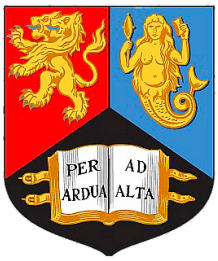
Improving the Performance of Nisin as a Natural Food Additive through the use of Nanotechnology

by

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Abstract

Nisin is the most famous natural food antimicrobial used in many countries. However, the direct introduction of nisin into food products decreases the duration of its antimicrobial activity, limiting its widespread use in the food industry. Nisin-loaded nanostructures are effective tools which prolong nisin's antimicrobial performance during the shelf life of food products due to their sustained release properties. However, further investigation is still needed to deliver nisin with easy-to-prepare, cost-effective, food-grade nanocarriers in food. Additionally, novel approaches need to be followed to develop smart nisin carriers (e.g. stimuli-responsive delivery) and thus deliver nisin on demand in food systems in case of contamination or unmaintained cold chain during the storage of food products.

In this project, Polyion Complex (PIC) nanoparticles were used as an easy and affordable platform to develop novel nisin-delivery systems. In each chapter, using different anionic polymers, a straightforward electrostatic complexation method was followed to prepare nisin-loaded PIC nanoparticles. Firstly, using commercially available Carboxymethylcellulose (CMC), a new example of a cheap and food-grade nisin-carriers displaying sustained antimicrobial activity properties (Chapter 2). Secondly, hyaluronic acid (HA) was employed as an enzymatically degradable (by hyaluronidase) polymer to encapsulate nisin (Chapter 3). Thus, the first example of enzyme-responsive nisin carriers was developed against hyaluronidase-producing foodborne pathogens (i.e. *Staphylococcus aureus*). Finally, a synthetic thermoresponsive polymer, poly(Valine-Proline-Glycine-Valine-Glycine-OH), was employed to prepare PIC nanoparticles loading with nisin (Chapter 4). In this way, the first example of thermoresponsive nisin-carrier was developed to achieve enhanced antimicrobial activity above a critical temperature (i.e. 40^{o} and above) at which specific foodborne pathogens (e.g. *Clostridium perfringens*) can rapidly grow in food.

All nanoparticles prepared in this project were characterised by DLS, zeta potential, TEM, and encapsulation efficiency experiments. Additionally, special antimicrobial activity experiments were designed to monitor sustained (Chapter 2) or responsive antimicrobial activity properties (Chapters 3-4) of these nanoparticles against relevant gram-positive foodborne pathogens and spoilage organisms.

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List of Abbreviations

ACF	Autocorrelation function
BHIB	Brain Heart Infusion Broth
CFU	Colony forming unit
CMC	Carboxymethylcellulose
D_H	Hydrodynamic diameter
DLS	Dynamic light scattering
DS	Degree of substitution
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
GPC	Gel permeation chromatography
GRAS	Generally regarded as safe
GSH	Glutathione
HA	Hyaluronic acid
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	High performance liquid chromatography
kcps	Kilo counts per second
LasB	Elastase B from Pseudomonas aeruginosa
LCST	Lower critical solution temperature
Mw	Molecular weight
MWCO	Molecular weight cut-off
NMR	Nuclear magnetic resonance
OD	Optical density
PdI	Polydispersity index
Pol-B	Polymyxin B
PSS	Poly(styrene sulfonate)
poly(VPGVG)	poly(Valine-Glycine-Alanine-Valine-Glycine)
TEM	Transmission electron microscopy
TSB	Tryptic soy broth

CHAPTER 1 - Introduction

Overview

Foodborne diseases (also referred to as food poisoning) are a growing public health problem. Millions of people fall ill, and many die due to the consumption of unsafe/contaminated food.[1] Therefore, food safety has become an increasingly significant concern. In this regard, as an important component of food safety, the concept of food preservation has significant importance all around the world. For the purpose of ensuring food safety as well as food quality, food preservatives are commonly employed against foodborne bacteria and spoilage microorganisms in the food industry.[2,3] In recent years, consumers' expectation from food processors is to commonly use natural food additives in food production instead of synthetic preservatives, which is also known as minimally processed food.[4] Consequently, natural food preservatives have drawn increasing attention in recent years.

As a natural food additive, nisin, a type of bacteriocin produced by *Lactococcus lactis,* is one of the most important antimicrobial agents widely employed in the food industry.[5,6] It has a broad spectrum inhibitory effect against foodborne pathogens (e.g. *Listeria monocytogenes* and *Bacillus cereus)* as well as spoilage microorganisms (*Pseudomonas fluorescent* and *Clostridium spp.).*[7] Although nisin is an effective bio-preservative to enhance food safety and quality, its efficacy can be reduced in food, particularly as a result of its interaction with food components such as proteins, lipids and enzymes.[8,9] To overcome this problem, nanotechnology has been used to develop delivery systems such as nanoliposomes and nanocapsules, and these systems have exhibited their important roles in minimizing interference of food components, eventually increasing the bioavailability of nisin in food systems through sustained release mechanisms.[10–12] However, further research is still necessary to develop novel nisin carriers due to both some drawbacks associated with existing nano-delivery approaches (e.g. high cost, complexity, safety concerns),[12] and the absence of attractive carriers

providing food systems with nisin on demand (i.e. responsive delivery).[11] Therefore, this project aims to not only develop cheap and food grade nisin carriers, but also achieve responsive nisin delivery in food.

The main objective of this chapter is to provide a concise review of topics relevant to this research. For this purpose, firstly, the importance of microbial food safety and the necessity of natural food bio-preservation will be highlighted. Second, background information on nisin will be provided in detail by pointing out all potential factors influencing its activity in food, followed by the existing approaches for the delivery of nisin in food. Finally, the chosen nano-delivery platform to develop novel carriers for nisin will be introduced.

1. Microbiological Food Safety and Quality Concerns and Food Preservation

Foodborne diseases have been a global issue, not only causing severe damages to public health but also impeding socioeconomic development by straining healthcare systems and thus harming national economies, even reputation of countries, tourism and trade for many years.[13] Food contamination with microbial pathogens is the major reason for foodborne diseases, generally known as food poisoning. Food products are generally a suitable environment for bacterial growth. Due to very ubiquitous character of pathogenic microorganisms, many types of food can be easily contaminated as a result of poor hygiene practices.[14] Upon contamination, these pathogens can grow rapidly and some of which can produce their toxins in food products that are not properly stored. Consuming such contaminated products result in food poisoning, which is, as such, one of the main public health concerns in today's world. According to a scientific review published by the Food Standards Agency (FSA)[15], it was estimated that 2.4 million people suffered from foodborne illnesses in the United Kingdom in 2018. Additionally, The U.S. Centre for Control and Prevention (CDC) estimates that around 48 million cases of foodborne illness and 3000 deaths occur annually in the United States.[16]

Pathogenic bacteria species	Common food products associated with contamination
Bacillus cereus	Cooked rice, meat
Campylobacter spp.	Poultry, unpasteurised milk
Clostridium botulinum	Fish, meat
Clostridium perfringens	Cooked meat, poultry
Staphylococcus aureus	Ham, poultry, dairy products
Shigella spp.	Eggs, salads
Escherichia coli	Meat, raw milk
Listeria monocytogenes	Soft cheeses
Salmonellae	Meat, poultry, eggs
Yersinia enterocolitica	Milk, poultry

Table 1 Common foodborne pathogens and food products associated with food poisoning.[14]

Apart from health issues, foodborne diseases place a financial burden on society. For instance, it was estimated that the financial cost of foodborne diseases were £9.1 bn a year in the UK.[17] Therefore, the microbiological safety of food products is an unrecognisable challenge for all parties involved from production to consumption, particularly for governments and food processors.

On the other hand, microbial activity of various organisms, such as *Bacillus spp.*[18] and *Pseudomonas spp.*[19], in food can lead to undesirable changes in sensory characteristics such as texture, smell, or appearance, called as food spoilage or food deterioration.[1] Spoiled food products may not always be dangerous to eat; nevertheless, sensory characteristics of a food products are usually regarded as a main quality criterion, and therefore spoilage commonly causes them to be rejected for human consumption. Due to microbial food spoilage, it is estimated that about one fourth of food products produced are lost in the world.[16] Therefore, microbial food deterioration significantly impacts food quality and shelf life.

In accordance with the importance of food safety and quality concept, it is essential to protect food from its contamination with microorganisms at any stage, from production to consumption. As many types of food, particularly dairy products, are excellent media providing pathogens with nutrients and suitable condition in terms of parameters such as pH, water activity and redox potential, many

pathogenic and spoilage bacteria can be easily present and grow in food.[21] For the purpose of producing safer foodstuffs, in addition to hygienic practices during food processing, various methods such as thermal processing, high pressure, and lowering of water activity have been applied.[3,11] Nonetheless, it has been commonly reported that the presence of pathogens could be effectively controlled upon heat treatments[22], which is the main reason why thermal processing remains the most common method of food preservation. Since adequate thermal processing or cooking is the most important and one of the simplest methods to eliminate pathogenic bacteria from food, numerous food products, particularly dairy products, are often disinfected or sterilised through pasteurisation and ultra-high temperature (UHT) treatment.[23]

However, products inadequately reheated, undercooked meals, ready-to-eat products, products stored under inappropriate conditions, and re-contaminated products after thermal treatment have been commonly identified as high-risk factors of food poisoning and even foodborne outbreaks.[24,25] On the other hand, thermal treatments have several limitations, including high installation and maintenance cost, reduced nutritional value in food products, affected sensory characteristics of food. Consequently, food antimicrobials, usually defined as food additives, have drawn significant attention as an alternative food preservation method to prevent microbial growth in food, eventually enhancing food safety and quality.[11] According to their sources, food antimicrobials can be classified into two groups, synthetic and natural antimicrobials (**Table 1**).

Synthetic antimicrobials are effective food preservatives due to their growth inhibition properties against various bacteria, yeast, and moulds. However, their possible carcinogenic, toxic, and allergenic effects limit their widespread use in food.[27] Furthermore, and more importantly, changes in people's lifestyles in recent years have had considerable impact on their food purchasing behaviour as well as on their eating habits and preferences; therefore, consumers usually prefer to buy minimally

processed products, free from synthetic preservatives.[28,29] Consequently, in recent years, food processors have had a tendency to use natural preservatives in order to both ensure food safety and meet consumer demands. In this respect, Nisin is the most well-known and best-studied antimicrobial additive,[30] and it is the focus of this project.

Synthetic food antimicrobials	
E200-202	Sorbic acid and Potassium sorbate
E210-213	Benzoic acid and benzoates
E214-219	Hydroxybenzoates
E220-228	Sulphur dioxide and sulphides
E239	Hexamethylene tetramine
E242	Dimethyl decarbonate
E243	Ethyl lauroyl arginate
E249-252	Nitrites and nitrates
E280-283	Propionic acid and propionates
E285	Sodium tetraborate
Natural food antimicrobials	
E234	Nisin
E235	Natamycin
E1105	Lysozyme

Table 2 Food antimicrobials approved by European Food Safety Authority (EFSA).[26]

2. General Properties of Nisin

2.1. Structure

Nisin, a cationic antimicrobial peptide composed of 34 amino acid residues, is produced by several strains of *Lactococcus lactis.* It is in the group of Class 1 bacteriocins, also knowns as lantibiotics. It is one of the most famous small lantibiotics (3510 Da), characterized by the presence of the unusual amino acids (lanthionine, β-methyllanthionine) as well as dehydro residues (dehydroalanine, dehydrobutyrine) (**Figure 1**).[31,32] Nisin A, one of the three natural nisin variants, has five internal disulphide bridges and it has an amphiphilic character with a hydrophobic N-terminal and a hydrophilic C-terminal. Nisin Z, the closest natural variant of nisin A, has also similar structure and differs from nisin A by single amino acid residue at position 27; histidine in nisin A and asparagine in nisin Z.[33] Due to the more polar side chain of asparagine compared to histidine, this substitution

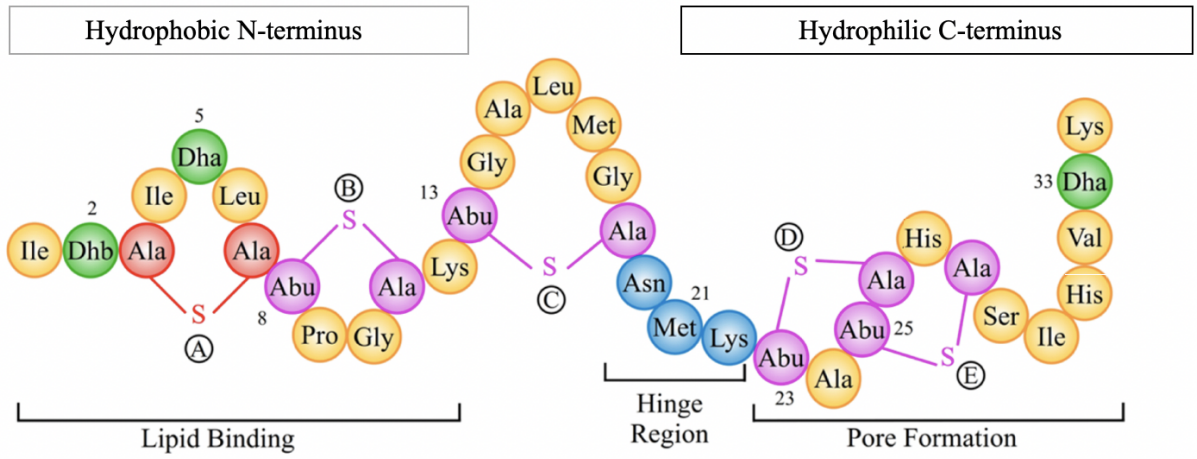


Figure 1 The structure of Nisin, composed of N and C-terminus containing one lanthionine and two (β-methyl) lanthionine rings (A, B, and C), and rings (D and E), linked with a flexible hinge region. Reprinted from reference [37].

The polycyclic-ring structure of nisin plays an important role in maintaining the rigidity of the peptide and protecting it from potential negative factors such as proteolytic enzymes and thermal disruption.[35] Unlike other proteins, Nisin does not contain aromatic amino acids, and therefore it has no absorbance at 280 nm.[36]

2.2.Mode of Action

The antimicrobial action of nisin has multiple character, starting with the binding of positively-charged nisin to the negatively-charged bacteria membrane by electrostatic attraction.[38] Then, the hydrophobic N-terminus of nisin allows interaction with the bacterial lipid II membrane, followed by pore generation in the cell membrane, leading to the ultimate interruption of cell-wall biosynthesis and eventually inhibition or death of the bacteria (**Figure 2**).[39] Due to this lipid II-specific antimicrobial mechanism, nisin usually displays much lower activity against gram-negative bacteria. This is because of the main difference between gram-positive and gram-negative bacteria, the presence of a thin peptidoglycan layer and an outer lipid membrane in the cell wall of gram-negative

bacteria. As such, this layer acts as a protective barrier preventing nisin from reaching the inner membrane and interacting with lipid II.[40] Consequently, nisin has a great inhibitory effect against Gram-positive bacteria species such as *Clostridium perfringens*[41], *Bacillus spp.*[42] and *Listeria monocytogenes*[43] Nevertheless, the combination of nisin with chelating agents (e.g. EDTA and citrate monohydrate) causing destabilisation of the outer membrane has been reported to exhibit remarkable inhibition against growth of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*.[40,44,45]

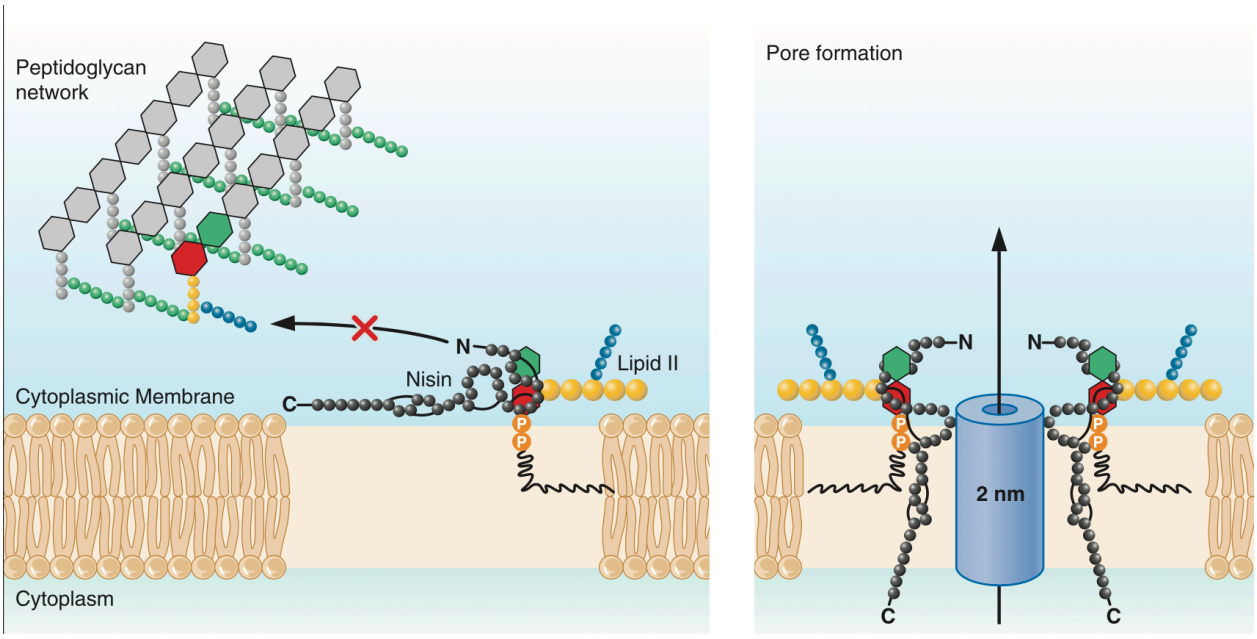


Figure 2 Penetration of the bacterial cell membrane by Nisin. Reprinted from reference [46].

