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Rapid communication

One-week *in vivo* sustained release of a peptide formulated into *in situ* forming implants

Marianne Parent[a,*], Igor Clarot[a], Sébastien Gibot[b], Marc Derive[c], Philippe Maincent[a], Pierre Leroy[a], Ariane Boudier[a]

[a] Université de Lorraine, CITHEFOR, EA 3452, Nancy, France
[b] Université de Lorraine, INSERM U1116, Vandœuvre-lès-Nancy, France
[c] INOTREM, Vandœuvre-lès-Nancy, France

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ABSTRACT

The LR12 peptide has been reported to reduce the size of infarct and improve both cardiac function and survival in myocardial infarction in murine models, after daily repeated intraperitoneal injections. In order to protect peptide from degrading and to prolong its release, *in situ* implants based on biocompatible biodegradable polymers were prepared and both *in vitro* and *in vivo* releases were evaluated after subcutaneous administration to Wistar rats. A progressive and complete release was obtained *in vitro* in 3 weeks. *In vivo*, a 7-day sustained release was demonstrated after administrating the formulation once; bioavailability was improved by protecting the peptide against the degradation identified as a dimerization through disulfide bond formation. As a conclusion, *in situ* forming formulations are a suitable alternative for the therapeutic use of this peptide.

With 17.3 million deaths per year (a number expected to grow to more than 23.6 million by 2030), cardiovascular disease is the leading global cause of death. Related direct and indirect costs are estimated to be higher than $320.1 billion, including health expenditures and loss of productivity (Mozaffarian et al., 2015). As the immune system and the inflammation are now recognized as key players in the establishment and exacerbation of cardiovascular diseases, new therapeutic strategies have been emerging to control them. In this context, the triggering receptor expressed on myeloid-cells-1 (TREM-1) has been identified as an interesting target. This immune-receptor is expressed by neutrophils, macrophages and mature monocytes, and acts as an amplifier of the innate immune response during both infectious and sterile inflammation (Bouchon et al., 2001; Gibot et al., 2009; Zhou et al., 2013). Recently, the modulation of TREM-1 signaling by an inhibitory dodecapeptide (LR12, Fig. 1A) reduced the size of infarct and improved both cardiac function and survival in a murine model of myocardial infarction (Boufenzer et al., 2015). In that study, LR12 (5 mg/kg) was intraperitoneally (IP) administered once a day for 5 days. This therapeutic scheme should be advantageously replaced by a single injection of a sustained-release formulation. Moreover, LR12 oxidizes spontaneously in aqueous media with a short half-life ($t_{1/2}$) (approximately 14 h in phosphate buffer saline (PBS) *in vitro*, and 0.5 h in blood *ex vivo*): a disulfide bridge is formed between two peptides, generating a dimer (Fig. 1B). While still to be confirmed by further investigation, preliminary experiments on cells suggested that the dimer is devoid of pharmacological activity. Consequently, the formulation should both sustain LR12 release for at least a 5-day period and, as much as possible, protect the drug from degrading by dimerization.

In this contribution, the *in vitro* and *in vivo* release of LR12 (both the monomer and its main product of degradation, *i.e.* the dimeric form) from *in situ* forming implants have been evaluated. *In situ* forming implants are liquid formulations which, when injected into aqueous environments, precipitate as solid polymeric matrices entrapping the drug (Parent et al., 2013a). Sustained releases obtained with *in situ* forming implants have been described in the literature for a wide range of drugs with various physicochemical properties. Herein, poly-lactide-co-glycolide (PLGA, Resomer RG502H, 50:50 ratio LA:GA) and poly-lactide (PLA, Resomer R203S) were used as biocompatible biodegradable polymers due to their frequent use in *in situ* forming formulations and their lack of toxicity. Polymers were solubilized (18.7% w/w) in triacetin (TA, 74.8% w/w) before adding the drug (6.5% w/w). The concentration

* Corresponding author at: Université de Lorraine, CITHEFOR, EA3452, 5 rue A. Lebrun, BP 80403, F–54001, Nancy Cedex, France.
E-mail address: marianne.parent@univ-lorraine.fr (M. Parent).

