



Single oral dose of cannabinoid derivate loaded PLGA nanocarriers relieves neuropathic pain for eleven days

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Received 24 December 2016; accepted 17 July 2017

Abstract

Neuropathic pain, resistant to opiates and other drugs, is a chronic/persistent state with a complex treatment and often poor efficacy. In this scenario, cannabinoids are increasingly regarded as a genuine alternative. In this paper, and in an experimental animal model of neuropathic pain, we studied the efficacy of three kinds of PLGA nanoparticles containing synthetic cannabinoid CB13: (i) plain nanoparticles (PLGA); (ii) particles coated with PEG chains (PLGA + PEG) and (iii) particles possessing hydrophilic surfaces obtained by covalently binding PEG chains (PLGA-PEG). The optimized formulation, CB13-PLGA-PEG, showed high drug loading (13%) and small size (<300 nm) with a narrow distribution and controlled surface properties (near-neutral zeta potential and stable PEG corona). Animal nociceptive behavioral studies were conducted by paw pressure and acetone tests. Versus the free CB13, CB13-PLGA-PEG nanoparticles showed a very noticeable analgesic efficacy with the longest sustained pain-relieving effect, lasting up to eleven days after one oral dose. © 2017 Elsevier Inc. All rights reserved.

Key words: PLGA nanoparticles; Synthetic cannabinoid; Oral administration; Neuropathic pain

Neuropathic pain is a chronic/persistent state resulting from injury to the nervous system due to trauma, chronic inflammation, viral infection, or metabolic disturbances, i.e., diabetes.¹ Drug associations in the routine clinical treatment of neuropathic pain are frequently used, including the common use of tricyclic antidepressants. Other antidepressants, such as duloxetine or venlafaxine, anticonvulsants and opioids, are also commonly used.

Nevertheless, neuropathic pain treatment is complicated and often poorly efficacious in the majority of patients. It is resistant to opiate analgesics^{2,3}; antidepressants, particularly tricyclic antidepressants, contribute to a poor side-effect profile and limited patient tolerance³ due to their anticholinergic, antihistaminergic and antiadrenergic properties. The efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) in treating neuropathic pain

has been questioned by many clinicians and indeed, such drugs are not recommended for treating neuropathies.

It is evident that new, effective chronic pain management drug therapy is required and that there is a drive to find more effective treatments.

In recent years there has been growing clinical evidence on the efficacy of cannabis and synthetic cannabinoid agonists in chronic pain states. Most recently, *Science* published a special issue dealing with pain research. The section: "The future of pain research"⁴ indicates that hints are emerging that cannabis could be one of today's pain management alternatives.

Cannabinoids are moderately effective in treating neuropathic pain and new synthetic cannabinoids presenting fewer side effects have been developed. This includes the potent cannabinoid

Financial support information: This work received funding from: Consejería de Innovación, Junta de Andalucía (Spain) (project P09-CTS-5029) and European funds; Fondo de Investigación Sanitaria (PI13/02659); the Spanish Ministerio de Economía y Competitividad (SAF2015-68647-R), CIBERSAM G18; Junta de Andalucía (CTS-510, CTS-7748, CTS-480) and 2015 NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation.

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<http://dx.doi.org/10.1016/j.nano.2017.07.010>

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receptor CB1/CB2 agonist 13 (CRA13 or CB13), which reverses neuropathic mechanical hyperalgesia.⁵ Like other cannabinoid compounds, CB13 is a highly lipophilic drug and belongs to class II compounds (low solubility and high permeability) of the Biopharmaceutics Classification System. In fact, in preclinical studies this drug was orally administered dissolved in a non-aqueous solvent or dispersed in an aqueous phase by the aid of dispersing agents such as Cremophor®.^{5,6} In addition, due to CB13's high lipophilic nature, Trevaskis et al⁷ studied the influence of food on its oral bioavailability. They demonstrated that the quantity and composition of food can enhance CB13 oral bioavailability by stimulating lymphatic transport.

The development of new techniques enabling lipophilic drugs to be administered orally is still a major challenge. In the search for solutions to these problems, nanotechnology-based drug delivery systems are promising tools. Moreover, the use of nanocarriers in the management of pain is a novel and exciting area of research, with a great potential for growth and clinical benefit.⁸

We have recently developed surface-modified PLGA nanoparticles and solid lipid nanoparticles intended for oral CB13 administration.^{9,10} In vitro and ex vivo mucoadhesive properties were enhanced using several mucoadhesive actives such as chitosan or Eudragit®. Nevertheless, in vivo biodistribution assays revealed that most particles accumulated in the liver and spleen, indicating that nanoparticles did not prevent the opsonization process.⁹

In this respect, polyethylenglycol (PEG)-coated nanoparticles appear to be the most promising strategy for several reasons as they: (i) promote nanoparticles mucoadhesion; (ii) stabilize the nanoparticles in digestive fluids; (iii) avoid plasma protein adsorption; (iv) minimize the interaction with phagocytic cells; and (v) increase the blood circulating time.^{11,12}

The objective of the present work is, therefore, to undertake practical research into the potential of PLGA nanoparticles as oral delivery systems for CB13. For this purpose, free and nanoparticle-encapsulated CB13 was orally administered at different doses to an animal model of neuropathic pain.

We prepared three kinds of PLGA nanoparticles that possessed either unmodified hydrophobic surfaces or hydrophilic surfaces. These were obtained by coating them with PEG chains that were either adsorbed or covalently attached.¹³

Methods

Synthesis of the PLGA-based nanoparticles

PLGA-based nanoparticles were prepared by a modified nanoprecipitation (NPP) method.¹⁴ Briefly, a weighed amount of PLGA or PLGA-PEG was co-dissolved with Span® 60 in acetone. 5 mL of this acetonic solution was then added dropwise under magnetic stirring to 15 mL of a Pluronic® F-68 aqueous solution (0.5% w/v). The acetone content was evaporated at room temperature (r.t.) for 4 h. Finally, the nanoparticles suspension was collected by ultracentrifugation. To prepare PEG coated-PLGA (PLGA + PEG) nanoparticles, PLGA nanoparticles were incubated in a PEG (4.5% w/v) (see Supplementary Materials).