2.3.History of Nisin as a food preservative

Nisin was first identified in 1928 in fermented milk cultures used for cheese production. Initially, although nisin was marketed as an antimicrobial agent for medical and veterinary purposes, it was found non-effective due to its low solubility at neutral pH and its immediate digestion.[47] Following, owing to its digestibility and heat stability at low pH, it came to the forefront as an ideal candidate for a food preservative. In 1951, Hirsch observed that nisin-producing starter cultures were able to prevent clostridial gas formation and that was the first evidence for the use of nisin as food

preservative. Then, in 1969, Nisin was first approved by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) as a food-grade antimicrobial.[48,49]

As a food preservative, nisin is the first bacteriocin introduced commercially, marketed as Nisaplin™, and therefore, it is widely used in food industry. In food manufacturing, there are two main approaches for the application of nisin for food preservation: (i) the use of nisin-producing Lactic acid bacteria as a nisin source, (ii) the direct addition of purified or semi-purified free nisin to food products. The direct addition of nisin as an aqueous solution to a wide range of liquid or solid food products have provided proper antimicrobial distribution in the food and is thus mostly preferred.[50,51] Use of nisin in various products is governed by the FAO/WHO Codex Alimentarius,[52] and the maximum permitted levels of nisin in certain food products are listed in **Table 3.**

Nisin is often used as a preservative in pasteurized food products but not fully sterilised during production, thus protecting the food from outgrowth of spores that can survive after pasteurisation.[47] The first area of use for nisin as a food additive was against clostridial spoilage in processed cheese. In addition, *L. monocytogenes*, *S. aureus, B. cereus* and *Cl. botulinum* are among the Gram-positive bacteria that have been commonly studied to be inhibited by nisin in milk and milk products.[49] Apart from the products listed in Table 3, since nisin does not show antimicrobial activity against yeasts, it has potential to be used in alcoholic beverages like wine to control the growth of Lactic acid bacteria.[53]

Food Category	Max. Level
Flavoured fluid milk drinks	12.5 mg/kg
Processed cheese	12.5 mg/kg
Clotted cream (plain)	10 mg/kg
Liquid egg products	6.25 mg/kg
Edible casings	7 mg/kg
Heat-treated processed meat	25 mg/kg
Cereal and starch-based desserts	3 mg/kg
Ready-to-eat soups and broths	5 mg/kg
Salads	5 mg/kg

Table 3 The maximum permitted levels of nisin in different food categories.[52]

Nowadays, there are strong permissions and legislations emphasizing the safety and applicability of nisin as a food additive. It is in the Generally Regarded as Safe (GRAS) category and has been permitted by the United States Food and Drug Administration (FDA) as a food preservation method for more than 40 years.[12] Additionally, According to Annex II of Regulation (EC) 1333/2008, nisin was recognized as a natural food additive by the European Union for applications in various food categories and assigned the number E234. Consequently, besides great antimicrobial potential, its natural and harmless character have contributed to nisin to become the most famous food preservative around the world, which explains its current and largely use in more than 50 countries.[6] Moreover, nisin has drawn attention in various platforms other than food applications due to its safe and effective antimicrobial properties. For example, it has been explored as an alternative antimicrobial to overcome drug resistance problems, and used in biomedical applications.[48]

Due to all these important characteristics of nisin, almost all mechanisms regarding stability and antimicrobial activity of nisin has been extensively studied and well documented. In this regard, numerous factors influencing the outcome of nisin activity within a food system have been reported in the literature as follows.

2.4.Factors influencing antimicrobial activity and stability of Nisin

Generally, for many food preservatives, their direct introduction into food systems is considered as the main reason for their limited stabilities against possible chemical and physical degradation. In this respect, since the application of nisin in food systems is commonly based on its direct addition, its degradation has been commonly reported in various food systems.[12,54–56] Due to its amphiphilic character, it can be adsorbed on food matrix and food macromolecules and eventually undergo proteolytic degradation. Therefore, the antimicrobials effectiveness and stability of nisin is usually dependent on the type of food in which nisin is used. Particularly, since proteolytic activity can be high in food that had not been pasteurized, the use of nisin in such foods may result in a rapid reduction in the antimicrobial efficacy of nisin. As exemplified by the study of Aasen et al.[8], over 80% of total antimicrobials added (Nisin and Sakacin P) were rapidly degraded in cold-smoked salmon. For instance, the presence of nisin is usually disturbed by glutathione, titanium dioxide, protease and sodium metabisulfite found in meat tissue and therefore, applications of nisin in its free form in meat product have been insufficient to ensure food safety for longer periods. Additionally, the pH of meat products (5.5-5.8 for fresh meat) is not sufficient for the solubility of nisin to show activity at high performance in all meat matrices.[57] Consequently, the use of nisin in meat products is less effective in comparison to other food systems, particularly dairy products.

Surprisingly, although nisin has been largely employed in the dairy industry for many years, it was also reported that the activity of nisin is decreased by interference with milk components. For instance, the negative effect of milk fat on the antilisterial activity of nisin was confirmed by Meena and co-workers.[58] Particularly, in most dairy products that are not pasteurized, or that have been minimally processed, nisin may be degraded by proteolytic enzymes.[47,59]

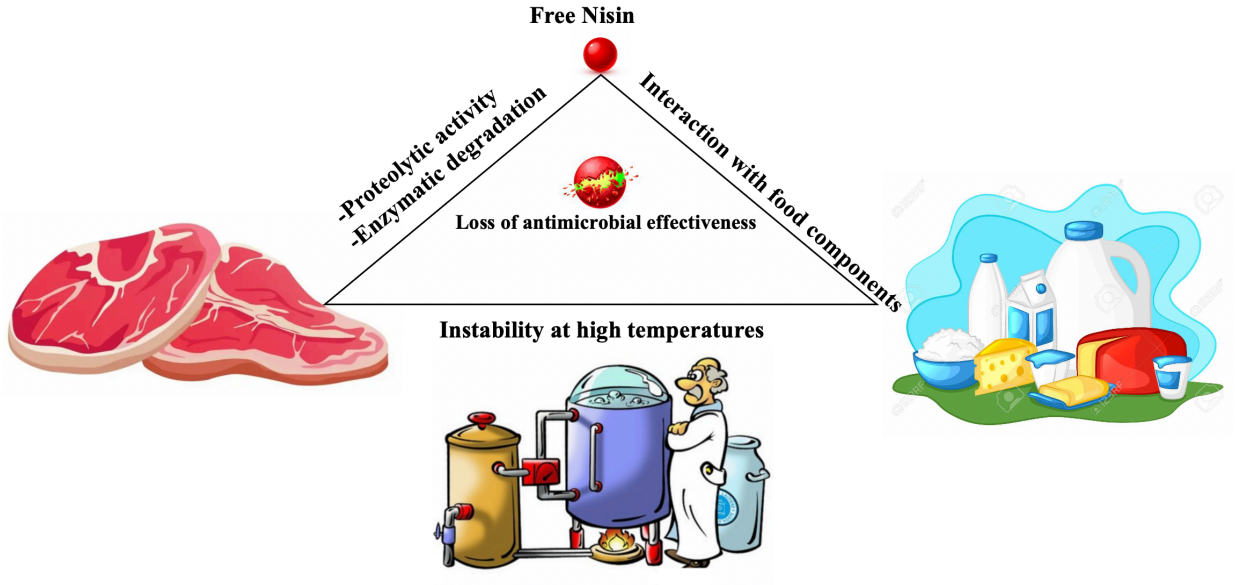


Figure 3 Schematic representation of the factors decreasing the antimicrobial activity of nisin in food.

It has also been reported that nisin's antimicrobial effect is sometimes prevented by a nisin-degrading enzyme (dehydroalanine reductase), nisinase. This enzyme has been reported in *Lactobacillus plantarum, L. lactis susp. lactis, L. lactis subsp. cremoris, Enterococcus faecalis, Bacillus cereus and Streptococcus salivarius subsp. Thermophilus.*[60] Apart from the food-related factors, some external factors influencing the antimicrobial activity of nisin have also been reported. For instance, the antagonist effect of common salt on the sporastatic activity of nisin was observed against *Bacillus spp.*[61], while the addition of NaCI caused an increase in bactericidal activity against *L. monocytogenes*[62].

Although nisin is considered as a heat stable bacteriocin, its inactivation has been reported after heat treatment of food products containing nisin. Generally, the thermal stability of nisin is higher in acidic food products. An example of this pH dependent thermal stability was reported by Delves *et al.*,[63] . In this study, the antimicrobial activity of nisin considerably was lost in food products with low acid (pH 6-7) at the end of the thermal process of 3 min at sterilisation temperature (121°C). Furthermore, losses of up to %40 in the activity of nisin was reported during the cheese (pH 5.6-5.8) production at

variable temperatures between 70-105°C.[6] On the other hand, in another study conducted with liquid eggs having a pH of 7.3-7.8, no temperature-related loss of activity was reported; on the contrary, significant antimicrobial efficacy of nisin was observed after the pasteurisation process. Overall, in comparison to nisin in low acid foods, nisin can exhibit its excellent antimicrobial activity in high acid foods regardless of the temperature application. This is probably because some food products with high pH may provide nisin with good heat stability through their food matrices.

The solubility of nisin is also pH-dependent, and it has been commonly reported that the solubility of nisin is better at low pH. As an example, a considerable decrease was observed in the solubility of nisin upon increasing pH from 2.2 (56 mg/ml) to 6.0 (1.5mg/ml) and 8.5 (0.25mg/ml).[64] Nevertheless, this limited solubility of nisin has not been considered as a major problem in food manufacturing as its max. level in food products usually does not exceed 0.025mg/ml,[6] as could be seen in Table 3 above.

In conclusion, the food preservation performance of nisin is directly dependent on several factors associated with the type of food and food manufacturing processes. Therefore, since the overall goal of this thesis is to improve performance of nisin in food, the main focus of this project will be to protect nisin from the adverse environment and its interaction with food components. Related to this purpose, various nanoencapsulation strategies have been reported to protect nisin in food. Therefore, the current state of the art in the approaches to protecting nisin will be reviewed.

3. Integration of Nisin into Nanostructures

In food systems, encapsulation is an important strategy for the protection of functional molecules such as antimicrobials, flavours, colours, fatty acids, and vitamins from various factors in food systems, such as oxidation, adsorption, precipitation, denaturation, light, temperature, diffusion, and biochemical interactions.[11,65] Generally, compared to the microencapsulation systems, nanometric-

scale encapsulation systems are more effective due to their high surface area, which can increase the distribution and bioavailability of antimicrobials throughout the food. Therefore, the use of nanotechnology to regulate and manipulate nisin for enhanced capacities in the food industry have dramatically expanded over the past decade. Since the direct introduction of nisin into food products is considered as the main factor influencing nisin' efficacy, encapsulation studies at the nanometric scale offer a wide range of approaches to enhance the utility of nisin in the food industry.[10,66,67]

One of the aims of this chapter is to expand the knowledge of the literature by exploring developed nanotech-based carriers of nisin in food. Therefore, and put this thesis in context, an overview of the recent developments in nanotechnology-based delivery systems for nisin in food will be discussed, pointing out the current challenges and limitations in the field. Here, it should be noted that only studies based on the use of nisin in food systems will be reviewed, not the systems developed for antimicrobial packaging and biomedical applications involved. In this regard, there are two approaches that have been frequently applied and proposed to the food sector, lipid-based nanostructures and polymeric nanoparticles (**Figure 4**).

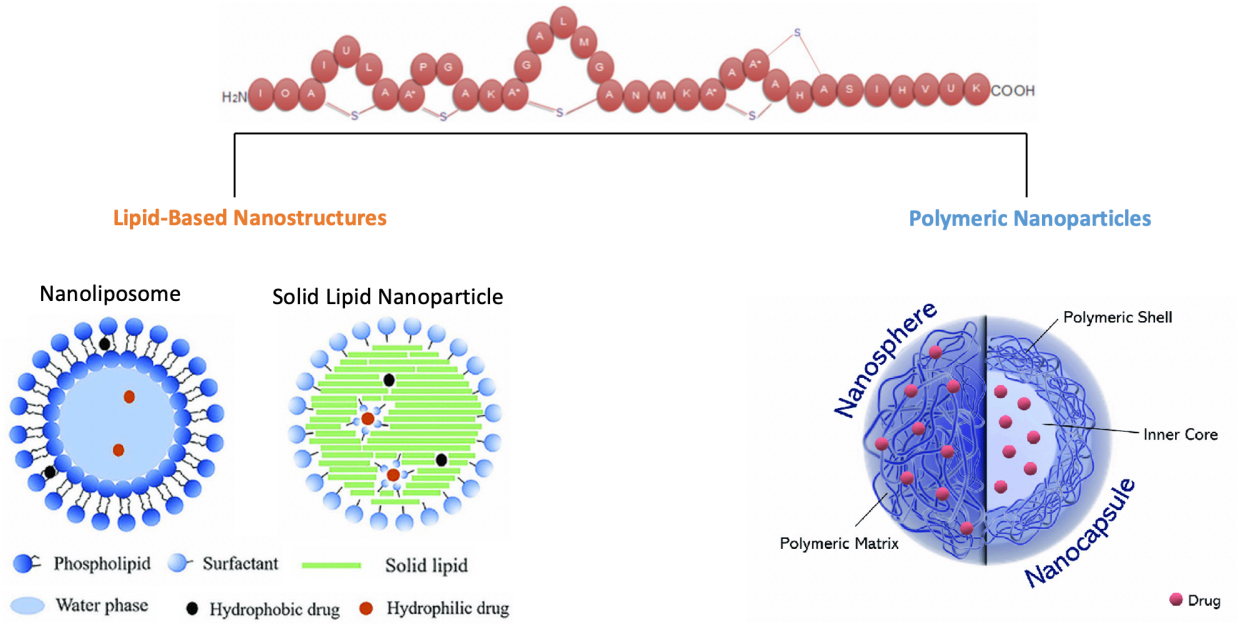


Figure 4 Nanostructures used for the delivery of Nisin in food systems Adapted from reference [68,69]

