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ORIGINAL ARTICLE

Evaluation of peptides release using a natural rubber latex biomembrane as a carrier

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Abstract
The biomembrane natural (NRL—Natural Rubber Latex), manipulated from the latex obtained from the rubber tree *Hevea brasiliensis*, has shown great potential for application in biomedicine and biomaterials. Reflecting the biocompatibility and low bounce rate of this material, NRL has been used as a physical barrier to infectious agents and for the controlled release of drugs and extracts. The aim of the present study was to evaluate the incorporation and release of peptides using a latex biomembrane carrier. After incorporation, the release of material from the membrane was observed using spectrophotometry. Analyses using HPLC and mass spectroscopy did not confirm the release of the antimicrobial peptide [W^6]Hylin a1 after 24 h. In addition, analysis of the release solution showed new compounds, indicating the degradation of the peptide by enzymes contained in the latex. Additionally, the release of a peptide with a shorter sequence (Ac-WAAAA) was evaluated, and degradation was not observed. These results showed that the use of NRL as solid matrices as delivery systems of peptide are sequence dependent and could to be evaluated for each sequence.

Keywords Drug delivery · Peptides · Natural rubber latex · Biomaterial

Introduction

Natural latex

Natural rubber latex (NRL) is extracted from the *Hevea brasiliensis*, a native tree from Brazil. NRL is a colloidal system containing 50% water, 4–5% non-rubber (such as proteins and lipids), and 30–45% rubber particles (*cis*-1,4-polyisoprene) (Fig. 1) (Neves-Junior et al. 2006; Nawamawat et al. 2011). The molecular structure of the NRL molecules is sustained by *trans*-isoprene units connected to long-chain *cis*-1,4-isoprene molecules. Latex shows positive qualities, including barrier protection, tensile strength, elasticity, comfort and fit. In addition, NRL obtained from rubber cream is used as a renewable, low-cost and biocompatible polymer.

Recently, many studies have employed the extracted latex from *H. brasiliensis* in biomedical applications. The latex used in these applications is NRLb (Natural Rubber Latex Biomedical), which avoids the use of chemicals, such as sulfur and carbamates (De Pinho 2004). NRLb was developed because the previous method had many occurrences of allergic reactions and cytotoxicity. NRLb is biocompatible and stimulates angiogenesis, cellular adhesion and the formation of extracellular matrix, promoting tissue replacement and repair (Azevedo Borges et al. 2014; Frade et al. 2004). NRLb has also been used as a passive biomembrane, which acts in bone repair. In this experiment, the latex biomembrane was applied onto bone fractures, preventing the migration of epithelial and connective tissue and facilitating the migration of regenerative cells (Ereno et al. 2010).

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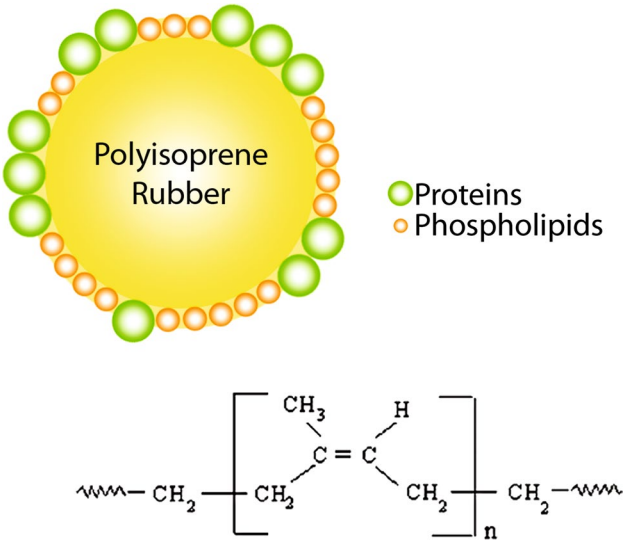

Fig. 1 *Cis*-isoprene monomer and proposed model comprising a mixed layer of proteins and phospholipids surrounding the latex particle

