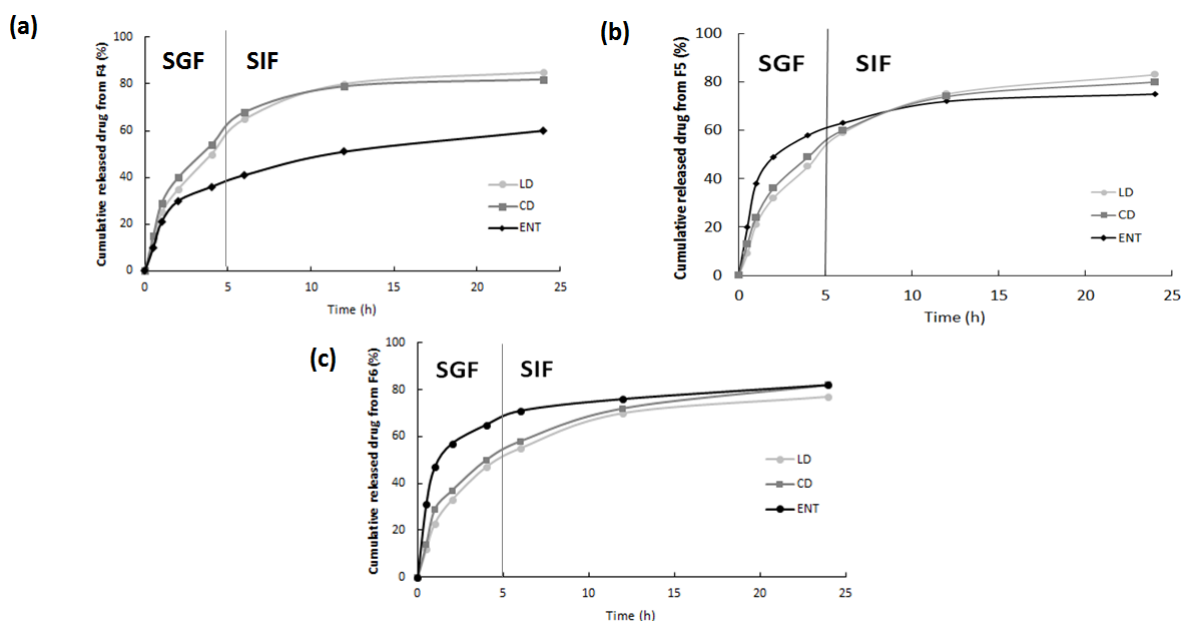


Figure 8. *In vitro* release profiles of LD, CD, and ENT from (a) F4, (b) F5 and (c) F6 in SGF for 5 h followed by release into SIF for 24 h at 37 °C (n=3).

The new formulations were also able to achieve sustained delivery of all three PD drugs, releasing up to 80% of the drugs within 24 h. This new coated formulation could modify release profile of multiple drugs regardless of their hydrophilicities by altering their PLLA/PCL ratio and through the use of an additional coating technique. To elucidate the mechanism of drug release from these coated microcapsules, the release data were fitted to zero order kinetics (cumulative amount of drug released vs time),^[47] first-order kinetics (log cumulative percentage of drug remaining vs time),^[48] and Higuchi's release kinetics (cumulative percentage of drug released vs square root of time)^[49]. **Table 2** summarizes the r^2 correlation values for all three fits and the corresponding release rate constants. From the data, Higuchi's equation has the best fit of all. Sample F4 had the lowest k values for all drugs as compared to F5 and F6 because of the PLLA/PCL ratio effects that were earlier discussed. Among the three samples, F6 had the closest k values for all three drugs. The total amount of LD, CD and ENT in the commercial tablet (Stalevo®-100) is 100, 25 and 200 mg, respectively. As F6, with a drug ratio of 4:1:8 (Table S1, supporting information) similar to commercial tablets, had the closest k values for all three drugs (Table 2), F6 could potentially be exploited for management of PD in patients. This would be further evaluated through *in vivo* pharmacodynamics studies. In terms of the weight of F6 required in patients, this would correspond to approximately 1.82 g of this microcapsules to meet the same amount of drugs as the commercial tablet – Stalevo®-100. Our spray-coated microcapsule may therefore be a promising oral drug delivery system to achieve sustained and controlled release of multiple drugs for the management of Parkinson's disease.