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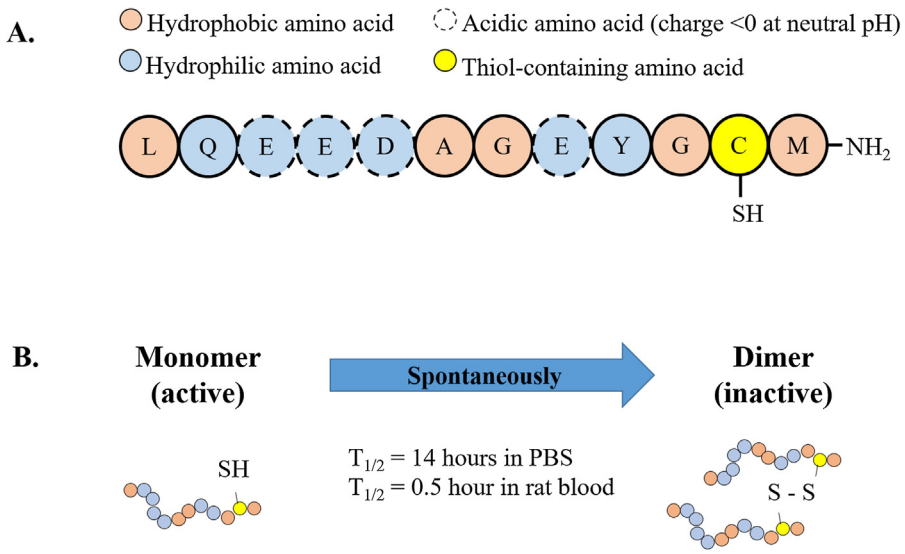

Fig. 1. A) Murine sequence of LR12 monomer peptide (Mr 1341 g/mol, calculated pI ~ 3.6) and B) main pathway of degradation by dimerization.

of peptide used in the formulation allowed to subcutaneously inject a dose of 80 mg LR12/kg to the animals. This can be compared to the total dose of 25 mg/kg of free LR12 administered *via* several IP injections in the study, which demonstrated the benefit of LR12 in myocardial infarction (Boufenzer et al., 2015). This dose is compatible with a slow delivery of LR12 from the reservoir formulation during the time of experiment, and it is likely without safety issues. Additionally, with this concentration, the viscosity of the formulation remained suitable for an easy injection.

Formulations were prepared and *in vitro* release experiments were performed in physiological buffer saline according to previously reported protocols (Parent et al., 2013b). An HPLC-UV method was developed, then validated for selectivity, precision, accuracy and linearity according to the FDA guidelines, and used to quantify LR12 (monomer and dimer) released in the aqueous medium (Fig. 2). The chromatographic system was the same as previously described (Parent et al., 2016). The elution was isocratic (86/14% v/v of water/acetonitrile + 0.1% trifluoroacetic acid) with 20 µL of injected sample. The detection was set at 220 nm. Linearity was verified for monomer and dimer between 1.0 and 50.0 µg/mL ($y = 14.30x - 17.55$ and $R^2 = 0.994$ for monomer, $y = 14.15x - 11.87$ and $R^2 = 0.996$ for dimer). Peptides remaining in the implants were also quantified with the same method after

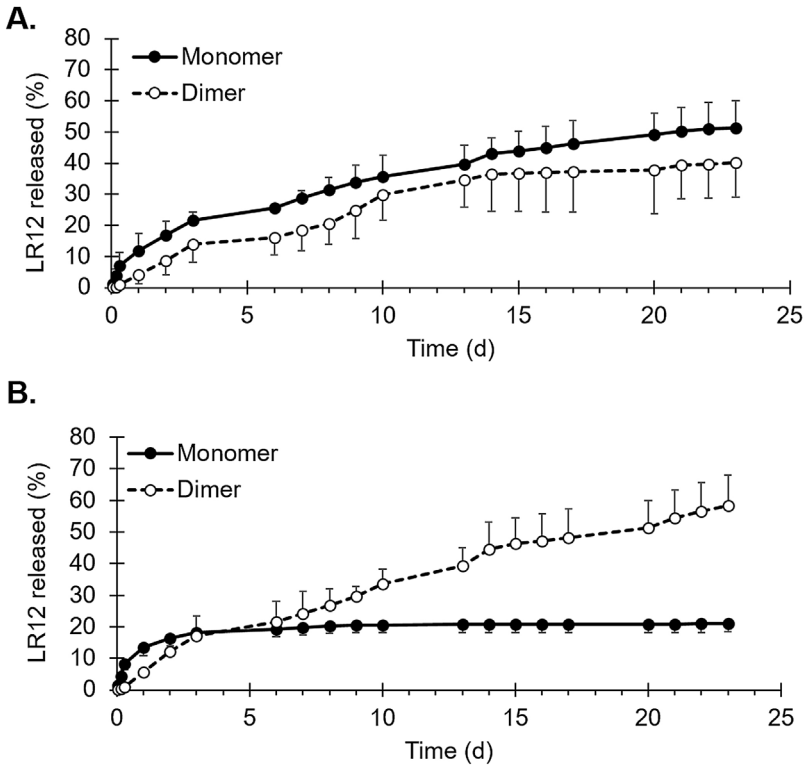

Fig. 2. *In vitro* release profiles of LR12 (monomer and dimer) obtained from *in situ* implants made of TA and of either PLGA (A) or PLA (B). Results are presented as mean ± sd of three experiments.

being extracted (dissolution of implants in ethyl acetate then liquid–liquid extraction with PBS added with 0.1% trifluoroacetic acid, recoveries of 102.5 ± 6.2% for the monomer and 100.3 ± 4.2% for the dimer, n = 3).