In another study, De Pinho (2004) investigated the use of natural latex biomembrane with 0.1% polylysine in conjunctival reconstruction in adult New Zealand rabbits. Histological analysis revealed satisfactory recovery in 60% of eyes with biomembrane, while in the control group (without biomembrane) only 20% satisfactory recovery was observed. The average number of vessels per optical field in surgical wound eyes with biomembranes was twice the number of vessels in eyes with bare sclera (control group). These data suggested that the latex biomembrane favors conjunctival scarring and neoangiogenesis. Balabanian et al. (2006) investigated the biocompatibility of natural latex implanted into the bony alveolar cavity after dental extraction in rats. These authors observed that this biomaterial is biologically compatible and accelerated bone formation. Moreover, de Oliveira et al. (2003) used latex biomembrane with polylysine as a temporary implant for the closure of the tympanic biomembrane. The biomembrane was placed on the external face of the edges of the tympanic biomembrane and temporal fascia on the inner face thereof. These authors concluded that NRLb could be used as a temporary implant in myringoplasty, improving the vascularization of the remaining tympanic biomembrane. Sader et al. (2000) observed that the NRLb stimulates neovascularization and organized tissue growth in different organs and tissues. NRLb has been classified as an innocuous material that is not rejected by the body. In other biological/biomedical applications, NRL membranes have also been employed for the treatment of pressure ulcers (Frade et al. 2006) and diabetic ulcers (Frade et al. 2004). These authors observed that NRL membranes are highly effective dressings, primarily reflecting the debriding potential and angiogenicity of this material.

In addition, recent studies have demonstrated the use of natural latex extracted from *H. brasiliensis* as a matrix for the release of molecules, nanoparticles, vegetable extracts, antibiotic, anti-inflammatory and nicotine (Herculano et al. 2006; Guidelli et al. 2013; Azevedo Borges et al. 2014; Dias Murbach et al. 2014; Barros et al. 2015; Suksaeree et al. 2012).

Reflecting the characteristics described above, rubber tree latex is an excellent candidate for the solid matrix delivery of molecules, such as peptides.

Hylin a1

Hylin a1 (IFGAILPLALGALKNLIK-NH_2) is an antimicrobial peptide isolated from the skin secretions of the frog *Hypsiboas albopunctatus*, commonly found in the Cerrado region of Brazil (Castro et al. 2009; Crusca et al. 2011). Hylin a1 showed activity against several types of fungi as well as Gram-positive and Gram-negative bacteria (Castro et al. 2009; Crusca et al. 2011; (Da Silva et al. 2013). Hylin a1 increases permeability in the cell membrane of these microorganisms, resulting in cell lysis and death (SF Alves et al. 2015).

Release of peptides

The controlled peptide release has been developed on different material. The release of osteogenic growth peptide (OGP) was evaluated using bacterial cellulose biomembranes (Saska et al. 2012) and nanostructured mesoporous silica (Mendes et al. 2013). In the other study, chitosan nanoparticles was used as carriers for the release of anti-tumor peptides (Medeiros et al. 2014). Notably, although several studies have used latex for drug release, only two peptides were evaluated using NRL, the oxytocin (de Barros et al. 2016) and the desmopressin (de Barros et al. 2017).

In the present study, we evaluated the release of the peptides using latex biomembrane as a carrier. The release of this peptide from the latex biomembrane could promote the development of antimicrobial dressings that stimulate tissue repair and avoid infection through the substances contained in the latex.

Materials and methods

The NRLb (mixture of two clones RRIM 600 and PB 235) used in the present study was a commercial high-ammonia latex obtained from the BDF Rubber Latex Co. Ltd. (a producer and distributor of concentrated rubber latex, Guarantã, Brazil), comprising approximately 60% dry rubber content

(DRC), 4–5% non-rubber constituents, such as proteins, lipids, carbohydrates, and sugars, and 35% water (Herculano et al. 2009; Dias 2014). After extraction, ammonia was used to maintain the latex in liquid form. The deproteinization of the NRL was achieved through centrifugation at 8000×*g*. After centrifugation, the cream fraction was redispersed to obtain the desired 60% dry rubber content latex and subsequently washed twice through centrifugation to reduce the cytotoxic protein content in the solution. The NRL has 0.22% of proteins in its composition, 27% of these proteins are removed during the deproteinization process for obtaining the NRLb with 0.16% of proteins (de Barros et al. 2016).

Peptide synthesis, purification and preparation

The [W^6]Hylin a1 (IFGAIWPLALGALKNLIK-NH_2) peptide was synthesized and purified according to a previously published methodology (Crusca et al. 2011). Additionally, a five-residue peptide (Ac-WAAAA-NH_2) was synthesized to evaluate whether the degradation of the peptide in the latex membrane was sequence dependent. In this sequence, four Ala residues were incorporated, as Ala is the simplest amino acid and has optical isomerism; to avoid electrostatic interactions, the N-terminus was acetylated and the C-terminus was amidated. The peptides were designed and synthesized to contain a tryptophan residue for evaluation using spectroscopy at 280 nm.

The peptides were purified, and homogeneity was determined using analytical HPLC and electrospray mass spectrometry. Prior to use, the peptide was fully solubilized at a concentration of 1000 µg/mL in sterile deionized water containing 0.1% acetonitrile and stored in a freezer at − 80 °C.