3.1. Encapsulation of Nisin in Lipid-Based Nanostructures

Firstly**, nanoliposomes**, sphere-shaped small artificial vesicles composed of amphipathic lipids arranged in one or more concentric bilayers with aqueous phase inside and between the lipid bilayers, are among first promising studies on the delivery of nisin and wide range of applications due to their ability to incorporate lipophilic, amphiphilic and/or hydrophilic compounds.[70] Therefore, as an amphiphilic bacteriocin, nisin has been incorporated into nanoliposomes to reduce or prohibit its affinity to food components such as fat and protein surfaces, thereby increasing its accessibility to bacterial cells and eventually its bactericidal efficacy.[71] For example, counts of *Listeria innocua* in cheese milk were kept below the detection limit over the storage a period of 6 months as a result of prolonged antimicrobial activity of encapsulated nisin in hydrogenated phosphatidylcholine.[72] In another study, when compared to free nisin, a better inhibitory effect of partially purified soybean phosphatidylcholine liposome-encapsulated nisin was observed in cheese against pathogenic *Listeria monocytogenes* during storage.[73]

In addition, **solid lipid nanoparticles** (SLNs) are another lipid-based nanomaterial studied in delivery of nisin in food products, composed of solid lipids as a matrix material and suitable colloidal carriers for loading both lipophilic and hydrophilic agents. They showed similar properties with nanoliposome as they also protect nisin from the food environment and prolong its bioactivity. For instance, it was reported that the duration of the inhibitory effect of nisin-loaded SLNs against bacterial growth of *Listeria monocytogenes* (20 days) was considerably longer in comparison to that of free nisin (3 day) (**Figure 5**).[74]

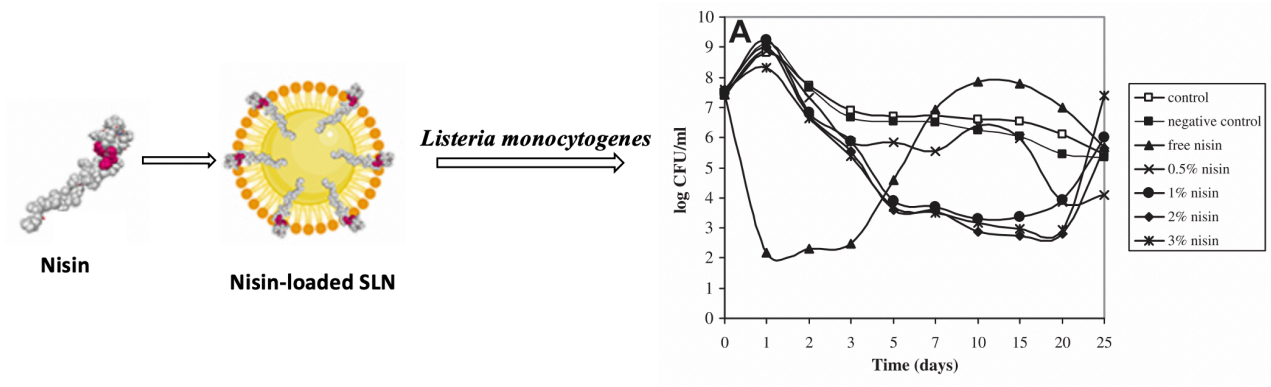


Figure 5 Time-dependent changes in the counts of *L. monocytogenes* incubated with free nisin and nisin-loaded solid lipid nanoparticles (SLN) for 25 days. Adapted from reference [75]

Despite the effective roles of these nanostructures in prolonging nisin's stability and antimicrobial activity, some drawbacks and limitations associated with these encapsulation approaches have been previously revealed. For instance, the interaction of nisin with liposomal membranes has been commonly reported as the main limitation of liposomal delivery approaches, causing membrane disruption.[76,77] On the other hand, the release rate of nisin from these nanostructures showed significant variations during the shelf life of food products. For example, it was reported that the no inhibitory effect of nisin-loaded SLNs against bacterial growth in milk was observed until third day of incubation due to insufficient amount of released nisin from SLNs (**Figure 5**). This finding is a good example reflecting the necessity of a sufficient amount of available (released) nisin in food to maintain the microbiological safety and quality of a food product from production to consumption.

In addition, the high sensitivity of liposomes to external factors such as variations in pH, temperature, and osmotic pressure has been regarded as a main drawback of liposomes. Since decreased stability in the food environment may limit the retention of the encapsulated nisin, the antimicrobial release rate from these unstable nanoliposomes may not be desirable profile to prolong the shelf life of food products. As exemplified in a study conducted by Laridi et al.,[71] a considerable decrease was observed

in the stability of liposomes loading with nisin due to high-fat content in milk, and the quantity of released nisin increased with increasing milk fat content.

Furthermore, low encapsulation efficiency and subsequent antimicrobial release stemming from their limited stabilities have been regarded as another drawback of nanoliposomes.[11] Therefore, some efforts have been paid to increase stability and encapsulation efficiency of nisin-loaded nanoliposomes by coating them with polysaccharides, such as chitosan[78] and pectin[79] (**Figure 6**). In this way, although, the shelf stability and nisin-loading capacity of nanoliposomes were enhanced, the methodologies used were more expensive and more complex, which may not be applicable at industrial level. Consequently, techniques employed in the preparation of nanoliposomes have been mainly considered complex and expensive,[80] which has been one of the main motivations for the development of polymeric nanoparticles for the delivery of nisin in food.

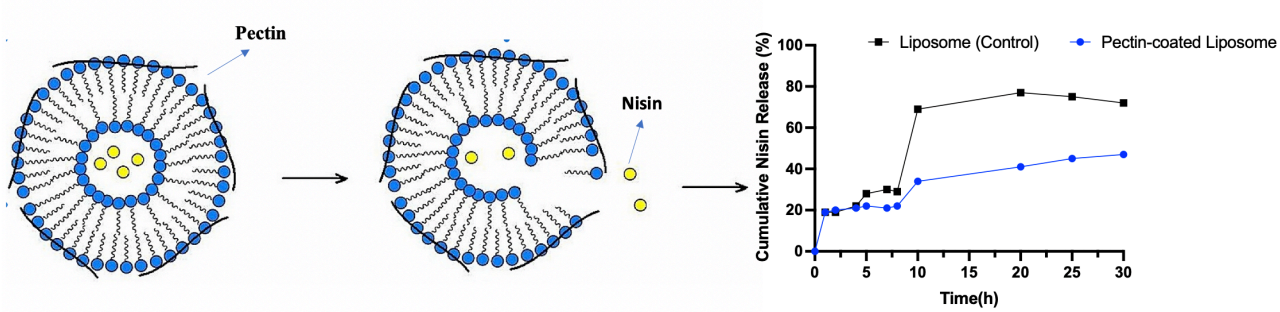


Figure 6 The release profiles of nisin from uncoated (control) and pectin-coated nanoliposome. Adapted from reference [79]

3.2. Delivery of Nisin with Polymeric Nanoparticles

As an important agent of non-covalently assembled structurally organised nanomaterials, polymers have drawn significant attention due to their ability to create void spaces or compartments where encapsulated cargo can be caged to minimise undesired interactions. Therefore, polymers have been

commonly employed for developing nanomaterials, which have significant roles in encapsulating, protecting and delivering nisin in food systems. In this regard, the first example of nisin-loaded polymeric nanoparticles was prepared using poly-L-lactide (PLA),[81] and sustained release of nisin was achieved. However, the employed technique in this study, CO2 anti-solvent precipitation, is not suitable in terms of affordability and safety because it is very expensive, and PLA is not in the GRAS (Generally Regarded as Safe) category.[12] Consequently, researchers have tended to employ food-grade encapsulating materials in nisin delivery systems over the past decade.

As an encapsulating material in the GRAS category, zein, a plant protein biopolymer, has been used to encapsulate nisin by a spray-drying process.[82] However, since this technique is required using an organic solvent that limiting nisin's encapsulation efficiency due to differences in the solubility of protein and the polymer.[12] In addition, a recent study has reported a new nisin-delivery system, zein-modified starch nanoparticles via microfluidic chip.[83] However, encapsulated nisin in these nanoparticles did not exhibit long-lasting antimicrobial activity against *L. monocytogenes* during the storage of cheese samples. Therefore, alternative biopolymers have been employed to access food-safe nisin carrier providing long-lasting antimicrobial activity.

Chitosan, sodium alginate, and carrageenan are three of the most preferred FDA-approved biopolymers in nisin delivery systems. Due to their natural, biocompatible, biodegradable, and non-toxic properties, they played a fundamental role in developing food-grade nanomaterials loading with nisin.[84] Another important property of these biopolymers is their ionisable groups in their monomeric repeating units. Chitosan is a cationic polysaccharide, while sodium alginate and carrageenan are anionic biopolymers.[84] Their charged natures have made them useful polyelectrolytes in cost-effective and bio-safe encapsulation methodologies. As an example, ionotropic gelation is a frequently employed technique to encapsulate and deliver a wide range of bioactive agents.[85] This

technique is simply based on electrostatic activity between oppositely charged polyelectrolytes. When a bioactive molecule is added to the reaction, it can be entrapped between polymeric chains (**Figure 7**). Due to cationic nature of nisin, the encapsulation of nisin with this technique usually requires a combination of chitosan and at least one anionic biopolymer. Therefore, a variety of chitosan-based ternary (i.e. polymer-nisin-polymer) nisin-loaded nanostructures have been developed with this technique as follows.

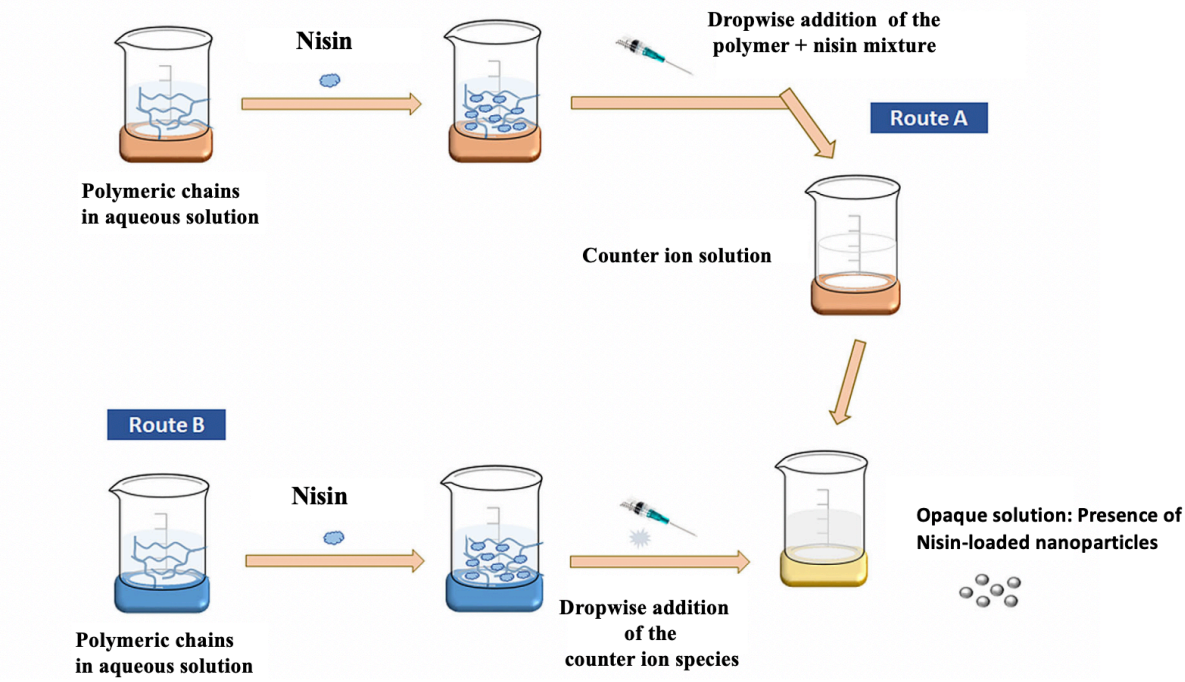


Figure 7 Schematic representation of ionotropic gelation method used for nisin's encapsulation. Adapted from reference [86].

3.2.1. Chitosan-based ternary nanostructures loading with nisin

Besides its excellent properties described above, such as biodegradability and non-toxicity, chitosan represents an antimicrobial character without the polypeptide structure. Moreover, it is an approved food additive in Europe, Japan, Korea, and the United States.[87] Therefore, it has been widely employed in developing nanostructures with enhanced antimicrobial properties for food applications.

Nanocapsules are one example of such nanomaterials, systems which drug is retained in the internal liquid compartment and confined to a cavity surrounded by a polymer membrane. In the literature, the only example of nanocapsules for encapsulation of nisin was prepared as a result of ionotropic complexion of natural biopolymers, chitosan and carrageenan.[88] Since the combination of these oppositely charged polyelectrolytes led to formation an ionotropic hydrogel,[89] nisin was loaded in this inner-liquid compartment, and nanocapsules formed were stabilised by a surfactant (Tween 40). This way, Nisin-loaded nanocapsules demonstrated a significant role in the delivery of nisin in a manner such that the cargo delivered is protected from undesired interactions with the food matrix materials in tomato juice, hence its antimicrobial efficacy was maintained over a prolonged period, up to 6 months. Moreover, in vitro antimicrobial evaluation of these nanocapsules showed that they could effectively inhibit the growth of gram-negative bacteria such as *Salmonella enterica* and *Pseudomonas aeruginosa*, due to synergetic antimicrobial effect of nisin and chitosan.

Further examples of chitosan-based delivery systems have been developed using anionic alginate, and **nisin-loaded nanoparticles** have been synthesised by a two-step protocol, dropping chitosan solution to alginate solution containing nisin.[90–92] In all these studies, it was found that nisin-loaded nanoparticles showed better in-vitro inhibitory effect against foodborne pathogens and spoilage organisms as compared to free nisin. For example, Nisin-loaded chitosan-alginate nanoparticles prepared by Zimet and co-workers[90] caused more delay in the growth of *L. monocytogenes* at 4ºC than free nisin (**Figure 8**). It was also reported that these nanoparticles could show sustained antimicrobial activity during the storage period of various food products, such as milk[92], cheese[91] and lean beef [90]. Moreover, no unwanted sensory changes were not observed in food products upon addition of nisin-loaded chitosan/alginate nanoparticles.[91] Thus, chitosan/alginate nanoparticles have been proposed to the food sector as a promising food preservation method.

Apart from the polysaccharide anionic biopolymers, chitosan was also complexed with poly-y-glutamic acid (y-PGA),[93] peptide-based natural anionic biopolymer. Similarly with chitosan/alginate nanoparticles, chitosan/poly(PGA) nanoparticles showed better in-vitro antimicrobial activity against both gram-negative (e.g. *Escherichia coli*) and gram-positive (e.g. *Staphylococcus aureus*) as compared to free nisin. Importantly, in all these studies, the duration of antimicrobial efficacy of nisin nanoparticles was extended, which is because nisin is slowly released from these nanoparticles.

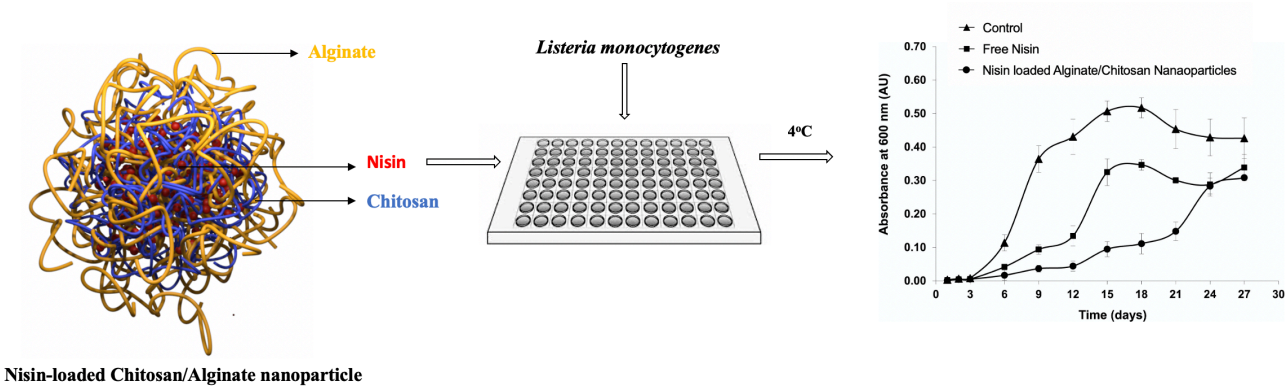


Figure 8 In-vitro antimicrobial evaluation of Nisin-loaded Alginate-Chitosan nanoparticles during the incubation *L. monocytogenes* at 4°C for 30 days. Increases in the absorbance (600 nm) represent bacterial growth. Adapted from reference[90] .

