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A physico-chemical approach to the study of genipin crosslinking of biofabricated peptide hydrogels

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ABSTRACT

Peptide-based hydrogels have been widely used for both tissue engineering approaches and therapeutic drug delivery mainly thanks to their tissue-like water content and tunable physicochemical properties. In particular, the modulation of the hydrogel chemical structure influences hydrogel properties. The most common approach for tuning hydrogel properties in terms of cell adhesion or release efficiency of entrapped compounds is chemical crosslinking. In this study Fluorenylmethyloxycarbonyl (Fmoc)-tripeptide hydrogels were synthetized in the presence of Genipin as a crosslinker using a biocatalyzed reverse hydrolysis reaction occurring between FmocPhe and Phe_2 in the presence of *Pseudomonas fluorescens* lipase. Infrared Attenuated Total Reflection (IR-ATR), Liquid Chromatography coupled to Mass Spectrometry (LC-MS) and Scanning Electron Microscopy (SEM) investigations were used to investigate the interaction of Genipin with Fmoc-peptides as well as with their precursors. The whole instrumental platform allowed us to identify two crosslinked products that had never been reported previously and provide deeper insight into the use of natural crosslinkers for peptide hydrogel modification.

1. Introduction

In the last years, the synthesis of peptide-based hydrogels has attracted a growing interest, mostly thanks to their promising uses in biotechnological and pharmaceutical applications [1,2]. Peptide-based hydrogels are formed through the self-assembly of amphiphilic peptidic precursors, which can be triggered by an external stimulus. Such a stimulus may be physical, chemical or biological, *i.e.* an enzymatic reaction [3]. In particular, hydrogels obtained from low molecular weight peptides (LMWP) show high levels of biocompatibility and biodegradability while allowing to modulate the activity of many well-known drugs [4]. Bioactive substances may be entrapped inside the hydrogel and employed for *in vivo* and *in vitro* applications [5,6]. Peptide-based materials can fold into ordered secondary structures that are readily recognized by cell systems [7,8]. Many peptide-based hydrogels are injectable, an interesting feature for developing *in vivo* applications [9,10]. In this type of context, hydrogels should be stable under physiological conditions, and, above all, possess mechanical properties comparable to those of the target tissue [11]. In order to improve the hydrogel rheological properties, different crosslinking agents, such as glutaraldehyde, *N*-hydroxysuccinimide esters and carbodiimide, may be exploited to form chemical interconnections between the peptidic components of the material [12,13]. Among them, Genipin, a natural substance extracted from *Gardenia jasminoides*, is an interesting crosslinking agent [14] since it is highly biocompatible and reacts straightforwardly with primary amine groups producing blue-colored materials [15]. Additionally, Genipin is being successfully employed to obtain hydrogels, based on materials such as chitosan, cellulose and fibrin, with tuneable properties for tissue engineering applications [16–18].

Recently, we biosynthesized Fmoc-tripeptide-based hydrogels in the presence of Genipin and observed that, depending on its concentration, the rheological properties of the resulting material were significantly enhanced [19]. Such results were justified by the different morphological characteristics of the crosslinked hydrogels. Moreover, Genipin-crosslinked hydrogels were suitable for the entrapment of model drugs and for controlling their *in vitro* release [19,20].

On the basis of such findings, that are particularly promising for future applications, the aim of this work was to focus on how Genipin interacts with the different peptidic components of the hydrogel material and on which molecular species are formed.

LC–MS measurements confirmed for the first time the presence of Fmoc-tripeptides as the main reaction products of the biocatalyzed

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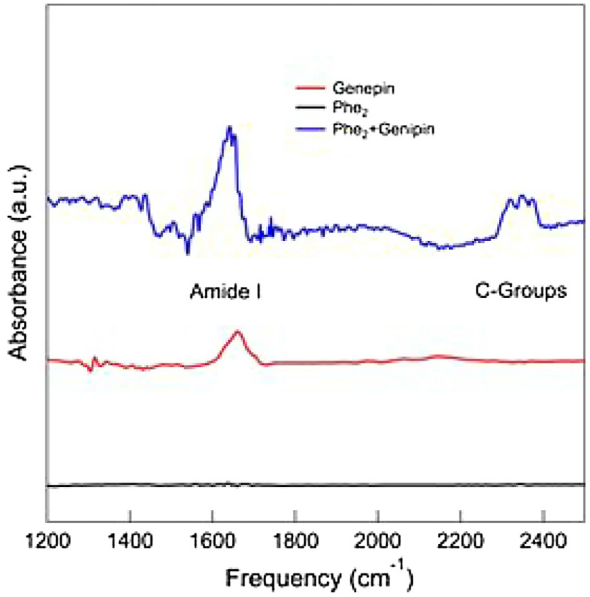

Fig. 1. Absorbance of Phe_2 (black line, bottom), Genipin (red line, middle) and Phe2 + Genipin 5 h after their mixing and interaction (blue line, top). All curves have been vertically shifted for the sake of clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