Biomembrane preparation

The preparation of the biomembrane involved the mixture of 0.5 mL liquid latex and 0.5 mL of a solution containing 5 mg of peptides or BSA, homogenization and deposition on a circular plate 22.30 mm in diameter, followed by drying for 24 h at 37 °C.

SEM

The surface morphology of the NRLb membranes was evaluated by Scanning Electron Microscopy (SEM) (EVO 50-Zeiss®, Germany) at an accelerating voltage of 20 kV and a take-off angle of 35° (using gold as conductor material). Three aleatory areas were analyzed.

Release studies

The release of the peptide was initially evaluated according to Dias Murbach et al. (2014). The biomembrane was immersed in 10 mL of deionized water under stirring at a controlled temperature of 37 °C. Measurements were made using a spectrophotometer at 280 nm. The first measurement was obtained at $t = 0$ min, and subsequent measurements were obtained until 24 h. The concentration of released peptide was determined using the Lambert–Beer equation. The release was also analyzed by HPLC using a 15.0 × 0.46 cm Shimadzu Kromasil C18 column and UPLC using a 2 × 30 cm C8 column. In addition, UPLC/mass spectrometry (MS) using an Ion Trap Amazon SL—Bruker® was performed to confirm the identity and stability of the released material.

The BSA release assay was made by UPLC were performed using a linear gradient of 5–95% (v/v) of solvent B (0.036% TFA in acetonitrile) for 30 min (solvent A: 0.045% TFA in H_2O) at a flow rate of 0.2 mL/min through a C8 column with UV detection at 220 nm.

Peptide degradation assay

A peptide degradation assay was performed to verify the presence of the enzymes contained in the latex. In the first experiment, the biomembranes were incubated for 24 h in 5 mL of deionized water at 37 °C. Subsequently, the biomembranes were removed from the water, and a solution contained the substances released from the latex was obtained (serum). Next, 1 mg of [W^6]Hylin a1 was added to the serum. HPLC and MS/MS spectra (Ion Trap Amazon SL—Bruker®) were obtained to evaluated the stability of the peptide. In the second experiment, three biomembranes content 5 mL of serum were incubated in 300 mL of water. The membrane was removed and the serum was liophylized and 50 mg of solid was obtained. After the liophylization, 5 mg of solid and 1 mg of [W6]Hylin a1 was dissolve with 1 mL of water and analysed by mass spectrometry. This last solution contain three times more latex material than the first experiment.

Results and discussion

Solid phase peptide synthesis

In the present study, a peptide analogous to the [W^6]Hylin a1 (IFGAIWPLALGALKNLIK-NH_2) was synthesized. The peptide [W^6]Hylin a1 has a tryptophan, instead of a leucine, residue at position 6. This substitution enables the use of spectrophotometry techniques to examine this molecule without altering its antimicrobial activity (Crusca et al. 2011). The peptide was obtained with 97% purity, and its identity was confirmed by mass spectrometry.

SEM

The SEM micrographs of the NRL biomembrane (Fig. 2a), the peptide powder (Fig. 2b) and an NRL containing the peptide (Fig. 2c) showed that part of the peptide was retained on the surface of the NRL, as evidenced by grains present in the SEM micrograph.

Release studies

[W^6]Hylin a1 release

Figure 3 shows the release from latex membranes with or without the [W^6]Hylin a1 peptide under the same experimental conditions. The increase in absorbance at 280 nm from the latex membrane without peptide indicated the release of various proteins containing tryptophan or tyrosine. This release solution contained proteases among other proteins (Azevedo Borges et al. 2014). The increase in the absorbance at 280 nm from the latex containing the peptide compared with the pure biomembrane indicated the potential release of the peptide.

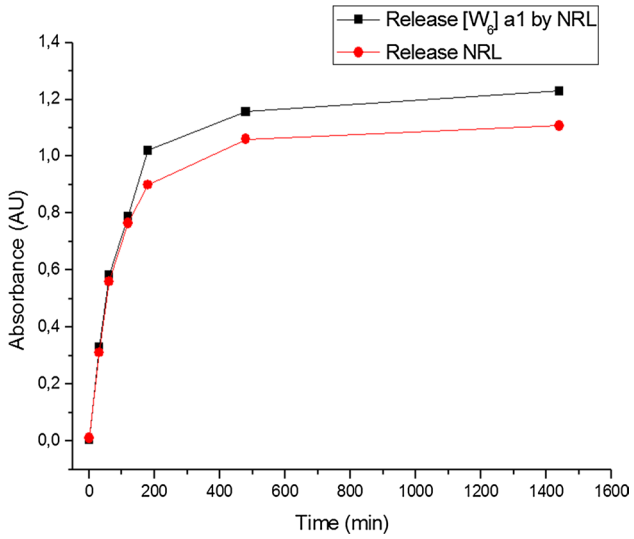

Fig. 3 Release curves for the pure NRL and [W^6]Hylin a1/NRL biomembranes obtained in the UV region at 280 nm. The following bi-exponential function was applied: $y(t) = y_0 + A_1 e^{-t/\tau 1} + A_2 e^{-t/\tau 2}$, where $y(t)$ is the amount of [W^6]Hylin a1 in the NRLb at a given time

