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ANTIMICROBIAL PEPTIDE ENCAPSULATION AND SUSTAINED RELEASE FROM POLYMER NETWORK PARTICLES PREPARED IN SUPERCRITICAL CARBON DIOXIDE

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Abstract

Antimicrobial peptide loaded poly(2-hydroxyethyl methacrylate) particles were synthesized in supercritical carbon dioxide *via* one-pot free-radical dispersion polymerisation of 2-hydroxyethyl methacrylate and a cross-linker. Discrete particles with a well-defined spherical morphology and a diameter as low as 450 nm have been obtained in mild conditions. The encapsulation and release of the peptide were confirmed by antimicrobial tests that demonstrated for the first time a sustained release of the peptide from poly(2-hydroxyethyl methacrylate) microgels prepared by one-pot dispersion polymerization in supercritical carbon dioxide and then dispersed in water.

1. Introduction

Nowadays, an increasing number of therapeutic peptides and proteins are approved by the Food and Drug Administration (FDA), and many others are being developed at various stages including pre-clinical or clinical trials [1]. Historically, the importance of the proteins for human beings was highlighted by the human genome studies; most of genes were found to be responsible for protein encoding [2]. Having said the importance of proteins for human body, it was also revealed that they can be used as therapeutics to treat different kinds of diseases for instance genetic and degenerative disorders, protein malfunction and enzyme deficiencies. When the peptide/protein based drugs are compared to conventional drugs, they can be more active, may exhibit lower toxicity and weaker drug-drug interactions [3]. As an example, protein-based drugs offer momentary actions which make them safer than existing therapies such as gene therapy where random or permanent genetic alterations occurs [4]. Considering all the advantages of proteins, a vast number of studies turned to formulate active pharmaceutical ingredients based on peptides or proteins. However, peptides and proteins exhibit some drawbacks such as a rapid degradation or inactivation, a low bioavailability and a low permeability towards biological membranes [5]. Therefore, researches are focused on the idea of encapsulating them to increase their bioavailability, protect them from harsh environmental conditions and more importantly insure their controlled release over a course of time [6].

Among the valuable available peptides, one emerging family is the antimicrobial peptides (AMPs) developed to circumvent the global increase of antibiotic resistance and address the major infectious public health problems [7]. Roughly all types of *Staphylococcus aureus* species show resistance towards the conventional antibiotics such as penicillin and methicillin [8]. Therefore, to find solutions against the resistant bacteria holds a great importance in the research area. Studies focussing on the development of alternative antibiotic agents such as gene-coded AMPs are thus numerous [9]. Among them, temporins are short AMPs having up to 14 amino acids which are derived from amphibians and showed high bactericidal efficiency. This group was named from the frog called *Rana temporaria* from which the AMP was initially extracted. Temporin A (TA) and Temporin L (TL) are active against Gram-positive bacteria, *Candida* species, fungi and have the ability to bind and permeate both artificial and biological membranes. TAs and TLs differ in the range of their antimicrobial activity as well as their toxicity. TLs were found to possess the strongest activity against fungi, Gram-positive and Gram-negative bacteria in comparison to other temporins. However, it was found that TLs were able to bind and permeate the membrane of mammalian cells (erythrocytes and cancer cells) [10]. TLs are toxic on human erythrocytes thus necessitate for the synthesis of analogues. The amino acid sequence of the TL and its analogue Pro^{3}TL is given below. Replacement of the Glutamine (Gln) residue in the third position with Proline (Pro) showed superior antimicrobial activity and lower haemolytic activity compared to native TLs [11].

TL: H-Phe^1-Val^2-$\underline{\text{Gln}}^3$-Trp^4-Phe^5-Ser^6-Lys^7-Phe8-Leu^9-Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$

Pro^3-TL: H-Phe^1-Val^2-$\underline{\text{Pro}}^3$-Trp^4-Phe^5-Ser^6-Lys^7-Phe8-Leu^9-Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$

Nevertheless, there is a need to encapsulate such AMP in order to control their release and time of action. Polymer network particles that have nano/micro scale size prepared by either physical or chemical cross-linking are referred as nano/microgels. These hydrogels were proven to be very promising candidates as drug carriers owing to their high loading capacity and stability [12]. Moreover, nano- or microgels hold a great promise due to their combinatorial properties between nanoparticles and hydrogels. They are highly hydrophilic and able to swell, whereas they have small size and large surface area as particles. Nano/microgels for protein/peptide delivery systems can be composed of either homo- or copolymers. 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), N-isopropyl acrylamide (NIPAAM), acrylic acid (AA) and methacrylic acid (MA) monomers are extensively used to form 3D polymer matrices. The polymer matrix will define the affinity for water whereas the crosslinking degree modulates the swelling of the network, therefore have an impact on the release behaviour of the encapsulated molecules [3]. Hydrogels of poly(HEMA) present excellent biocompatibility and can act as potential carriers in drug delivery, dental, ophthalmic, and neural tissue engineering applications [13,14]. Poly(HEMA) networks can be produced by free radical dispersion polymerization of HEMA monomer in the presence of a cross-linker and appropriate stabilizer. As previously mentioned, peptides and proteins have a sensitive structure which can be degraded easily by temperature and upon exposure to solvents leading to the loss of their activity [4,15]. In order to encapsulate them efficiently without altering their activity, a variety of production methods have been studied [16,17] including supercritical carbon dioxide processes [18]. Supercritical carbon dioxide ($scCO_2$) is a green alternative for the conventional solvents. It is non-toxic, non-flammable and has low critical point (31.1 °C and 73.8 bar) [19]. More interestingly, $scCO_2$ goes to gaseous state upon depressurisation which enables to produce formulations as dry powders offering better stability in comparison to liquid formulations [18].

