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Suggested Procedures for the Reproducible Synthesis of Poly(d,l-lactide-co-glycolide) Nanoparticles Using the Emulsification Solvent Diffusion Platform

Shadabul Haque^{1,2}, Ben J. Boyd^{1,2}, Michelle P. McIntosh¹, Colin W. Pouton¹, Lisa M. Kamin-skas^{1,3,*} and Michael Whittaker^{1,2,*}

¹Drug Delivery Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Pde, Parkville VIC, 3052, Australia; ²ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Melbourne, VIC, 3052, Australia; ³School of Biomedical Sciences, University of Queensland, St Lucia QLD, 4072, Australia

Abstract: Background: Poly(d,l-lactide-co-glycolide) (PLGA) based biodegradable nanoparticles are of key interest for the development of controlled release drug delivery systems and for other biomedical applications. It has been reported that PLGA polymers can be converted into colloidal nanoparticulate systems by various techniques, such as emulsification-diffusion, emulsification-evaporation, interfacial deposition, salting out, dialysis and nanoprecipitation. Emulsification-evaporation with water immiscible solvents including dichloromethane and chloroform has been the preferred method for the synthesis of PLGA nanoparticles due to the low boiling point and limited water solubility of these solvents. We and others, however, have found that when water-immiscible solvents are used for the synthesis of PLGA nanoparticles, particle aggregation, non-uniform particle size and multimodal size distribution are commonly encountered problems. This suggests that the synthesis of PLGA nanoparticles using water immiscible solvents is highly sensitive to small procedural variations that affect overall reproducibility.

Objective: This study presents a simple and robust procedure for the preparation of PLGA nanoparticles with very small batch to batch variability (<5% variability in size (z-average) as determined by dynamic light scattering).

Results: The results showed that the emulsification solvent diffusion method teamed with partially water-miscible solvents, such as ethyl acetate, is a versatile approach for the preparation of PLGA nanoparticles with highly reproducible sizes (between 50 and 400 nm) and zeta potentials (between -30 and +30 mV), with relatively narrow polydispersity.

Conclusion: Emulsification-diffusion with ethyl acetate is, therefore, a more reliable alternative to several existing procedures for the reproducible and refined synthesis of PLGA nanoparticles.

Keywords: PLGA, nanoparticles, drug delivery, ethyl acetate, emulsion, solvent diffusion.

1. INTRODUCTION

Over the last two decades, research efforts in pharmaceutical technology have been directed towards the use of biocompatible and biodegradable polymers for drug delivery applications. Among these polymers, poly(lactide-co-glycolide) (PLGA) has gained a lot of attention, in part because these materials have been approved by various

regulatory agencies for human use [1, 2]. PLGA degrades in the body by the hydrolytic cleavage of the ester bond to form the biological by-products of respiration, lactic acid and glycolic acid [1-3]. Critically the rate of this degradation can easily be tuned *via* the copolymer composition [4, 5]. Lactic acid is produced endogenously and is easily metabolized by the Krebs cycle, while glycolic acid is a product of plant photosynthesis [1, 2]. Translation of PLGA copolymers into useful colloidal drug delivery systems is usually achieved from pre-formed PLGA copolymer using top-down techniques such as emulsification-diffusion, emulsification-evaporation, interfacial deposition, salting out, dialysis and nanoprecipitation with or without the use of stabilizers [2, 3].

Of these, the emulsification based methods have been most widely used for the preparation of PLGA nanoparticles [2, 6-9]. Depending upon the solvent used they are sub-

*Address correspondence to these authors at the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, 381 Royal Pde, Parkville, VIC, 3052, Australia; Tel: +61 3 990 39266; Fax: +61 3 990 39582; E-mail: michael.whittaker@monash.edu
School of Biomedical Sciences, University of Queensland, St Lucia QLD, 4072, Australia; Tel: +61 7 3365 3124; Fax: +61 7 3365 1766; E-mail: l.kaminskas@uq.edu.au