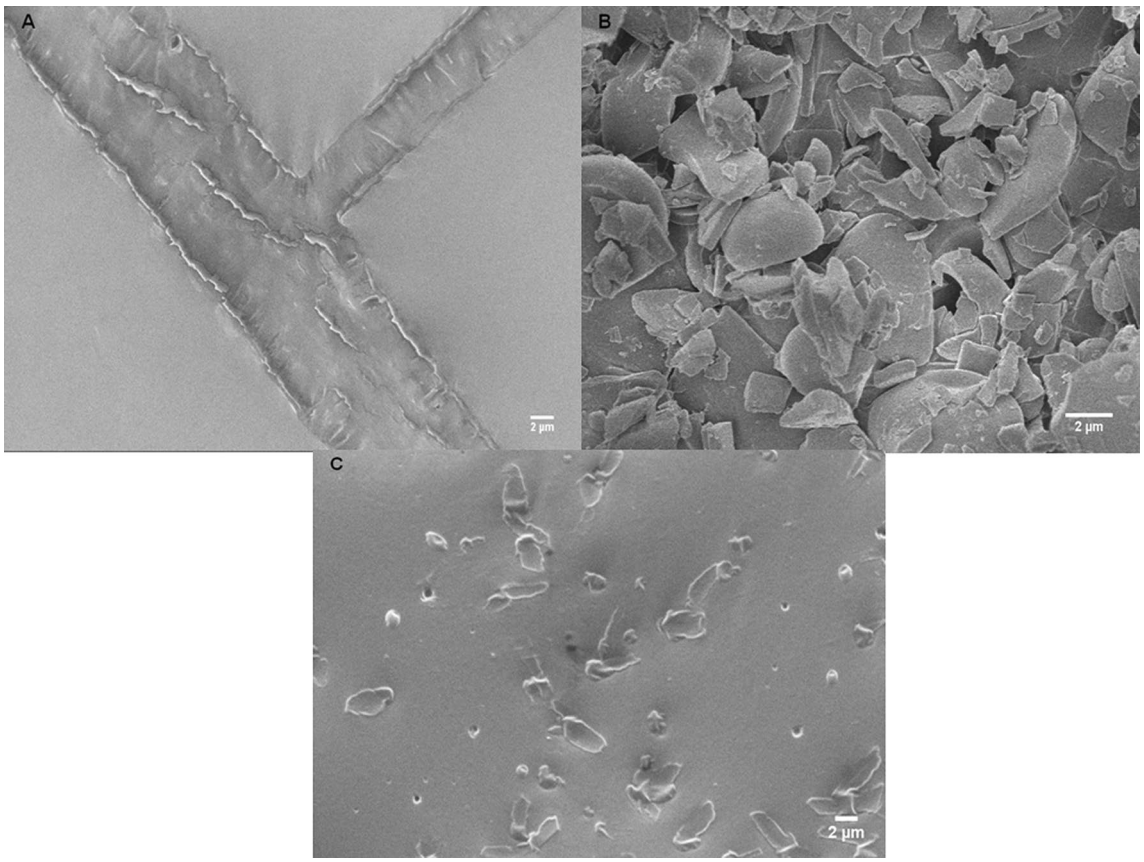

Fig. 2 Representative SEM micrograph of: **a** the NRL biomembrane; **b** [W^6]Hylin a1 powder and **c** an NRL containing the peptide [W^6]Hylin a1

To confirm the peptide release from the latex, the solution was evaluated using HPLC. Comparing the chromatographic peptide release profiles of the latex solution without (Fig. 4a) and containing [W6]Hylin a1 (Fig. 4b) to the pure peptide profile (Fig. 4c), we observed the absence of a [W^6]Hylin a1 (16.3 min) peak in the release assay for the latex solution containing the peptide, indicating that the peptide was not present. In addition, the presence of peaks that were absent in the pure latex release samples were observed (10.5; 11 and 18 min).