CB13 vehiculization capabilities

Drug content into the nanoparticles was determined by a previous validated and verified HPLC method.¹⁵ Briefly, 5 mg of lyophilized nanoparticles was accurately weighed. 1 mL of acetonitrile was then added to dissolve the particles. After this, 10 µL of the previously-filtered solution was injected into the HPLC system for CB13 detection (see Supplementary Materials).

CB13 release experiments were performed using the three PLGA-based nanoparticles. 4 mg of nanoparticle samples was suspended in 15 mL of a release medium: hydrochloric acid media, pH = 2.0 ± 0.1 or phosphate buffered saline, PBS, pH = 7.4 ± 0.1 to simulate gastric and intestinal conditions, respectively (see Supplementary Materials).

In vivo studies of CB13-loaded nanoparticles in an animal neuropathic pain model

Animals

Adult male Harlan Sprague–Dawley rats weighing 250–300 g were provided by the Experimental Unit of the University of Cádiz (registration number ES110120000210). All the experimental protocols were approved by the Committee for Animal Experimentation at the University of Cádiz (Spain) and they complied with the International Association for the Study of Pain ethical guidelines.¹⁶ All procedures relating to animal care and use conformed to European Ethical Standards (86/609-EEC) and Spanish Law (RD 1201/2005). All efforts were made to minimize animal suffering.

Drugs

All formulations were administered orally by gavage in a volume of 2 mL/kg. Control animals received DMSO or blank nanoparticles (with no drug) in the same amounts of loaded nanoparticles corresponding to a CB13-equivalent dose of 1.7, 3.4 and 6.8 mg/kg, respectively.

Neuropathic pain model

Chronic constriction injury (CCI) was used as a model of neuropathic pain because it induces clinical signs of hypersensitivity that mimic human conditions of neuropathic origin. CCI was produced as previously described^{17,18} after anesthetizing the rats with an intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine. The left sciatic nerve was exposed at the mid-thigh level proximal to the sciatic trifurcation, and four chromic gut (4/0) ligatures were tied loosely around the nerve, 1.0 to 1.5 mm apart, so that the vascular supply was not compromised. Sham operations were performed in the same manner but with no nerve ligation.

Nociceptive behavioral assessment

Paw Pressure Test. Mechanical threshold was determined using the paw pressure test.^{19,20} Briefly, increasing pressure was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile, Comerio, Italy) with an initial 30 g of pressure. Two measurements were taken for each paw at 5-min intervals and the average value was determined, with a 250 g cut-off applied to prevent damage to the paw. Mechanical hypersensitivity is indicated by a reduction in the pressure provoking withdrawal. Nociceptive behavior was assessed in both

Table 1

Characterization of plain and PEGylated PLGA nanoparticles (mean \pm standard deviation (SD); n = 3) (theoretical drug loading (DL) = 16.5% w/w).

Formulation	D (nm) \pm SD	PdI \pm SD	ZP (mV) \pm SD	EE (%) \pm SD	DL (%) \pm SD
PLGA	196.0 \pm 12.6	0.188 \pm 0.024	-31.4 \pm 5.89	73.4 \pm 8.0	12.1 \pm 0.4
PLGA + PEG	654.4 \pm 185.5	0.315 \pm 0.051	0.45 \pm 3.18	79.4 \pm 9.0	13.1 \pm 0.6
PLGA-PEG	207.6 \pm 24.5	0.262 \pm 0.107	-24.97 \pm 4.11	80.1 \pm 8.2	13.2 \pm 0.5

the ipsi- and contralateral paws. The test was performed by an experimenter who was unaware of the treatment condition.

Acetone test. To assess the sensitivity to non-nociceptive thermal stimuli, a drop of acetone (100 μ L) was placed gently on the plantar surface of the ipsilateral hind paw. Acetone was applied alternately five times, with a 5 min delay between each successive application. Responses were monitored for 1 min after acetone application and they were graded on a 4-point scale as described previously^{16,21}: 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking of the paw; 3, repeated flicking of the paw with persistent licking directed at the ventral side of the paw. The cumulative scores were then obtained by summing the four scores for each rat and dividing by 5 (the number of assays). The responses were scored by an observer who was unaware of the treatment condition.

Experimental protocols

Experiment 1: Evaluation of the effectiveness of PLGA nanoparticles (plain) in the CCI model. On day 7 after surgery, PLGA (1.7–6.8 mg/kg) was orally administered once. Mechanical pain hypersensitivity was evaluated using the paw pressure test at 0.5, 3, 9 and 24 h after administration. Sensorial threshold was evaluated once a day on days 3 and 5. The same protocol was used for free CB13 (3.4 mg/kg, p.o.). This time schedule was designed based upon pilot experiment results.

Experiment 2: Evaluation of the effectiveness of PLGA + PEG nanoparticles (PEG adsorbed) in the CCI model. To study the effect of PLGA + PEG, the same protocol as that used for PLGA was employed. The selection of this time schedule was also based upon pilot experiment results.

Experiment 3: Evaluation of the effectiveness of PLGA nanoparticles (PEG covalently bind) in the CCI model. 7 days after surgery, PLGA-PEG (1.7–6.8 mg/kg) was orally administered once. Mechanical pain hypersensitivity was evaluated using the paw pressure test at 0.5, 3, 9 and 24 h after administration. Sensorial threshold was also assessed once a day on days 3, 5, 7, 9 and 11. The same protocol was used for free CB13 (3.4 mg/kg, p.o.). This time schedule design was based upon pilot experiment results. The acetone test was also performed 2 days after surgery in both groups.

Results

Preparation of the CB13-loaded PLGA, PLGA + PEG and PLGA-PEG nanoparticles

The nanoparticles containing CB13 were successfully obtained by the nanoprecipitation method. The choice of a nanoencapsula-

tion method is based on the drug solubility. It is based on interfacial deposition of a polymer after displacing a water-miscible semipolar solvent from a lipophilic solution containing CB13 and PLGA. This method provides high encapsulation efficiency for drugs presenting low water solubility.²²

Table 1 illustrates the size characteristics of the nanoparticles obtained. The three formulations: PLGA; PLGA + PEG; and PLGA-PEG nanoparticles containing CB13 presented mono-disperse profiles and narrow size distributions.