classified into emulsification–diffusion and emulsification–evaporation [6, 10]. The distinction between these two methods is a result of the choice of solvent. In the emulsification evaporation approach, PLGA is dissolved in a water-immiscible and volatile solvent, such as dichloromethane (DCM) or chloroform [2, 10]. In contrast, partially water-miscible solvents such as Ethyl Acetate (EA), benzyl alcohol, propylene carbonate (PC) and methyl acetate are used in the emulsification-diffusion technique [2, 10]. The basic steps of the two methods are the same (i) emulsification of a PLGA dispersed phase in an emulsifier-containing aqueous phase by exposure to a high-energy source, such as an ultrasonic device or homogenizer, (ii) solvent evaporation or solvent diffusion from emulsion droplets into the aqueous phase, and (iii) solidification of emulsion droplets into the PLGA nanoparticles [10]. However, the bulk of the literature suggests that DCM, which has a low boiling point (39.8°C) and negligible water solubility, is the preferred solvent of choice for the preparation of PLGA nanoparticles [3, 6, 10]. We and others though, have found that when DCM was used as the organic phase solvent for the synthesis of PLGA nanoparticles, a significant proportion of the batches prepared were compromised by particle aggregation, non-uniform particle sizes and multimodal size distribution [9, 11–13]. Further, we also found that the average particle size of each batch was consistently higher than reported literature values. These observations suggested that reproducible synthesis of PLGA nanoparticles by emulsification evaporation using DCM is only possible under very specific optimized conditions. Furthermore, this method is very sensitive to changes in process variables, such as the grade of the PLGA used (co-polymer ratio/ molecular mass), polymer concentration, concentration of the stabilizers, stirring speed of the homogenizer/ power settings of the sonicator, phase volume ratio and geometry of the mixing vessel.

To overcome this barrier, we optimized the emulsification solvent diffusion method for the preparation of PLGA nanoparticles exhibiting a wide range of sizes and charges. We found that the emulsification solvent diffusion approach (with EA as solvent) is a versatile platform for the preparation of PLGA nanoparticles over a size range of 50 to 400 nm, and charge range of -30 to +30 mV that exhibit a narrow polydispersity index (PDI) and are able to be prepared with high batch to batch reproducibility.

2. MATERIALS AND METHOD

2.1. Materials and Reagents

PLGA [50:50 L:G, Mw 24,000–38,000 (Resomer RG503H) and 75:25 L:G, Mw 76,000–115,000, (Resomer RG756)], Polyvinyl alcohol (PVA, 87–90% hydrolyzed, average mol wt 30,000–70,000) and Mowiol® 4-88 (Mw~31,000) were purchased from Sigma Aldrich, Australia. Ultrapure chitosan chloride (CS, Protasan® UP CL113, 75%–90% deacetylation, molecular weight 50–150 kDa) was purchased from NovaMatrix (FMC BioPolymer, Drammen, Norway). Poloxamer 188 (commercially named Pluronic F68 (PF68), Mw 9000–10000) was obtained from Croda International (Snaith, UK). All the solvents used were of AR grade supplied from Merck.

2.2. Instrumental Techniques

The particle size analysis, size distribution and zeta potential were determined in MilliQ water using Zetasizer (model: Nano ZS, Malvern Instruments, UK) employing the supplied DTS software. The particle size and morphology of the PLGA nanoparticles were also determined by transmission electron microscopy (TEM) (model: Technai, FEI Company, Germany). Briefly, a drop of nanoparticles was placed on the copper grid and allowed to dry at room temperature. After drying and removal of excess fluid, the copper grids were examined by TEM.

3. EXPERIMENTAL

3.1. Preparation of PLGA Nanoparticles

PLGA nanoparticles of size range 50–400 nm and charge range -30 to +30 mV were prepared by emulsification solvent diffusion method with ethyl acetate as solvent and PF68, PVA and chitosan as stabilizers (Fig. 1). The following describes the method used to prepare the nanoparticle library: PLGA was first dissolved in EA and then this solution was added dropwise to surfactant solution under mild to vigorous magnetic stirring. The pre-emulsion was then either homogenized using a T18 ULTRA-TURRAX homogenizer with S18N-19G Dispersing element (IKA, China) or *via* short sonication using a VibraCell sonicator (IKA, China). Following the pre-emulsification, water was added to the emulsion to promote diffusion of EA into the external phase, leading to the formation of PLGA nanoparticle. Finally, the organic phase was evaporated with continuous magnetic stirring for 10–12 hr. The reproducibility of all synthetic methods was tested by preparing several batches of each type of nanoparticles.