In addition, the mass spectrometry analysis of the solution from latex containing the peptide did not show the presence of a peptide with the theoretical molecular weight of [W^6]Hylin a1, which is 1937.4 mol/g (data not shown). This finding suggests two possibilities: the peptide was retained in the polymeric network of the biomembrane, or [W^6]Hylin a1 was degraded.

The hypothesis of peptide retention in the polymeric mesh was initially accepted. The specific interaction between the peptide and components of the latex is possible. Secondary structure measurements of the [W^6]Hylin a1 displayed a typical spectrum of a disordered structure in aqueous solution, but in the presence of TFE or micelles it presented a higher amphyphatic helical content (Crusca et al. 2011). This amphipathic structure could interact with the lipids of the latex and avoid peptide release. There are two potential models for the structure of the NRLb rubber latex particle surface. The first, traditional model shows a particle surrounded by a double-layer of proteins and phospholipids, and another recently proposed model comprises a mixed layer of proteins and phospholipids surrounding the latex particle (Nawamawat et al. 2011). In both models, phospholipids are present. The interaction between antimicrobial peptides, such as Hylin a1, and phospholipid vesicles has been previously described (Brogden 2005; Ferreira Cespedes et al. 2012; Lorenzón et al. 2012). In this interaction, the peptides are initially bound parallel to a lipid bilayer, oriented perpendicular to the membrane (Brogden 2005; Melo et al. 2009). The interaction with the phospholipid chains could retain the peptide bound in the bilayer.

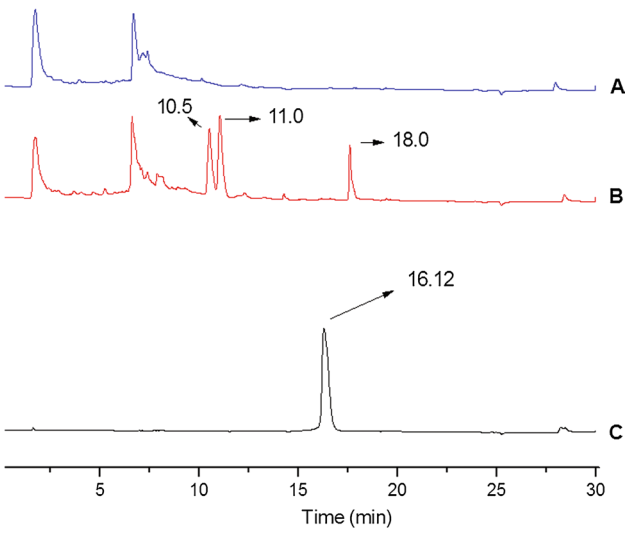

Fig. 4 HPLC profile of (A) release solution from the pure latex and (B) release solution from the latex containing [W^6]Hylin a1 after 24 h and (C) solution of standard peptide. The analysis was conducted using a reverse phase Phenomenex Jupiter C18 column (150 × 4.6 mm), packed with spherical 5 mm particles with 300 Å pore size, with a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Solvent B: 0.036% TFA in acetonitrile; solvent A: 0.045% TFA in H_2O

Although the retention of the peptide in the latex is possible, the enzymatic degradation of the peptide is also a possibility. The release of the [W^6]Hylin a1 peptide was not observed in the analysis of the HPLC profiles (Fig. 4b). However, other compounds that were not present in the pure latex release (Fig. 4a) were observed (Fig. 4b). These compounds could have been produced by the proteolytic degradation of the peptide. Previous studies have shown that latex contains many enzymes, including cysteine proteases (Peng et al. 2008) and serine proteases (Lynn and Clevette-Radford 1986), which could promote peptide degradation. However, the release of BSA (Bovine serum albumin), a larger protein than the [W^6]Hylin a1 peptide, has been observed (Herculano et al. 2009). The researchers only used UV analysis to confirm the release of BSA, and this nonspecific method is not sufficient to confirm the release of the compounds. To confirm these data, the release of BSA was repeated and aliquots of the solution were evaluated using UPLC. Figure 5a shows the release of 88% of the BSA contained in the latex after 12 h. The presence of BSA in the release solution was confirmed by the increase in the peak eluted at 6.3 min (Fig. 5b), the same retention time for BSA standards. These data indicated that: (1) the retention of the peptide in the latex is unlikely, as BSA is a much larger molecule than the peptide; and (2) the BSA is not degraded by the proteolytic enzymes in the latex.

Notably, according to Lynn and Clevette-Radford (1986), the serine proteases contained in the latex showed reduced ability to degrade BSA protein and hemoglobin. This finding could explain the presence of BSA in the latex release solution.