In vitro experiments demonstrated a 3-week sustained release of LR12 monomer from PLGA implants (Fig. 2A), without any burst, as expected when using a solvent with low water solubility such as TA (Camargo et al., 2013). LR12 dimer also appeared progressively in the medium. The release profile from PLA implants was different (Fig. 2B): the monomer release reached a plateau after 3 days, while the concentration of the dimer increased until the end of the experiment. Dimer appears, resulting from the degradation of monomer, which can occur either inside the implant or in the aqueous medium after its release. In the conditions of the *in vitro* release test, non-formulated LR12 monomer spontaneously oxidized within time to form the disulfide dimer, with a short half-life of 14 h. Moreover, at the end of the release experiments (after 3 weeks), remaining LR12 (<5.0% of the initial load) was extracted and was shown to be mainly under the monomer form (95.0 ± 1.3% for PLGA and 93.4 ± 1.1% for PLA). The very low proportion of dimer inside the implants suggests that the degradation of LR12 likely occurs in the release medium rather than inside the formulations. To conclude, *in situ* implants offered a sustained *in vitro* release of LR12 monomer up to 3 weeks, while efficiently protecting the drug from dimerization.

In a previous study, blood concentrations of LR12 (monomer and dimer) after intraperitoneal injection (5 mg/kg) to male Wistar rats were monitored (Parent et al., 2016). The same protocol and method were applied to quantify LR12 in blood after one single subcutaneous injection of *in situ* implants (80 mg LR12/kg) to male Wistar rats (280–350 g). Rats were subcutaneously injected with *in situ* forming formulation prepared with PLGA or PLA (final dose of 80 mg LR12/kg, 21 G needle used for injection). Under isoflurane anesthesia, blood was collected 1 h, 1, 3, 7 or 14 days after the treatment, before sacrificing the animal and retrieving the implants for residual LR12 quantitation. Three animals were used for each time point for each polymer (total = 30 rats). Blood samples were treated and analyzed with the HPLC-fluorescence method as previously reported (Parent et al., 2016). Remaining LR12 was also extracted from the retrieved implants and quantified as described above for the *in vitro* study. At autopsy, no sign of irritation or inflammation of the tissues surrounding the implants was observed, whatever the polymer used (Representative photograph of extraction is supplied in Supplementary data S1).

After one single administration of *in situ* forming implants, LR12 monomer was detected at therapeutic concentrations (between 100 and 400 ng/mL) for a week (Fig. 3). The area under the concentration-time curve ($AUC_{0 \rightarrow 7days}$) was calculated using the trapezoidal rule and was normalized by the received dose (Table 1). The highest observed plasma concentration (C_{max}) and the time required to reach C_{max} (T_{max}) were obtained from the concentration-time curves. Results indicated that *in situ* forming formulations significantly improved LR12 monomer bioavailability by a factor 50, without any difference between PLGA and PLA. This can be explained by the sustained-release properties of the *in situ* forming formulations, which increase the circulation time of LR12, and by the protection of monomer from degradation because it was encapsulated into the polymeric matrices. Although LR12 remaining inside the implants was mainly under the monomer form, the proportion of dimer inside the *in vivo* implants increased within time (4.3 ± 1.7% at day 1 to 27.3 ± 2.4% at day 7 for PLGA and 3.5 ± 3.0% at day 1 to 26.1 ± 10.9% at day 7 for PLA). After one week, more than 95% of the initial load was released. Compared with these *in vivo* results, better LR12 protection (>90% remaining as monomer after 3 weeks in the implants) and more sustained release (up to 3 weeks) with *in situ* forming implants were observed *in vitro*.