4. RESULTS AND DISCUSSION

The nanoparticles described in this study (Fig. 1, Scheme I), were composed of PLGA (50:50 L:G, Mw 24,000–38,000) and PF68. To prepare these nanoparticles, 50 mg of PLGA was dissolved in 7.5 mL of EA and added dropwise (100 drops/min, 23 gauge needle) to PF68 solution (1% w/v, 10 mL) under vigorous magnetic stirring (900–1000 rpm). The pre-emulsion was vortexed for 60 sec and then homogenized with a T18 ULTRA-TURRAX homogenizer with S18N-19G Dispersing element (IKA, China) at 12000 rpm for 5 min at room temperature (23–25°C). Following homogenization, water (20 mL) was added to the emulsion to promote diffusion of EA into the external phase, leading to nanoparticle formation. Finally, the organic phase was evaporated at room temperature (23–25°C) with continuous magnetic stirring (800 rpm) for 10–12 hr. The average size (z-average) and zeta potential of these systems [determined via dynamic light scattering (DLS)] was ~160 nm and ~-30 mV, respectively (Table 1). The TEM images also suggested that the average size of these systems was less than 200 nm (Fig. 2a). In another variant, (Fig. 1, Scheme II), the pre-emulsion after vortexing (as described above) was sonicated using a VibraCell sonicator (IKA, China) at 30% power using ¼” tip for 25 sec on ice. The average size was lower (~140 nm) but the reproducibility of the method in terms of size, PDI and zeta-potential was similar to Fig. (1, Scheme I) & Table 1.

Fig. 1. Schematic representation of the synthesis of various types of PLGA nanoparticles by the emulsification solvent diffusion method. **Step 1)** PLGA was dissolved in ethyl acetate (scheme I-VI). **Step 2)** PLGA dissolved in ethyl acetate was added drop wise to aqueous surfactant solution under continuous magnetic stirring (scheme I-VI). **Step 3)** The pre-emulsion was vortexed for 60 sec (scheme II-IV & VI), *exception scheme V*. **Step 4)** The pre-emulsion was sonicated using a VibraCell sonicator using a 1/4" tip (scheme II-VI) on ice or homogenised with a T18 ULTRA-TURRAX homogeniser with S18N-19G dispersing element at room temperature (23-25°C) (scheme I & V), *step 3 for scheme V*. **Step 5)** Following homogenisation or sonication, distilled water was added to the emulsion to promote diffusion of ethyl acetate into the external phase and the organic phase was allowed to evaporate at room temperature (23-25°C) with continuous magnetic stirring to form different types of the nanoparticles (scheme I & V), *step 4 for scheme V*. **Step 6)** The nanoparticles were centrifuged at 11000-12000 rpm for 30-45 min to get 50 nm PLGA nanoparticles (only scheme VI).

Table 1. Physicochemical characteristics and batch to batch variability of PLGA nanoparticles prepared using the optimized procedure (represented as mean $\pm$ sd, n=5 batches prepared on different days).

Nanoparticles	PLGA (Amount and Concentration in EA)	Surfactant (Amount and Concentration in DW)	Mean Particle Size (z-Average) (nm) $\pm$ S.D.	Mean PDI $\pm$ S.D.	Mean Zeta Potential (mV) $\pm$ S.D.
Anionic Scheme I	50 mg (6.66 mg/mL)	100 mg (PF68) (10 mg/mL)	164.1 $\pm$ 8.5	0.09 $\pm$ 0.00	-28.1 $\pm$ 1.8
Anionic Scheme II	100 mg (10 mg/mL)	200 mg (PF68) (10 mg/mL)	140.0 $\pm$ 7.5	0.11 $\pm$ 0.04	-31.0 $\pm$ 3.0
Uncharged Scheme III	30 mg (3.33 mg/mL)	250 mg (PVA) (25 mg/mL)	169.7 $\pm$ 7.5	0.10 $\pm$ 0.02	-1.3 $\pm$ 0.8
Cationic Scheme IV	30 mg (3.33 mg/mL)	10 mg (CS) (1 mg/mL)	184.2 $\pm$ 7.6	0.17 $\pm$ 0.03	+30.2 $\pm$ 6.0
Anionic Scheme V	175 mg (29 mg/mL)	1000 mg (PF68) (100 mg/mL)	393.3 $\pm$ 28.9	0.22 $\pm$ 0.06	-18.0 $\pm$ 2.0
Anionic Scheme VI	100 mg (10 mg/mL)	200 mg (PF68) (10 mg/mL)	58.3 $\pm$ 1.9	0.13 $\pm$ 0.03	-26.5 $\pm$ 2.3