Another study showed that the NRLb was effective as a model for the release of oxytocin (de Barros et al. 2016). In this study, the degradation of oxytocin was not observed, even after 48 h. These data could be associated with the disulfide bridges connected to the two cysteine residues (Cys1 and Cys6) in the primary sequence, forming a ring (more rigid structure), and/or the specificity of the latex enzymes. Natural rubber latex membranes loaded with desmopressin peptide showed similar results and 60% of

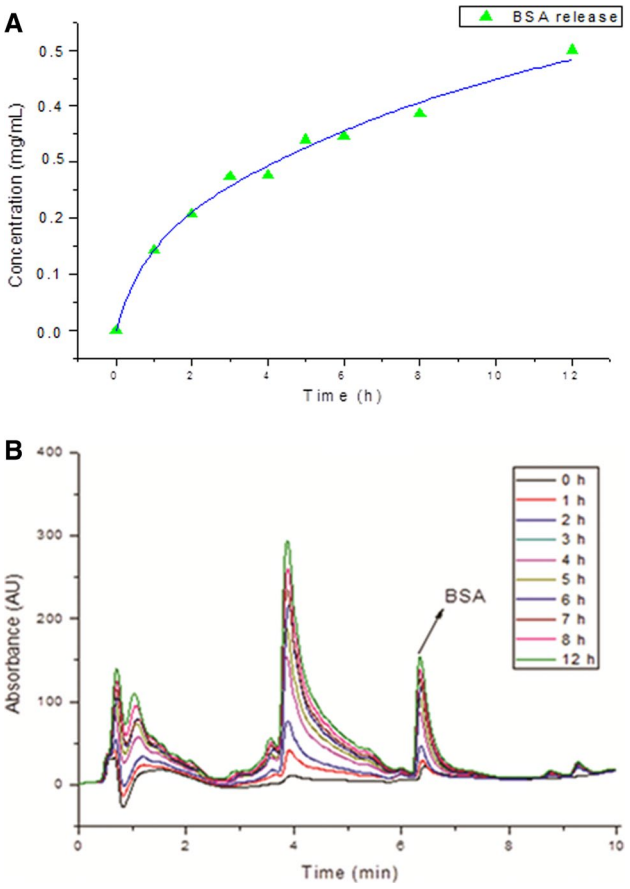

Fig. 5 **a** Curve of the release of BSA from BSA/NRLb. A bi-exponential function was applied: $y(t) = y_0 + A_1e^{-t/\tau 1} + A_2e^{-t/\tau 2}$, where $y(t)$ is the amount of BSA in the NRLb at a given time. **b** UPLC profiles of the release solution of the latex membrane containing BSA. The UPLC analyses were performed using a linear gradient of 5–95% (v/v) of solvent B (0.036% TFA in acetonitrile) for 30 min (solvent A: 0.045% TFA in H_2O) at a flow rate of 0.2 mL/min through a C8 column with UV detection at 220 nm

the peptide incorporated in natural latex was released up to 96 h (de Barros et al. 2017). In this manner, the data showed a dependence of the degradation with the peptide sequence.

[W^6]Hylin a1 peptide degradation assay

To determine whether degradation is responsible for the non-release of the peptide, the NRLb membrane was polymerized using 0.5 mL of latex suspension. This biomaterial was immersed in 5 mL of deionized water, and the latex serum was extracted after 24 h. A total of 1 mg of peptide was incubated in the latex serum, thereby avoiding interactions between the peptide and the polymeric chain. The presence or degradation of the peptide was then evaluated using UPLC. The peak at 4.2 min retention time, characteristic of [W^6]Hylin a1, decreased over time. In addition, a new peak was observed at 2.6 min,

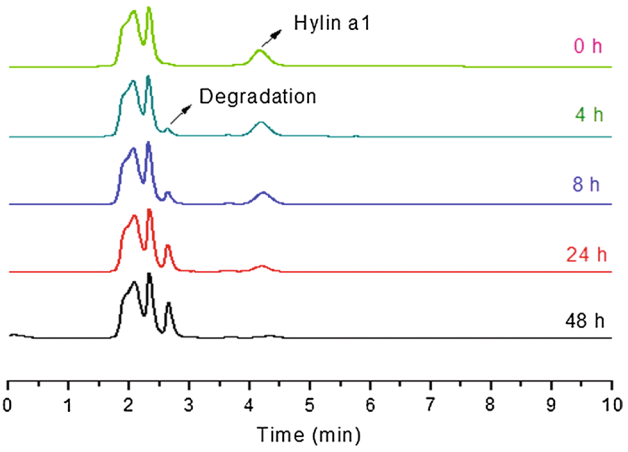

Fig. 6 UPLC profiles of the [W^6]Hylin a1 degradation assay at 0, 4, 8, 24 or 48 h