Some of us have previously demonstrated the successful synthesis of cross-linked poly(HEMA) particles in super critical carbon dioxide ($scCO_2$) using mild conditions (35 °C/300 bar) [20] and on their further redispersion and swelling in water thanks to the use of a fluorinated photocleavable stabilizer, i.e. poly(ethylene oxide) (PEO)-$h\nu$-poly(heptadecafluorodecyl acrylate) (PFDA) (PEO-$h\nu$-PFDA) block copolymer where $h\nu$ stands for a photosensitive moiety. This stabilizer was designed to perform dispersion polymerization in $scCO_2$ leading to nanoparticles of poly(HEMA) that can swell in water after photolysis and removal of the fluorinated block [21].

The aim of this present work is to apply this process to the one-pot encapsulation of AMPs in cross-linked poly(HEMA) particles and to demonstrate the possible controlled release behaviour of the antibacterial peptide from the resulting nanogels.

2. Experimental

2.1. MATERIALS

All chemicals are purchased from Sigma-Aldrich and used as received unless otherwise it is noted. 2,2'-Azobis(4-methoxy-2,4dimethylvaleronitrile) (V-70,Wako, 99%), 2-hydroxyethyl methacrylate (HEMA, Aldrich, 97%), Ethylene glycol dimethacrylate (EGDMA, Aldrich, 98%), Carbon dioxide (I5122,CO_2 N27 purity >99,999%, Air Liquide, Belgium), α,α,α-Trifluorotoluene (TFT, Aldrich, 99+%). Ultrapure water was produced by MilliQ plus 188 apparatus (Millipore). Bradykinin, Pro^{3}TL and *S. Aureus* were kindly provided by the Symbiose Biomaterials. The photocleavable diblock copolymer used as stabilizer, PEO$_{45}$-$h\nu$-PFDA$_{40}$ was synthesized according to the previously published method by Alaimo et al. [21].

2.2. METHODS

2.2.1. SYNTHESIS OF THE POLY(HEMA) PARTICLES IN SCCO2

Dispersion copolymerisation of HEMA and EGDMA was performed as previously described by Parilti et al. [20], except that the peptide was added to the reaction medium. Briefly, the high pressure stainless steel cell (82 ml, ToP Industrie) was charged with 8.2 ml of HEMA (10% v/v CO$_2$), 82 µl of EGDMA (cross-linker, 1% v/v monomer), 0.82 g of stabilizer (10% $w_{stabilizer}/V_{monomer}$), 0.187 g V-70 (1% w/v monomer) and 8.79 mg peptide (0.1% $w_{Bradykinin}$ or w_{Pro3Tl}/w_{HEMA}) and pressurized with CO$_2$ at 300 bar at 35 °C and stirred for one night. After total depressurization, the cell was opened and the dry particles collected as a powder. Then, particles dispersed in TFT were subjected to 10–30 min of UV-irradiation using a UV lamp (LOT-Oriel Arc Light Source Hg(Xe), power = 500 W) to cleave the fluorinated block as quantified by XPS (for more details see ESI) and obtain water dispersible particles as reported by Alaimo et al. [21].

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcis.2018.07.125.

2.2.2. ANTIBACTERIAL TEST

For the quantification of the biocidal activity of the particles the following multiple step protocol has been applied as follows:

> A *S. aureus* preculture has been prepared by taking a few bacteria spread on a Luria-Bertani agar gel (LBA), and placed in 10 ml of Luria-Bertani culture medium (LB) then incubated at 37 C overnight under agitation. Then, 2 ml of this solution was placed in 10 ml of fresh LB at 37° C under stirring until the optical density at 600 nm is equal to about 0.5;
>
> The samples to be tested (i.e. the free AMP, or 5 ml of the aqueous dispersion of loaded or empty particles), were added with 4.5 ml of water and 0.5 ml of the bacterial solution;
>
> Then at each time t, 10 µl of solution was picked out and placed in a tube containing 10 ml of LB culture medium; these tubes were placed on ice and then used for the drop plate method in order to count the colonies of viable bacteria. For this test, 3 × 10 µl were placed on an LBA gel putting the plates vertically so that the drops go down thanks to gravity. Once they were almost down, the plate was turned horizontally and incubated at 37 °C for 20 h and then the bacteria colonies visible to the naked eye were enumerated.

For these experiments, the LB solution was composed of 10 g/L of Bacto tryptone, 10 g/L of NaCl and 5 g/L of yeast extract. For the LBA solution, the composition was the same as for LB except that 15 g/L of agar was added.

2.3. CHARACTERISATION

Particles were analysed by scanning electron microscopy (SEM, JEOL JSM 840-A) to evaluate their morphology. Samples were prepared on SEM stubs and coated with Pt (30 nm). The size distribution of particles in suspension in TFT (before UV) and in water (after UV) were measured by dynamic light scattering (DLS) using a Delsa Nano C, Particle Analyzer, Beckman Coulter at 25 °C and the intensity of scattered light was detected at 165° to an incident beam. DLS measurements were done in triplicates in both TFT and water and each data set treated by the cumulant analysis.

