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Short Communication

Antimicrobial peptide encapsulation and sustained release from polymer network particles prepared in supercritical carbon dioxide

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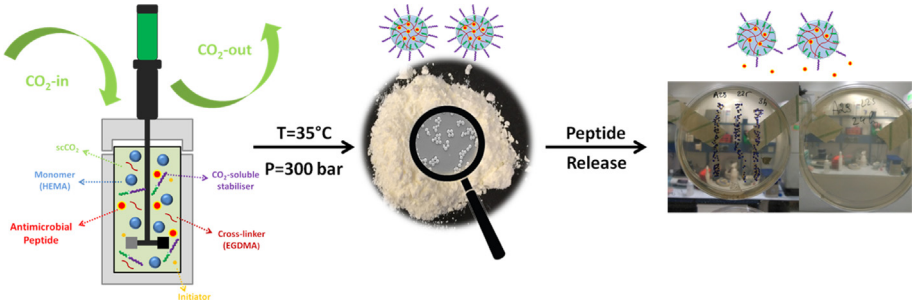
GRAPHICAL ABSTRACT



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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

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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^3TL 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-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-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 cross-linking 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 supercritical 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)-hν-poly(heptadecafluorodecyl acrylate) (PFDA) (PEO-$h\nu$-PFDA) block copolymer where hν 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-2,4-dimethylvaleronitrile) (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^3TL 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 $scCO_2$

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].