PF68 - Poloxamer 188, PVA - Polyvinyl alcohol, CS- Chitosan, EA- Ethylacetate, DW- Distilled water. Dynamic light scattering measurements were performed using a Malvern Zetasizer Nano Series running DTS software and operating a 4 mW He-Ne laser at 633 nm. Analysis was performed at an angle of 173° and a constant temperature of 25°C. The z-average particle size (d.nm) and polydispersity index (PDI) are reported. The PDI was used to describe the width of the particle size distribution. It was calculated from a Cumulants analysis of the DLS measured intensity autocorrelation function and is related to the standard deviation of the hypothetical Gaussian distribution (*i.e.*, $PSD = \sigma^2/ZD^2$, where σ is the standard deviation and ZD is the Z average mean size).

The reproducibility of all synthetic methods was tested by preparing at least 5 batches on different days (Table 1).

Following the optimization and characterization of these anionic PLGA nanoparticles, the platform was further validated for uncharged and cationic nanoparticles of similar size (~160 nm) using different stabilizers. PVA (87-90% hydrolyzed, average mol wt 30,000-70,000) and a mixture of chitosan [Protasan® UP CL113, 75-90% deacetylation, 50-150 kDa] and Mowiol (Mw ~31 kDa,) were used as stabilizers for the respective synthesis of uncharged and positively charged nanoparticles. For the preparation of these PLGA nanoparticles, 30 mg of PLGA (Mw 24,000-38,000) was dissolved in 9 mL of EA and added dropwise (50 drops/min, 23 gauge needle) into 10 mL of a 2.5% (w/v) aqueous solution of PVA (Fig. 1, Scheme III) to prepare uncharged nanoparticles, or a 10 mL solution containing 0.1% (w/v) chitosan and 1% (w/v) Mowiol (Fig. 1, Scheme IV) to prepare cationic nanoparticles under vigorous magnetic stirring (1000 rpm). The pre-emulsion was vortexed for one minute and then sonicated using a VibraCell sonicator using ¼" tip at 55% power for 150 secs or 60% power for 100 secs on ice, respectively. The size of both systems was between 160 and 180 nm, each with a narrow PDI (< 0.2) [determined by DLS and TEM (Table 1, Figs. 2b & 2c)], consistent with the anionic nanoparticles described above. The zeta potential was either -1.8 mV or +24.1 mV depending upon the stabilizer used (Table 1). Chitosan was unable to stabilize the PLGA nanoparticles in the absence of PVA as reported earlier by others [14].

To determine whether emulsification solvent diffusion with EA can also be used for the preparation of smaller and larger PLGA nanoparticles, anionic large (~400 nm) and small (~50 nm) PLGA nanoparticles were prepared using PF68 as a stabilizer. For the preparation of large particles, 175 mg of PLGA (Mw 24,000-38,000) was dissolved in 6 mL of EA and added dropwise (180 drops/min, 18-gauge needle) to 10 mL of a 10% (w/v) aqueous solution of PF68 under slow magnetic stirring (500-600 rpm) (Fig. 1, Scheme V). The pre-emulsion was sonicated using a VibraCell sonicator at 20% power using a ¼" tip for 8 sec, or homogenized with a T18 ULTRA-TURRAX with S18N-19G dispersing element (IKA, China) at 5000 rpm for 5 min at room temperature (23-25°C). The organic phase was allowed to evaporate following the addition of water (10 mL) to the emulsion with constant magnetic stirring (500-600 rpm). The size of the optimized batch was ~400 nm with a negative zeta potential (Table 1, Fig. 2d). The smaller ~50 nm PLGA nanoparticles were obtained in two steps (Fig. 1, Scheme VI). In step 1, 100 mg of PLGA (Mw 24,000-38,000) was dissolved in 10 mL of EA and added dropwise (60 drops/min, 23-gauge needle) to 20 mL of a 1% (w/v) aqueous solution of PF68 under vigorous magnetic stirring. The pre-emulsion was vortexed for one minute and then sonicated using a VibraCell sonicator at 60% power using a ¼" tip for 1 min and 10 secs on ice. In the next step, the ~100 nm PLGA nanoparticle suspension was centrifuged at 13000-13500 x g for 30-45 min to pellet large particles. The average size of the nanoparticles remaining in suspension was ~50 nm (Table 1, Fig. 2e).