Table 2. Kinetics of LD, CD and ENT from coated microcapsules.

Drug	Formulation	Coefficient (r^2)			Rate constant
		Zero-order	First-order	Higuchi equation	$K \text{ (h}^{-1/2}\text{)}$
LD	F4	0.7145	0.8694	0.9472	15.023
	F5	0.6436	0.8596	0.8826	17.245
	F6	0.7102	0.8717	0.9315	17.735
CD	F4	0.6923	0.8966	0.9526	15.815
	F5	0.704	0.8665	0.9150	17.052
	F6	0.752	0.9067	0.9310	17.345
ENT	F4	0.7214	0.8346	0.9290	12.249
	F5	0.6387	0.7865	0.8816	15.488
	F6	0.5305	0.7089	0.8032	16.615

3. Conclusion

Floatable microcapsules loaded with three different PD drugs, and with the same drug ratio as commercial PD tablets, were fabricated. Manipulating polymer ratio between PLLA and PCL can influence how these drugs were released. However, because of the hydrophobic nature of ENT, its release was greatly retarded. Coating of these microcapsules with ENT and PCL onto its shell was therefore introduced as a technique to load a portion of ENT onto the surface of the microcapsules. With this, the release profiles fitted well into the Higuchi's release kinetics and sample F6 was found to have similar release kinetics ($k = 17.23 \text{ h}^{-1/2}$) for all three PD drugs. This delivery system would be further evaluated through *in vivo* studies in comparisons against commercially available PD tablets.

4. Experimental Section

Materials: Poly-L-lactide (PLLA) (IV: 2.4, Purac), Polycaprolactone (PCL) (molecular weight 10 kDa, Sigma-Aldrich), and Polyvinyl alcohol (PVA) (molecular weight 30 – 70 kDa, Sigma-Aldrich) were used without further purification. LD, CD, ENT, Tween 20, HCl solution (37% v/v Fuming), acetic acid and ethyl acetate (ETA) were purchased from Sigma-Aldrich (Steinheim, Switzerland). Dichloromethane (DCM), acetonitrile (ACN), chloroform and tetrahydrofuran (THF) were purchased from Tedia Co. Inc. Olive oil (Pietro Coricelli) was used. All other chemicals and reagents used were of analytical grade. The simulated gastric fluid (SGF) (pH 1) was prepared by adding 0.1 M HCl solution to 0.02% (w/v) Tween 20. The simulated intestinal fluid (SIF) was prepared by mixing pH 6.8 phosphate buffer and 0.02% (w/v) Tween 80.

Preparation of multi drugs loaded hollow microcapsules: Encapsulation of free LD and CD in PLLA/PCL polymer mixture was performed using the water-oil-water ($W_1/O/W_2$) double emulsion method. The internal aqueous solution was prepared by adding 1 ml of 0.5M HCL to 50.0 mg of LD, 12.5mg of CD and vortex until fully dissolved. The resultant solution was then introduced drop-wise above the polymer solution (0.3 g of PLLA and 0.1 g of PCL dissolved in 5 ml of DCM) and then add 10 μ L of olive oil under magnetic stirring. The obtained mixture was added to 0.25% (w/v) PVA solution (50 mL) containing DCM (1 mL) and stirred using overhead stirring at 250 rpm for 4 min. For preparation of mixture of PVA solution (50 mL) containing DCM (1 mL), DCM (1 mL) was added into 0.25% (w/v) PVA solution (50 mL) using syringe, followed by stirring with overhead stirrer for one min to disperse it in PVA solution uniformly. Since DCM is not miscible in water, DCM was dispersed as small droplets.

The resultant emulsion was quickly transferred to rotary evaporator with the addition of 0.25 % (w/v) PVA solution (150 mL) to solidify the microcapsules. The microcapsules

obtained were centrifuged, washed with distilled water for three times, freeze dried and kept in a desiccator.

