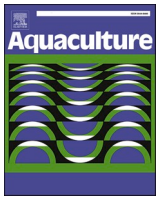
Aquaculture 613 (2026) 743334

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Characterization of PLGA nanoparticles for stabilization and controlled release of antimicrobial peptides into marine bivalves

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ARTICLE INFO

Keywords:
Antimicrobial peptides
Bivalves
Clams
Caerin
PLGA
Encapsulation

ABSTRACT

Nanoparticles (NPs) have revolutionized the field of medicine and veterinary for both detection and treatment of infection diseases, however, their utilization to supply drugs or bioactive metabolites to marine invertebrates has been insufficiently investigated. Antimicrobial peptides (AMPs) have gained increasing attention as potential alternatives to overcome the shortcomings associated with conventional therapeutic strategies. Using an emulsion/evaporation approach, spherical poly(lactic-*co*-glycolic acid) (PLGA) NPs charged with D-Caerin (D-CAE) peptide have been prepared. The obtained NPs showed a bimodal size distribution, with mean diameters of 130 ± 4 and 421 ± 19 nm. Among the tested formulation, NPs with a peptide/polymer ratio of 0.02 exhibited the best physico-chemical properties, achieving encapsulation efficiencies of 70 %. Release studies demonstrated that 73.5 % of the peptide was liberated from loaded the NPs within 40 h. Furthermore, the D-CAE peptide retained its antimicrobial activity following encapsulation in PLGA NPs and exhibited bactericidal effects against two prevalent pathogens in aquaculture species. Finally, it has been shown that peptide-loaded NPs are effectively ingested by clam post-larvae, suggesting the potential of peptide-PLGA NPs as biocompatible nanocarriers for delivery of AMPs into bivalves and other invertebrates.

1. Introduction

There is an urgent need for alternatives to traditional antibiotics in both human and veterinary medicine, and antimicrobial peptides (AMP) have emerged as promising candidates to address the limitations of conventional treatments (Robles Ramirez et al., 2024). The increasing development of antibiotic-resistant bacteria is one of the main challenges threatening livestock farming and aquaculture. Antimicrobial peptides (AMPs) are small host defense, generally amphipathic and cationic, peptides present in all kingdoms of life, which exhibit antimicrobial activity against a wide range of bacteria, viruses, fungi and parasites (Lazzaro et al., 2020). Most AMPs are derived from gene-encoded inactive precursor pro-peptides, which undergo several proteolytic and post-transcriptional modifications steps before becoming functionally active (Gan et al., 2021). The growing interest in AMPs as therapeutic agents has led to the development of a large variety of synthetic peptides, either mimicking natural ones or specifically designed through advanced computational pipelines (Huang et al., 2023).

The wide distribution of natural AMPs suggests that they play an essential role in microbial ecology and innate immunity of most organisms (Nuti et al., 2017; Shabir et al., 2018). An increasing number of new peptides isolated from different natural sources has been characterized for their bioactive properties (Ji et al., 2024). Peptides isolated from the skin glands of Australian tree frog have been well studied and some of them, including several versions of Caerin peptides, were identified as potent antimicrobial agents (Steinborner et al., 1998). Although most research on AMPs has focused on human health, their

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https://doi.org/10.1016/j.aquaculture.2025.743334
Received 18 July 2025; Received in revised form 1 October 2025; Accepted 20 October 2025
Available online 22 October 2025

application in veterinary medicine, particularly in aquaculture, has also been explored, raising considerable expectations for combating infections without the drawbacks associated with antibiotic overuse (Valdez-Miramontes et al., 2021; Xia et al., 2024). Several studies have investigated the use of AMPs as antimicrobial agents to improve health and disease resistance of aquaculture species, including invertebrates (Rodrigues et al., 2025; Gyan et al., 2020) and clams (Zhang et al., 2025). AMPs offer several advantages over conventional antibiotics, including a wide spectrum of action and the apparent difficulty for target pathogenic microorganisms to develop resistance. However, their practical application has been limited by their low stability and the challenges for their efficient supply (Yadav and Chauhan, 2024).

We have previously shown that peptides isolated from frog's skin, such as Caerin 1.1, as well as from sea bass and sole, exhibit both bactericidal and antiviral activity against fish pathogens (León et al., 2020). In addition, Xiao and colleagues reviewed diverse applications of Caerin peptides, highlighting their efficacy against antibiotic-resistant bacterial infections and various tumor cell lines and emphasized the need of strategies to enhance the stability and half-life of Caerin (Xiao et al., 2022). In this sense, we have previously demonstrated that the dextrorotatory enantiomeric version of the natural peptide Caerin 1.1, designed D-Caerin (D-CAE), not only retains potent antimicrobial activity, but also shows higher stability against proteolytic degradation, extreme pH or saline environments than the L-Caerin peptide (López-Sanmartín et al., 2023). D- Caerin has the same molecular weight, isoelectric point, hydrophobicity, and net charge than its corresponding natural L-Caerin counterpart. However, it is predicted to contain two-left-handed alpha-helices, whereas natural L-Caerin contains two right-handed alpha-helices (Fig.1), as was confirmed by comparison of the circular dichroism spectrum of both enantiomers (López-Sanmartín et al., 2023).