Consequently, regardless of the type of anionic polymer employed in these nanostructures, chitosan has had two fundamental roles in nisin delivery systems; one is its synergetic antimicrobial property showing with nisin. The second is that it increases the stability of nanoparticles, making the release of nisin slower and eventually enhancing the duration of nisin's antimicrobial efficacy.

Although it has been considered that chitosan could led to increase the encapsulation efficiency (EE) of nisin, the EE of nisin has been below 60% in the most of the chitosan-based delivery systems described above. There are few examples of chitosan-based nanoparticles with higher EE values; nevertheless, they required to use of an additional polymer and cross-linking agents for obtaining

nanoparticles with desirable characteristics (i.e. high EE and small size). As an example, nisin-loaded tripolymeric (chitosan-sodium alginate-pluronic F68) nanoparticles with EE of 88% were prepared by iotroponic pre-gelation followed by ionic cross-linking,[56] a relatively more complex system as compared to usual two-step polyelectrolyte complexation. On the other hand, interestingly, there has have considerable differences between EE of nisin in the chitosan-based nanoparticles prepared by same protocols. For instance, EE of nisin in chitosan/alginate nanoparticles was reported by Zohri et al.[92] as at least 90%, while this value was only 36% for another chitosan/alginate nanoparticle system prepared by Zimet et al.[90], prepared by same protocols. Considering the absence of a commercially available nisin delivery system in the food industry, such disagreements could be associated with the necessity of an easier, affordable, and reliable protocol for nisin encapsulation.

In addition, since EE is a critical parameter which may limit the undesired burst release in the dilution media (i.e. food)[94], encapsulated nisin could be released in a desired manner from nanoparticles with high EE, hence bioavailability of nisin could be extended. Consequently, for the purpose of optimizing a nisin delivery system with desirable properties (i.e. high EE and cost-effective) further efforts to encapsulate nisin have recently followed a more straightforward methodology without employing a cationic biopolymer.

3.2.2. Binary nanoparticles loading with Nisin

This approach is simply based on the preparation of Nisin-loaded nanoparticles using only one anionic polymer as an encapsulating material. In this way, nisin has been used as a main cationic polyelectrolyte and electrostatically complexed with an anionic biopolymer (**Figure 9A**). For example, there are only three studies reported within the last 5 years, Nisin-loaded pectin[80], soluble soybean polysaccharide (SSPS)[95], and ulvan[96] nanoparticles. Notably, compared to other nisin-delivery approaches described above, these nanoparticles displayed excellent antimicrobial

encapsulation efficiency; for example, the EE of nisin in pectin, SSPS, and ulvan nanoparticles was 100%, 99.8%, and 100%, respectively. Also, they were able to inhibit the growth of gram-positive foodborne pathogens such as *S.aureus* and *L.monocytogenes.* Overall, besides their desired properties (small size-high EE), mainly depending on their easy and inexpensive preparation method, these nanoparticles have been proposed to the food sector as the most appropriate nisin delivery system for food preservation.

Unsurprisingly, since anionic polymers used in these studies had no antimicrobial property, the inhibitory effects of these nanoparticles against gram-negative bacteria have been lower than that of chitosan-based nanoparticles. Furthermore, the initial in-vitro antimicrobial activity of such nanoparticles is generally lower than free nisin because of the limited availability of encapsulated nisin in the growth media. However, it can be expected that antimicrobial activity of encapsulated nisin lasts much longer as a result of inactivation of free nisin during the storage period of food products. As exemplified by the study of Luo and co-workers[95], although antimicrobial activity of Nisin-SSPS nanoparticles caused less growth inhibition against *S. aureus* and L. *monocytogenes (***Figure 9B***)*, these nanoparticles displayed more long-lasting food preservation performance in tomato juice as compared to free nisin at the same concentration (**Figure 9C**).

On the other hand, although it has been thought that the use of composite biopolymers (e.g. chitosan/alginate) are more effective of limiting the release of encapsulated bioactive,[97] the release profile of nisin from binary nanoparticles has been similar with chitosan-based ternary nanoparticles. For instance, as mentioned above, Nisin-SSPS nanoparticles displayed considerable potential to extend the antimicrobial efficacy of nisin due to their slow and sustained release properties in tomato juice (**Figure 8C**).[95] However, no further attention has yet to be paid to investigate the antimicrobial

release properties of these nanoparticles during shelf-life of food products in which nisin is frequently applied, such as dairy products.

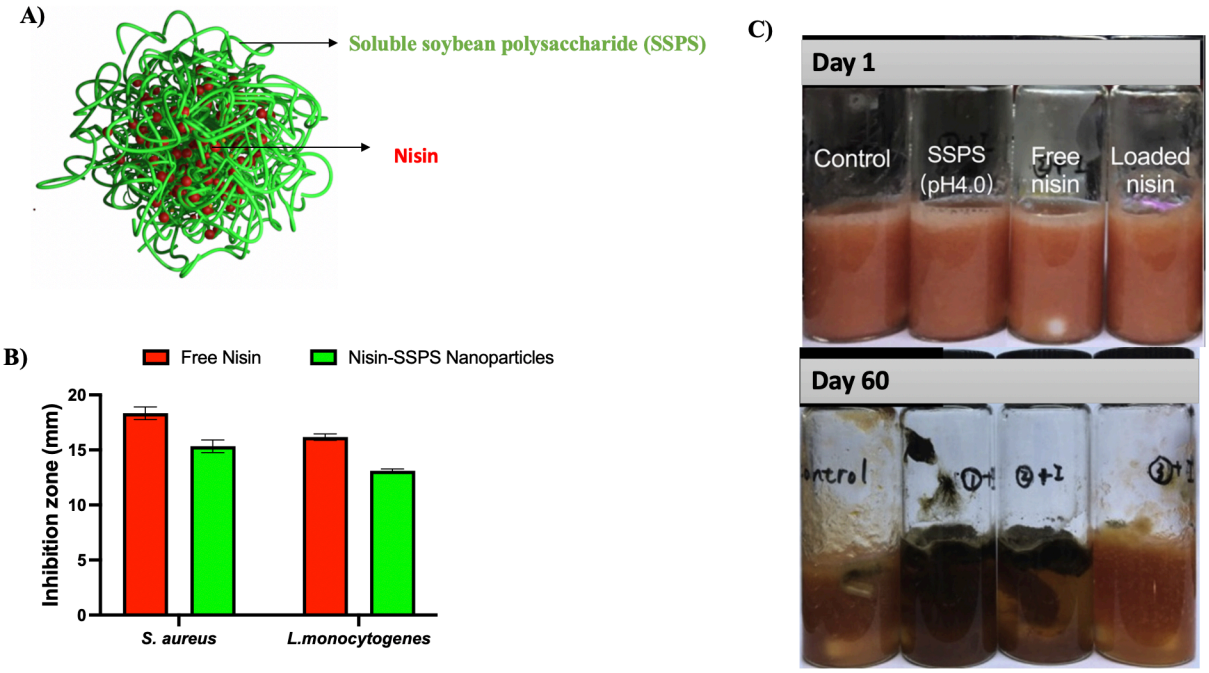


Figure 9 Encapsulation of Nisin using soluble soybean polysaccharide (SSPS) (A), in-vitro antimicrobial activity of Free Nisin and Nisin-SSPS nanoparticles (B), antimicrobial activity of free nisin, SSPS (polymer), and Nisin-SSPS nanoparticles during the storage of tomato juice for two months. Dark colour in the samples caused by bacterial putrefaction. Adapted from reference [95]

Overall, all delivery systems highlighted in this chapter have had the potential to prolong the antimicrobial efficacy and stability of nisin, despite the differences in their components (lipid, cationic/anionic polymer), techniques and costs. This is because encapsulated nisin is slowly released from these nanostructures, hence it is less affected from its undesired food interactions or enzymatic degradation in food systems in comparison to free/unencapsulated nisin. This unspecific release of bioactive agents from nanoparticles is also called **passive release** mechanism.[98] In particular, passive release of encapsulated drug in biopolymeric complex nanoparticles is mainly attributed to the swelling or slow degradation of encapsulating matrix, passive diffusion, and charge-charge interactions.[93] As such, nisin is continuously released in food systems, even in the uncontaminated food or products being kept at a safe temperature range. As a result, passively released nisin ultimately

may lost its effectiveness before possible contamination and eventually, microbial food safety and quality problems may occur.

To overcome this potential disadvantage of passive delivery systems, novel delivery approaches could play an important role in rapidly triggering the release of nisin from nanostructures in times of need, such as in the presence of serious pathogens. In this regard, **stimuli-responsive nanomaterials** are an excellent example of such delivery systems, and they are usually considered the most efficient and effective way of antimicrobial delivery against pathogenic organisms. In these systems, the release of encapsulated bioactive could be accelerated in response to specific stimuli which can be endogenous (e.g. pH and enzyme) or exogenous (e.g. temperature and light).[99–101] This approach has mainly been followed for the delivery of antimicrobials in biomedical applications, whereas it has never been applied for food antimicrobials, including nisin. Therefore, one of the main goals of this thesis is to develop novel carriers capable of accelerating nisin release by a stimuli-responsive delivery mechanism.

In conclusion, in the light of all these highlighted advantages, disadvantages, and limitations of existing nisin delivery systems, it can be seen clearly that complexation of cationic nisin with anionic polymers is the simplest and the cheapest encapsulation methodology. Nevertheless, the food industry has yet to create a commercially available nisin delivery system. Therefore, the use of alternative food-grade polymers for nisin's encapsulation and further antimicrobial evaluation of this type of nanoparticles in real food systems could provide a deeper understanding of their performance as a food preservative. On the other hand, since there is no reported study for the stimuli-responsive delivery of nisin in food systems, novel nano-carriers designed for this purpose could be game changers in food preservation. In order to fulfil these gaps either in the literature or in the food industry, nisin-loaded nanoparticles prepared by new anionic polymers were selected as an easy and cost-effective platform in this project.

Since this type of particles formed upon electrostatic complexation between cationic peptide or polymer and anionic polymer are called **PolyIon Complex (PIC) nanoparticles**,[102] from now onwards, this nanoplatform will be called as PIC nanoparticles in this thesis. In accordance with the context of this chapter and the aim of this thesis, upon the brief introduction of this nanoplatform, the current state of the art in the PIC nanoparticle-based passive and stimuli-responsive antimicrobial delivery systems will be reviewed. Finally, the applicability of PIC nanoparticles for developing novel nisin delivery systems will be discussed.

4. Polyion Complex (PIC) Nanoparticles: As a passive and responsive antimicrobial carrier

Due to the important properties of polyelectrolytes in dictating physical properties of biopolymers and mediating relevant biological processes, researchers have tended to use charge-charge interactions in novel platforms for the stabilisation and delivery of drugs and proteins. In this regard, coacervates are dense spherical aggregates, which can stem from associative phase separation in colloidal systems. For example, in polyelectrolyte systems, coacervates can be formed as a result of electrostatic associative interaction, leading to the formation of a matrix system having a loadable vesicular space for the delivery of biomolecules in an aqueous medium.[103] This complex structure formed between at least two oppositely charged polyelectrolytes is known as polyionic complexes (PIC)[102], a.k.a polyelectrolyte complexes (PECs)[104] or interpolyelectolyte complexes (IPECs) [105].

These complexes can present different morphologies depending on the topology of the polyelectrolytes used and the way these are assembled. For instance, nanogels consist of highly hydrated and cross-linked polyelectrolyte networks,[106,107] while complexation of ionic-hydrophilic block copolymers lead to formation of PIC micelles[108]. According to the types of components used in complexation, polymer-polymer[109], polymer-drug-polymer[110], and polymer-drug[111] complexes are

types of PIC particles which have been frequently studied for drug delivery and biomedical applications.

As a self-assembly system, PIC nanoparticles (drug-polymer type) are simply formed by non-stoichiometric combinations of a charged biomolecule with an oppositely charged polymer, usually consist of two parts; a neutral core and a shell formed by the excess polyion chain (**Figure 10**). The charged corona plays a fundamental role in the protection of nanoparticles from irreversible aggregation by repelling other PIC particles in solution.[112,113] In this way, a charged bioactive desired to be encapsulated can be easily entrapped to the neutral core of PIC nanoparticles. Thus, it can be stabilised by eliminating its undesired interactions in a solution. Therefore, the colloidal stability of PIC nanoparticles can be tuned by simply adjusting the relative content of polyelectrolytes.

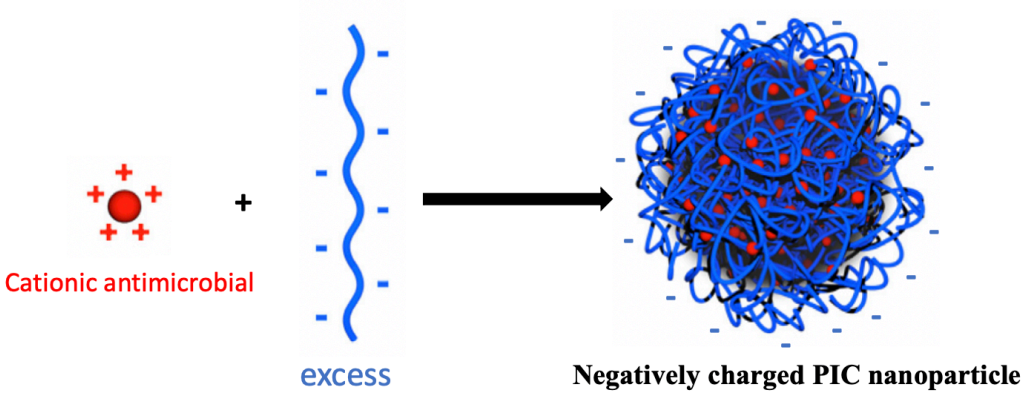


Figure 10 Assembly of negatively charged PIC nanoparticles loading with cationic antimicrobial in excess of anionic polymer

Considering optimization of a PIC nanoparticle system, in the literature, important factors that need to be optimised during the development of a PIC nanoparticle system such as density of the charges on the polyelectrolytes (PELs), the duration of the interaction, the concentration of the PELs, their mixing ratio, mixing order, ionic strength, and pH of the reaction medium have been well-

documented and well-understood. In this regard, the reader is referred to a specialised literature previously published by our group for more information about PIC particles[114].

While encapsulating a biomolecule, since it is important to maintain its bioavailability in the system, the character of the encapsulating component is a key in creating a delivery system providing a desired release profile from PIC particles. In this regard, PIC particles can be examined by dividing them into two approaches according to their release properties. The first approach is to use PIC particles as a **passive nano-delivery system**. This type of PIC nanoformulation involves anionic polymers which are generally intended to be used solely as carriers for the delivery of cationic antimicrobial peptides. Generally, most of those are polyelectrolytes degrading at a slow rate, water-soluble, biodegradable, without pharmacological and functional properties and therefore, their roles in PIC particle systems are often passive/structural.[115] For instance, nanoparticles formed by anionic poly(glutamic acid)[116] and poly(styrene sulfonate)[117] for the delivery of cationic antimicrobials ,amphotericin B and polymyxin B respectively, are good examples of PIC particles with passive delivery mechanisms. These nanoparticles exhibited promising potentials in both reducing the toxicity of cationic antimicrobials and releasing antimicrobials depending on their stabilities in physiological conditions.

In passive delivery systems, since the stability of PIC nanoparticles is a critical parameter affecting to antimicrobial release rate, the character of excipient polyelectrolyte (i.e. pharmacologically inactive) have important role in controlling the stability and eventually antimicrobial activity of PIC nanoparticles. In this regard, it was reported that antimicrobial activity of Pol-B-loaded PIC nanoparticles prepared with PSS of high Mw was lower than that of low Mw against *Pseudomonas aeruginosa* due to higher stability of Pol-B-PSS (high-Mw) PIC nanoparticles under the same

conditions (**Figure 11**).[118] Therefore, the antimicrobial activity of PIC nanoparticles can be simply adjusted depending on the degree of polymerisation of the inert component.