Spray-coating on microcapsules: The spray-coating was performed using MediCoat DES 1000 Benchtop Stent Coating System (Sono-TekCorp., Milton, NY). The coating process were conducted in a row-by-row sweeping manner. The settings are identical and include power for the frequency generator of 1.0W for coating layer 1, 1.4W for the remaining layers and an air shroud level of 2 psi. Table 1 shows each layer's spraying composition. Finally, the spray-coated microcapsules were dried in a vacuum oven at 40°C for 24 h.

Drug loading content: Microcapsules (10 mg) were accurately weighed and dissolved in 1 ml of DCM through sonication. SGF (10 mL) was then added, and mixed using a vortex at 300 rpm (n=3). The hydrophilic LD and CD partitions into SGF and the supernatant was analyzed using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) with 100% Acetic Acid (2% v/v) as mobile phase at wavelength 284 nm. The ENT is then re-dissolved in Sodium Dihydrogen Phosphate (60 %) / Methanol (40 %) Mixture to precipitate polymer. The supernatant is then taken and filtered through a 0.22 µm syringe filter. The resultant solution is then analyzed using RP-HPLC with Sodium Dihydrogen Phosphate (60 %) / Methanol (40 %) Mixture as mobile phase at wavelength 284 nm. The following equation was applied to calculate the drug loading (DL). $DL (\%) = \frac{\text{weight of the drug in microcapsules}}{\text{weight of the microcapsules}} \times 100 \%$

Morphological Analysis: The cross-sectional morphology of microcapsules prepared was observed with a scanning electron microscope (SEM, JEOL JSM-6360A). First, the microcapsules were mounted on carbon tape attached metal stub and cross-sectioned using a surgical blade to expose the internal cross sections and the microcapsules were subsequently coated with gold using a sputter coater (SPI-Module). To measure average capsule size, The Image J software was used to measure capsule size.

Raman mapping: Raman mapping was used for observing the polymer and drugs distribution within the microcapsules. Cross-sectioned microcapsule was put under a microscope objective with a laser power of 10 mW. Raman mapping measurements were carried out with a step size interval of 5 μm to form a grid map using a Raman microscope (NicoletTM isTM50, Thermo Scientific) equipped with a near-infrared enhanced deep depleted thermoelectrically Peltier-cooled CCD array detector and a high-grade Leica microscope. The pre-sectioned microcapsule was irradiated with a 785 nm near-infrared diode laser, and the back scattered light was collected by an objective lens. Measurement scans were collected in a spectrum range from 200 to 3200 cm^{-1} .

Confocal laser microscopy (CLMS): ENT distribution within microcapsule was analyzed with a confocal laser scanning microscope (CLSM, LSM710). The aqueous suspension containing particles were drop-wise added to a glass slide before sealing with a glass cover slip. All photos were obtained using 63 \times /1.40 oil objective lens, and the AxioCan MRm camera. Analysis of the images was done with the ZEN 2012 software.

Hydrolytic degradation: Microcapsules were weighed (50 mg) and placed in glass bottles filled with SGF (20 mL). Samples were incubated 37 $^{\circ}\text{C}$ with gentle shaking. At pre-determined time points, microcapsules were collected from the bottles. For water uptake study, the microcapsules were washed with distilled water, weighed, and dried to obtain the dry mass. The percentage of water uptake was calculated at pre-determined time point as the difference between the mass of the wet and dry microcapsules, measured at time t, and taken as a percentage of the dry weight. Each experiment was conducted in triplicate. To determine mass loss, microcapsule mass loss was calculated as the difference between the initial mass of the microcapsules and dry mass at time t, and calculated by dividing by the initial mass and dry mass. Finally, molecular weight of the microcapsules was measured using the Agilent GPC 1100 Series using a reflective index detector (RID) at 30 $^{\circ}\text{C}$. Chloroform used as solvent and the flow rate was 1 mL/min. Based on the solubility differences of the polymers in THF

(PCL is soluble in THF, while PLLA is not), the two polymers in the microcapsules were separated by the dissolution method. Each microcapsules (10 mg) were added in THF (1 mL) to dissolve the PCL. The mixture solution was evaporated at room temperature for 48 h. The remaining solvent in the solution was further dried in an oven at 40 °C for a 48 h. And then, chloroform (1 mL) added to dried PLLA and analyzed for GPC. Molecular weights of the microcapsules were calculated by the calibration curve using polystyrene standards (165-5000 kDa).