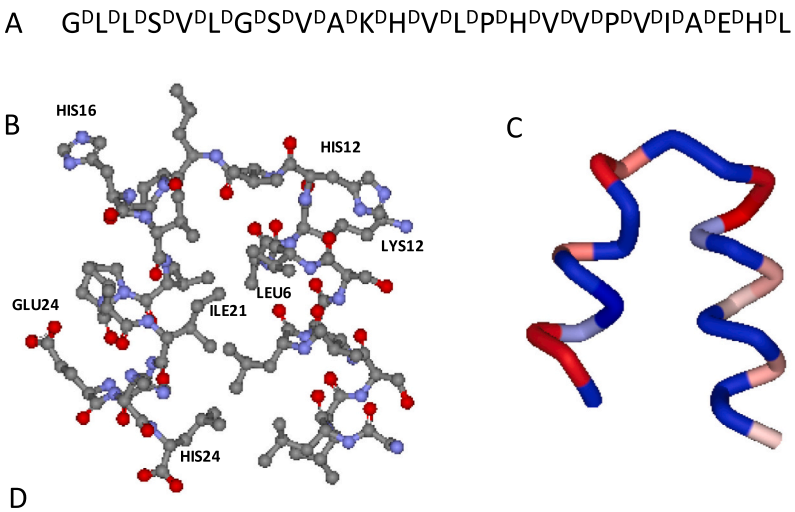
Encapsulation of AMPs in nanoparticle systems can enhance their stability and facilitate their delivery and oral administration (Mitchell et al., 2021). This approach is particularly useful for small-sized animals or those at larval or seed stages, for which other delivery systems are not-viable (Fajardo et al., 2022; Khan et al., 2024). Moreover, encapsulation of AMPs can protect the structural integrity of AMPs, which is typically compromised by peptidases produced by bacteria, or by enzymes present in the gastrointestinal tract or plasma of the target organisms. Biodegradable delivery systems based on poly(lactic-*co*-glycolic acid) (PLGA) have been widely used as nanocarriers for vaccines (Cui et al., 2025), antibiotics (Jeong et al., 2008; Arafa et al., 2020) and other pharmaceutical and bioactive compounds due to their safety, biocompatibility and biodegradability (Naskar et al., 2021; Alsaab et al., 2022).

In this study, PLGA nanoparticles loaded with the synthetic antimicrobial peptide D-Caerin (D-CAE) and its fluorescently labeled analogue (D-CAE-FAM) were obtained and characterized. The physical characterization of the nanoparticles (NPs), their encapsulation efficiency, peptide release kinetics, and antibacterial activity against several pathogenic bacteria were evaluated, and optimal parameters were determined. Furthermore, the uptake of these (NPs) by clam larvae was demonstrated, highlighting the potential of peptide-loaded PLGA NPs as biocompatible nanocarriers for the delivery of antimicrobial peptides into bivalves and other invertebrates.

2. Material and methods

2.1. Synthesis of peptides and structure prediction

Peptide D-Caerin (G{d-L}{d-L}{d-S}{d-V}{dL}G{d-S}{d-V}{d-A}{d-K}{d-H}{d-V}{d-L}{d-P}{d-H}{d-V}{d-V}{d-P}{d-V}{d-I}{d-A}{d-E}{d-H}{d-L}) and its corresponding FAM-tagged version (D-CAE-FAM) were synthesized by GenScript Corp. (Piscataway, NJ, USA), where FAM denotes the fluorescent dye 5-Carboxyfluorescein. Lyophilized peptides, with a purity higher than 95 %, as verified by HPLC, were reconstituted in sterile ultrapure water at a concentration of 10 mg mL^{-1}, aliquoted and stored at −20 °C until their use as previously described (López-Sanmartín et al., 2023). The main physico-chemical properties of the peptide were obtained using Heliquest Analysis module (Gautier et al., 2008), and the Protein Identification and Analysis Tools on the Expasy Server (Gasteiger et al., 2005). Spatial coordinates for D-CAE were



Physico-chemical properties					Polar residues + GLY (9 residues)		Nonpolar residues (16 residues)	
MW	PI	H	Z	GRAVY	Uncharged	charged	Aromatic	Special
2585.13	7,02	0,734	0	1,188	3HIS, 2 SER 2 GLY	1 LYS 1 GLU	0	2PRO

Fig. 1. Sequence, structure and main features of D-CAE peptide. Sequence (A), three dimensional structure highlighting functionally significant aminoacids (B); hydrophilic/ hydrophobic residues (C) and main characteristics (D) of the peptide Caerin (D-CAE). In panel C: Red tube lines represent hydrophilic residues and blue tube lines represent hydrophobic residues. In panel D: MW, Molecular Weigh; PI, Theoretical Isoelectric point; H, Hydrophobicity; Z, Net charge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

obtained by generating the mirror images of the spatial coordinates of each atom with Chem3D software. PEP-FOLD3 (Shen et al., 2014) and UCSF-Chimera (Pettersen et al., 2004) were used for predicting and visualizing 3D structure of the peptide D-CAE (Fig. 1).

2.2. Nanoparticle preparation

Peptide-loaded PLGA NPs were prepared by using an emulsion/evaporation method (Pinto Reis et al., 2006) with some modifications. The organic phase consisted of ethyl acetate, in which the solid polymer (50 mg) and the solid antimicrobial peptide (in varying amounts, as shown in Table 1) were dissolved. The aqueous phase consisted of 1.5 % *w/v* Poly vinyl alcohol (PVA), used as emulsifier. To prepare the nanoparticles, 5 mL of the organic phase containing the polymer and the peptide was added to 20 mL of the aqueous phase under magnetic stirring at 600 rpm. The resulting dispersion (25 mL) was stirred overnight in the dark to allow complete evaporation of the organic phase, leading to nanoparticle formation. To ensure full evaporation, the dispersion was further subjected to vacuum drying using an Eppendorf Concentrator Plus for 40 min at room temperature. The solid polymeric nanoparticles were then recovered by centrifugation at 13400 rpm for 30 min. After centrifugation, the nanoparticles were washed twice with water (2 mL for each Eppendorf tube), dried under vacuum for 2–3 h at room temperature, and stored in a freezer, protected from light. The supernatants were also frozen and stored in the dark for further determinations.

2.3. Emission fluorescence measurements

Fluorescence emission spectra of D-CAE-FAM were recorded using a Hitachi 2500 spectrofluorimeter connected to a Lauda flow thermostat. A quartz cell with a 10 mm path length was used. The excitation wavelength was set at $\lambda = 495$ nm, and spectra were registered from 500 to 650 nm. Both excitation and emission slit widths were 5 nm. To quantitatively determine the D-CAE-FAM concentration, calibration curves were generated in both MiliQ water and seawater (Suppl. Mat. 1). The concentration of the standard solutions ranged from $4.7{\cdot}10^{-3}$ to $3.75{\cdot}10^{-2}$ g L^{-1}. The studies of stability showed that D-CAE-FAM solutions remained stable for over 24 h in the dark in water at room temperature.