3. Results and discussion

3.1. PEPTIDE LOADED POLY(HEMA) PARTICLES IN SCCO$_2$

In a previous work, cross-linked poly(HEMA) nanoparticles were prepared by dispersion copolymerisation of HEMA and a cross-linker (EGDMA) in scCO$_2$ in mild conditions (35 °C, 300 bar) with the aid of PEO-$h\nu$-PFDA photocleavable diblock copolymer stabilizer with an emerging optimal composition about PEO$_{45}$-$h\nu$-PFDA$_{40}$. Indeed, by using 10% of this stabilizer, regular poly(HEMA) particles with a size as small as 279 nm are obtained (**Table 1**, entry 1) while a size of 400 nm is reached by decreasing the stabilizer content to 5% (**Table 1**, entry 2). Therefore, for the one-pot encapsulation of the guest peptide, the Bradykinin or the Pro^{3}TL were introduced to this original reaction mixture (**Table 1**, entries 3–5) following the experimental set-up depicted in **Fig. 1**.

Table 1

Characteristics of the synthesized poly(HEMA) particles.

Entry	Stabilizer (%,wt)	EGDMA (%, v)	Bradykinin (%, w_t)	Pro^{3}TL (%, w_t)	SEM		D_h[c] by DLS	
					D_n (nm)	C_v[a]	In TFT before UV	In water after UV
1	10	1			279	9	461 nm	4.1 µm
2	5	1			404	12	1.10 µm	4.4 µm
3	10	1	0.1		316	43[b]	771 nm	3.2 µm
4	10	2.5	0.1		436	34[b]	639 nm	3.0 µm
5	5	2.5		0.1	432	8	700 nm	3.8 µm

Monomer 2-hydroxyethyl methacrylate (8.2 ml, 10% v/v CO$_2$) and initiator V-70 (0.187 g, 1% w/v monomer) concentration and reaction conditions (35 °C/300 bar) were kept constant.

[a] C_v stands for the coefficient of variance which is calculated according to the equation (Standard Deviation/D_n) * 100 applied to SEM data.

[b] Bimodal distribution.

[c] Hydrodynamic diameter: average size on triplicates.

With the purpose to evaluate the impact of the presence of the peptide on the dispersion polymerisation reaction in scCO$_2$, Bradykinin a model peptide readily available and presenting no bactericidal activity has been firstly used, then Pro^{3}TL, the active antibacterial peptide, was introduced to the reaction mixture for encapsulation.

Initially, Bradykinin loaded particles were synthesized with two cross-linker concentrations (1% and 2.5%) using 10 wt% stabilizer. Although particles were obtained with well-defined morphology, they had a bimodal distribution which was clearly evidenced by SEM images (**Fig. S1**: Entries 3 and 4) and confirmed by very high C_v number (**Table 1**). Bradykinin was found to have a low solubility in HEMA, therefore some phase separations could explain this large dispersity. On the other hand, the one-pot encapsulation of Pro^{3}TL was carried out in the presence of 5 wt% of the PEO_{45}-hv$PFDA_{40}$ stabilizer. At the end of the polymerisation, monodisperse spherical particles with 432 nm diameter were harvested as a dry fine powder (**Fig. 1**). The presence of the peptide in the medium did not appear to affect the particles formation and size. In that case, the cross-linker content was also set to 2.5% in order to get a denser network to sufficiently trap the peptide inside the particles and insure a controlled release.

In order to remove the fluorinated block from the particles surface, they were dispersed in TFT and irradiated by UV. As previously reported, the UV-photolysis of the nitrobenzyl group at the blocks junction leads to the release of 57% of the hydrophobic block (PFDA) of the stabilizer in the TFT solution when the irradiation is kept for 30 min [21]. This percentage of the extracted PFDA was confirmed by XPS measurements (ESI). In case of peptide loaded particles, the irradiation time was reduced to 10 min. to prevent photodegradation of the peptide. In that case, a release of 41% of the PFDA block was determined by XPS. This partial removal of PFDA was sufficient to allow the particles dispersion and swelling in water. Indeed, as demonstrated by DLS measurements performed in water (**Table 1**, after UV), all the particles showed a significant size increase upon swelling in water as compared to the dry state (**Table 1,** SEM) or to TFT, a non-solvent of poly(HEMA) (**Table 1**, before UV) resulting in hydrogel particles of 3–4 µm.