Buoyancy test: A visual observation method was used to measure the buoyance of the various microcapsules.^[41] The microcapsules were added into in the transparent glass vials filled with SGF (20 mL). And then, the vials were placed under stirring at 250 rpm at 37 °C for 24 h. At each time point, the number of microcapsules floating on the SGF was counted visually. Finally, the percentage of floating microcapsules was calculated according to the ratio of the number of microcapsules floating on the SGF to the number of microcapsules used.

In vitro release study: Release study was conducted in both SGF and SIF for 24 h. Twenty milligram of microcapsules were added in SGF (20 mL) each. Both samples were placed in a 37 °C rotating incubator. The medium (10 mL) in each bottle was extracted and replaced with 10 ml new medium at different time points. The time points included intervals of hours within a 24-hour period. The drug content was analyzed using RP-HPLC.

Kinetics release profile: In order to analyze the kinetics of drug release, the release data were fitted to Zero-order, First-order and Higuchi equations.^[47-49]

Statistical analysis: Student's *t*-test was used to compare the groups. Statistically significant differences were considered when *p* value < 0.05. All data are expressed as the mean ± standard deviation from three independent experiments.

Supporting Information

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

Acknowledgements

The authors would like to acknowledge the financial support from the Singapore Centre on Environmental Life Sciences Engineering (SCELSE) (M4330001.C70.703012), the School of Materials Science and Engineering (M020070110), National Medical Research Council, Ministry of Health (NMRC/CIRG/1342/2012, MOH), the NTU-National Healthcare Group (NTU-NHG) grant (ARG/14012) and the Biomedical Research Council (A*STAR, Singapore).

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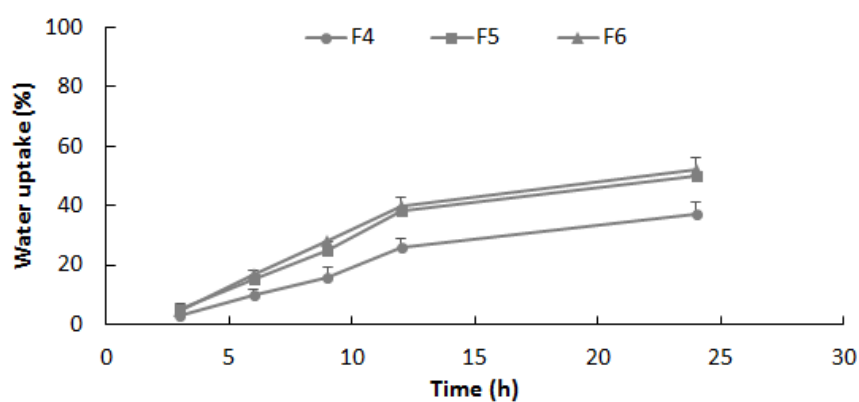
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Supporting Information

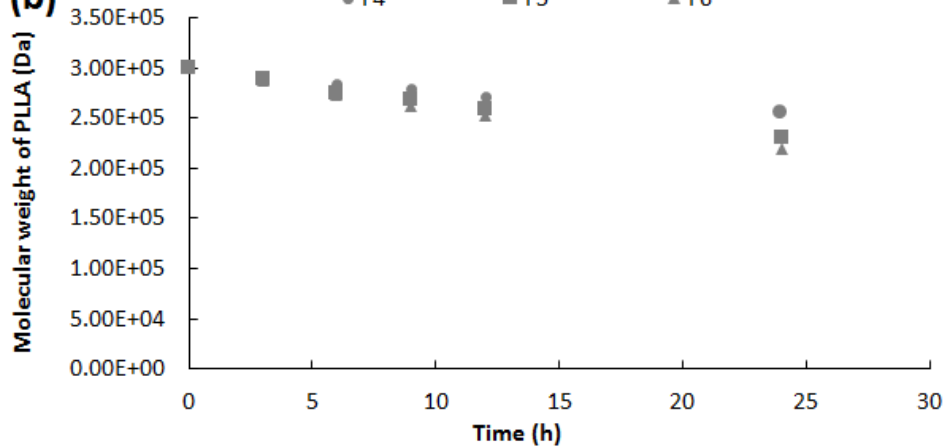
Multi-drug-loaded Microcapsules with Controlled Release for Management of Parkinson's Disease