2.4. Physical characterization of peptide-PLGA NPs

2.4.1. Zeta potential

Zeta potential, ζ, values were determined by measuring the electrophoretic mobility of polymeric nanoparticles based on their velocity using a laser Doppler velocimeter (LDV). The measurements were conducted with a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The temperature was maintained at 298.0 ± 0.1 K, and DTS1060 polycarbonate capillary cells were used. The PLGA NPs concentration in the aqueous dispersions was 0.05 g mL^{-1}. Each sample was measured at least ten times, and the average value was recorded.

Table 1
Physico-chemical characteristics of PLGA NPs synthetized with different initial amounts of D-CAE-FAM peptide and PLGA polymer.

Initial mg						Particle d_H (nm)		
D-CAE-FAM	PLGA	Pep/Pol_{ratio}	%R	% EE	ζ/mV	small	large	PDI
0	50	–	80 ± 5	–	−25 ± 4	–	432 ± 5	0.39 ± 0.07
0.5	50	0.01	87 ± 2	40 ± 4	−25 ± 3	124 ± 6	426 ± 15	0. 53 ± 0.04
1.0	50	0.02	91 ± 3	71 ± 3	−27 ± 3	130 ± 4	421 ± 19	0.42 ± 0.01
2.0	50	0.04	90 ± 3	70 ± 4	−21 ± 5	146 ± 5	460 ± 13	0.57 ± 0.06

Pep/Pol_{ratio}, peptide/polymer ratio; Synthesis yield, %R, encapsulation efficiency, %EE, hydrodynamic diameter, d_H, zeta potential, ζ, and polydispersity index, PDI. All the values are the mean of at least three determinations ± SD.

2.4.2. Dynamic light scattering

A Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used to determine the hydrodynamic diameter (d_H, *Z*-average) and the polydispersity index (PDI) of the PLGA NPs through dynamic light scattering (DLS) measurements. The analysis was conducted at a scattering angle of 90°. The samples and the cell were the same as those used in the Zeta potential measurements. At least ten repetitions were made for each sample and the average value was recorded.

2.5. Synthesis yield and encapsulation efficiency

Synthesis yield (%R) and encapsulation efficiency (%EE) of the different NPs used in this study were calculated using Eq. (1) and Eq. (2), respectively.

$$\%R = \frac{\text{mg NPs}}{(\text{mg of PLGA} + \text{mg of AMP})} \text{ x } 100 \quad (1)$$

Here mg NPs is the amount in mg of nanoparticles obtained in each synthesis, mg of PLGA and mg of AMP are the initial amounts of PLGA and AMP used in the preparation, respectively.

$$\%EE = \frac{\text{mg AMP encapsulated}}{\text{Total mg of AMP}} \text{x } 100 \quad (2)$$

where mg $AMP_{encapsulated}$ is the amount of AMP encapsulated and Total mg of AMP represents the total amount of AMP initially added in the NP preparation reaction. The amount of encapsulated AMP was determined by subtracting the amount of peptide remaining in the supernatant and washings from the initial quantity of AMP used in the preparation of the NPs. Fluorescence measurements and the calibration curves for the fluorescent D-CAE-FAM peptide (Suppl. Mat. 1) were used to determine the %EE.

2.6. In vitro release studies

Release experiments were carried out using the procedure previously described (López-López et al., 2019) with some minor modifications. In summary, a dispersion of the peptide-loaded NPs ([NPs] ≈ 1.5 g L^{-1}) was prepared in 2 mL of Milli-Q water (or filtered sea water (FSW)), and placed in a thermostated glass vial at room temperature (25 °C), under continuous magnetic stirring (200 rpm) in the dark. At defined time intervals, samples were centrifuged (13,400 rpm, 30 min). One milliliter of supernatant was collected and replaced with an equal volume of fresh of filtered sea water to maintain a constant NP concentration throughout the experiment. The collected sample was then analyzed to determine the peptide concentration by fluorescence emission measurements. Each experiment was performed in triplicate and the average value calculated.

2.7. Bacterial strains and culture conditions

The control bacteria *Micrococcus luteus* CECT245 and *Escherichia coli* (DH5α) were cultured at 37 °C in LB culture medium. The pathogenic strains *Vibrio anguillarum* (CECT 522 T), and *Pseudomonas anguilliseptica* (CECT 899 T) from the Spanish collection of type cultures, were cultured in marine broth (MB) and Tryptic Soy broth (TSB), respectively, at 26 °C. All the strains were incubated with rotatory agitation (150 rpm). Solid culture media were prepared by adding 1 % agar.

2.8. Determination of the antimicrobial activity of peptide charged PLGA NPs

Antimicrobial activity of the peptide-charged PLGA NPs was determined as previously described (Ali et al., 2023) with minor modifications and expressed as the lethal antibacterial concentration (LC99). The assays were carried out at room temperature in 1.5 mL Eppendorf tubes with agitation at 100 rpm. Bacterial suspension samples, 5 μL containing 10^4 CFU, were mixed with increasing quantities, between 2.5 mg mL^{-1} to 20 mg mL^{-1} of PLGA NPs containing 16 mg of Caerin per g of NP in a final total volume of 500 μL and incubated for 24 h. A control of NPs without the peptide was included to evaluate possible toxicity of the NPs. An assay without bacterial inoculum was also included to verify the absence of contaminant bacteria. After 24 h, aliquots of each sample were withdrawn, subjected to serial 10-fold dilutions, plated on agar plates with the indicated growth medium, and incubated overnight at 37 or 26 °C to determine the colony forming units (CFU). The lethal concentration (LC99), defined as the minimal peptide-PLGA NP concentration at which more than 99 % of the bacterial population is killed, was calculated.