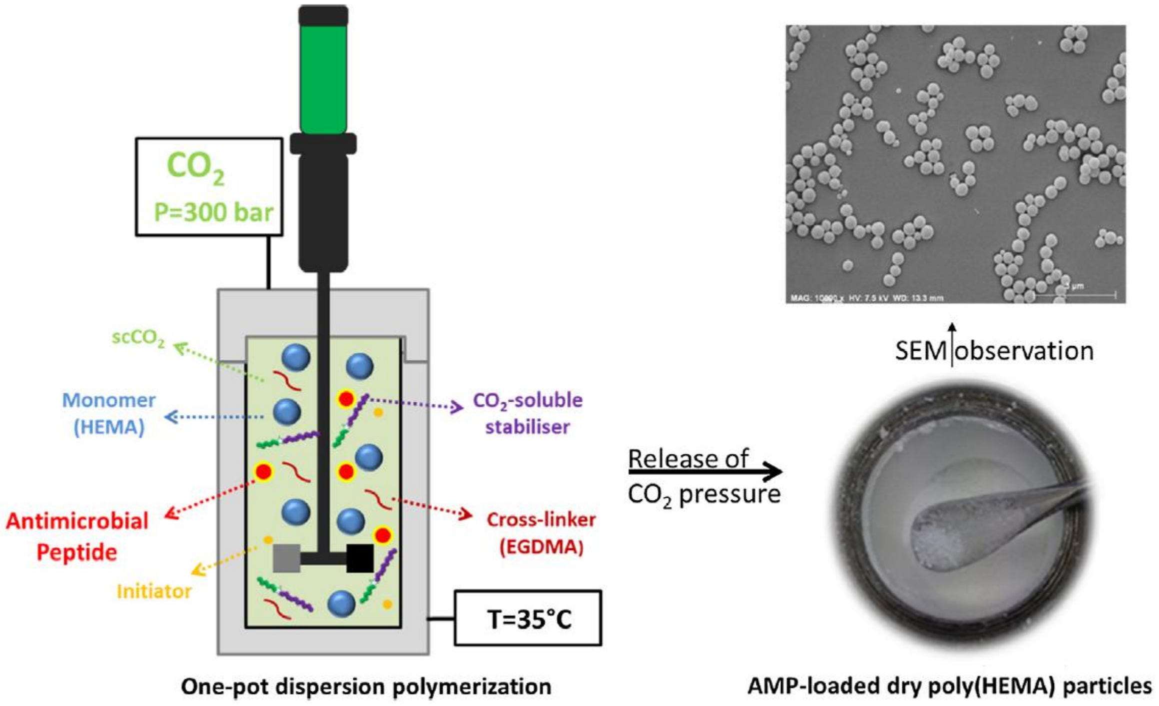

Fig. 1. Synthesis of the antimicrobial peptide (AMP)-loaded particles by one-pot dispersion polymerisation of HEMA in $scCO_2$. Left: scheme of the high-pressure stainless steel cell which is filled with the HEMA monomer (8.2 ml, 10% v/v CO_2), the EGDMA cross-linker (16.4 µl, 2.5% v/v monomer), the PEO_{45}-hv-$PFDA_{40}$ stabilizer (0.41 mg, 5% w/w monomer), the V-70 initiator (0.187 g, 1% w/v monomer), the AMP Pro^{3}TL (8.79 mg, 0.1% w/w monomer) and the CO_2 (P = 300 bar, T = 35 °C). Right: A picture of the dry powder obtained after 12 h of polymerization and complete depressurization of the cell and a scanning electron microscopy image of this powder (scale bar: 3 µm).

3.2. ANTIBACTERIAL TESTS ON EMPTY AND LOADED PARTICLES

In order to determine the potential of this encapsulation strategy, the bactericidal behaviour of the particles loaded with Pro^{3}TL have been evaluated against *Staphylococcus Aureus* and compared to the behaviour of empty particles. Some preliminary tests were first performed to validate the test and collect references. A first reference sample was performed by incubating the bacteria in water without particles. The number of remaining viable bacterial colonies was counted at regular time intervals and are reported in **Table S1**. This test confirms that the bacterial strains are able to multiply in aqueous medium in which the number of bacterial colonies is very large (Uncountable colonies (UC)). Similar behaviours are obtained when the bacteria are incubated with bradykinin, this peptide having no bactericidal effect. In contrast, an immediate and complete killing of the bacteria is observed when the free Pro^{3}TL peptide is incubated with the bacteria whatever its concentration (6.25 or 12.5 µg/mL).

Then, empty particles obtained from **Table 1** entry 1 after removal of the fluorinated block by irradiation of the particles in TFT were incubated at two concentrations with the bacteria (**Table S2** and **Fig. 2**). Note that in the **Fig. 2** which reports the same data as **Table S2**, an arbitrary number of colonies of 120 was selected when the number of colonies are impossible to count because they are too many (UC in **Table S2** means that there is no bactericidal effect). These empty particles at both concentrations did not present any bactericidal behaviour for 24 h meaning that the particles themselves do not lead to an antibacterial effect. This is also confirmed by using bradykinin-loaded particles. Regardless of the concentration, Bradykinin loaded particles (**Table 1**: Entry 3 and 4) showed similar behaviour as empty particles which did not present any bactericidal effect for 24 h (**Table S2** and **Fig. 2**). This first set of tests evidenced that the encapsulation carrier as produced by the scCO$_2$ process does not exhibit any effect on the bacteria growth and proliferation. Then, the particles loaded with ~0.1% of Pro^3-TL (**Table 1**, entry 5) were incubated at two concentrations, i.e. 56 mg and 112 mg of particles. The first assays (Test 1) for antibacterial tests were performed right after the irradiation and removal of the fluorinated segments (**Table S2** and **Fig. 2**).