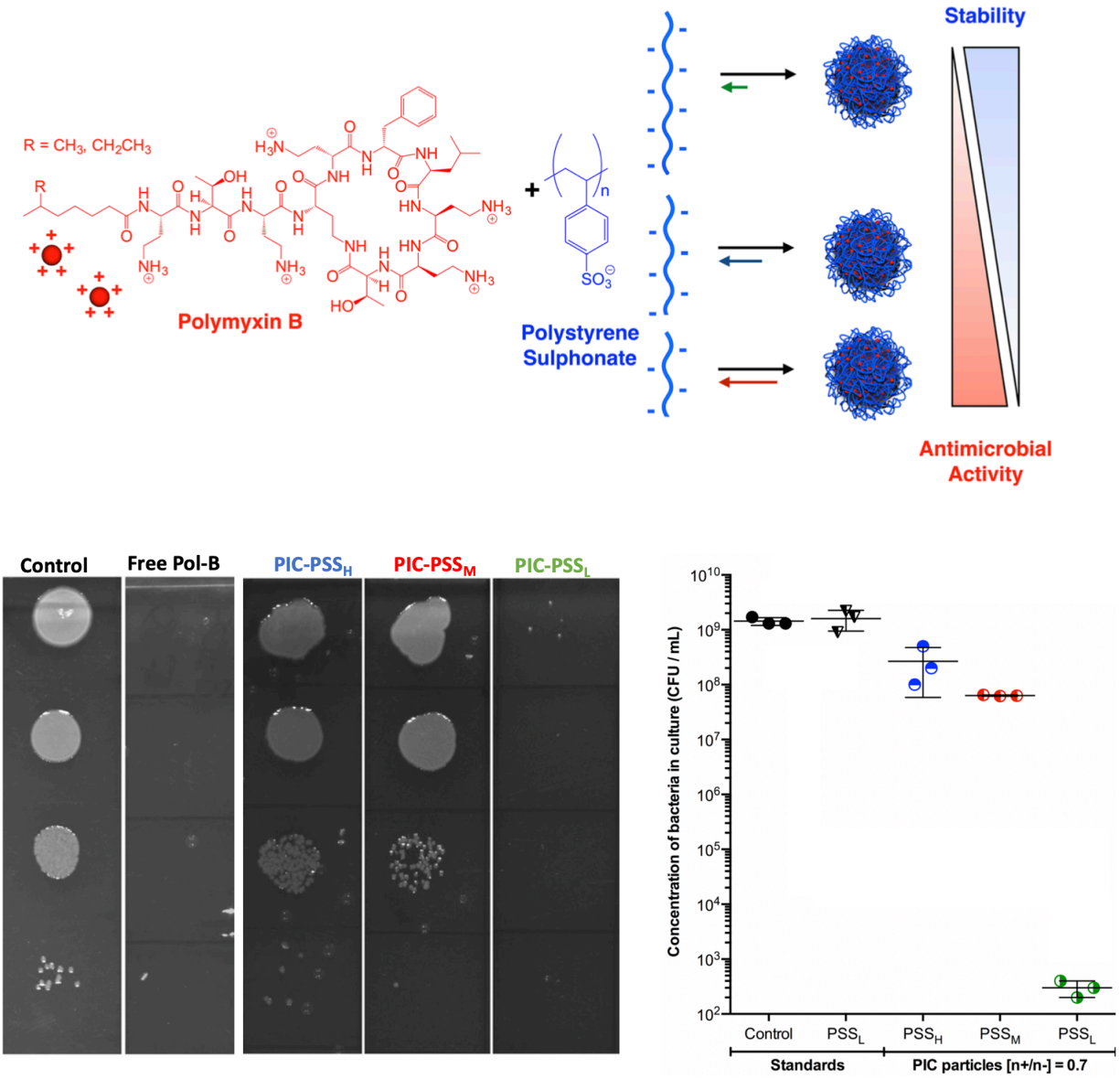


Figure 11 In-vitro antimicrobial activity free and polymyxin B-loaded PIC nanoparticles prepared from poly(styrene sulphonate) PSS with high (PSS_H), medium (PSS_M), low Mw (PSS_L). Representative agar plates represent the concentration of bacteria (*P. aeruginosa*) in the growth media treated by free and Pol B-loaded PIC nanoparticles after incubation of 24 h. Reprinted from reference [118]

Apart from the passive release properties of PIC particles, physicochemical characteristics of the components comprising this nanomaterial system have led to the development of functional PIC particles able to promote triggered release of the loaded antimicrobials, which is the second antimicrobial delivery approach and known as **stimuli-responsive PIC particles.**[119,120] This approach usually attempts to deliver antimicrobials in a smart and environmentally sensitive manner, which involves delivering antimicrobials unchanged to the target site of action at the right quantity, time and duration. In this regard, the stimuli-responsive feature of PIC nanoparticles has allowed the development of effective antimicrobial release systems in response to exogenous (e.g. temperature) or endogenous stimuli (e.g. enzyme and pH).

Firstly, since the formation of PIC particles is a process based on charge-charge interaction, **pH-responsiveness** is an inherent property of PIC delivery systems depending on the ionisable functional groups of the masking component in the PIC particle formulation. More specifically, since the charge density of polyelectrolytes is commonly dependent on the pH of the system in which PIC particles are found, pH-related disassembly of PIC particles has led to the development of pH-responsive PIC formulation, playing active role in delivery of antimicrobial agents.[21] For example, in a study conducted by Giannotti et al.,[121] pH-associated release of lysosomal enzyme a-Galactosidase A (a-Gal A) from *N*-trimethyl chitosan-a Gal A PIC particles at acidic pH was achieved as a result of disassembly of these particles at acidic pH values (4.5-5.5), which is directly related to neutralisation of a-Gal A stemming from changes in pH towards to its isoelectric point (5.7). In another study conducted by Cerchiara and co-workers,[122] PIC nanoparticles were designed by positively charged chitosan and negatively charged carboxymethylcellulose for the delivery of antibiotic vancomycin. This formulation played an important role in protecting positively charged vancomycin from its degradation and absorption in the stomach (pH:2); thus, the release was triggered in the colon (pH7.4) in which vancomycin shows antimicrobial activity against gastrointestinal pathogens.

Additionally, ternary PIC nanoparticles (polymer-drug-polymer) can be designed to deliver encapsulated drugs in a dual-responsive way. This drug delivery approach has been mainly employed in cancer treatments. For example, in a study conducted for the oral delivery of paclitaxel, PIC nanoparticles were prepared by cationic chitosan and anionic hyaluronic acid (HA) conjugated with PTX.[123] In this way, these PIC nanoparticles remained stable in the stomach's acidic environment (pH:3), while PTX was released due to the disassembly of PIC particles at a tumour cell pH of 7.4. Furthermore, since hyaluronidase is commonly found in acidic tumour matrix, degradation of HA by this enzyme caused triggered release of PTX. Consequently, this PIC nanoparticle system exhibited both pH-responsive and enzyme-responsive drug release properties.

Additionally, polymers designed for specific cleavage by an enzyme originating from a specific pathogen could play a pivotal role in the effective delivery of antimicrobial agents. For instance, certain hydrolytic enzymes (e.g. proteases and glycosidases) have been employed for prodrug delivery because they can cleave various types of natural biopolymers or synthetic amino acid sequences. This release behaviour is also called as targeted delivery [99], and a good example of this system developed by our group[124]. In this study, **enzyme-responsive** antimicrobial delivery was achieved through a polymer-polymer-based PIC particle system consisting of antimicrobial poly(ethyleneimine) (PEI) and a synthetic, selectively degradable anionic peptide by LasB, a virulent factor released by *Pseudomonas aeruginosa* during infection (**Figure 12**). These nanoparticles exhibited an enzyme specificity and therefore, they were not degraded by human elastase or non-virulent strains of *P. aeruginosa.* Consequently, these nanoparticles had a remarkable potential not only for the localised delivery of antimicrobials in the presence of relevant organism, but also reducing the possible toxicity of cationic antimicrobials derived from their undesired interactions with healthy organs.

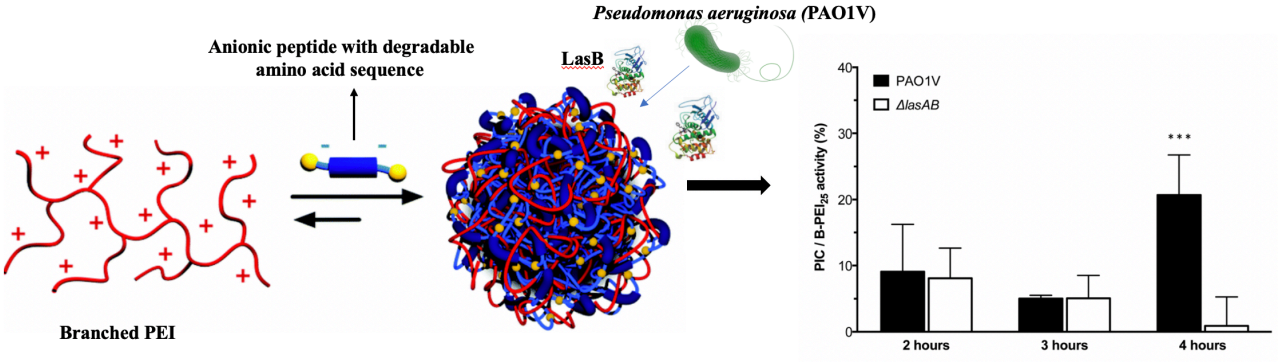


Figure 12 Enzymatic disassembly of PIC nanoparticles formed by the complexation of cationic antimicrobial PEI and an anionic peptide selectively degradable by LasB of *Pseudomonas aeruginosa.* PAO1V is the LasB producing strain, while *ΔlasAB* is the LasB-deletion strain of *P. aeuruginosa* Reprinted from reference [124]

Generally, since most of the natural polyelectrolytes commonly used in PIC formulations do not induce a considerable responsive activity against variations in the environment in which PIC particles are found, except for pH, researchers have been interested in the development of responsive PIC nanoparticles through a combination of natural biopolymers with environmentally sensitive polymers. As an example of environmentally sensitive materials, polymers exhibiting a lower critical solution temperature (LCST) have been widely used in various drug delivery systems. Such polymers are soluble below a critical temperature, while they are insoluble above that temperature.[125] Therefore, when they are implemented in drug delivery systems, their thermosensitive properties can be transferred to drug carriers and therefore, drug release can be triggered as a result of temperature-related structural changes (e.g. shrinkage).[126]

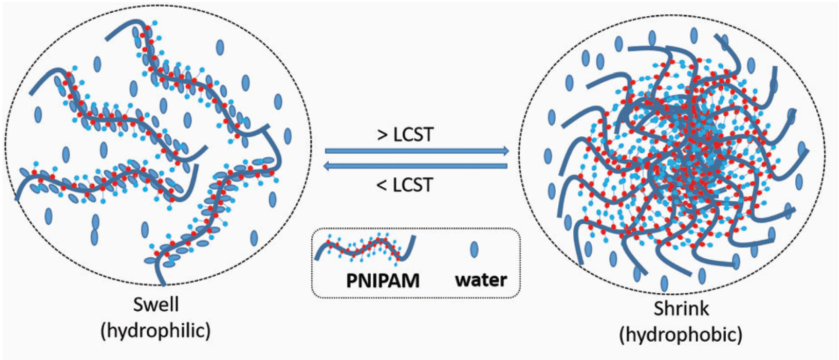


Figure 13 Graphical representation of thermoresponsive LCST behaviour of PNIPAAm. Reprinted from reference [127].

In this respect, Poly(*N*-isopropylacrylamide) or PNIPAAm, a synthetic polymer exhibiting LCST above 32°C, is the most closely studied polymer employed in thermoresponsive drug delivery applications (**Figure 13**).[128–130] For example, a ternary type of PIC nanoparticle system having a thermoresponsive character was synthesized by complexation of chitosan and sodium alginate grafted by Poly(*N*-isopropylacrylamide). In this study, drug-loaded PIC nanoparticle was stabilised by the hydrated PNIPAm chains below LCST of PNIPAAm, while drug release was considerably higher upon increasing the temperature from 25°C to 37°C (**Figure 14**). This result showed that these PIC nanoparticles delayed the release of the drug below LCST of PNIPAm, while drug release kinetics from these PIC nanoparticles was triggered as a function of temperature above its LCST.[131]

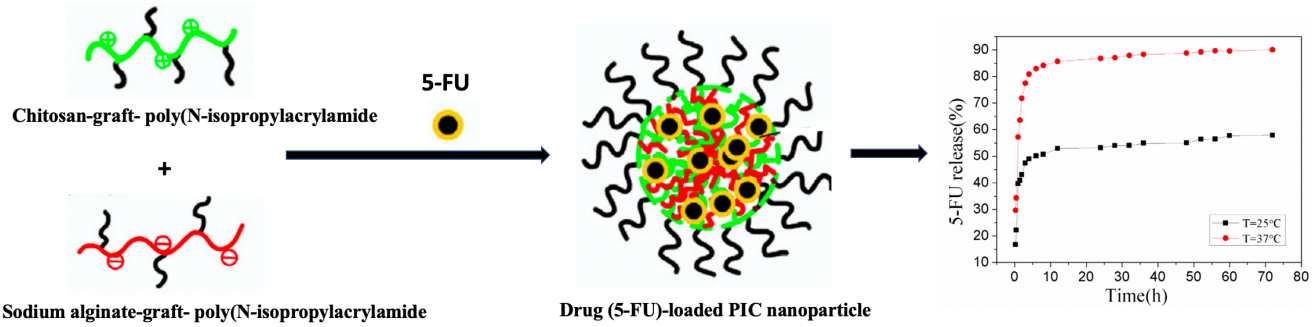


Figure 14 Assembly and drug release profile of drug-loaded PIC nanoparticle formed by cationic chitosan and anionic sodium alginate grafted by PNIPAAM. Reprinted from reference [131] .

In the light of all these provided information and examples, when considering the encapsulation and delivery of cationic nisin in food systems, PIC nanoparticles may offer important advantages that make them potentially attractive nisin carriers. Due to cationic nature of nisin, PIC nanoparticles can be simply assembled from the complexation of nisin with an anionic polymer; thereby, new polymers can be employed to access novel nanoparticles loading with nisin. Secondly, the size, charge, colloidal stability, and nisin-loading capacity of PIC nanoparticles may be simply optimised by adjusting the mixing ratio of polyelectrolytes. For instance, negatively charged PIC nanoparticles

loading with nisin can be prepared in excess of anionic polymer and thus, undesired interactions of nisin with negatively charged food ingredients (e.g. casein in dairy products)[132] during food storage may be prevented.

In addition, the selection of anionic polymer may have a fundamental role in adjusting the type of antimicrobial release profile in food systems. Firstly, to obtain a passive antimicrobial delivery system in food, characteristics of the encapsulating component can be exploited to obtain nisin-loaded PIC nanoparticles with different stabilities and antimicrobial activity. As such, since the stability of PIC nanoparticles prepared from a polymer with high Mw may increase the retention of antimicrobial cargo, stable nisin-loaded PIC particles may be prepared by employing an anionic polymer with high Mw. In this way, Nisin-loaded PIC nanoparticles may initially present at least an intermediate stability profile when introduced into the food systems to avoid a possible high premature release, which decreases the bioavailability of nisin in food systems. Consequently, such PIC nanoparticles may present prolonged release of nisin and eventually extended antimicrobial efficacy in food systems as compared to less stable encapsulation systems and free nisin.