These results show that PLGA nanoparticles of various size and charge can be prepared by emulsification solvent diffusion using EA as a solvent. The independent process variables such as the PLGA/ stabilizer concentration, stirring speed and power setting of the sonicator, and speed of the homogenizer were optimized by their systematic variation separately for the different nanoparticles. The batch to batch variability in particle size was <5% for Fig. (1, Schemes I-IV & VI), but slightly higher (7.3%) for Fig. (1, Scheme V) (large anionic nanoparticles). Polydispersity was generally <0.2 with the exception of the large anionic particles (Fig. 1, Scheme V, 0.22) and variability in PDI was 1 to 36%. The variability in zeta potential was 6 to 20% for charged nanoparticles and 61% (0.3-2.28 mV) for the uncharged nanoparticle. These results suggest that the optimized method described here results in batch to batch variability that is considerably lower than other reported procedures. For example, McCall and Sirrianni reported a batch to batch variation of over 32% in particle size across 6-7 batches of PLGA nanoparticles prepared by emulsion evaporation using DCM as a solvent [11]. The authors did not report the zeta potential or PDI however based on particle size variation disclosed, these batches had broad size distribution [11]. Others reports have similarly indicated lack of uniformity and batch-to-batch size variability in PLGA nanoparticles synthesized using DCM [9, 12, 15, 16].

The reproducibility of the optimized procedures was also tested by changing the grade of PLGA or replacing a fraction of PLGA with fluorescently labeled PLGA. PLGA nanoparticles of almost similar size [$n=2$ (Fig. 1, Scheme I - 188 nm, < 15% variability), (Fig. 1, Scheme II - 147 nm, < 5% variability)] and PDI [$n=2$ (Fig. 1, Scheme I - 0.08, 11.4% variability), (Fig. 1, Scheme II - 0.09, 23% variability)] were obtained using an alternative grade of PLGA [75:25, Mw -76000-115000, (Sigma Aldrich, Australia)] by Fig. (1, Schemes I and II). Likewise, the variation in the amount of PLGA and stabilizer by ± 25 wt % in Fig. (1, Scheme II) was not found to have any substantial impact on size ($n=2$, 164 nm, 17% variability) or PDI ($n=2$, 0.06, 44% variability) of nanoparticles. Similarly, the substitution of a fraction of PLGA polymer (~5 wt %) with a fluorescently labelled PLGA of slightly lower molecular weight (Fig. 1, Schemes I-VI) did not result in significant changes in the size of nanoparticles (Fig. 1, Scheme I - 159 nm, 3% variability; Fig. 1, Scheme III - 186 nm, 9% variability; Fig. 1, Scheme IV - 159 nm, 13% variability; and Fig. 1, Scheme VI - 60 nm, 3% variability).

These observations are in agreement with previous reports which have shown that partially water-soluble solvents favour the formation of small monodisperse PLGA nanoparticles, whereas large polydisperse nanoparticles are obtained with water-immiscible solvents (e.g., DCM) [9, 12, 15, 16]. This is due to differences in the mechanism of formation of PLGA nanoparticles by the two solvents. In the formation of nanoparticles by the emulsification solvent diffusion method, the dispersed phase (i.e., EA) containing PLGA and continuous aqueous stabilizer phase is in a state in which the EA is partitioned between a polymer rich phase and a water-rich phase [15]. The addition of water post emulsification alters the partitioning of the EA and facilitates the diffusion of EA into the aqueous phase which leads to