When a low concentration of the particles loaded with Pro^3-TL (56 mg of particles, thus ~5.6 µg/mL of AMP) is incubated with the bacteria, no bactericidal effect is observed after 24 h in strong contrast to free Pro^3-TL which immediately kills the bacteria already at a concentration of 6.25 µg/mL (**Fig. 2**). This evidences that the AMP is well encapsulated in the particles and is released slowly during the first 24 h so that no bactericidal concentration is reached for this period. A deactivation of the peptide could also account for this loss of activity but doubling the concentration of particles to 11.2 mg/ml (~11.2 µg/mL of AMP) against the same amounts of bacteria led to a clear recovery of the bactericidal effect after 1 h which becomes significant after 4 h of release evidencing the gradual release of the active peptide from the nanogel particles. The incubation time was extended till 24 h leading to further release of the peptide and allowed the complete killing of the bacteria after 5 h (**Fig. 2**). This proves that the encapsulated peptide is still active, and able to diffuse slowly out of the particles to reach a minimal concentration in solution leading to the bactericidal effect. These data show for the first time that the developed process allows an efficient encapsulation of the peptide while keeping its bactericidal activity, and its sustained release from the particles.

A second run of assays was performed after storing the dry particles at room temperature for 10 days after UV treatment (Test 2) (**Fig. 3**A, **Table S3**). In this test 2, the concentration of particles has been doubled, i.e. 11.2 mg/ml and 22.5 mg/ml, as compared to test 1 to accelerate the biocidal effect. **Fig. 3**A further confirmed the gradual release of the encapsulated Pro^3-TL from the nanogel particles for both concentrations (11.2 mg/ml and 22.5 mg/ml). As the concentration of the particles increases (from 11.2 mg/ml to 22.5 mg/ml),the amount of released peptide in the same period of time is increased and leads to faster bactericidal effect.

Complete killing of the bacteria is observed between 5 and 6 h of incubation time for the highest concentration (22.5 mg/ml, ~22.5 µg/mL of AMP) while more than 7 h of incubation is required for the lower concentration (11.2 µg/mL of AMP). Nevertheless, after 24 h of release, all the bacteria are killed even at the lower concentration.

Comparing tests 1 and 2 (**Figs. 2** and **3**A) with the same number of particles, the kinetics of release appears slower in the test 2 performed after 10 days of storage. Therefore, a third test was performed in triplicates after 2 months of storage of the particles in the dry state in glass vial at room temperature. The results of this test 3 are reported in **Fig. 3**B and **Table S4** and indicate that the bacteria are still proliferating after 24 h for the lowest concentration (11.2 mg/ml particles, ~11.2 µg/mL of AMP), and that the kinetic tests performed simultaneously are reproducible, regardless of the quantity of microgels. In the case where a larger amount of microgels is used (22.5 mg/ml, ~22.5 µg/mL of AMP), the decrease in the number of bacterial colonies is progressive with a population number equals to zero after 24 h of release. Nevertheless, once again, the bactericidal effect appears after a slightly longer time period than in the previous tests 1 and 2 performed with the same batch of particles but at early stages after their preparation. From these measurements, we can conclude that the peptide is well encapsulated in the particles which control its release. Nevertheless, it appears that the peptide stability and/or its release rate is affected by the prolonged storage of the particles. Further studies will have to be done to clarify the origin of this phenomenon.

Nevertheless, the complete killing of the bacteria is still observed for 24 h of release for a storage time of 2 months showing the sustained release of the AMP thanks to the developed encapsulation process.