In the literature, only a few studies deal with *in vivo* peptide sustained delivery with *in situ* forming formulations. A 48 h – *in vivo* release was for example reported for enfuvirtide, an anti-HIV fusion inhibitor of 36 amino acids, when incorporated into implants made of PLGA and a mixture of DMSO and triacetin (Kapoor et al., 2012). A sustained effect of *S*-nitrosoglutathione, a nitric oxide donating tripeptide, was also observed over the same time length, using PLGA implants with *N*-methyl-2-pyrrolidone (NMP) as solvent (Parent et al., 2015). Regarding leuprolide acetate (9 amino acids), a 14-days *in vivo* release in rats was described from PLGA/NMP implants (Mashayekhi et al., 2013), but similar marketed formulations (Eligard®) allow therapeutic efficiency in humans from 1 to up to 6 months after a single administration. In this study, *in situ* forming formulations administered to healthy rats demonstrated their ability to deliver the dodecapeptide LR12

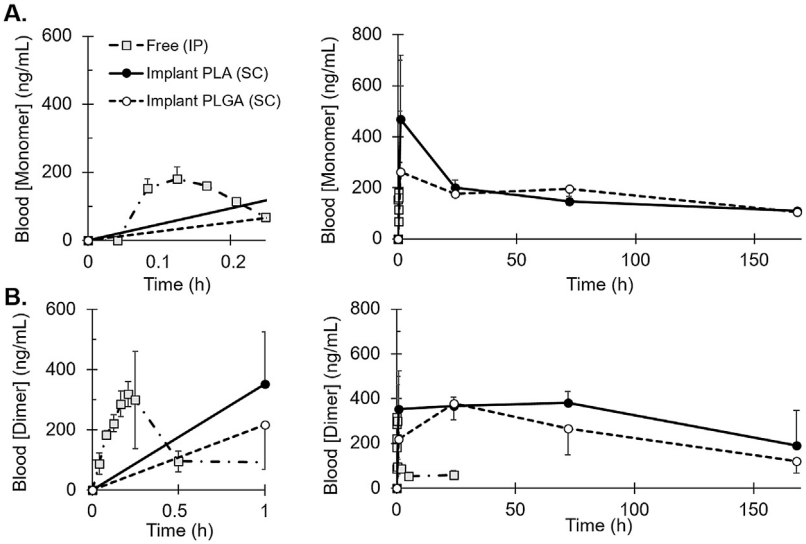

Fig. 3. Blood concentrations of LR12 (monomer form, panel A and dimer form, panel B) after administration of LR12 monomer (unformulated, 5 mg/kg, intraperitoneally *vs* formulated into *in situ* implants, 80 mg/kg, subcutaneously). Limit of quantification of the method was 50 ng/mL. All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for the use of experimental animals, and protocols were approved by the regional and national ethical committees (project "Slow-release", APAFIS#1146-2015071313458604 v3).

Table 1
Pharmacokinetic parameters of LR12 after administration to male Wistar rats.

		Intraperitoneal injection of LR12 monomer	Subcutaneous injection of LR12 monomer-*in situ* implants	
			PLGA	PLA
Monomer	$AUC_{0\rightarrow 7days}$ ($ng\,mL^{-1}\,h$)	18	17 467	17 388
	AUC/Dose ($L^{-1}\,h\,g$ body weight)	4	218	217
	T_{max} (h)	0.08	1	1
	C_{max} (ng/mL)	181 ± 35	261 ± 95	468 ± 252
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Dimer	$AUC_{0\rightarrow 7days}$ ($ng\,mL^{-1}\,h$)	197	38 414	57 499
	AUC/Monomer dose ($L^{-1}\,h\,g$ body weight)	39	480	719
	T_{max} (h)	0.25	24	24
	C_{max} (ng/mL)	300 ± 162	379 ± 74	367 ± 39

according to a smooth and sustained profile at therapeutic concentrations for 7 days. As a result, this single injection could be an interesting alternative to the current therapeutic scheme (free drug 5 mg/kg, daily injection for 5 days) proposed in myocardial infarction (Boufenzer et al., 2015). PLGA formulations should probably be preferred over PLA ones, because they will be degraded faster, while offering the same monomer bioavailability in this case, but with lower burst and less dimer blood exposure (dimer AUC/dose increased by 12 for PLGA implants compared to IP administration and by 18 for PLA ones, Table 1). Modifications of the peptide itself or of the formulation could also be envisaged to further increase the *in vivo* duration of release. For example, the PEGylation of a natural polysaccharide enhanced the *in vivo* mean retention time from 1.0 h to 2.8 days, and this result was drastically improved (up to 13 days) when the conjugate was formulated into PLGA *in situ* implants (Shi et al., 2014, 2015).

To conclude, this study demonstrates that *in situ* formulations are promising candidates for the therapeutic use of LR12, a TREM-1 inhibitory dodecapeptide useful in many conditions involving inflammation and exacerbated immune response. However, pharmacokinetics of LR12 could be modified in pathological situations, for example by the apparition of the soluble form of TREM-1 in the blood (Boufenzer et al., 2015). The benefit of *in situ* formulations for LR12 treatment should therefore be confirmed in animals suffering from myocardial infarction for example.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2017.02.046.

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