On the other hand, in order to develop a PIC nanoparticle formulation for responsive antimicrobial delivery, firstly, specific conditions in which antimicrobial release is required should be determined. Then, a functional encapsulating polymer should be chosen or designed according to these conditions. For example, food products are an inherently excellent environment for the growth of various pathogens that can produce enzymes and show different temperature-dependent growth profiles, leading to food safety as well as quality problems. Therefore, from the microbiological food safety perspective, responsive antimicrobial (i.e. nisin) release could be valuable in response to the growth of undesired pathogens in food and abnormal increases in the storage temperatures of food products.18 With this in mind, an anionic polymer which is degradable by a pathogen-associated

enzyme or an anionic polymer with a thermosensitive character can be employed to prepare novel PIC nanoparticles loading with nisin. Considering that there is no reported study based on the responsive delivery of nisin in food systems, these nanoparticles could be the first examples of responsive antimicrobial delivery systems in food, thereby fulfilling the main gap in the literature**.**

5. Conclusions

Improving the performance of nisin is of great importance for the prevention of microbial food safety and quality problems. To do this, nanoencapsulation of nisin is the most effective way to enhance its antimicrobial effectiveness during the storage of food products. Encapsulation approaches discussed in this chapter generally provide various nanostructures protecting nisin from its undesired interactions and delivering it in a sustained manner in food systems. Due to the absence and potential commercial opportunity of an effective nisin carrier in the food industry, over the past decade, there has been an increasing trend towards the development of food-safe, cost-effective, easy-to-prepare nisin delivery systems. In this regard, in terms of affordability and scalability, the complexation of cationic nisin with an anionic polymer is the most suitable methodology to encapsulate nisin in polymeric nanoparticles; in other words, nisin-loaded PIC nanoparticles. However, there are few studies following this approach, and more attention needs to be paid to investigating the antimicrobial activity of such nanoparticles while storing food products. Therefore, new anionic polymers could be exploited to access new, cost-effective nisin-loaded PIC nanoparticles, and their antimicrobial activities could be monitored in relevant food systems with nisin use.

Additionally, responsive delivery mechanism systems can play an important role in either protection of nisin or the achieving rapid antimicrobial release, thereby preventing possible rapid microbial growth in food. However, the lack of a responsive nisin carrier in food systems is the main gap in the literature. In the previous section, the feasibility of PIC nanoparticles for the development of

responsive nisin delivery system in food has confirmed .Therefore, one of the milestones of this thesis will be to design novel PIC nanoparticles capable of delivering nisin in response to the presence of specific foodborne pathogens or abnormal increases in the storage temperature of food products.

6. Objectives

This thesis is centred around the development of Nisin-loaded PIC nanoparticles using anionic polymers that have never been employed for the delivery of food antimicrobials (**Figure 15**). Firstly, to prepare one of the most affordable nisin delivery system, using a cheap, food-grade (in GRAS category), and commercially available biopolymer will be employed (Chapter 2). Critical parameters affecting PIC nanoparticle formation (see reference[133]) will be optimised to access promising (i.e., small size, low polydispersity, high encapsulation efficiency, good colloidal stability) PIC nanoparticles. Finally, sustained antimicrobial activity properties of these nanoparticles will be investigated to monitor their long-term antimicrobial properties against the growth of foodborne pathogenic (i.e. *Staphylococcus aureus*) and spoilage bacteria (i.e. *Bacillus subtilis*) in a real food product in which nisin is widely used (i.e. milk).

In addition, PIC nanoparticles will be exploited to develop novel antimicrobial delivery systems in food by using an enzyme-degradable polymer to encapsulate nisin (Chapter 3). For this purpose, an anionic polymer (i.e. Hyaluronic acid) degradable by specific foodborne pathogens (e.g. *S. aureus*) will be employed an encapsulating agent. In this way, the release of nisin will try to be triggered in food in the presence of these pathogens. Firstly, enzyme (i.e. hyaluronidase) susceptibility of PIC nanoparticles will be explored by in-vitro nisin release studies. Then, the responsive antimicrobial activity of these nanoparticles will be evaluated in the absence and presence of target foodborne pathogens (e.g. enzyme-producing *S. aureus*) in microbiological growth media and food product relevant with the contamination of target pathogens.

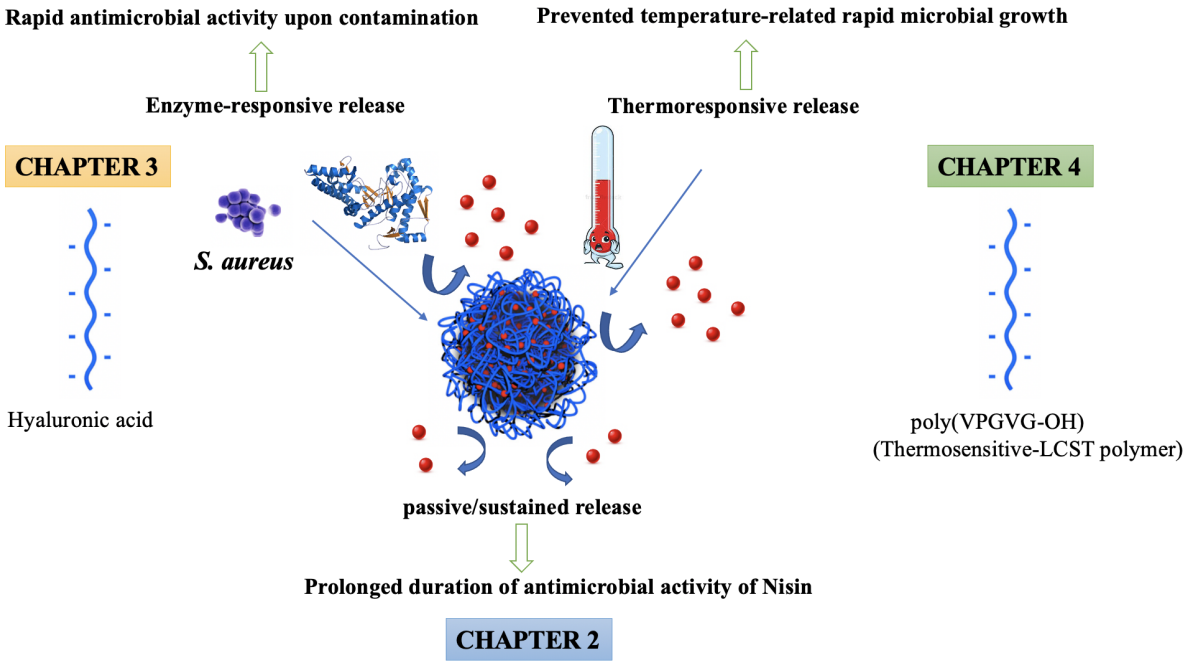


Figure 15 Schematic representation of Nisin-loaded PIC nanoparticles capable of displaying passive, enzyme-responsive, and thermoresponsive antimicrobial release properties in food systems

Finally, an anionic thermosensitive polymer exhibiting LCST behaviour will be synthesised to encapsulate nisin and provide PIC nanoparticles with thermoresponsive character (Chapter 4). Characterisation of these nanoparticles will be done at different temperatures to understand their thermoresponsive behaviour, and thermoresponsive domain (i.e. transition temperature). Thereafter, the rational design of the antimicrobial activity experiments will be designed according to thermoresponsive behaviour of these nanoparticles. For this purpose, antimicrobial evaluation of these nanoparticles will be carried out mimicking temperature abuse during storage of a model food system against relevant foodborne organisms (i.e. *Clostridium perfringens*) capable of proliferating above these nanoparticles' thermoresponsive domain.

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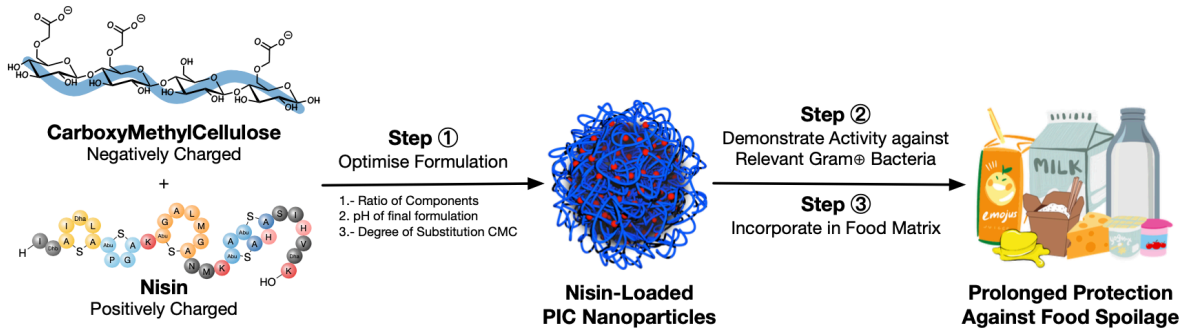
CHAPTER 2

Antimicrobial Activity of Nisin:Carboxymethylcellulose PIC nanoparticles

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Overview: In this Chapter, we present our first attempt to prepare nisin-loaded PIC nanoparticles. Considering that a cost-effective, food-grade and effective nisin delivery system is necessary and could create a commercial opportunity in the food industry in the near future, we decided to start our investigations using Carboxymethylcellulose, a natural, food-safe (in GRAS category), commercially available, cheap anionic polysaccharide. PIC nanoparticles were assembled from this anionic polymer and cationic nisin following a straightforward methodology based on electrostatic interactions at low ionic strength under mild conditions.



These nanoparticles were characterized in terms of their size, charge, encapsulation efficiency, and morphologies. Polyelectrolyte concentrations and pH were explored to mediate electrostatic activity between nisin and CMC and thus obtain nanoparticles with higher encapsulation efficiency. Also, as an important parameter affecting CMC's charge density, different types of commercially available CMC with various degree of substitution (DS) values

allowed us to increase the encapsulation efficiency of nanoparticles. Overall, protocols, techniques, and observations derived from this study contributed to creating and expanding our expertise on the preparation of PIC nanoparticles loading with nisin.

In addition to characterisation and optimisation steps, the paper presented in this chapter covers the study of antimicrobial evaluation of Nisin:CMC PIC nanoparticles, carried out in a microbiological growth media and in milk. Due to their passive release properties, these nanoparticles displayed limited in-vitro inhibitory effect against food spoilage organism *Bacillus subtilis* in the short term. Finally, a controlled contamination experiment designed in this study revealed that these nanoparticles could show sustained inhibitory effect against foodborne pathogen *Staphyloccocucs aureus*. To sum up, this study has provided a new nisin-loaded PIC nanoparticle system that can be employed in the food industry as a cheap and effective food preservation method.

Contributions: T.C. and P.F.-T. conceived the project and designed the nanoparticle preparation and characterization. T.C., C.A, H.O. and T.M. designed the antimicrobial experiments. P.F.-T., H.O. and T.M. secured funding. T. C. performed all experiments. T.C. and P.F.-T. analysed the data and wrote the paper, with all other authors contributing to the final version of the manuscript.

Antimicrobial Activity of Nisin:Carboxymethylcellulose PIC Nanoparticles

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Abstract

Nisin is a cationic antimicrobial peptide used as a natural food preservative against gram-positive bacteria. However, interactions with food components make the widespread use of Nisin in the food industry limited. In the present work, we report the first use of Carboxymethylcellulose (CMC), a food additive commonly used as a viscosity modifier and stabilizer, to protect Nisin from its undesired interactions in food and extend its antimicrobial activity. First, we report the synthesis of Nisin:CMC PIC nanoparticles following an easy methodology and optimize them considering the effect of various parameters including polyelectrolyte concentration, pH and degree of substitution. In particular, we evaluated how these parameters affected the size, charge and, more importantly, the encapsulation efficiency of these nanomaterials. Using this methodology, we have been able to prepare set of negatively charged Nisin:CMC PIC nanoparticles with over 80% encapsulation efficiency and spherical morphology. Then, we demonstrate that the prepared Nisin:CMC PIC nanoparticles show an inhibitory effect *in-vitro* against *Bacillus subtilis*, a representative gram-positive microorganism commonly found in food products. Finally, a controlled contamination experiment was performed to evaluate the antimicrobial activity of these nanomaterials against *Staphylococcus aureus*, a major foodborne pathogen, using milk as a representative natural food system. Our results suggest that these Nisin:CMC PIC nanoparticles can extend the antimicrobial effect of this food preservative, reducing the growth of this microorganism by almost 5 orders of magnitude and thus preventing the proliferation of this foodborne pathogen in this food matrix.

Key words: Biopreservation; Natural preservatives; Nisin; Sustained Antimicrobial Activity; PolyIon Complex Nanoparticles.

1. Introduction

Due to the high prevalence of foodborne diseases – nearly one in 10 people fall ill after eating contaminated food that can lead to over 420 000 deaths – developing new strategies that can minimise the proliferation of foodborne pathogens is a high priority (WHO, 2015). However, modern consumers of food demand that products look appealing for longer while requiring minimally processed products with no “chemical” preservatives. As such, there is an increasing reliance on food preservatives of natural origin (Tiwari et al., 2009). Nisin is one of the most important of these natural preservatives being currently used in more than 50 countries as a suitable food preservation method due to its significant inhibitory effect against foodborne pathogens, particularly gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* (Benmechernene et al., 2013). However, the use of Nisin in its free form leads to reduced efficacy and bioavailability of Nisin due to its interaction with food components and its possible enzymatic degradation, leading to reduced shelf life of food products and potential food poisoning (Ibarra-Sánchez et al., 2020). Encapsulation of Nisin has been proposed as a viable route to protect Nisin from its undesired interactions in food systems. Systems that have been currently investigated include liposomes (Boualem et al., 2013; Laridi et al., 2003), solid lipid nanoparticles (Prombutara et al., 2012) and, particularly, chitosan-based ternary nanostructures (Alishahi, 2014; Bernela et al., 2014; Chopra et al., 2014; Lee et al., 2018; Niaz et al., 2018; Wang, 2017; Zohri et al., 2010).

In this work we report the preparation of Nisin-Carboxymethylcellulose PolyIon Complex nanoparticles (Nisin:CMC PIC nanoparticles) as an alternative, cost effective methodology of encapsulation of this natural preservative. More importantly, we demonstrate the ability of these PIC nanoparticles to extend the antimicrobial activity of Nisin in a model system (ie. Milk) against a relevant food pathogen (ie. *S. aureus*). Due to Nisin's cationic nature, PIC particles, formed by the electrostatic interaction between two oppositely charged polyelectrolytes (Insua, Wilkinson, et al., 2016), are particular suited for the encapsulation of this natural preservative (Alishahi, 2014; Bernela et al., 2014; Chopra et al., 2014; Krivorotova et al., 2016; Luo et al., 2019; Niaz et al., 2018; Novickij et al., 2016; Zohri et al., 2010). Here, we used CMC, an anionic cellulose derivative used in the food industry as a viscosity modifier and stabilizer (Casaburi et al., 2018; Tabari, 2017), as a cheap and readily available anionic excipient. In our approach, Nisin:CMC PIC nanoparticles are prepared simply by mixing both components in a suitable buffer and used without further purification. First, the preparation of Nisin:CMC nanoparticles was optimised by tailoring the ratio between both components. Then, the effect of pH and CMC degree of substitution on the size, charge and encapsulation efficiency of these Nisin:CMC PIC nanoparticles was evaluated and we demonstrate that the prepared Nisin:CMC PIC nanoparticles present a spherical morphology. Next, the antimicrobial activity of these Nisin:CMC PIC nanoparticles against *Bacillus subtilis*, a representative gram-positive microorganism commonly found in food products, was evaluated *in-vitro*. Finally, a controlled contamination experiment was performed to evaluate the antimicrobial activity of these nanomaterials against *S. aureus*, a major foodborne pathogen, using milk as a representative food system. This microorganism is commonly found in milk products and is the most common cause of foodborne diseases among gram positive bacteria. Our results suggest that the reported Nisin:CMC PIC nanoparticles can extend the antimicrobial activity of this natural preservative and inhibit the proliferation of *S. aureus* in milk following contamination of milk products.

2. Materials and Methods

2.1. Materials

Nisin (Ultrapure Nisin-≥%99 purity) was purchased from Handary S.A. (Brussels, Belgium). Sodium Carboxymethylcellulose (CMC) (Average M_w 250,000) with the degree of substitution (DS) of 0.7, 0.9 and 1.2 (represented as CMC 0.7, CMC 0.9 and CMC 1.2, respectively) and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich Co. Nylon 0.45 μm syringe filters were bought from Camlab Ltd. Fluorescamine was purchased from Acros Organics™. Formvar Carbon support (300 mesh) Transmission Electron Microscopy (TEM) grids were purchased from Labtech International Ltd. All other chemicals used were of analytical grade. Brain heart infusion broth (BHIB) and Tryptic Soy Broth (TSB) were purchased from Fisher Scientific (United Kingdom). UHT skimmed milk was bought from a local market in Birmingham, United Kingdom. *S. aureus* ATCC 6538 and *B. subtilis* NCIMB 3610 were kindly provided by Biochemical Engineering, University of Birmingham, United Kingdom.