Fig. 2. TEM image of the **a)** PLGA PF68 nanoparticles (scheme I & II), **b)** PLGA PVA nanoparticles (Scheme III), **c)** PLGA CS nanoparticles (Scheme IV), **d)** anionic PLGA PF68 nanoparticles (scheme V), and **e)** anionic PLGA PF68 nanoparticles (scheme VI).

the formation of a local region of supersaturation at the liquid-liquid interface (diffusion-stranding), provoking phase transformation and PLGA aggregation in the form of solid colloidal nanoparticles [15]. The mechanism by which diffusion of EA induces PLGA aggregation and nanoparticle formation has been attributed to the formation of new smaller nanosized globules and globule size reduction, likely due to interfacial turbulence generated during rapid diffusion of EA from the internal to the external aqueous phase, but the exact mechanism remains unclear [7, 8, 15]. It has been reported that when partially miscible solvents like EA are used, the solvent water exchange is bidirectional due to the mutual diffusion of the solvent phase into the water phase, and the water phase into the solvent phase (the ‘exchange ratio’) [8, 15]. A small exchange ratio has been found to produce a diminutive local supersaturation region near the interface to form smaller and more uniformly sized PLGA nanoparticles [8, 15]. In comparison, a large exchange ratio leads to an exponential increase in particle size [8, 15]. EA has the lowest exchange ratio (even amongst different partially water-miscible solvents) and therefore it plays an important role in explaining the high reproducibility and size uniformity of the PLGA nanoparticles [8, 15].

Organic solvents of low water solubility (e.g., DCM, chloroform) lead to a slow precipitation of emulsion droplets [8, 15]. In addition, due to the lack of interfacial turbulence, no globule size reduction occurs and therefore nanoparticles of different sizes are formed after DCM evaporation from the surface of the emulsion droplets [7, 8]. Due to these reasons, solvent evaporation is more sensitive to changes in process variables. Further, a high solvent-polymer affinity (e.g., chloroform and PLGA) has been found to produce a large supersaturation region at the liquid-liquid interface to form larger and non-uniformly sized nanoparticles [7, 8, 15].

Thus, the choice of the solvent is a key factor in determining the size & PDI of nanoparticles and the batch to batch reproducibility [15]. Finally, the interaction between the stabilizers and organic solvents also plays a major role in determining the physiochemical properties of PLGA nanoparticles [8, 12]. During the synthesis of PLGA nanoparticles, the stabilizers are adsorbed on the interfacial area (liquid-liquid interface) in between the diffusion and/or evaporation of solvents from emulsion droplets [8, 12]. Due to the high interfacial tension of DCM (28.3 dyn/cm), the stabilisers (irrespective of the concentration) are unable to prevent the coalescence of emulsion droplets leading to variations in their particles size and multimodal size distribution [8, 12, 14, 16]. In contrast, the combined impact of low interfacial tension of EA (1.7 dyne/cm) with the aqueous phase and the protective effect of the stabilizers produces stable emulsion droplets to form small and uniformly sized PLGA nanoparticles [8, 12, 16].

CONCLUSION

In this study, a range of PLGA nanoparticles (50 to 400 nm and -30 to +30 mV) was prepared and characterized using the emulsification solvent diffusion method with ethyl acetate as solvent and PF68, PVA and chitosan as stabilizers. The results highlighted that irrespective of the stabilizer used, the emulsification-diffusion method with ethyl acetate can be used for the synthesis of different types of PLGA nanoparticles with narrow size distribution and minimum batch to batch variation in physiochemical parameters, namely particle size and zeta potential. The results further showed that the optimized methods described above are sufficiently robust to withstand minor changes in process variables without altering the size, size uniformity and zeta potential of nanoparticles. Emulsification-diffusion with ethyl

acetate is, therefore, a more reliable alternative to several existing procedures for the reproducible and refined synthesis of PLGA nanoparticles.

LIST OF ABBREVIATIONS

CS	=	Chitosan
DCM	=	Dichloromethane
DLS	=	Dynamic Light Scattering
EA	=	Ethyl Acetate
PDI	=	Polydispersibility Index
PF68	=	Poloxamer 188
PLGA	=	Poly(d,l-lactide-co-glycolide)
PVA	=	Polyvinyl Alcohol

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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