which increased over time (Fig. 6). This new peak was collected and subjected to mass spectrometry. The mass spectrum of this material showed two peaks (810.5 g/mol to $Z = 2$ and 540.6 g/mol to $Z = 3$), indicating a compound with a molecular weight of 1619 mmol/L and the sequence AIWPLALGALKNLIK-NH_2. These data indicate the enzymatic degradation of [W^6]Hylin a1. In the control experiment using only water, the peptide was not degraded (Fig. 7). The peptide in aqueous solution showed no significant degradation, obeying the linear function: $= a + bx$, where y is the amount of peptide in the biomembrane at a given time (x), and constants a and b are the angular coefficient and linear coefficient, respectively. The values for constants a and b were equal to 0.199 and -1.399×10^{-4},

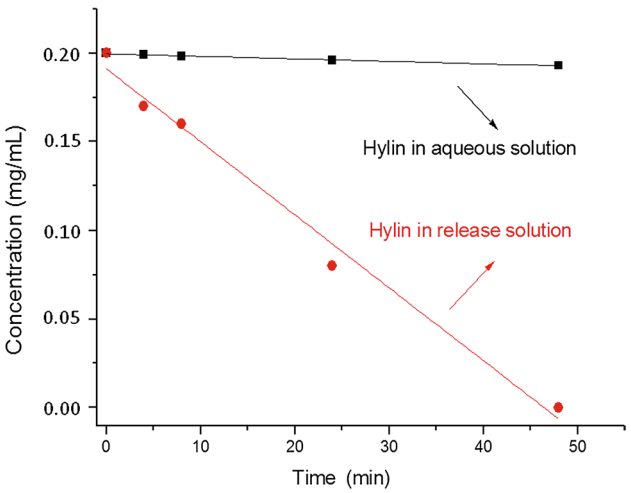

Fig. 7 Analysis of [W^6]Hylin a1 degradation in aqueous and latex serum solutions. The black and red lines indicate the peptides in the aqueous and natural latex release solutions, respectively. The mass was obtained by UPLC (Fig. 6) at 220 nm

respectively. Thus, the peptide present in the latex release solution showed 100% degradation within 48 h (Fig. 7). In this experiment, constants *a* and *b* were equal to 0.191 and − 0.004, respectively. These results suggest that degradation is initiated from the production of the membrane containing the peptides.

To confirm the observed proteolysis, another sample was incubated in a higher latex serum concentration (3 times more concentrated) for 5 min and subsequently evaluated using LC–MS. More generally, our results revealed the existence of additional fragment peptides beyond that found previously in Fig. 7. The mass spectrometry analysis of the solution revealed the presence of peptides: LALGALKNLIK-NH_2 (1152.4 g/mol; $Z = 1$); KNLIK-NH_2 (614.4 g/mol; $Z = 1$); NLIK-NH_2 (485.6 g/mol; $Z = 1$); and LIK-NH_2 (372.3 g/mol; $Z = 1$). These peptide are fragments of the hydrolysis of peptide obtained in Fig. 7 (AIWPLALGALKNLIK-NH_2). Additionally other fragments were obtained containing the N-terminal region of peptide: IFGAIWPLA (986.6 g/mol and 494.3 g/mol for $Z = 1$ and 2, respectively); IFGAIWPLALGALKN (792.5 g/mol; $Z = 2$); and LKNLIK (728.5 g/mol; $Z = 1$). These results indicated the presence of more than one kind of enzymes. These data confirm the presence of proteases in the latex serum, which are responsible for the degradation of the [W^6]Hylin a1 peptide.

To confirm the dependence of degradation on the peptide sequence, the release and degradation of Ac-WAAAA-NH_2 was evaluated. The amino acid Trp in peptide Ac-WAAAA was used to allow the UV detection at 280 nm. The Ala was choice because in the smaller amino acid with chiral center. Latex containing the peptide was prepared by adding 5 mg of Ac-WAAAA-NH_2 to 0.5 mL of the latex