Jong-Suep BAEK, Chee Chong CHOO, Cheng QIAN, Nguan Soon TAN, Zexiang SHEN, Say Chye Joachim LOO*

(a)



(b)



(c)

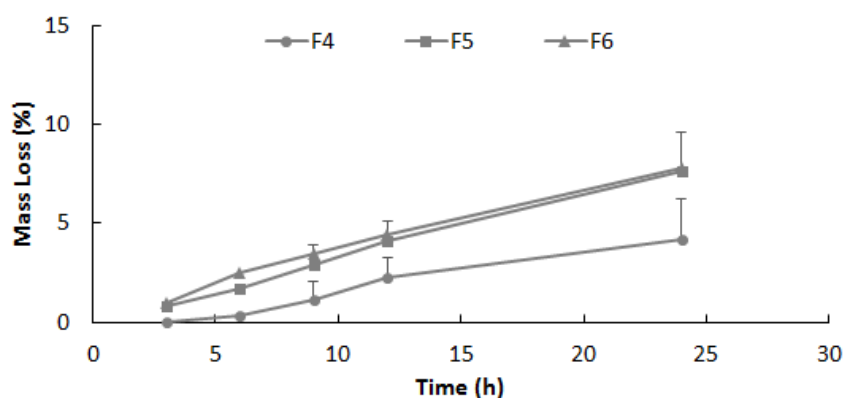
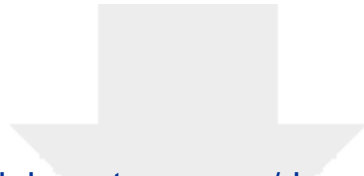


Figure S1. (a) Water uptake, (b) change in molecular weight of PLLA and (c) mass loss of the degrading spray-coated microcapsules as a function of incubation time (n=3).

Table S1. Kinetics of LD, CD and ENT from coated microcapsules (n=3).

	LD	CD	ENT
F1	4.9 ± 0.3	0.6 ± 0.3	3.8 ± 0.3
F2	5.0 ± 0.2	0.6 ± 0.1	3.9 ± 0.1
F3	5.2 ± 0.2	0.8 ± 0.4	6.0 ± 0.3
F4	5.3 ± 0.2	1.1 ± 0.4	8.3 ± 1.6
F5	5.4 ± 0.3	1.4 ± 0.4	10.2 ± 1.9
F6	5.5 ± 0.4	1.5 ± 0.4	11.4 ± 2.2

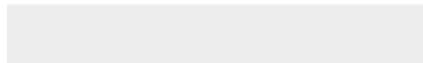




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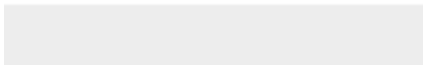
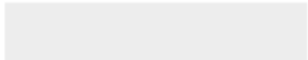
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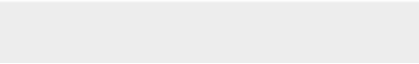
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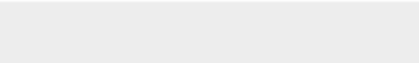

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EVALUATION:

Reviewer's Responses to Questions

Please rate the importance of this submission.

Reviewer #1: (no response)

Please rate the originality of this submission.

Reviewer #1: (no response)

Please rate the scientific and technical content of this submission.

Reviewer #1: (no response)

Please rate the length of this submission.

Reviewer #1: (no response)

COMMENTS TO AUTHOR:

Reviewer #1: Interesting paper and I recommend publication in Small.