PLGA + PEG nanoparticles presented the largest size, which may be attributed to the adsorption of the PEG chains onto the PLGA surface in the form of one or more layers.²³ This PEG adsorption could, perhaps, be attributed to van der Waals forces, hydrogen bonding, apolar interactions, etc., and not by an electrostatic interaction. The other two formulations presented a smaller diameter (\approx 200 nm).

ζ value was found to be lowest (-31.4 ± 5.9 mV) in the case of plain PLGA nanoparticles due to their carboxyl end groups. PLGA + PEG nanoparticles have shown the highest ζ values ($\approx +0.5$ mV), perhaps due to the displacement of the diffuse ionic layer onto the particle surface to greater distances. The change in ζ values is a simple means for estimating the extent of surface shielding provided by PEG. These almost neutral ζ values suggested the highest PEG chain density on particle surface.^{24,25}

Finally, the geometry and surface of the particles and the quality of the PLGA suspensions did not vary significantly when they were loaded with CB13. No presence of aggregates or bulky sediments was observed in any of the formulations. Figure 1, A–D) show SEM images of different PLGA nanoparticles assayed. As it can be seen, particles kept their size and spherical morphology. Nevertheless, we observed some capillary bridging between PLGA + PEG individual particles. It is probably due to an incomplete washing process before collecting nanoparticles by ultracentrifugation (PEG bridges).

Encapsulation efficiency (EE %) and drug loading (DL %) were determined by directly dissolving nanoparticles in acetonitrile (Table 1). We found values of around 80% for EE and 13% for DL, respectively. This high drug incorporation efficiency may be attributed to the organically soluble nature of CB13 that prevents partitioning into the aqueous phase, thus increasing drug entrapment in nanoparticles during polymer deposition. The high DL (%) values that were found enable the same amount of drug to be delivered with less polymer. The method used for nanoencapsulation resulted in a significant enclosure of CB13 and the process was found to be highly reproducible.

As the values between batches were not significantly different ($P > 0.05$), PEG coating did not influence the encapsulation, probably due to the process being performed in an aqueous solution where the drug is not soluble. Similar results were

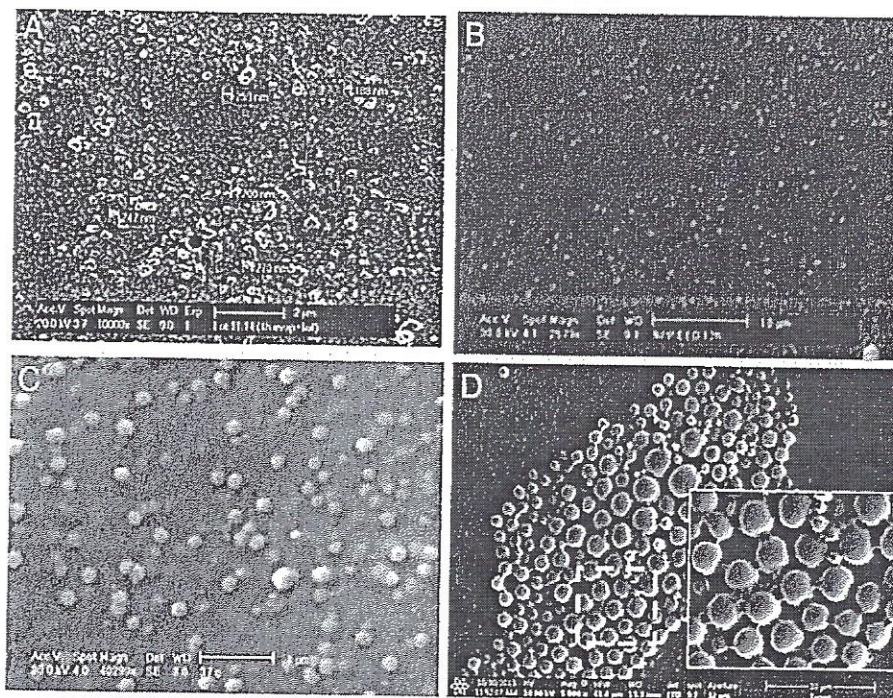


Figure 1. Scanning electron microscopy (SEM) micrographs corresponding to: (A) blank plain-PLGA nanoparticles (from Ref. 10); (B) CB13 plain-PLGA nanoparticles (from Ref. 15); (C) blank PLGA-PEG nanoparticles; and (D) blank PLGA + PEG nanoparticles.

obtained for PLGA-PEG. Indeed, the hydrophilic character of PEG kept it oriented toward the aqueous phase, while the hydrophobic core of PLGA can entrap the hydrophobic drugs.

Since EE% of three formulations was similar, we investigated a possible interaction between drug to PLGA or PEG or both. For this purpose a spectroscopy analysis using Fourier transform infrared (FT-IR) was carried out (see Supplementary Materials). Results revealed no significant differences in the frequencies of the bands in the three nanoparticle formulations compared to the single substances (CB13 and the components of the polymeric matrix) that could indicate the presence of any kind of interaction such as hydrogen bond, for example. In addition, CB13 cannot act as hydrogen donor in order to form hydrogen bond with PLGA or PEG.

The *in vitro* release of CB13 from nanoparticles was evaluated. CB13 release profiles were obtained by graphing the cumulative percentage of the drug released with respect to the amount of CB13 encapsulated as a function of time. The experiment was performed over 15 days.

Figure 2 collects the *in vitro* CB13 release profiles at pH 2.0 (Figure 2, A) and at pH 7.4 (Figure 2, B) from: PLGA, PLGA + PEG and PLGA-PEG nanoparticles. Independently of the release medium pH, a similar release profile can be observed. Remarkably, at pH 2.0 the drug release kinetics can be considered slower. Drug release was markedly inhibited in acidic environments, e.g., < 10% drug release after 2 h. The lowest release profile was obtained for plain PLGA nanoparticles. The presence of PEG chains determined a faster drug release. For PLGA + PEG and PLGA-PEG \approx 6% drug release was obtained after 30 min.