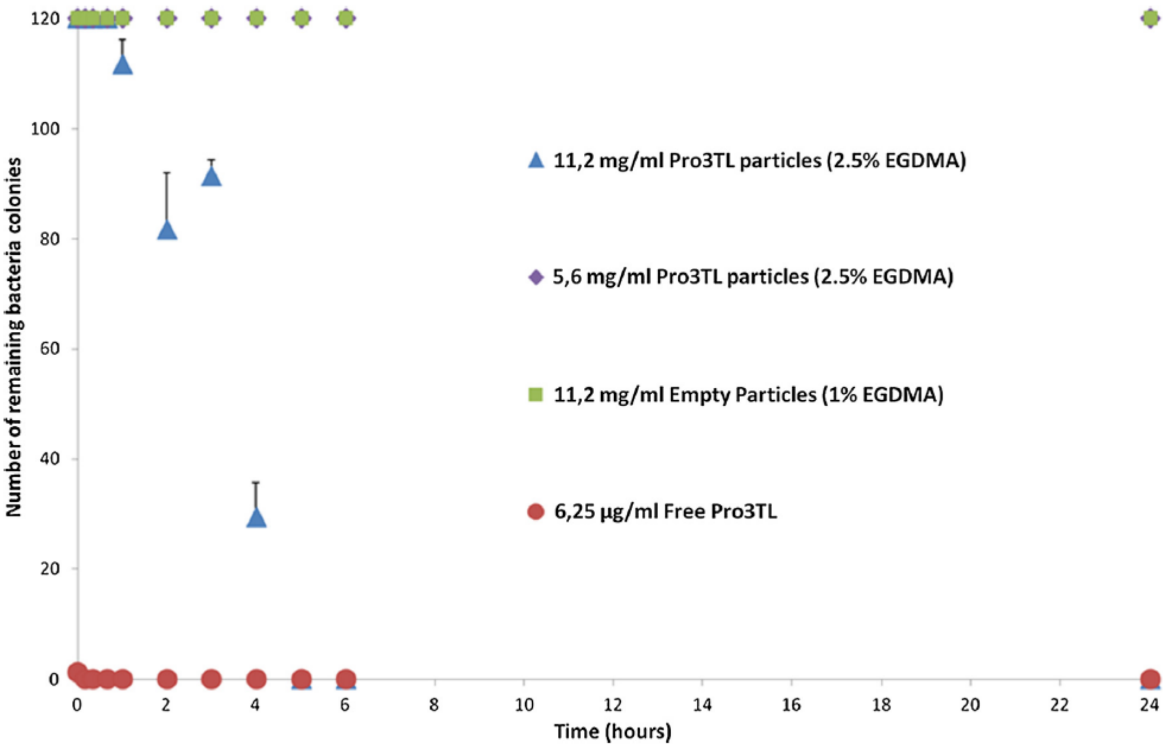

Fig. 2. Bactericidal test against *Staphylococcus Aureus* incubated with free Pro^{3}TL 6.25 µg/mL (circle), empty particles 11.2 mg/ml (square), and Pro^{3}TL-loaded particles 5.6 mg/ml (diamond) or 11.2 mg/ml (triangle) performed directly after synthesis and UV irradiation of the particles (Test 1). When the number of colonies is uncountable because they are too large (no bactericidal effect), an arbitrary value of 120 was chosen in this figure.

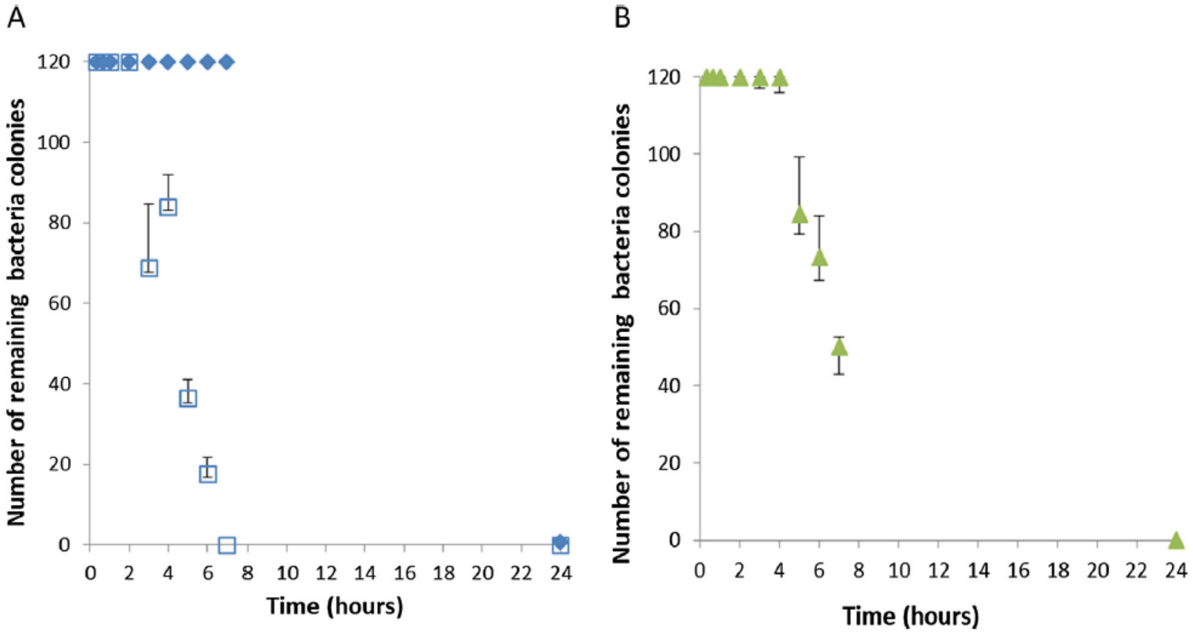

Fig. 3. Bactericidal test against *Staphilococus Aureus* incubated with Pro^{3}TL-loaded particles (A) after 10 days of particles storage, particles concentration of 11.2 mg/ml (diamond) and 22.5 mg/ml (square) (Test 2) (B) after 2 months of particles storage, particles concentration of 22.5 mg/ml (Test 3). When the number of colonies is uncountable because they are too large (no bactericidal effect), an arbitrary value of 120 was chosen in this figure.

Conclusions

In this work, we reported a novel, facile process for the encapsulation of antimicrobial peptide in well-defined spherical poly (HEMA) cross-linked particles *via* one-pot free radical dispersion polymerization carried out in $scCO_2$. Based on previous studies [20,21], peptide loaded poly(HEMA) particles were successfully synthesized in the presence of PEO_{45}-$h\nu$-$PFDA_{40}$ using mild conditions (35 °C/300 bar). At low concentration of stabilizer (5 wt%), discrete monodisperse peptide loaded poly(HEMA) particles could be easily obtained with a size of 400 nm. After only 10 min. of UV treatment of these particles, the fluorinated block of the stabilizer could be partially extracted and the particles could be redispersed and swollen in water, resulting into micron-sized hydrogels which was evidenced by DLS measurements. The antibacterial tests revealed the sustained release of the AMP from these poly(HEMA) particles swollen in water. In contrast to free AMP, the antibacterial effect was delayed which confirms the efficient encapsulation of the AMP. The robustness of the developed one-pot encapsulation method was proven even if a slight decrease of the bactericidal efficiency was observed for prolonged storage time. However, concrete hypothesis could not be drawn with respect to data in hand and would require further investigations beyond the scope of this first study. We believe that this study opens a pathway to encapsulate the active pharmaceutical ingredients that are heat and solvent labile especially such as peptides in mild conditions using $scCO_2$ as a green polymerisation medium.