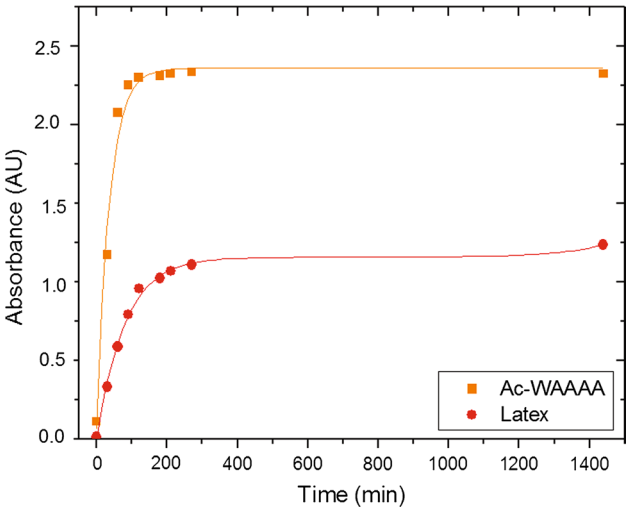

Fig. 8 Release curve for the pure NRL and Ac-WAAAA/NRL biomembranes obtained by absorbance in the UV region at 280 nm. A bi-exponential function was applied: $y(t) = y_0 + A_1 e^{-t/\tau 1} + A_2 e^{-t/\tau 2}$, where $y(t)$ is the amount of Ac-WAAAA in the NRLb at a given time

suspension, followed by drying at 37 °C. Figure 8 shows the results of the release studies from latex with and without the Ac-WAAAA-NH_2 peptide, under the same experimental conditions. The increase in the maximum absorbance at 280 nm for latex containing the peptide compared with the pure biomembrane indicated the release of the peptide. This shift was larger than that observed for [W^6] Hylin a1.

To confirm the release of Ac-WAAAA-NH_2, the chromatographic profiles of the pure latex release solution (Fig. 9a), latex containing Ac-WAAAA-NH_2 (Fig. 9b) and a pure sample of the peptide (Fig. 9c) were obtained.

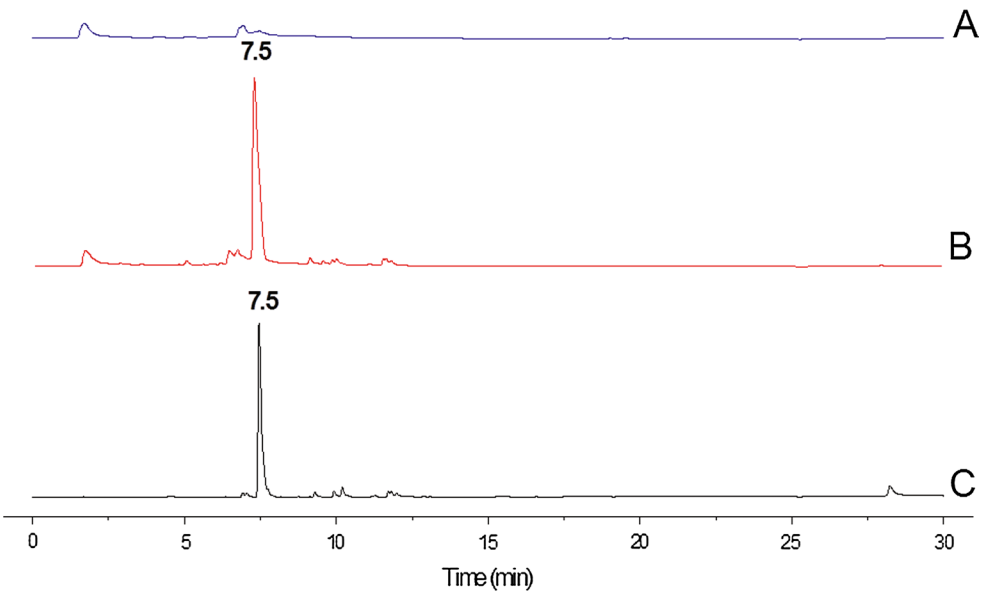

Fig. 9 HPLC profile of (A) release solution from pure latex, (B) release solution from latex containing the Ac-WAAAA peptide and (C) a standard Ac-WAAAA solution. The HPLC analyses were performed using a linear gradient of 5–95% (v/v) of solvent B (0.036% TFA in acetonitrile) for 30 min (solvent A: 0.045% TFA in H2O) at a flow rate of 1.0 mL/min through a C18 column with UV detection at 220 nm

These chromatograms revealed the release of peptide with a retention time of 7.5 min. In addition, after incubation, other materials were not detected in the chromatographic profile. Thus, the degradation of this pentapeptide was not observed.

Taken together, these data showed that the activity of the enzymes in the latex membrane is dependent on the amino acids in the peptide.

Conclusion

The release of the [W^6]Hylin a1 antimicrobial peptide from latex membranes is not likely. This result reflects the enzymatic degradation of the peptide by enzymes in the latex membrane. Some of the proteases identified in the latex, such as cysteine and serine proteases, have been previously described, and the presence of these enzymes could explain the observed peptide degradation. The results obtained showed that the release of peptide and protein using latex membrane as solid matrices is sequence dependent and could to be evaluated for each sequence.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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