At pH 7.4 CB13 release from both PLGA-PEG and PLGA + PEG nanoparticles was faster than from PLGA nanoparticles. A biphasic release pattern of CB13 was observed from

PLGA-PEG and PLGA + PEG nanoparticles, where the initial 24 h period released 43% and 23% of drug followed by a sustained release to a total of 90% and 82%, respectively, over 15 days of assay.

CB13 release from PLGA nanoparticles was the slowest. In this case drug release was progressive, to a total of 58% after 15 days. It is possible that CB13 strongly interacts with the PLGA matrix, thus retarding the release capability, and that the PEG can increase the wettability of the polymeric surface and matrix, contributing to the increase in drug release.

These differences may be attributed to the hydrophilic character of the PEGylated nanoparticles thanks to the existence of the PEG chains on the nanoparticle surface which accelerated the degradation (hydrolysis) rate by improving buffer penetration into the nanoparticle matrix.^{10,25-28} Thus, surface modification of PLGA nanoparticles with PEG could be advantageously used in order to modulate drug release (nanoparticle erosion) kinetics.

In vivo studies of CB13 loaded PLGA nanocarriers in an animal neuropathic pain model

Results for *in vivo* assays are collected in Figures 3-6. In all cases asterisks indicate a significant difference compared to saline (* $P < 0.05$ vs. free CB13 control group, # $P < 0.05$ vs. PLGA + PEG control group, & $P < 0.05$ vs. free CB13 (3.4 mg/kg)). (0 h: pre-drug administration) (see Supplementary Materials).

Experiment 1: CB13-loaded PLGA nanoparticles

The effect of three doses of CB13-loaded PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) was explored in the paw pressure test in the ipsi- and contralateral hindpaw. This effect was compared with those from free CB13 (3.4 mg/kg) (Figure 3).

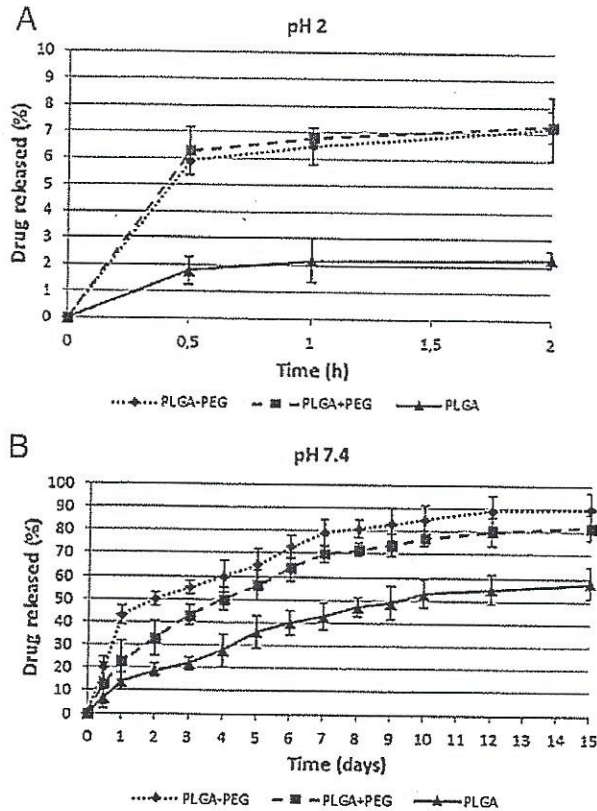


Figure 2. Cumulative release in vitro of CB13 from plain PLGA nanoparticles (Δ); PLGA-PEG (\diamond) and PLGA + PEG (\blacksquare) nanoparticles ($n = 3$; error bar: SD).

In agreement with previous data,⁵ free CB13 showed a significant analgesic effect in comparison with its control group at 0.5, 3 and 9 h in the ipsilateral hind paw (Figure 3, A). The peak effect was found at 3 h after drug administration.

The three doses of PLGA nanoparticles explored showed a significant and dose-dependent effect at 0.5, 3, 9 and 24 h compared with its respective control group in the ipsilateral hind paw (Figure 3, B). The highest effects were found at 9 and 24 h. This analgesic effect was kept up to 3 days after drug administration (Figure 3, B).

In the comparison of free CB13 versus CB13-loaded PLGA nanoparticles in the effect on the ipsilateral hind paw (Figure 3, C), we found that free CB13 had a superior analgesic effect at the beginning of the treatment (0.5 and 3 h compared with PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13). However, CB13-PLGA nanoparticles showed a higher analgesic effect at later time points. That is, at 9 h and 24 h and at 1–5 days CB13-PLGA nanoparticles 3.4 and 6.8 mg/kg CB13 had a significant effect compared with free CB13. CB13-PLGA nanoparticles 1.7 mg/kg also showed a higher analgesic effect versus free CB13 at 1–5 days. Therefore, free CB13 is effective for the first 9 h and the CB13-loaded PLGA nanoparticles are effective for 3 days when exploring the ipsilateral paw.

No significant effect was seen in any experimental group when exploring the sensorial sensitivity of the contralateral paw (Figure 3, D–F) suggesting that the analgesic effect of CB13-loaded PLGA nanoparticles and free CB13 is restricted to neuropathy.

Experiment 2: CB13-loaded PLGA + PEG nanoparticles

The effect of three different doses of CB13-loaded PLGA + PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) was explored in the paw pressure test in the ipsi- and contralateral hind paw over time (Figure 3, C and D). This effect was compared with those from free CB13 (3.4 mg/kg) (Figure 4). We first assessed the effect in the ipsilateral hind paw. Free CB13 showed a similar effect to that previously described (Figure 4, A). PLGA + PEG nanoparticles at 3.4 mg/kg CB13 significantly increased the pain threshold 3 h after administration (Figure 4, B). The significant analgesic effect was maintained for 5 days. At 6.8 mg/kg CB13 PLGA + PEG nanoparticles showed a significant analgesic effect from 9 h and continued for 5 days. The analgesic profile effect of PLGA + PEG nanoparticles at 3.4 and 6.8 mg/kg CB13 was very similar. The peak effect was found between 9 h and 3 days. No significant analgesic effect was found for the PLGA + PEG nanoparticles at a dose of 1.7 mg/kg CB13 (Figure 4, B).