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Supplementary data

QUANTIFICATION OF THE EXTRACTED FLUORINATED STABILIZER BY XPS

The particles before and after UV treatment have been analysed by X-ray photoelectron Spectroscopy (XPS) in order to quantify the percentage of released fluorinated stabilizer. XPS analyses were performed on a SSX 100/206 spectrometer (Surface Science Instruments - USA) equipped with a monochromatized micro-focused aluminium X-ray source (powered at 20 mA and 10 kV). A flood gun set at 6 eV and a Ni grid placed 3 mm above the sample surface were used for charge stabilisation. The angle between the surface normal and the axis of the analyser lens was 55°; in these conditions, the probed depth was of the order of 5 nm. The molar fractions were calculated from the peak areas normalized on the basis of the acquisition parameters and sensitivity factors provided by the manufacturer. The calculations exclude hydrogen which is not detected by XPS. Carbon and fluorine spectra recorded second time after the analysis in order to validate that the sample was not degraded by X-ray source. The data analysis was performed with the CasaXPS program (Casa Softwares, Teignmouth, UK).

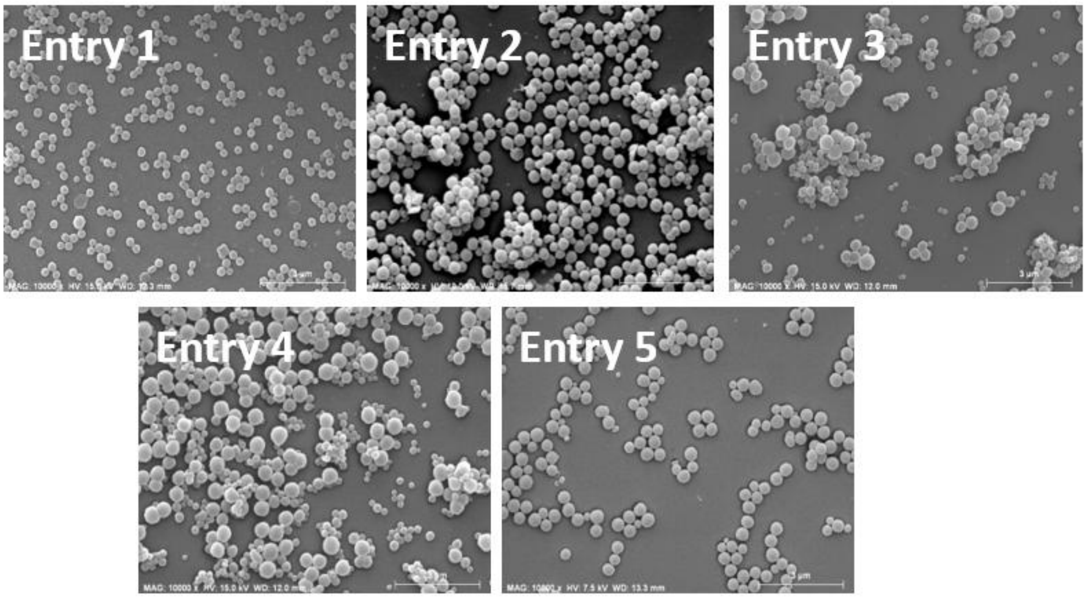

Figure S1: SEM images of the poly(HEMA) cross-linked nanoparticles obtained in supercritical carbon dioxide ($scCO_2$) from the reaction conditions described in **Table 1**. Entries 1 and 2 are particles obtained without AMP with 10 % and 5% of stabilizer respectively. Entries 3 and 4 are particles loaded with bradykinin with 1% and 2.5% cross-linker content respectively. Entry 5 corresponds to Pro3TL-loaded particles.

Table S1: Biocidal activity of free peptides against S. Aureus (UC stands for uncountable colonies = large number of remaining colonies due to no bactericidal effect)

Time	**Water**	**Free Bradykinin (µg/ml)**		**Free Pro^{3}TL (µg/ml)**	
		6.25	**12.5**	**6.25**	**12.5**
<10	UC	UC	UC	2 0 2	0
10	UC	UC	UC	0	0
20	UC	UC	UC	0	0
40	UC	UC	UC	0	0
1 hr	UC	UC	UC	0	0
2 hrs	UC	UC	UC	0	0
3 hrs	UC	UC	UC	0	0
4 hrs	UC	UC	UC	0	0
5 hrs	UC	UC	UC	0	0
6 hrs	UC	UC	UC	0	0
24 hrs	UC	UC	UC	0	0

Table S2: Number of remaining bacterial colonies corresponding to various incubation times. A solution of *Staphylococcus Aureus* incubated with AMP loaded poly(HEMA) particles having 2.5% of cross-linking agent EGDMA. (UC stands for uncountable colonies = large number of remaining colonies due to no bactericidal effect): Test 1