2.9. Capture and ingestion of peptide charged-PLGA NPs by postlarvae of the Japanese clam Ruditapes philippinarum

To evaluate the effective capture and ingestion of peptide charged-PLGA NPs by the clam *Ruditapes philippinarum,* batches of 30 *Ruditapes philippinarum* postlarval samples, with sizes ranging between 0.5 and 1 mm, were placed in 24-multiwell plates with a capacity of 3 mL per well and incubated with increasing concentrations (between and 0.01 and 100 μg mL^{-1}) of PLGA-NPs charged with D-CAE-FAM peptide, in a final volume of 1 mL of FSW. PLGA NPs containing 16 mg of D-CAE-FAM per g of NP were dispersed in ultrapure water at the indicated concentrations by several cycles of vortex-agitation and incubation in a sonication bath. Postlarvae were incubated for 72 h with the indicated quantity of NPs in an orbital shaker at 80 rpm. Tests were carried out in duplicate and a control experiment with clam postlarvae incubated in FSW without addition of NPs was simultaneously performed. After 24 h and 72 h of incubation, clam postlarvae were collected, rinsed with fresh seawater and examined under a stereomicroscope and a fluorescence microscope, Olympus BX61, with an Olympus DP73 camera and equipped with a specific FITC filter (Ex. 470 ± 20, Em. 530 ± 25).

2.10. Electron microscopy

The morphology of the peptide-loaded nanoparticles was examined using Scanning Electron Microscopy (SEM, Hitachi S5200) and Transmission Electron Microscopy (TEM, Talos S200). Aqueous solutions of the samples ([NPs] = 1.5 g L^{-1}) were dropped onto a monocrystalline silicon grid for SEM analysis and onto a copper grid coated with a carbon film for TEM imaging. The samples were then air-dried at room temperature prior to observation.

2.11. Cytotoxicity assay in human cell lines

T84 CRC human cells ((ATCC, Manassas, VA, USA cells) were seeded in 48-well plates at densities of 5 × 10^3 cells per well in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Sigma-Aldrich, Madrid, Spain). Cultures were maintained at 37 °C in 5 % CO_2 atmosphere. After 24 h, the cells were exposed to an increasing range of concentrations (0.2–50 μg mL^{-1}) of D-CAE for 3 and 48 h. Cell proliferation was assessed using a modified sulforhodamine B (SRB) colorimetric assay. Cells were fixed with 10 % trichloroacetic acid (TCA) at 4 °C for 20 min, stained with 0.4 % SRB in 1 % acetic acid for 20 min at room temperature under agitation, and washed. Bound dye was solubilized in 10 mM Trizma® (pH 10.5), and absorbance was measured at 492 nm (EX-Thermo Multiskan, Waltham, MA, USA). Cell viability (%) was calculated as: % Proliferation = ((OD sample − blank) / (OD negative control − blank)) × 100.

3. Results and dicussion

3.1. Physicochemical characteristics of D-Caerin and optimization of peptide-loaded nanoparticles formulation

The main physico-chemical properties were analyzed using using Heliquest module and protein Identification and Analysis Tools on the ExPASy server as described in Section 2.1 (Fig. 1D). The three-dimensional structure was predicted with PEP-FOLD3 software, a tool specially adapted for short peptides, and visualized using Chimera (Fig. 1). D-Caerin is predicted to contain two left-handed alpha-helices, whereas the L-Caerin contains two right–handed alpha-helices, as previously demonstrated by NMR studies (Wong et al., 1997). Similar observation can be withdrawn for the corresponding CD spectra of these peptides, as previously demonstrated (López-Sanmartín et al., 2023).

PLGA nanoparticles were synthesized at different peptide/polymer ratios (Pep/Pol ratio) and both the peptide encapsulation efficiency and the physico-chemical characteristics of the obtained NPs were evaluated. This characterization was based on the fluorescence emission spectroscopic properties of D-CAE peptide, tagged at the N-terminal end with the fluorescent dye 5-Carboxyfluorescein (D-CAE-FAM). The results are summarized in Table 1.

As shown in Table 1, the synthesis yield (%R) follows an increasing trend with the polymer/peptide ratio (Pep/Pol_{ratio}): %R (PLGA) < %R (PLGA/D-CAE-FAM 0.01) < %R (PLGA/D-CAE-FAM 0.02) ≈ %R (PLGA/D-CAE-FAM 0.04). The encapsulation efficiency (%EE), determined as described in Section 2.5, increases with the amount of peptide, until reaching a saturation limit value of 71 ± 3 %. Moreover, the influence of the Peptide/polymer ratio on Z potential, size and polydispersity index was investigated. All formulations show a negative Z potential value, which is characteristic of PLGA polymeric nanoparticles (López-López et al., 2019). These Z potential values are indicative of moderate colloidal stability (Ma et al., 2023). PLGA nanoparticles without encapsulated AMP exhibit a unimodal size distribution with an average hydrodynamic diameter of 432 ± 5 nm. In contrast, the NPs with encapsulated D-CAE-FAM exhibit a bimodal size distribution, characterized by the presence of both a smaller and larger-sized particle populations (Suppl. Mat. 2). This distribution profile suggests a higher degree of size heterogeneity in the AMP-loaded PLGA nanoparticles relative to their unloaded counterparts nanosystems. A slight increase in the hydrodynamic diameter of both populations is observed as the initial AMP mass used in the synthesis increases. This increase can be attributed to the adsorption of the peptide onto the surface of the NPs and may also be promoting surface reorganization, leading to greater hydrodynamic expansion. Moreover, as shown in Table 1, the NPs without D-CAE-FAM have a PDI of 0.39 ± 0.07, indicating a relatively homogeneous size distribution. The incorporation of the peptide leads to an increase in the mean PDI value, suggesting greater heterogeneity in the NPs size distribution. This increase could be due to variable adsorption of the peptide onto different particles, resulting in a greater diversity of sizes. These values are in the same order of magnitude of those observed for PLGA NPs obtained in other studies, that exhibit variable sizes, which has been described to be highly influenced by factors such as polymer concentration, organic solvent, temperature, aqueous phase ionic strength, agitation rate or gauge of the needles (Huang and Zhang, 2018). Based on the data shown in Table 1, the Pep/Pol_{ratio} ratio of 0.02 was selected for the formulation of NPs to be used in the following experiments, since at this ratio the obtained NPs exhibited the best synthesis yield, higher encapsulation efficiency, and higher homogeneity in size distribution.