Free CB13 showed an effect similar to that previously described. Its effect was superior to all doses of PLGA + PEG nanoparticles 0.5 and 3 h after administration. Free CB13 also showed a higher analgesic effect than 1.7 mg/kg of PLGA + PEG nanoparticles after 9 h (Figure 4, C). PLGA + PEG nanoparticles (6.8 mg/kg CB13) showed a significant antinociceptive effect compared with free CB13 after 9 h. As before, PLGA + PEG nanoparticles (3.4 and 6.8 mg/kg CB13) were more effective at reducing the pain threshold than free CB13 24 h, and 3 and 5 days after administration (Figure 4, C).

This indicates that CB13-loaded PLGA + PEG nanoparticles have a mechanical antihypersensitivity effect that lasts for up to 5 days.

No effect with regard to the contralateral hind paw was found in any group (Figure 4, D–F).

Experiment 3: CB13-loaded PLGA-PEG nanoparticles

The effect of three doses of CB13-loaded PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) was explored in the paw pressure test in the ipsi- and contralateral hind paw over time. This effect was compared with those from free CB13 (3.4 mg/kg) (Figure 5). Free CB13 showed a similar effect to that previously found in the ipsilateral hind paw (Figure 5, A). PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) showed a dose-dependent analgesic effect over time when exploring the ipsilateral hind paw (Figure 5, B). PLGA-PEG nanoparticles (1.7 mg/kg CB13) showed a significant analgesic effect 9 and 24 h as well as at days 5 and 9 after administration. 3.4 mg/kg CB13 PLGA-PEG nanoparticles showed a significant effect from 3 h until 9 days. The highest PLGA-PEG nanoparticles dose (6.8 mg/kg CB13) showed an analgesic effect from 0.5 up to 11 days. The greatest effects were found between 24 h and 3 days (Figure 5, B).

Free CB13 showed an effect similar to that previously found. It displayed a superior analgesic effect to PLGA-PEG nanoparticles at the beginning (0.5 h for all the doses of PLGA-PEG nanoparticles and after 3 h for PLGA-PEG nanoparticles 1.7 and 3.4 mg/kg CB13) (Figure 5, C). Free CB13 also had a significant effect versus PLGA-PEG nanoparticles (1.7 mg/kg CB13) after 9 h. After this point, the profile was the contrary. PLGA-PEG nanoparticles (1.7 mg/kg CB13) had a significant effect compared with free CB13 after 3 and 5 days. Similarly, the

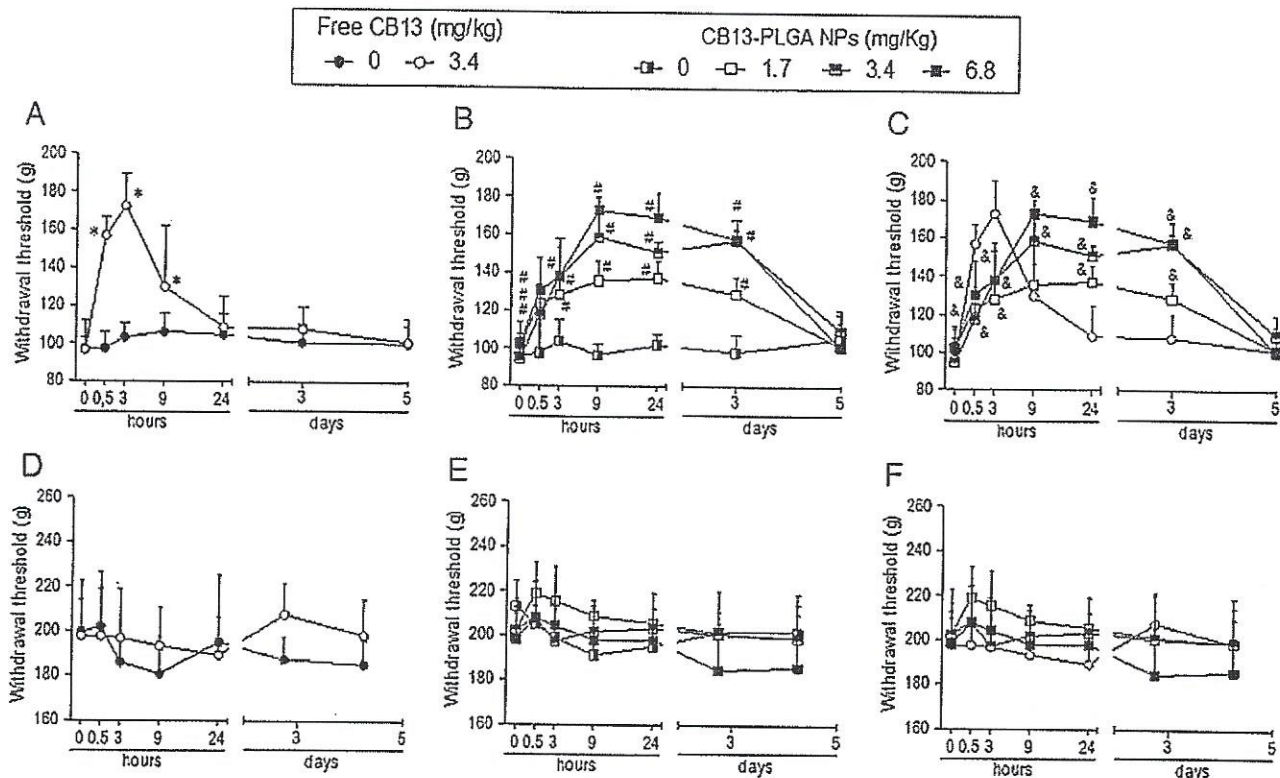


Figure 3. Effects in the paw pressure test in the CCI model in rat of CB13-PLGA nanoparticles and free CB13 (p.o.). (A) Free CB13 (3.4 mg/kg) vs. control group; (B) CB13-PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (C) free CB13 (3.4 mg/kg) vs. CB13-PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the ipsilateral hind paw ($n = 8-10$). (D) Free CB13 (3.4 mg/kg) vs. control group; (E) CB13-PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (F) free CB13 (3.4 mg/kg) vs. CB13-PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the contralateral hind paw ($n = 8-10$).

highest doses of PLGA-PEG nanoparticles (3.4 and 6.8 mg/kg CB13) showed a superior effect 24 h–7 days after administration. The 6.8 mg/kg dose of CB13 PLGA-PEG nanoparticles was significantly more effective than free CB13 9–11 days after oral administration (Figure 5, C).