Time	**Water**	**Empty Particles 1% EGDMA mg/ml**		**Brad. loaded particles 1% EGDMA mg/ml**		**Brad. loaded particles 2.5 % EGDMA mg/ml**		**Pro^{3}TL loaded particles 2.5 % EGDMA mg/ml**			
		5,6	**11,2**	**5,6**	**11,2**	**5,6**	**11,2**	**5,6**	**11,2**		
<10 min	UC	UC	UC	UC	UC	UC	UC	UC	UC		
10 min	UC	UC	UC	UC	UC	UC	UC	UC	UC		
20 min	UC	UC	UC	UC	UC	UC	UC	UC	UC		
40 min	UC	UC	UC	UC	UC	UC	UC	UC	UC		
1 h	UC	UC	UC	UC	UC	UC	UC	UC	107	116	112
2 h	UC	UC	UC	UC	UC	UC	UC	UC	79	93	73
3 h	UC	UC	UC	UC	UC	UC	UC	UC	92	88	94
4 h	UC	UC	UC	UC	UC	UC	UC	UC	22	33	33
5 h	UC	UC	UC	UC	UC	UC	UC	UC	0	0	0
6 h	UC	UC	UC	UC	UC	UC	UC	UC	0	0	0
24 h	UC	UC	UC	UC	UC	UC	UC	UC	0	0	0

Table S3: Number of remaining bacterial colonies corresponding to various incubation times. A solution of *Staphylococcus Aureus* incubated with AMP loaded poly(HEMA) particles having 2.5% of cross-linking agent EGDMA. (UC stands for uncountable colonies = large number of remaining colonies due to no bactericidal effect): Test 2 after 10 days of storage.

Time	Water	Pro^3-TL loaded particles 11,2 mg/ml			Pro^3-TL loaded particles 22,5 mg/ml		
10'	UC	UC			UC		
20'	UC	UC			UC		
40'	UC	UC			UC		
1h	UC	UC			UC		
2h	UC	UC			82	73	51
3h	UC	UC			89	75	88
4h	UC	UC			39	39	31
5h	UC	UC			17	22	14
6h	UC	UC			0		
24h	UC	1	0	1	0		

Table S4: Number of remaining bacterial colonies corresponding to various incubation times. A solution of *Staphylococcus Aureus* incubated with AMP loaded poly(HEMA) particles having 2.5% of cross-linking agent EGDMA. (UC stands for uncountable colonies = large number of remaining colonies due to no bactericidal effect): third test performed after 2 months of particles storage.

Time	Water	Pro^3-TL loaded particles 11,2 mg/ml			Pro^3-TL loaded particles 22,5mg/ml								
		1st	2nd	3rd	1st			2nd			3rd		
20'	UC	UC	UC	UC	UC			UC			UC		
40'	UC	UC	UC	UC	UC			UC			UC		
1h	UC	UC	UC	UC	UC			UC			UC		
2h	UC	UC	UC	UC	UC			UC			UC		
3h	UC	UC	UC	UC	UC			UC			UC		
4h	UC	UC	UC	UC	UC			UC			UC		
5h	UC	UC	UC	UC	77	74	79	80	81	75	72	80	101
6h	UC	UC	UC	UC	64	65	70	66	58	61	61	81	78
7h	UC	UC	UC	UC	42	/	46	49	55	41	51	47	52
24h	UC	UC	UC	UC	0	0	0	0	0	0	0	0	0

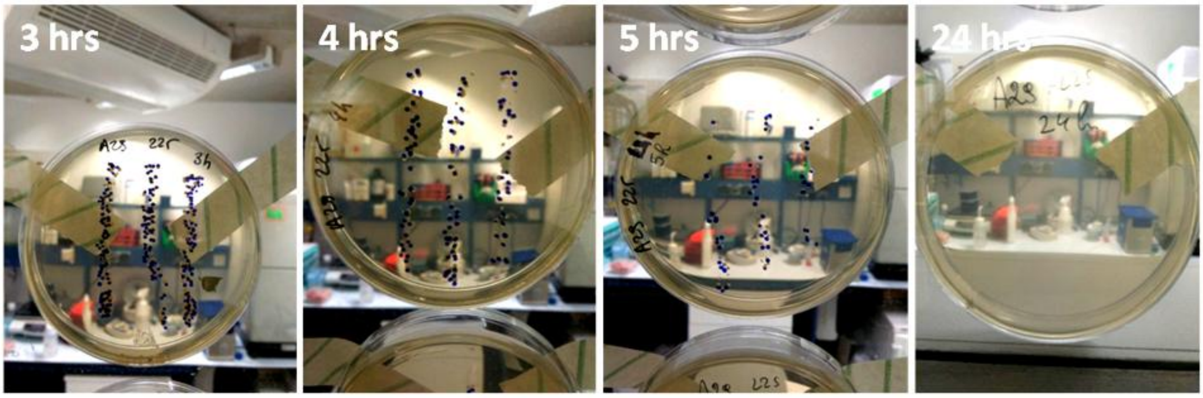

Figure S2: Illustration of the decay of bacterial colonies as a function of the release time (3 hours, 4 hours, 5 hours, 24 hours) for a concentration of particles of 22,5 mg/ml which equals to ~22,5 µg/ml of AMP.

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