The selected nanosystems were analyzed by electron microscopy (Fig.2). The obtained NPs exhibited a yield of 94 % ± 2.

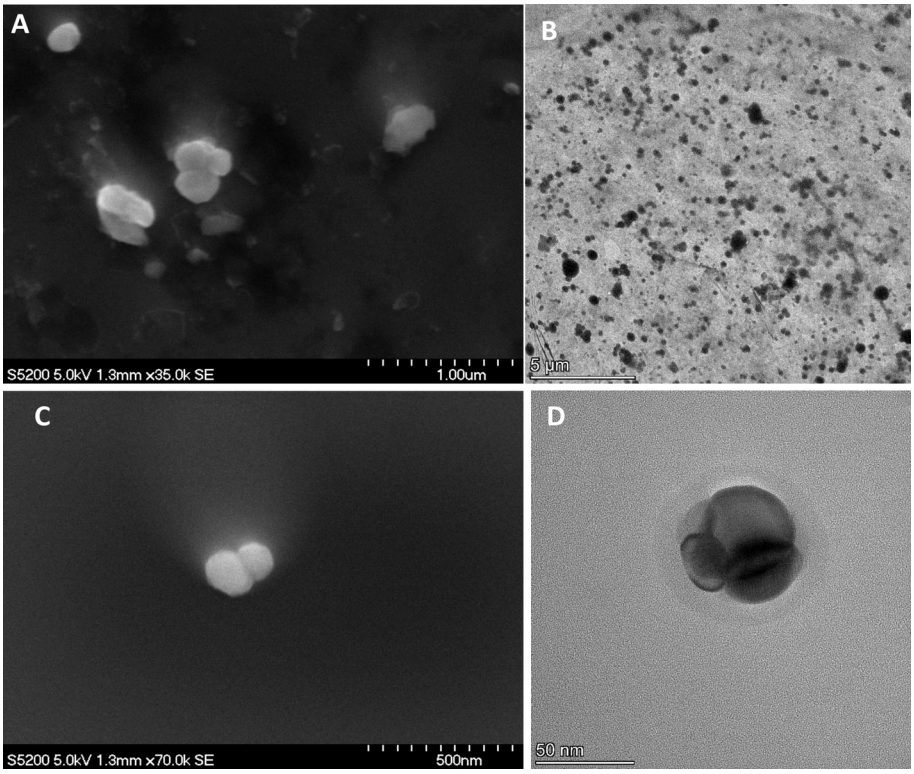


Fig. 2. Electron microphotograph images of peptide-charged PLGA NPs. NPs were formed by emulsifying 50 mg of PLGA and 1 mg of D-CAE, dissolved in ethyl acetate (Pep/Pol ratio of 0.02), into 5 mL of an aqueous PVA solution (1.5 % *w/v*), following the procedure detailed in M&M. Representative images obtained *via* scanning electron microscopy, SEM, (A, C) and transmission electron microscopy, TEM, (B,D) are shown.

Characterization by DLS and Z potential measurements, revealed hydrodynamic diameter, polydispersity index and Z potential comparable to those observed for PLGA-DCAE-FAM 1.0 ($d_H = 131 \pm 5$, 450 ± 18 nm, PDI = 0.40 ± 0.03 and $\xi = -26 \pm 4$ mV). The heterogeneous size (Fig. Suppl. Mat. 2) is also evident in the electron microscopy images (Fig. 2), which show a certain degree of aggregation and an approximately spherical morphology.

3.2. *In vitro* AMP release from the PLGA nanoparticles

Release of the AMP from the PLGA NPs was evaluated by measuring *in vitro* the cumulative D-CAE-FAM released peptide in Milli-Q water and FSW at 25 °C, following the procedure outlined in the Experimental section and the calibration curves obtained by fluorescence emission intensity measurements (Fig Suppl. Mat. 1). The results, expressed as percentage of peptide released are shown in Fig. 3.

The maximum percentage of peptide released in Milli-Q water was 73.5 %, reached at approximately 40 h. A rapid increase in the peptide release is observed during the first 2 h, during which approximately 56 % is released. After this initial phase, the release rate decreases, and approaches a plateau, at which the maximum concentration of released peptide is attained. This release profile is known as biphasic and is often associated to systems in which the drug is located in different areas of the nanocarrier: surface and core of the nanosystem. The kinetic analysis of the release profile has been performed by fitting the experimental data to first order kinetics using the following Eq. (3) (Espíndola et al., 2022):

$$\%D-CAE-FAM_{released} = a\cdot\left(1-exp\left(-k_{surf}\cdot t\right)\right)+b\cdot\left(1-exp\left(-k_{core}\cdot t\right)\right) \quad (3)$$

where k_{surf} and k_{core} are the first-order rate constants corresponding to the release of the D-CAE-FAM peptide from the surface and the core of the PLGA NPs, respectively, while a and b are adjustable parameters

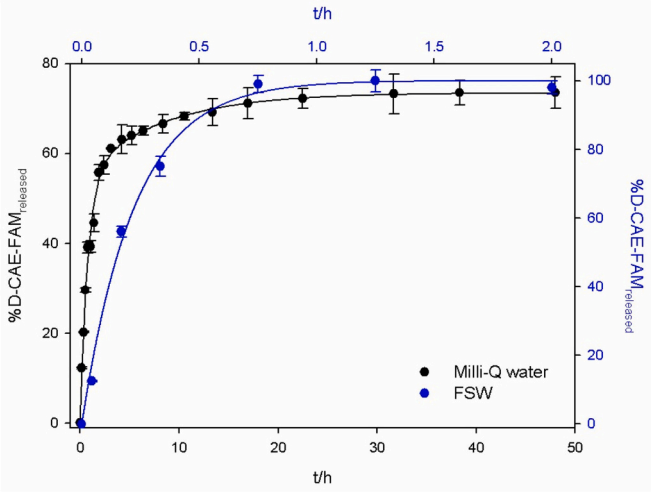


Fig. 3. Cumulative release of D-CAE-FAM from PLGA nanoparticles (PLGA/D-CAE-FAM 0.02) in Milli-Q water and FSW at 25 °C. Solid lines correspond to the fitting of the experimental data to a first order kinetics using Eq. (3). The experimental values are expressed as the mean ± SD ($n = 3$).

related to the AMP released from the surface and the core of the nanocarriers, respectively. The theoretical data obtained from the fitting show good agreement with the experimental results (Fig. 3). The calculated release rate constants are shown in Table 2.