When exploring the effect of these CB13-nanosystems in the contralateral hind paw, we found a significant effect of PLGA-PEG nanoparticles (3.4 mg/kg nanoparticles) 24 h after administration versus control group and versus free CB13 (Figure 5, D and F). Similarly, the highest dose of PLGA-PEG nanoparticles (6.8 mg/kg CB13) had a significant analgesic effect from 3 h to 5 days versus control group (Figure 5, D). Furthermore, 6.8 mg/kg of CB13 PLGA-PEG nanoparticles displayed a significant effect versus free CB13 24 h and 3–7 days after administration (Figure 5, F).

Furthermore, we explored the effect of PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) in the acetone test (ipsilateral hind paw) 2 days after oral administration (Figure 6). As before, this effect was compared with that from free nanoparticles (3.4 mg/kg). The highest doses of PLGA-PEG nanoparticles (3.4 and 6.8 mg/kg CB13) showed a clear reduction in the acetone score versus control group and versus free CB13. Compared with its corresponding control group, no effect was found in the dose of free CB13 (3.4 mg/kg) explored.

Overall, CB13-loaded PLGA-PEG nanoparticles show the longest mechanical antihypersensitivity effect of the three preparations of nanoparticles evaluated. That is, PLGA-PEG nanopar-

ticles have a consistent analgesic effect for up to 11 days. Furthermore, PLGA-PEG nanoparticles are also effective in thermal hypersensitivity in the neuropathic animal model studied. Finally, the highest dose of PLGA-PEG nanoparticles evaluated (6.8 mg/kg CB13) seems to increase the pain threshold in the non-injured (contralateral) paw.

Discussion

At this point, it can be concluded that neuropathic pain relief with CB13 can be clearly modulated using PLGA nanoformulations. It is quite noticeable that both free CB13 and the three nanosystems assayed showed the same analgesic potency while therapeutic response exists. The main difference lies in the fact that CB13-loaded nanoparticles with an optimal design maintain the analgesic effect for up to 11 days, while free CB13 exerts pain control for 9 h. It could, therefore, be expected that the longest analgesic effect (up to 11 days) of CB13-PLGA-PEG could make this formulation a good candidate for chronic pain management.

In the present work, we have used PEG for nanoparticle surface modification. Versus the free drug, the huge difference observed for CB13-PLGA-PEG on the behavioral and pharmacological effect of CB13 suggests an increased oral bioavailability. The enhanced access to the intestinal lymphatics after oral administration, as well as the capacity of PLGA matrices to control drug delivery, may explain this extremely long therapeutic response.