--

Dr. Jose Oliveira, Editor-in-Chief

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Author's Response To Reviewer Comments

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Response to Reviewers' Comments

The authors would like to take this opportunity to thank the Editor and Reviewers for their time taken to review this manuscript. Our responses (R:) to the reviewers' comments are shown below in blue fonts. The amendments are highlighted in yellow in the revised manuscript.

Reviewer #1: In this manuscript, the authors developed a poly-L-lactide (PLLA) and poly (caprolactone) (PCL) based mix hollow microcapsules as LD, CD and ENT (4:1:8) combination delivery system for Parkinson's disease treatment. From the results, the platform could control and sustain the release of the three drugs via adjust the ratio of PLLA/PCL and the coating formulation. However, as a sustained and controlled formulation, the in vivo release profile is highly different from that of the in vitro one. Therefore, there are no significant advantages of the hollow microcapsules compared with commercialized tablet based formulation. I do not recommend acceptance of this manuscript in small. It could be suitable for a more specialized journal after addressing following issues.

R: Yes, the authors agree that in vitro release may not always correlate to the in vivo release; and this goes the same for many other in vitro studies for pharmaceutical delivery. This study is, however, a starting point in the development of such a technology. At least, we have shown, for the first time, the ability to control the release of these three Parkinson's disease drugs (i.e. LD, CD, ENT) in a controlled in vitro environment that simulates the gastric environment; and in comparison against the in vitro release for commercial tablets.

Major issues:

In vivo pharmacokinetics should be studied to evaluate the superiority of coating hollow microcapsules compared with existing formulation.

R: The aim of this work was to investigate how this newly developed drug delivery system can be tuned to control the release of three Parkinson's Disease drugs (i.e. two hydrophilic drugs (Levodopa and Carbidopa) and one hydrophobic drug (Entacapone)) that would be comparable to non-sustained releasing commercial tablets. The scope was to validate this release through in vitro studies, and not in vivo studies. Although this manuscript does not report on the in vivo pharmacokinetics, we believe that these results would still be of interest to the readers of SMALL, because of the use of a novel controlled delivery system that can potentially be used to manage Parkinson's Disease, while improving patient compliance (through sustained release). We have also compared the release results with commercial tablets (as control), i.e. Stalevo®, through the in vitro studies.

Minor issues:

1. The abbreviation of "levodopa (LP)" in Line 32 page 2 should be forwarded in Line 15 page 2 as present for first time of "levodopa"

R: The first abbreviation of levodopa (LD) in Line 32 page 2 has been changed to line 15 page 2.

2. The unit of x-axis of Figure 4(a) should be revised as "Time (h)"

R: The unit of x-axis of Figure 4 (a) has now been revised as Time (h).

3. I am not sure how the authors mixed 50 mL of 0.25% (w/v) PVA solution containing 1 mL of DCM in the preparation of multi drugs loaded microcapsules process. Please state details.

R: The following sentence has been added to the Experimental section on Page 20:

'For preparation of mixture of PVA solution (50 mL) containing DCM (1 mL), DCM (1 mL) was added into 0.25% (w/v) PVA solution (50 mL) using syringe, followed by stirring with overhead stirrer for one min to disperse it in PVA solution uniformly. Since DCM is not miscible in water, DCM was dispersed as small droplets.'

Reviewer #2: The authors have described a new sustained release formulation of Levodopa, Carbidopa and Entacapone - the three drugs most commonly used to treat Parkinson's Disease. The authors claim that they are able to encapsulate the three drugs in gastric floating spray-coated microcapsules which then lead to a sustained and controlled release of these drugs. Overall the paper is well written, the experiments have been carefully performed and all the possibilities have been carefully addressed. The paper is likely to be of interest to the readers of this journal, and I recommend publication provided the authors take care of the following points:

1) "...commercial tablets that do not enable controlled, sustained release capabilities" " commercially available oral medications do not address the above issues. Instead , they are laden with several limitations such as non-controlled release rates of drug ..."

The FDA has already approved a Levodopa- Carbidopa sustained release capsule called RYTARY(Impax Pharmaceuticals) for the treatment of Parkinson's Disease. It is incorrect to claim that no drug has been formulated so far to address the issue of sustained release. It is however true that the triple combination of Levodopa- Cabadopa- Entacapone is not available yet in the sustained-release format. The authors best reword their claim.