Peptide release in filtered seawater (FSW) is complete and significantly faster than in Milli-Q water: 100 % of the AMP is released within 1 h. The increased salt content, *i.e.*, the higher ionic strength of the FSW solution, induces an earlier rupture of the nanocarrier compared to the Milli-Q water. This disruption occurs so rapidly that no distinction can

Table 2
Values of the release rate constant of the peptide from PLGA nanoparticles in different media at 25 °C.

Media	k_{surf}/h^{-1}	k_{core}/h^{-1}
Milli-Q water	1.3 ± 0.1	0.12 ± 0.04
FSW	4.5 ± 0.2	4.5 ± 0.1

be observed in the release rates of the peptide located on the surface *versus* that in the core of the nanoparticles (Table 2).

The kinetics observed for D-CAE-FAM release from PLGA nanoparticles reveal a rapid release profile, which is further accelerated in a seawater environment. Nevertheless, the encapsulation system is effective in protecting the peptide during delivery, enabling the AMP to reach bactericidal concentrations within 2–4 h (Fig. 3). PLGA NPs have been widely used to encapsulate antibiotics (Jeong et al., 2008) and other bioactive metabolites, including bioactive peptides. As an example, Silva et al. (Silva et al., 2013) observed about 30 % release of long peptides from PLG NPs after 1 h incubation at 37 °C. Similarly, Ali et al. (Ali et al., 2023) concluded that encapsulation of the SAAP-148 AMP in PLGA NPs resulted in high diffusion rates and sustained release of peptide for up to 21 days in PBS at 37 °C. NPs of PLGA with other *co*-polymers, such as poly (ethylene glycol) methyl ether (PEG) or Acid–Polyvinylpyrrolidone (PVP) have been described for entrapment of ciprofloxacin (Watcharadulyarat et al., 2023) and β-carotene (Liu et al., 2022). In both cases, incorporation of a copolymer in the nanoformulation resulted in a reduced the particle size and prolonged release.

3.3. Assessment of the antimicrobial activity of D-Caerin loaded-PLGA nanoparticles

The bactericidal activity of the D-CAE, both soluble (D-CAE) and encapsulated in PLGA nanoparticles (D-CAE-PLGA), over four different bacterial strains, including typical aquaculture pathogenic species, was evaluated by incubating the selected bacteria with the free suspended peptide and with D-CAE-PLGA NPs for 24 h and determining the concentration of microorganisms after the treatment by counting the colony forming units (CFU), as detailed in Materials and Methods. The encapsulation of D-CAE in PLGA NPs was carried at a Pep/Pol_{ratio} of 0.02, which resulted to be the best ratio obtained for this formulation.

Antimicrobial activity was expressed as the lethal concentration (LC99), that is the minimal concentration of D-CAE-PLGA NPs or free suspended D-CAE at which 99 % or *more* of the initial bacterial inoculum *is killed (*Table *3)*. The obtained results revealed that D-CAE-PLGA NPs have a strong inhibitory effect on the growth of all the bacterial strains studied. NP lethal concentrations (LC99) were 2.52 mg mL^{-1} and 11 mg mL^{-} for *E. coli* and *M. luteus*, respectively, while lethal concentrations (LC99) of 5.1 mg mL^{-1} and 5 mg mL^{-1} were observed for the pathogenic strains *V. anguillarum and P. plecoglosicida*. The corresponding lethal concentration for free suspended D-CAE was between 41 ± 4 μg mL^{-1} for *E. coli* and 91 ± 3 μg mL^{-1} for *M. luteus.*

Considering that the content of encapsulated peptide was 16.3 μg per mg of NP, the susceptibility observed in the tested bacteria against D-CAE loaded in PLGA NP is approximately equivalent to that observed for free suspended D-CAE. Empty NP, without the peptide, had no significant effect over any of the bacterial strains at the studied concentrations, in agreement with data reported for the low cytotoxicity of this biodegradable polymer, generally considered as safe and approved by Food and Drug Administration (FDA) and the European Medicine Agency (EMA) in various formulations as therapeutic delivery vehicles as reviewed by Alsaab and coworkers (Alsaab et al., 2022).

Table 3
Antimicrobial activity of D-CAE and D-CAE-PLGA NPs.

LC99	*E. coli*	*M. luteus*	*V. anguilarum*	*P. plecoglosicida*
D-CAE-PLGA (mg mL^{-1})	2.5 ± 0.2	5.6 ± 1.2	5.1 ± 0.6	5.0 ± 0.4
D-CAE (μg mL^{-1})	41 ± 4	91 ± 3	77 ± 4	70 ± 5

LC99 Lethal concentration (LC99) of PLGA-D-CAE NPs and the corresponding free suspended D-CAE peptide over different bacterial strains. D-CAE concentration per NP is 16.3 μg/mg NP. All the values are the mean of three determinations ± SD.