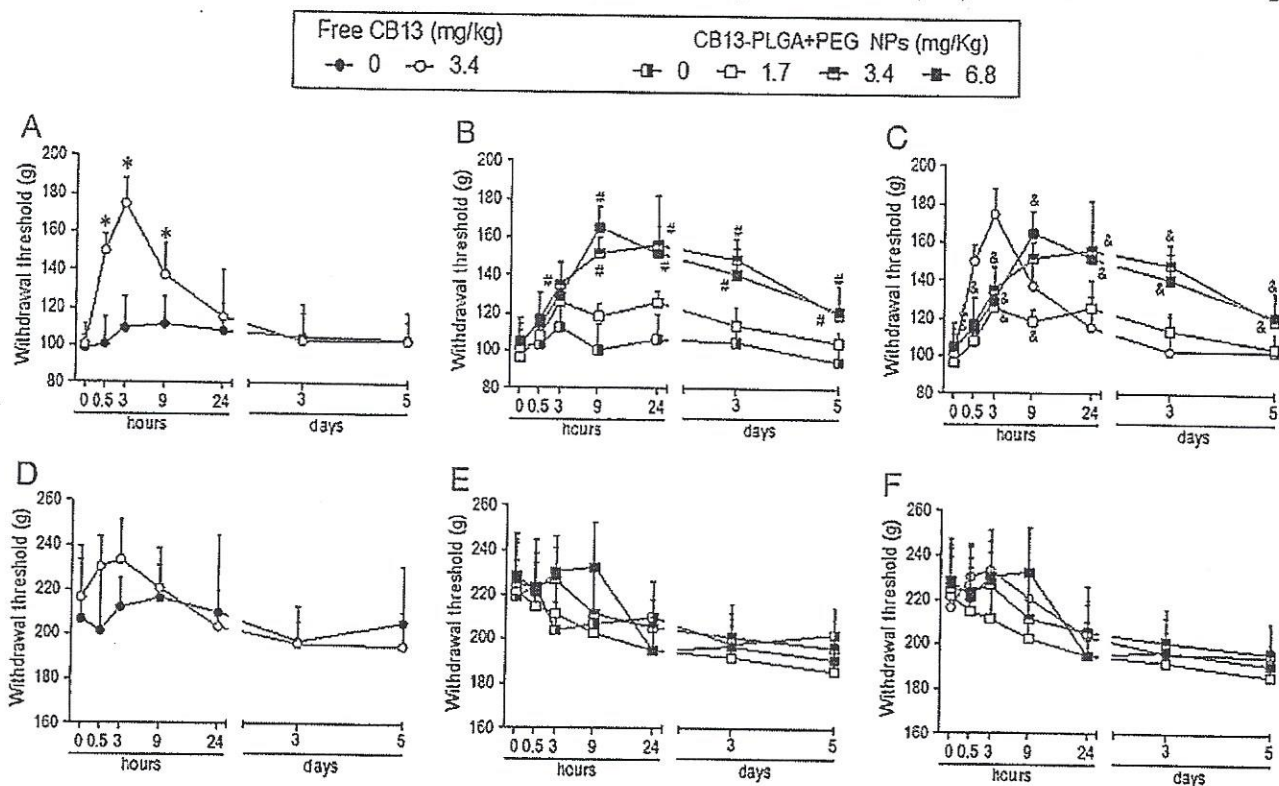


Figure 4. Effects in the paw pressure test in the CCI model in rat of PLGA + PEG nanoparticles and free CB13 (p.o.). (A) Free CB13 (3.4 mg/kg) vs. control group; (B) CB13–PLGA + PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (C) free CB13 (3.4 mg/kg, p.o.) vs. CB13–PLGA + PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the ipsilateral hind paw ($n = 7-4$). (D) Free CB13 (3.4 mg/kg) vs. control group; (E) CB13–PLGA + PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (F) free CB13 (3.4 mg/kg) vs. CB13–PLGA + PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the contralateral hind paw ($n = 7-4$).

It is known that CB13 physicochemical profile and lipoprotein affinity (80% of CB13 plasma protein binding is due to association with plasma lipoproteins) promote lymphatic transport. Accessing lymphatics, first-pass CB13 metabolism is circumvented and its oral bioavailability substantially enhanced.⁷

A variety of nanoparticle delivery systems targeting intestinal lymphatic include various fatty acid mono-, di- and triglycerides in their composition in order to promote association with lymph lipoproteins. Recently, Attili-Qadr et al²⁹ reported a significantly enhanced effect of orally-administered docetaxel when this latter is contained within nanocapsules containing a mixture of glyceryl tributyrates, oleoyl polyoxyglycerides and PLGA. In this case, the authors hypothesized that nanocapsules were transported into the intestinal lymphatics after receiving a surface coating of apoproteins and phospholipids during their passage across the enterocytes (in effect, the nanocapsules became “lipoproteinized”).

The PLGA nanoparticles assayed in the present work did not include any kind of fatty acid in their composition. We suggest that, due to its affinity to lipoproteins, the CB13 being released from nanoparticles from the start can itself “lipoproteinize” the nanosystems. It is also important to notice that PLGA nanoparticles assayed contain a significant amount of Span®60 (33% w/w related to PLGA). The presence of this non-ionic lipophilic surfactant can also provide a suitable micro-environment for improving oral bioavailability by reducing interfacial surface tension, enhancing

the penetration of hydrophobic drugs and promoting intestinal lymphatic transport after oral administration.³⁰⁻³²

Nonetheless, other factors concerning nanoparticles must also be taken into account. The particles’ size and surface properties suggest that a large proportion of an absorbed dose might be expected to drain into intestinal lymphatic capillaries.³³ Recent literature³³⁻³⁵ points out the main factors for the passive lymphatic targeting of nanoparticles: biocompatible and biodegradable components, carrier and drug stability, zeta potential and hydrophobicity. For targeting lymphatic vessels, size and hydrophobicity seem to be the most important nanocarrier design criteria.

In the case of anionic plain CB13–PLGA, it is expected that nanoparticles will be partially degraded by gastric pH¹¹ and that they will encounter high electrostatic repulsive forces from the negatively-charged intestinal mucus, leading to faster clearance.³⁶⁻³⁸ A sufficient amount of nanoparticles was, however, viable as the therapeutic response increased from 9 h to 3 days, as compared to free CB13.

PLGA + PEG neutral nanoparticles are expected to show a high PEG density on the nanoparticles’ surface, protecting them from gastric pH and enzymes. When compared to plain nanoparticles, the presence of a dense PEG coating was translated into a threefold increase in diameter. This increase would, however, be less evident through the GI tract due to a partial loss of PEG chains. A higher mucus penetration of these nanoparticles