2.2. Instrumentation

Dynamic light scattering (DLS) measurements were carried out in a Zetasizer Nano ZS (Malvern Instruments Ltd). All described samples in this chapter were analyzed by using same conditions: temperature= 25 ºC, run duration= 60 s, fixed measurement position= 4.65 mm and automatic attenuation. Zeta potentials were recorded 30 times at 140 V. A FLUOstar Omega (BMGLabtech Gmbh) microplate reader was used to measure fluorescamine reactions. TEM images of Nisin:CMC PIC nanoparticles were acquired on a Jeol 1400 Bio TEM.

2.3. Preparation of Nisin:CMC PIC nanoparticles

Nisin:CMC PIC nanoparticles were prepared by adopting a protocol previously reported by our group.(Insua, Liamas, et al., 2016). Firstly, stock solutions of Nisin (0.3 mM, pH 4) and CMC 0.7, CMC 0.9 and CMC 1.2 (2.5 mM, pH 7) were prepared in 5 mM HEPES, and all stock solutions were filtered through 0.45 μm nylon syringe. Thereafter, PIC samples were prepared by drop-wise addition of cationic Nisin solutions to anionic CMC solutions under stirring to obtain final Nisin concentrations of 0.2, 0.4, 0.6, 0.8 and 1 $mg{\cdot}mL^{-1}$ while CMC concentrations were equal in all samples, 0.33 $mg{\cdot}mL^{-1}$. Finally, the

pH of the reaction mixture was adjusted to 4.5, 5.5 and 6.5, and samples were incubated under stirring at 500 rpm/rt. for 24 h.

2.4. Estimation of encapsulation efficiency

The encapsulation efficiency of Nisin in Nisin:CMC PIC nanoparticles was carried according to the percentage of available Nisin nanoparticle samples to react with fluorescamine dye. To this end, firstly, a standard curve of Nisin-fluorescamine conjugates was established by reacting 0.15 ml of Nisin solution at different concentrations (dissolved in 5 mM HEPES) with 0.15 ml of fluorescamine dye (2 mM) (dissolved in methanol) for 30 minutes and measuring fluorescence at 355 nm (excitation) and 460 +\-10nm (emission) (**Figure S1A**) (Held, 2001; Weigele et al., 1972). 5 mM HEPES buffer was used as blank reagent. Having established a standard curve of Nisin, Nisin:CMC PIC nanoparticles were analyzed following the same procedure (**Figure S1B)**. Encapsulation efficiency (%) was estimated using (**Equation 1**)

$$\frac{[mg{\cdot}mL^{-1}]_{total\ Nisin}-[mg{\cdot}mL^{-1}]_{available\ Nisin}}{[mg{\cdot}mL^{-1}]_{total\ Nisin}} * 100 \qquad \textbf{Equation 1}$$

2.5. TEM of Nisin:CMC PIC nanoparticles

5 µL of a suspension of Nisin:CMC PIC nanoparticles were deposited on the surface of the TEM grid and it was left to dry at ambient temperature covering from dust. Once dried, 5 µL of a 1 mg·mL^{-1} solution of phosphotungstic acid in distilled water were used to stain the nanoparticles. The excess of staining solution was gently removed at the end of the 1 minute staining period.

2.6. Growth curves of B. subtilis in the presence of Nisin:CMC PIC nanoparticles

50 µl aliquots of *B. subtilis* in BHIB (OD_{600} = 0.1) were loaded in the designated positions of a 96-well microplate. Then, to determine the minimum inhibitory concentration (MIC) of Nisin for *B. subtilis*, these bacterial cultures were mixed with 50 µl of Nisin (in 5 mM HEPES buffer pH 5.5) at different concentrations, together with 50 µl of 5 mM HEPES buffer and with 50 µl of sterile BHIB. All samples were prepared in triplicate, with mean values and standard deviations reported. The microplate was incubated at 30 ºC, and the OD_{600} was monitored every hour for 18 h. After incubation, the same protocol was followed using 50 µl of free Nisin at the double amount of MIC value or Nisin:CMC PIC nanoparticles to compare the antimicrobial activity of free and encapsulated Nisin at the same concentration. At the end of the 18 h incubation, total growth inhibition was calculated using **Equation 2**. Since no growth was observed when *B. subtilis* was treated with free Nisin, data was normalized to give 100% inhibition for this condition.

$$Abs\left[\frac{OD_{600}\ untreated - OD_{600}\ sample}{OD_{600}\ untreated}\right] * 100 \qquad \textbf{Equation 2}$$

Onset of growth (**Figure S6B**) and rate of growth (**Figure S6C**) were calculated by fitting OD_{600} to a segmental line regression (**Figure S6A**). These fittings were done using GraphPad Prism version 8.0.0, GraphPad Software, San Diego, California USA, www.graphpad.com.

2.7. Antimicrobial activity Nisin:CMC PIC nanoparticles against *S. aureus* in skimmed milk

Nisin solutions (in 5 mM HEPES buffer) at different concentrations were added to skimmed milk. Then, these Nisin-containing milk samples were artificially contaminated with an overnight culture of *S. aureus* ATCC 6538 (OD_{600} = 0.75 in Tryptic Soy broth). Samples were incubated at 25 ºC for 24 h. After incubation, enumeration of *S. aureus* was carried by calculating Colony Forming Unit (CFU·mL^{-1}) using the Miles and Misra technique (Munsch-Alatossava et al., 2007). Also, the initial amount of bacteria in contaminated milk samples was determined by enumerating *S. aureus* before incubation for 24 h. MIC was determined to be that of the milk sample containing the least amount of Nisin in which no bacterial growth was observed. Next, milk samples were treated with free Nisin or Nisin:CMC PIC nanoparticles at the double amount of MIC value and incubated at 4 ºC for 0, 2, 4, 8, 10 days. At the end of each incubation period, samples were artificially contaminated with an overnight culture of *S. aureus* (OD_{600} = 0.75 in TSB) and incubated at 25 ºC for 24 h. Finally, CFU·mL^{-1} were calculated for each sample. All samples were prepared in triplicate.

3. Result and discussion

3.1. Optimizing Nisin Concentration and Ratio of Components

The main goal of this work was to develop a cheap and efficient method to prepare Nisin-loaded nanoparticles. As mentioned above, due to the cationic nature of Nisin, PIC particles are an ideal platform to develop Nisin-loaded nanomaterials (Insua, Wilkinson, et al., 2016). Particles can be prepared by simply mixing Nisin, which carries 4-6 positive charges depending on the pH, with suitable negatively-charged polyelectrolytes. Nisin-loaded PIC particles derived from soybean polysaccharides (Luo et al., 2019) and pectin (Krivorotova et al., 2016; Novickij et al., 2016) had already been described in the literature, together with ternary systems which often carry chitosan as an additional cationic antimicrobial (Alishahi, 2014; Bernela et al., 2014; Chopra et al., 2014; Niaz et al., 2018; Zohri et al., 2010). Here, we wanted to evaluate the potential of CMC to form Nisin-loaded PIC nanoparticles. CMC is an FDA-approved food additive in the Generally Regarded as Safe (GRAS) category, also approved for human consumption by the EU with E number E234. CMC is used as a viscosity modifier and stabilizer in a range of food products. From a formulation perspective, CMC's charge density and solubility can be easily tuned by changing the degree of substitution of carboxymethyl groups per glucose monomer. CMCs with different degree of substitution and molecular masses are available commercially, representing a cheap, versatile and widely available anionic polysaccharide (Ntazinda et al., 2014).

With these considerations in mind, our initial efforts to develop Nisin:CMC PIC nanoparticles relied on commercially available CMC with an average Mw of 250 KDa and a degree of substitution of 0.7. First, we evaluated the effect that the ratio between Nisin and CMC had on the size, charge (**Figure 1**) and encapsulation efficiency (**Figure 2**) of the formed particles. Particles were prepared by adding different amounts of a solution of Nisin at pH 4 (to ensure full protonation of amine and imidazole groups in Nisin) to a solution of CMC at pH 7 (to ensure full deprotonation of carboxylic acids).[†] The final concentration of CMC in the particles was 0.33 mg·mL^{-1}. In this first screening, the pH of the final solution was adjusted to pH 4.5 to maximize the degree of protonation of Nisin without significantly affecting CMC anionic charge. Samples were incubated at room temperature for 24 h under stirring at 500 rpm and then characterized (**Figure S2**)

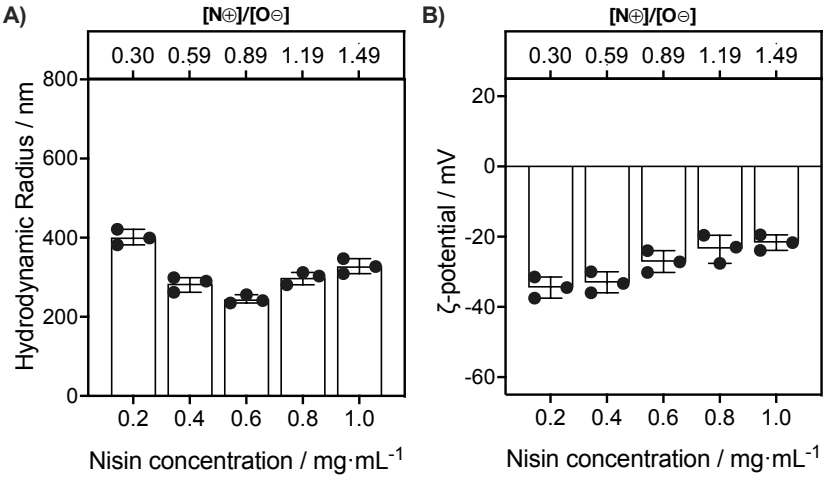


Figure 1 The effect of Nisin concentration in the sample on the size (A) and ζ-potential (B) of Nisin:CMC PIC nanoparticles. Final concentration of CMC (DS 0.7) was equal in all samples (0.33 mg·mL^{-1}). Final pH of the samples was 4.5. All replicates (●) together with mean value (hollow bars) shown. Ratio between positive charge in Nisin and negative charge in CMC shown above each graph. Data shown is the average of 3 technical replicates.

[†] Simulations of the degree of protonation using Marvin Sketch pKa plugin suggested that while an overall charge of 5 was observed for Nisin at pH 4.5 and 5.5, representing all 6 cationic residues protonated, and the terminal carboxyl group deprotonated, Nisin's charge will be reduced to 4.5 at pH 6.5. CMC will be almost completely fully deprotonated at pH 4.5-6.5.

All formulations tested resulted in the formation of particles in the nanometer range, as calculated by DLS (**Figure 1A**). However, no clear correlation could be observed between the amount of Nisin used in the sample and the size of the nanoparticles. At the lowest concentration of Nisin used (0.2 mg·mL^{-1}), nanoparticles of 400 nm were formed. The size of the nanoparticles decreased with increasing amounts of Nisin, with the smallest nanoparticles (244 nm) formed when 0.6 mg·mL^{-1} of Nisin were used. Particle size then increased with increasing amounts of Nisin, with nanoparticles of 328 nm formed when 1 mg·mL^{-1} of Nisin was used. This trend was consistent with the calculated ratio between cationic and anionic charges in the samples mixed, that was close to 1 for samples prepared at 0.6 mg·mL^{-1}. At this point, the number of electrostatic interactions in the sample should be maximized. Lower concentrations of Nisin would promote electrostatic repulsion between the anionic charges of the excess of CMC used, leading to larger particle sizes. Similarly, at higher concentrations of Nisin electrostatic repulsion between cationic moieties in Nisin would lead to bigger particle sizes. Interestingly all of the nanoparticles were negatively charged when their ζ-potential was analysed (**Figure 1B**) suggesting that a CMC corona was being displayed for all of these Nisin:CMC PIC nanoparticles. Such arrangement could suggest that not all Nisin was being encapsulated.

In order to characterize how much free Nisin was present for each of the samples, the number of available amines was calculated using a fluorescamine assay (Held, 2001; Weigele et al., 1972). In this fluorogenic assay, the number of amines can be correlated with an increase in fluorescence at 460 nm following incubation with fluorescamine. This fluorescence was then correlated with a calibration curve made with increasing amounts of Nisin (**Figure S1**) to calculate the amount of available Nisin and thus the encapsulation efficiency (**Figure 2A**). It should be noted that this method can underestimate this encapsulation efficiency, as some of the dye can diffuse to the core of the nanoparticles where Nisin is encapsulated. Despite this limitation, it was interesting to see that for all these nanoparticles the encapsulation efficiency was between 60-70%, suggesting that there was free Nisin in solution. A good correlation (R^2 = 0.95) could be observed between the size of the nanoparticles and encapsulation efficiency, with smaller nanoparticles formed when the amount of Nisin encapsulated was higher.

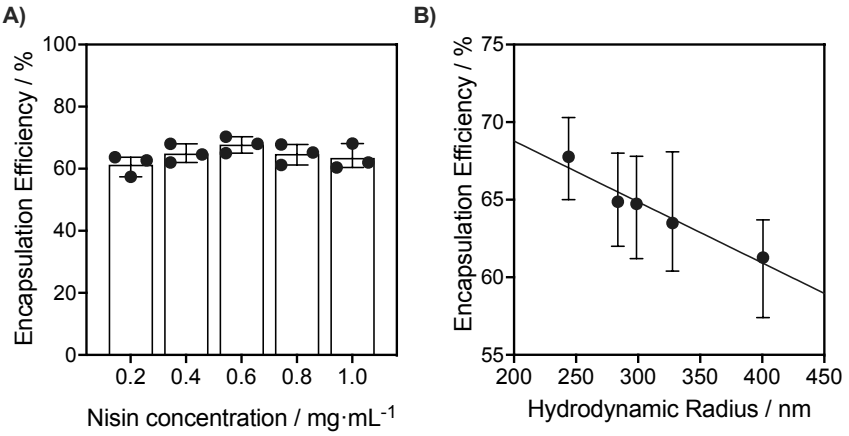


Figure 2 (A) The effect of Nisin concentration in the sample on the encapsulation efficiency of Nisin:CMC PIC nanoparticles. All replicates (●) together with mean value (hollow bars) shown. (B) Correlation between encapsulation efficiency and size of the Nisin:CMC PIC nanoparticles. Mean (●) together with range shown. Final concentration of CMC (DS 0.7) was equal in all samples (0.33 mg·mL^{-1}). Final pH of the samples was 4.5. Data shown is the average of 3 3 technical replicates.