Although the mechanism of action of antimicrobial peptides is not well known (Valero et al., 2020; Mookherjee et al., 2020) there are many evidences that demonstrate the antimicrobial activity of a big number of peptides from different sources. Caerin peptides, both the natural and D-artificial isoform have shown activity against pathogenic virus and bacteria usual in aquaculture (León et al., 2020; López-Sanmartín et al., 2023; Cuesta et al., 2021), but their entrapment in polymeric NPs has not been previously described. Ali and coworkers (Ali et al., 2023) described the sustained local release of the synthetic peptide SAAP-148, entrapped in PLGA NPs, providing higher selectivity and stronger antibacterial response against antimicrobial resistant (AMR) planktonic bacteria than the free peptide. Similarly, Silva et al. (Silva et al., 2013) observed that encapsulation of a synthetic long peptide in PLGA nanoparticles was crucial for its efficient antitumoral activity. In the case of D-Caerin, both free and encapsulated formulations of the peptide are demonstrated to be functional and to exhibit high antimicrobial activity against the target bacteria, as shown in this study.

3.4. Uptake of peptide-loaded nanoparticles by Ruditapes philippinarum postlarvae

To check the ability of clam larvae to capture and uptake PLGA NPs charged with the desired entrapped peptide and possible toxicity, *R. philipinarum* postlarvae specimens were incubated with different concentrations of D-CAE-FAM-PLGA NPs, as detailed in the materials Section 2.9. FAM-tagged NPs were incorporated by clam postlarvae at all the concentrations assayed. Clams were observed under a stereomicroscope after 24 h and 72 h to assess potential toxicity. This revealed that peptide-loaded nanoparticles produced no adverse effects in clams with 100 % of the specimens remaining viable at all the concentration tested, showing their siphons out and filtering normally (Suppl. Mat 3).

Observation by optical fluorescence microscopy allowed to observe multiple fluorescent emission points and an intense fluorescence signal in the digestive gland region of the larvae (Fig. 4). The fluorescence was still visible 72 h after particle supply, confirming the successful ingestion and retention of the NPs carrying the tagged D-CAE-FAM peptide, which were not rejected nor eliminated at pseudofeces.

Exogenous feeding of bivalves involves the capture of particles suspended in the surrounding water. However, filtering organisms can be very selective, regarding size and nature of the particles they filter (Cognie et al., 2003; Rosa et al., 2018). Ward and Kach (Ward and Kach, 2009) studied the uptake of fluorescently labeled, 100-nm polystyrene beads by mussels and oysters and observed that particle assimilation efficiency is about 15 % for particles <1 μm in size, and that this efficiency is enhanced by the generation of particle aggregates usually produced during delivery. Similarly, active ingestion and long retention times of NPs of gold (Joubert et al., 2013), ZnO (Saidani et al., 2023) and fluorescent dyed nanoplastics (Zhou et al., 2023) has been described in different species of clams. A recent study carried out in tumor human cell lines elucidates the movement of PLGA-NP in cells, demonstrating their retention and subcellular localization within intracellular vesicles (Singh et al., 2023). Other nanosystems based in sodium octenyl succinate starch and methylcellulose microparticles have been used for encapsulation of antimicrobial peptides and feeding of clams, demonstrating the antimicrobial activity of the obtained nanosystems and their ability to improved survival of *Ruditapes philippinarum* larvae challenged with *Vibrio crassostreae* (Zhang et al., 2025). An interesting approach uses a microalga for the recombinant production of an AMP and its

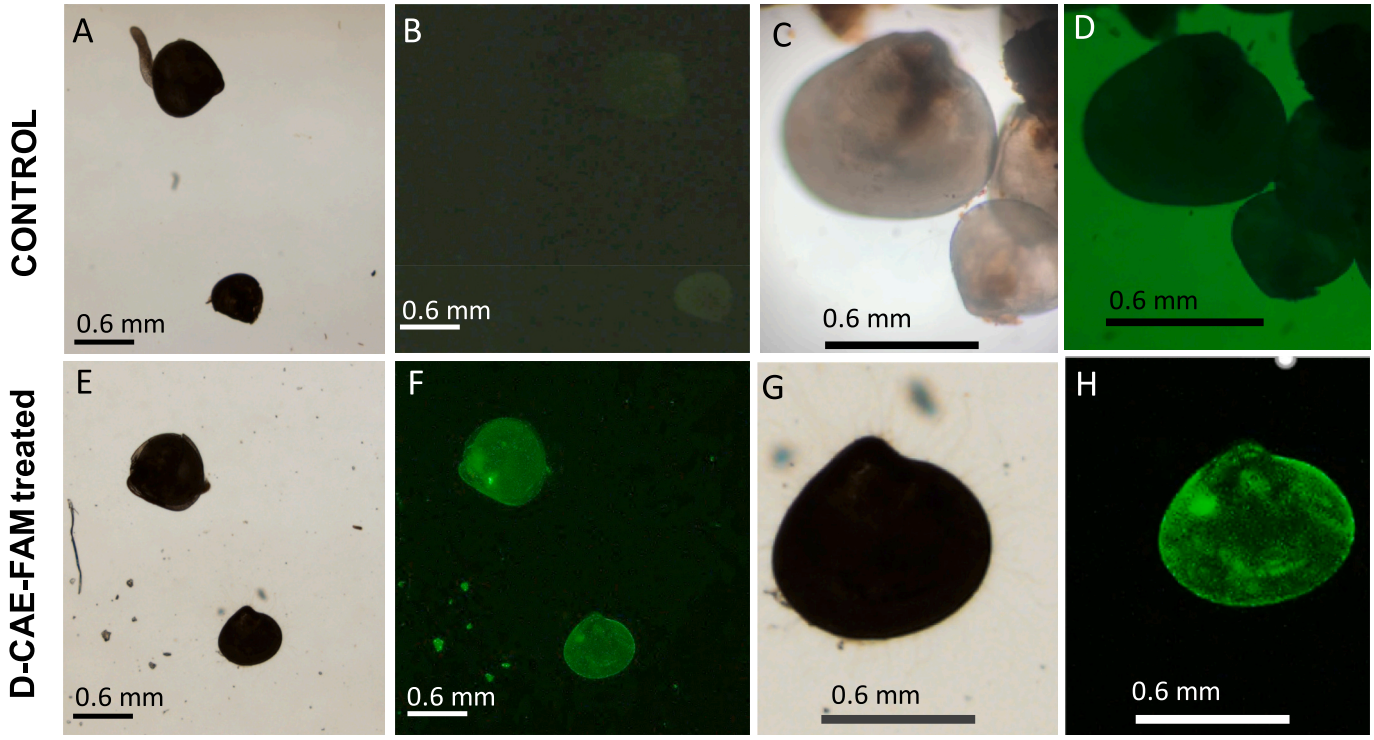


Fig. 4. Optical bright field (A, C, E and G) and fluorescence (B, D, F, H) microscopy images of *R. philipinarum* postlarvae. CONTROL untreated (A-D) and specimens treated with FAM-D-CAE-PLGA NPs (1 μg mL^{-1}) for 72 h (E-H) are shown. The scale bar is 0,6 mm for all pannels. Filter for fluorescence FITC (Ex. 470 ± 20, Em. 530 ± 25).