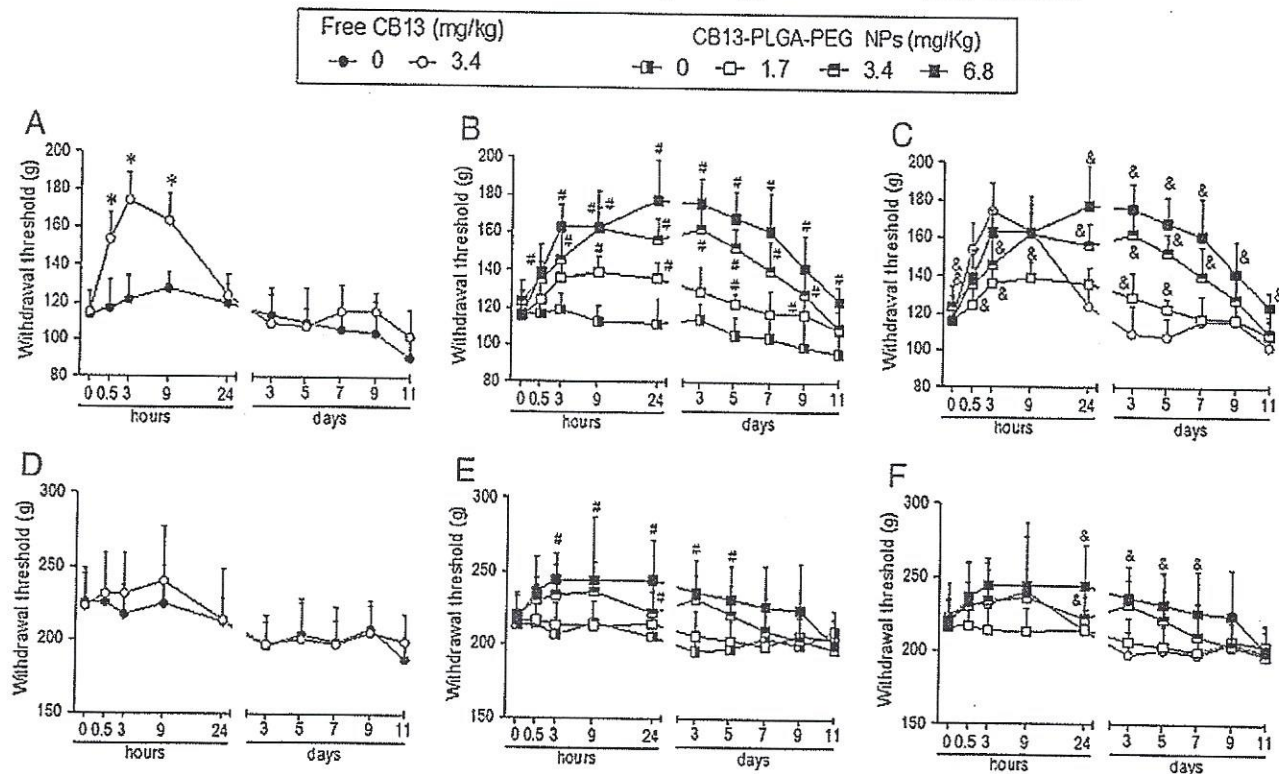


Figure 5. Effects in the paw pressure test in the CCI model in rat of CB13-PLGA-PEG nanoparticles and free CB13 (p.o.). (A) Free CB13 (3.4 mg/kg) vs. control group; (B) CB13-PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (C) free CB13 (3.4 mg/kg) compared with CB13-PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (D) Free CB13 (3.4 mg/kg) vs. control group; (E) CB13-PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (F) free CB13 (3.4 mg/kg) compared with CB13-PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the contralateral hind paw ($n = 8-10$).

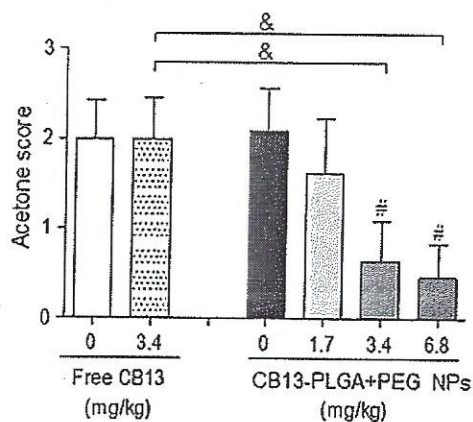


Figure 6. Effect of CB13-PLGA-PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg, p.o.) and free CB13 (3.4 mg/kg, p.o.) in the ipsilateral hind paw in the acetone test in the CCI model in rat. Results are expressed as the mean \pm S.D. ($n = 7-9$).

is expected, followed by a smoother passage through enterocytes and finally a facilitated access to intestinal lymphatics, which would promote CB13 activity for 5 days.

Curiously, CB13-PLGA + PEG (1.7 mg/kg) nanoparticles did not show a significant analgesic effect at any time. This could be due to a combination of circumstances: (i) a small CB13 amount released from nanoparticles, not enough to achieve similar

blood levels to free CB13; (ii) bigger particle size than plain or PLGA-PEG nanoparticles (600 nm versus 200 nm); (iii) near-neutral ζ -potential values; (iv) a possible PEG loss preventing nanoparticles diffusion across mucus and then; (v) a higher or faster nanoparticle clearance.

Finally, moderated anionic PLGA-PEG nanoparticles showed an analgesic effect that lasted for as long as 11 days.

Difference in activity of PLGA + PEG and PLGA-PEG nanoparticles can be explained by the PEGylation procedure. For PLGA + PEG nanoparticles, PEG chains are expected to be non-strongly anchored on the surface of nanoparticles. This way, it is more than likely that PEG chains go away from nanoparticles surface in the GI tract. Once in the blood stream, a progressive PEG loss can be followed. This can be translated into a higher opsonization process and a fast clearance from the body. In short, PLGA-PEG nanoparticles with stable PEG chains anchored onto the surface of the nanoparticles presented a half-life higher than PLGA + PEG nanoparticles.

As explained in Trevaskis et al,³³ nanoparticle size and surface properties suggest that a large proportion of an absorbed dose might be expected to drain into intestinal lymphatic capillaries. Trevaskis et al³³ state that: "In general, few studies report nanoparticle bioavailability using detailed pharmacokinetic analyses of exposure after oral administration and the extent of lymphatic transport of nanoparticles has rarely been quantified directly. Whether sufficient quantities of nanoparticles

are absorbed to deliver a typical therapeutic load is therefore less clear". Moreover, Lammers et al³⁹ suggest that, beyond targeting and beyond numbers, nanomedicine works and what counts is patient benefit.

In this sense, we have focused our investigation on the therapeutic effect of PLGA nanosystems in pain relief management using an animal model. Results clearly showed an ultra-long analgesic effect by means of using oral CB13–PLGA–PEG nanoparticles.

Conclusions

We have developed biodegradable nanosystems with different surface properties varying PEG surface density and anchoring. The three examples of PLGA nanosystems presented here performed the following task: sustained and prolonged neuropathic pain relief after just one oral dose of PLGA nanoparticles containing CB13.

CB13–PLGA–PEG nanoparticles presenting small size, near-neutral ζ -potential values and stable PEG coating exert an analgesic effect for 11 days after administering just one oral dose. This noticeable difference in therapeutic effect duration suggests an enhanced passive lymphatic targeting followed by systemic drug delivery. Therapeutic effect was clearly dose-dependent after orally administering nanoparticles. Furthermore, CB13–PLGA–PEG nanoparticles presented a delayed analgesic effect of up to 0.5 h, 3 h or 9 h as a function of CB13 doses (6.8, 3.4 or 1.7 mg/kg, respectively).

The overall results may advocate the feasibility of reducing the dose and suggest that CB13–PLGA–PEG nanoparticles may be an exciting new therapeutic option for the treatment of neuropathic pain.

Moreover, these results can open up a new perspective for the future of therapeutic cannabinoid uses, not only with regard to its use in pain management, but also with regard to its wide therapeutic spectra.

Acknowledgments

We are very grateful to Mr. José Antonio García Partida for his excellent technical assistance. We also thank the Universidad de Sevilla's CITIUS Microscopy and Biology Services for their technical assistance

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2017.07.010>.

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