R: The authors have now revised the Introduction and included the following sentences in Page 4:

At present, there are only a few medications that are commercially available to patients, and these include Sinemet®, Stalevo®, and Rytary®. Unfortunately, Sinemet® and Stalevo® are non-sustained releasing medications, and do not address to the above issues. Although Rytary® has sustained release capabilities, it contains only two of the three PD drugs, i.e. LC/CD.

2) "It is hypothesized that this microcapsule delivery system would provide controlled, and a more sustained, release of PD drugs as compared to commercially available PD tablets"

The authors have compared their drug to the commercially available Stalevo-100 which is not a sustained release tablet. It would be interesting to see the comparison with a commercially available Levodopa- Carbidopa sustained release capsule (like RYTARY).

R: Recent papers have also reported that a combination of Carbidopa and Entacapone with Levodopa exhibited significant higher bioavailability of Levodopa rather than those formulations containing Levodopa and Carbidopa alone. For this reason, we encapsulated both Carbidopa (CD) and Entacapone (ENT), as dopa-decarboxylase inhibitor and catabolism-methylation [catechol-O-methyltransferase (COMT) inhibitor respectively, into the delivery system, and chose Stalevo-100 as the control formulation because it contains all three drugs (LD, CD, ENT). As such, we compared our formulation against a non-sustained release formulation in Stalevo-100.

3) The formulation mentioned in this paper releases the drugs very slowly (upto 80%) over a span of 24 hours. Have the authors considered if this rate of drug release is clinically suitable for management of PD symptoms?

R: Our group is currently conducting in vivo studies, including pharmacokinetics and pharmacodynamics studies, with the best formulation obtained from the in vitro results reported in this paper. With this, we would have a better understanding on whether this slow drug release would be clinically suitable to manage PD symptoms. From the in vivo studies, we would therefore be able to fine-tune the release of Levodopa to achieve a clinically meaningful therapeutic concentration of Levodopa in human plasma. The method to manipulate drug release from this delivery system is likewise reported in this manuscript.

4) Minor errors of grammar and punctuation are seen. Proof-reading of manuscript is recommended.

R: We have engaged a native English speaking person to run through the manuscript. All changes are highlighted in yellow in the manuscript.

Reviewer #3: Please follow unit format of the small journal.

R: Yes. We have followed the requirements of the journal. In experimental section, quantities of reactants, solvents are also included in parentheses.

Please arrange Figure 1 to show the time and legends.

R: The labelling of the formulations and time has now been included in Figure 1.

Table 1 should be revised as to the black-white format.

R: Table 1 has now been revised to the black-white format.

Please include repetition number in Figure 3 legend and in supporting data (Figure S1 and Table S1).

R: Repetition number (n=3) has now been included in Figure 3 and supporting data.

References are out of date, then please update the references.

R: Some of references are important for the explanation of our results. For example, references 1 – 17 include the mechanism and role of three drugs used in this work for a treatment of Parkinson's disease. Similarly, 43 – 45 references are the first papers that created the equations to calculate release rates of three drugs. Nevertheless, the authors have included other more recent papers to ensure that a good spectrum of references within the scope of this work is reflected in this manuscript. The following have

now been included:

- [13]. D. E. Payer, M. Guttman, S. J. Kish, J. Tong, J. R. Adams, P. Rusjan, S. Houle, Y. Furukawa, A. A. Wilson, I. Boileau, *Neurology*. 2016, 86, 224.
- [14]. A. Ciesielska, N. Sharma, J. Beyer, J. Forsayeth, K. Bankiewicz, *PLoS One*. 2015, 10, e0122708.
- [15]. N. Fernandez, D. Carriedo, M. Sierra, M. J. Diez, A. Sahagun, A. Calle, A. Gonzalez, J. J. Garcia, *Eur Neuropsychopharmacol*. 2005, 15, 505.
- [22]. A. Hsu, H. M. Yao, S. Gupta, N. B. Modi, *J Clin Pharmacol*. 2015, 55, 995.

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