delivery into mussels, achieving promising results in controlling *Vibrio* infections (Wang et al., 2023).

D-CAE-FAM PLGA NPs were non-toxic to clam larvae, which remained viable and exhibited a high degree of mobility after the treatment (Suppl. Mat. 3). The efficient uptake of PLGA NPs and their aggregates observed in this study, along with their demonstrated non-toxicity, confirms that PLGA NPs can be biocompatible non-toxic adequate nanocarriers for delivering antimicrobial peptides into bivalve larvae. The non-toxicity of the PLGA polymer is very well stablished; It is generally recognized as safe and has received approval from both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use in various therapeutic delivery formulations, as reviewed by Alsaab and colleagues (Alsaab et al., 2022). In addition, a cytotoxicity assay performed on human cell lines confirmed that D-Caerin is non-toxic to animal cells (Suppl Mat. 4).

The NPs size distribution obtained in our study (Table 1) is adequate to feed clams. Several studies have demonstrated that particles with more of 500 μm are not uptaken by bivalves, being particles under 25 μm the preferred ones. This has been particularly studied for metals, metal oxides and plastic nanoparticles, which have received an enormous interest for both industrial and medical applications but have also risen increasing concerns for their potential undesirable effects in the environment and aquatic and terrestrial biological systems (Zhou et al., 2023; Fernández and Albentosa, 2019). Unlike polyethylene and polypropylene, poly(lactic-*co*-glycolic acid (PLGA) is a synthetic, biodegradable polymer, which breaks down into lactic acid and glycolic acid, which are naturally metabolized. PLGA NPs are very attractive for their ability to encapsulate hydrophilic and hydrophobic drugs, their safety, biodegradability and lack of toxicity, having being approved by FDA for various clinical applications (Guo et al., 2023; Marquina et al., 2023; Shakya et al., 2023).

4. Conclusions

Nanoparticles (NPs) have revolutionized the field of medicine and veterinary for both detection and treatment of infectious diseases. Using an emulsion/evaporation method, D-CAE peptide-loaded PLGA NPs have been prepared. D-CAE-PLGA NPs with a Pep/Pol_{ratio} of 0.02 exhibited the best physico-chemical characteristics, ($\xi = -26 \pm 4$ mV) synthesis yield (94 %), encapsulation efficiencies of (70 %), and adequate size range ($d_H = 131 \pm 5$, 450 ± 18 nm). Besides, peptide release kinetic revealed that the obtained PLGA nanoformulations showed an adequate liberation rate (73.5 % after 40 h and 100 % after 2 h in Milli-Q water and FSW, respectively), which make them particularly suited as nanocarriers for the delivery of antimicrobial peptides into bivalve larvae. The antibacterial activity of the NPs was tested against several bacteria, including two prevalent pathogens in aquaculture species, the causal agents of vibriosis and pseudomoniasis. The uptake test confirmed that the NPs effectively ingested with no apparent toxicity of the peptide-charged NPs for clams (Suppl. Mat. 3) and the effective ingestion and retention of tagged-NPs, even after 72 h after the treatment.

Among the main challenges to be addressed for the efficient application of AMPs to combat infections in aquaculture species are their inherent instability and low bioavailability. These limitations can be mitigated through encapsulation in nanocarriers, such as PLGA NPs, which can protect AMPs from unfavorable external physiological conditions and enable their controlled release. These conclusions anticipate the important role that polymeric biocompatible, biodegradable nanoparticles can play in veterinary and aquaculture as a promising technology for animal health. Specifically, this study demonstrates that D-Caerin encapsulated in PLGA nanoparticles could serve as an effective system for the control of vibriosis in *Ruditapes* cultures. This formulation has shown no toxicity to clams or human cell lines, exhibited good encapsulation capacity and has been effectively taken up by clam seeds. However, further detailed studies are required to assess its practical

application, particularly through *in vivo* challenge experiments to assess survival rates and the long-term effects of the nanosystem.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.743334.

CRediT authorship contribution statement

Ana Molina-Márquez: Writing – review & editing, Methodology, Investigation. **Manuel López-López:** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Racio Pereira-Rengel:** Writing – review & editing, Methodology. **Monserrat López-Sanmartín:** Writing – review & editing, Methodology, Investigation. **Elena Domínguez:** Investigation. **Rocío Jaramillo:** Investigation. **Pilar López-Cornejo:** Writing – original draft, Supervision, Funding acquisition. **José Antonio Lebrón:** Investigation. **Gloria Perazzoli:** Investigation. **José Carbajo-Cobacho:** Investigation. **Rosa León:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Funding

This research was financially supported by grant PID2022-140995OB-C21 (CARACAS), funded by MCIN/AEI/ 10.13039/501100011033; and Consejería de Educación y Ciencia de la Junta de Andalucía- USE (FQM-206).

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data.

Acknowledgments

The authors acknowledge F. Varela from the Microscopy Service of the University of Seville (CITIUS) for assistance with the electron microscopy study, and C. Melguizo and M.C. Prados from the University of Granada for their support with the cytotoxicity assays. Funding for open-access publishing was provided by the University of Huelva / CBUA.

Data availability

Data will be made available on request.

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