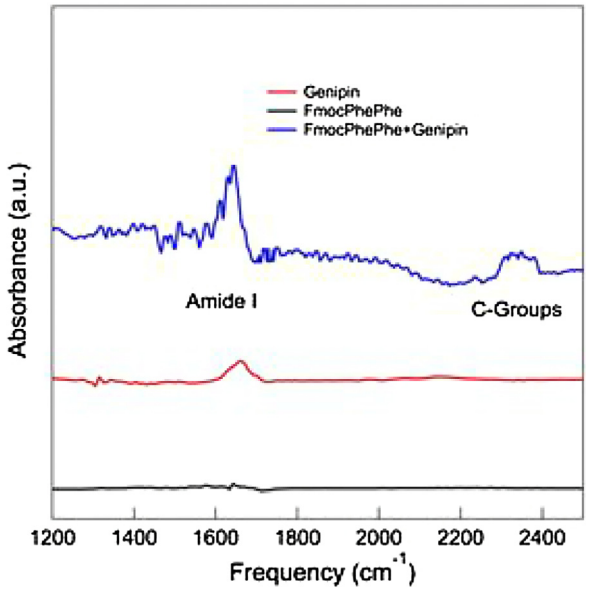

Fig. 2. Absorbance of Fmoc-Phe_2 (black line, bottom), Genipin (red line, middle) and Fmoc-Phe_2 + Genipin after 5 h from their mixing and interaction (blue line, top). All curves have been vertically shifted for the sake of clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reverse hydrolysis reaction occurring between Fmoc-Phe and Phe_2 in the presence of *Pseudomonas fluorescens* lipase. Moreover, Infrared Attenuated Total Reflection (IR-ATR) and Liquid Chromatography coupled to Mass Spectrometry (LC–MS) investigations were applied to investigate the chemical interaction of Genipin with Fmoc-peptides and their precursors. The whole instrumental platform allowed us to identify two crosslinked products that were never reported previously.

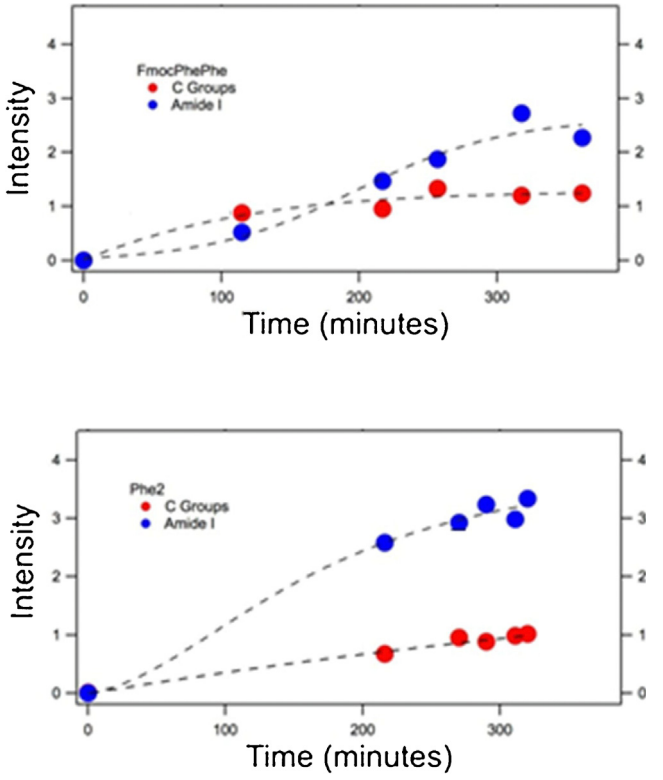

Fig. 3. Intensity of C-Groups (red) and Amide I (blue) IR absorption bands vs time of FmocPhe_2 (top) and Phe_2 (bottom) incubated with Genipin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Materials

Fluorenylmethyloxycarbonyl-phenylalanine (Fmoc-Phe, > 99%), Fluorenylmethyloxycarbonyl-diphenylalanine (Fmoc-Phe_2, > 99%) and diphenylalanine (Phe_2, > 99%) were purchased from Bachem GmbH (Weil am Rhein, Germany). Lipase from *Pseudomonas fluorescens* (PFL, ≥20.000 U/mg) and all other chemicals and solvents were obtained from Sigma Aldrich (St. Louis, MO, USA) and used without further purification.

2.2. Biosynthesis of Fmoc-tripeptide hydrogels

For hydrogel preparation, equimolar quantities of Fmoc-Phe and Phe_2 were suspended in a mixture containing 1 mL of H_2O and 420 µL of 0.5 M NaOH. Successively, pH was adjusted to 7 by the addition of 0.1 M HCl. Then, a fixed amount (100 µL) of lipase solution (50 mg/mL) was added and the mixture was incubated at 30 °C for 30 min.

For the preparation of crosslinked hydrogels, 100 µL of a Genipin solution (5 mM) were added to the suspension of the precursors adjusted to pH 7 before the addition of the PFL solution. The mixture was incubated at 30 °C for 30 min.

2.3. IR measurements

Mid-Infrared Spectroscopy measurements were performed through an Attenuated Total Reflectance (ATR) infrared system based on a ZnTe crystal and mounted in a Jasco Michelson interferometer.

The concentration of all aminoacid and peptide samples was the same and equal to 6.67 µmol/mL.

The ratio between sample and Genipin was 100:1. The