3.2. Increasing Encapsulation Efficiency – Effect pH

Having identified conditions for the preparation of Nisin:CMC PIC nanoparticles, we wanted to increase the encapsulation efficiency of the system, to minimize the amount of free Nisin available and therefore its degradation when used in a food matrix. We postulated here that increasing the pH of the final solution would increase the degree of deprotonation of CMC, increasing its charge density and therefore facilitating its interaction with Nisin. However, at pHs higher than 6 the charge density in Nisin will be reduced, as a result of the partial deprotonation of the two histidine residues (Rollema et al., 1995).[†] Thus, the best conditions identified above (0.6 mg·mL^{-1} Nisin, 0.33 mg·mL^{-1} CMC) were repeated

but in this case, the pH of the final solution was kept also at 5.5 and 6.6 (**Figure 3**, **Figure S3**). Once again, Nisin:CMC PIC nanoparticles were formed under all the conditions evaluated, with barely any differences in size between the nanoparticles prepared at pH 4.5 and those prepared at pH 5.5 (**Figure 3A**). However, a significant increase in particle size was observed for those prepared at pH 6.6 (584 nm) possibly due to the increase repulsion between CMC side chains as a result of the increased charge density at this pH (**Figure 3B**). This increase in charge density resulted in a decrease in the ζ-potential as the pH of the solution was increased. Interestingly, while an increase in encapsulation efficiency could be observed at pH 5.5. (76% vs 68% at pH 4.5), the encapsulation efficiency was then reduced at pH 6.5 (50%) (**Figure 3C**). We believe this loss of efficiency in the encapsulation of Nisin is due to a decrease in the charge density of this natural food preservative as the pH of the solution is increased.[†]

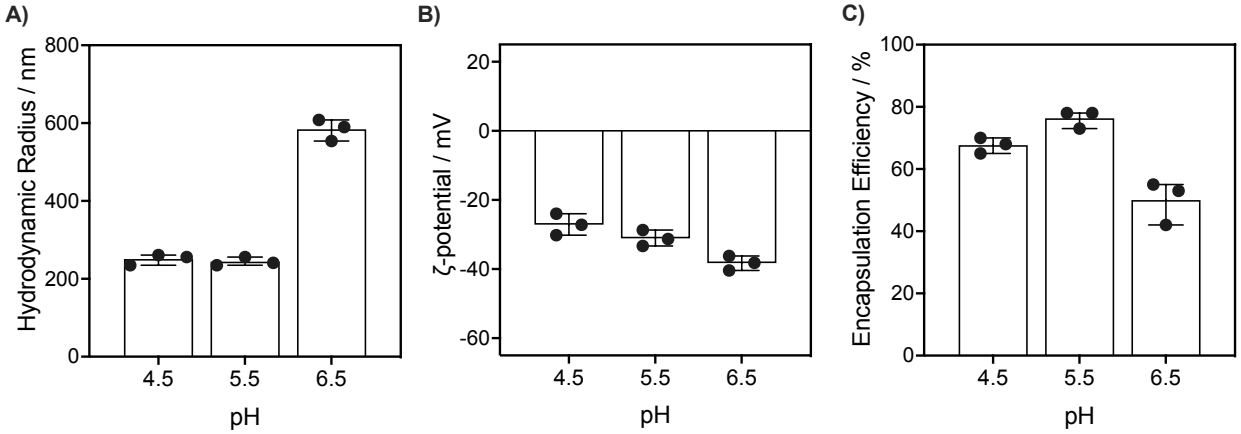


Figure 3 The effect of final pH on the size (A), ζ-potential (B) and encapsulation efficiency (C) of Nisin:CMC PIC nanoparticles. Final concentration of CMC (DS 0.7) (0.33 mg·mL^{-1}). Final concentration of Nisin (0.6 mg·mL^{-1}). All replicates (●) together with mean value (hollow bars) shown. Data shown is the average of 3 technical replicates.

3.3. Increasing Encapsulation Efficiency – Effect of degree of substitution

Seeing how pH, and therefore the charge density of Nisin, had a significant effect of the encapsulation efficiency of Nisin in these Nisin:CMC PIC nanoparticles, we decided to investigate the effect of increasing the charge density of CMC. To this end, in addition to CMC with a degree of substitution of 0.7 used so far (**PIC0.7** from now on), samples were prepared using CMC with degrees of substitution of 0.9 and 1.2 (**Figure 4**, **Figure S4**). Similar to the effect of pH, almost no effect on the particle size was observed when a CMC with a degree of 0.9 (**PIC0.9**) was used (both samples resulted in Nisin:CMC PIC nanoparticles of ~244 nm, **Figure 4A**). Increasing the degree of substitution in CMC to 1.2 (**PIC1.2**) then resulted in bigger nanoparticles (~388 nm), possibly again due to increased repulsion between the negative charges in CMC. This increase in charge density resulted in Nisin:CMC PIC nanoparticles with smaller ζ-potentials (**Figure 4B**) and, more importantly, with increasing degrees of encapsulation efficiencies, that was now 89% for the nanoparticles prepared using CMC with a degree of substitution of 1.2 (**Figure 4C**). This loading efficiency is in line with that reported for the other binary Nisin-loaded PIC particles reported in the literature (Krivorotova et al., 2016; Luo et al., 2019).

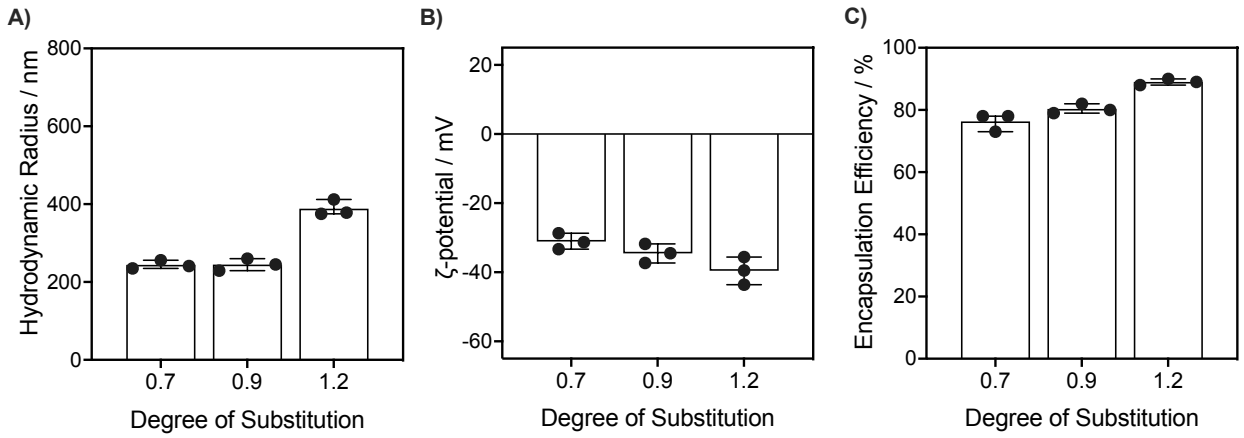


Figure 4 The effect of degree of substitution on the size (A), ζ-potential (B) and encapsulation efficiency (C) of Nisin:CMC PIC nanoparticles. Final concentration of CMC in each sample (0.33 mg·mL^{-1}). Final concentration of Nisin in each sample (0.6 mg·mL^{-1}). Final pH of the samples was 5.5. All replicates (●) together with mean value (hollow bars) shown. Data shown is the average of 3 technical replicates.

At this stage we decided to investigate also the morphology of the formed particles. Seeing how degree of substitution was the parameter that had a biggest effect on the encapsulation efficiency of these Nisin:CMC PIC nanoparticles, these samples prepared at pH 5.5 with CMC with different degrees of substitution (**Figure 4**) were deposited on a grid an imaged by TEM (**Figure 5**, **Figure S5**). In agreement with previous reports on this type of nanoparticles (Insua, Wilkinson, et al., 2016), all three Nisin:CMC PIC nanoparticles presented a spherical morphology, with sizes calculated by TEM in agreement to those found by DLS (**Table S1**). Based on these results, these samples (**PIC0.7**, **PIC0.9** and **PIC1.2**) were used for all subsequent work.

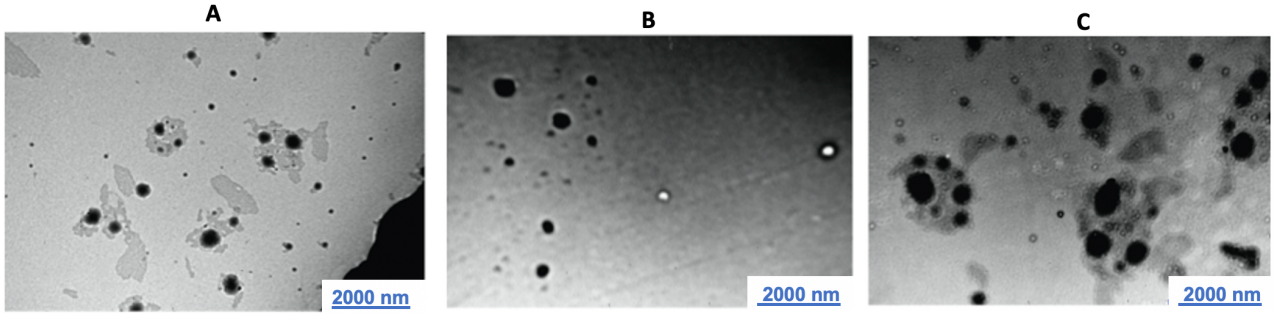


Figure 5 Representative TEM micrographs for Nisin:CMC PIC nanoparticles prepared with CMC with a degree of substitution of 0.7 (A), 0.9 (B) and 1.2 (C). Final concentration of Nisin in each sample (0.6 mg·mL^{-1}). Final pH of the samples was 5.5.

3.4. Antimicrobial activity of Nisin:CMC PIC nanoparticles against *B. subtilis*

Now that we had access to a range of formulations that resulted in Nisin:CMC PIC nanoparticles with encapsulation efficiencies of Nisin above 75% (**Figure 4**), we decided to investigate their antimicrobial activity against a relevant food-spoilage organism. At this point, we considered if these nanoparticles showed an antimicrobial activity against *B. subtilis*, a representative gram-positive bacteria. The endospores of this ubiquitous microorganism can survive most common sterilization procedures, except autoclaving and ultra-high temperature (UHT) treatment and, as such, *B. subtilis* is commonly found in food products such as pasteurized milk (Foods, 1980). To demonstrate that these Nisin:CMC PIC nanoparticles had an antimicrobial effect against this microorganism, *B. subtilis* was incubated in the presence and absence of these nanoparticles, and the optical density at 600 nm (OD_{600}) monitored as a proxy of microbial growth. Conditions were adjusted so that the Nisin concentration in all samples (25 μg·mL^{-1}) was twice the minimum inhibitory concentration of this food additive towards this microorganism, determined using the same experimental procedure (**Figure 6A**).

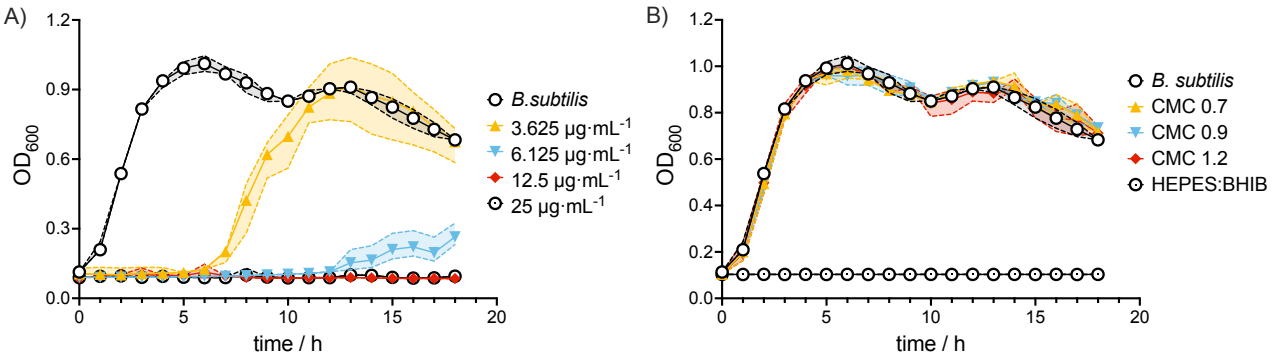


Figure 6 (A) Changes to the optical density at 600 nm of *B. subtilis* cultures in BHIB incubated in the absence (○) and presence of Nisin at different concentrations. (B) Changes to the optical density at 600 nm of *B. subtilis* cultures in BHIB incubated in the absence (○) and presence of CMC with a degree of substitution of 0.7 (▲), 0.9 (▼) and 1.2 (◆). Final CMC concentration 0.82 mg·mL^{-1}. Changes in OD_{600} for growth media (⊙) in the absence of bacteria, shown for comparison. Mean values shown. Error bars represent range. Data shown is the average of 3 technical replicates.

As expected, free Nisin was able to completely inhibit the growth of *B. subtilis* under these conditions (**Figure 7A**). Conversely, none of the CMCs employed had an inhibitory effect in the growth of this microorganism (**Figure 6B**). Interestingly, all Nisin:CMC PIC nanoparticles showed an inhibitory effect in the growth of *B. subtilis*, causing a delay in the lag phase and suggesting that Nisin was being released under these conditions. Growth inhibition (**Figure 7B**) was calculated by comparing the OD_{600} values after 18 h of incubation and normalized to the optical density of untreated *B. subtilis* cultures as described in the Materials and Method section above (Section 2.6). In this assay, **PICo.7** showed the longest antimicrobial effect, with no growth of bacteria being observed for approximatel 11h (**Figure 7A** and **Figure S6B**). **PIC1.2** however had the lowest growth inhibition after 18 h (**Figure 7B,** 29±4 %). This poor performance by **PIC1.2** seemed to be the result of an early onset of growth when *B. subtilis* was incubated with this Nisin:CMC PIC nanoparticle (6.6±0.4 h, **Figure S6B**) and a higher rate of growth (0.039±0.001 OD_{600}·h^{-1}, **Figure S6C**). Notably, **PICo.7** had a higher rate of growth than **PICo.9** (0.023 vs 0.018 OD_{600}·h^{-1}, **Figure S6C**) which meant that, despite a delayed onset of growth for **PICo.7**, both formulations showed similar growth inhibition after 18 h, 67±8 % and 69±5% respectively (**Figure 7B**).

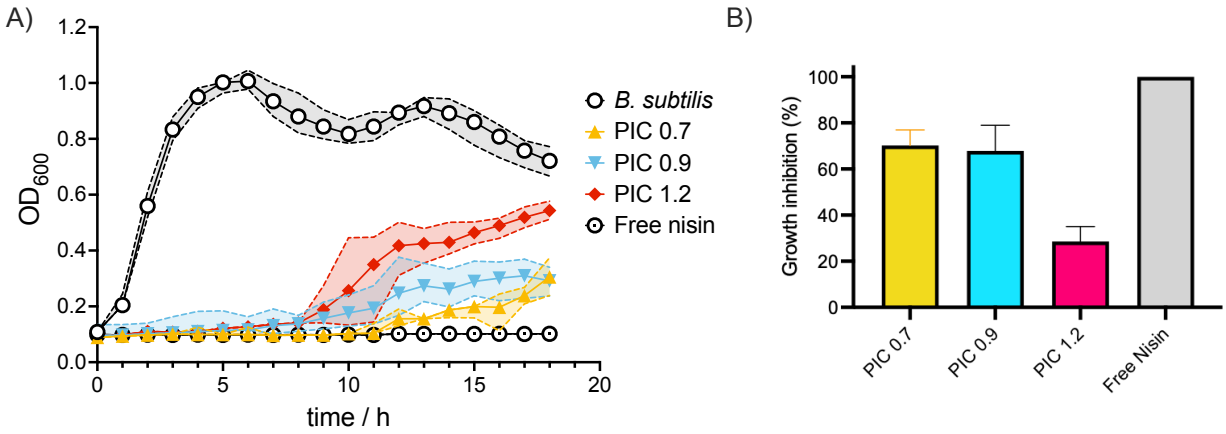


Figure 7 (A) Changes to the optical density at 600 nm of *B. subtilis* cultures in BHIB incubated in the absence (○) and presence of Nisin (⊙) and Nisin:CMC PIC nanoparticles prepared with CMC with a degree of substitution of 0.7 (▲), 0.9 (▼) and 1.2 (◆). Final concentration of Nisin in each sample (25 µg·mL^{-1}). B) Growth inhibition (%) caused by free Nisin or Nisin:CMC PIC nanoparticles after 18 h incubation. Nisin:CMC PIC nanoparticles prepared at 0.33 mg·mL^{-1} of CMC and 0.6 mg·mL^{-1} of Nisin, at pH 5.5. Data shown is the average of 3 biological replicates, each having 3 technical replicates. Mean values shown. Error bars represent range.

3.5. Antimicrobial activity of Nisin:CMC PIC nanoparticles against *S. aureus* in skimmed milk

Having identified that these Nisin:CMC PIC nanoparticles were showing an antimicrobial effect in a laboratory setting, we wanted to explore their antimicrobial activity in a real food system, milk in particular. As mentioned, Nisin is commonly used as a preservative in dairy products, where its antimicrobial activity and stability are decreased due to unwanted interactions with the ingredients in milk (Ibarra-Sánchez et al., 2020). To demonstrate that the Nisin:CMC PIC nanoparticles above had an antimicrobial effect in this food matrix, and could prolong the antimicrobial effect of this food preservative, a controlled contamination experiment was designed. In brief, skimmed milk stored at 4 °C containing free Nisin or Nisin:CMC PIC nanoparticles was spiked with *S. aureus* at different times, to mimic the potential contamination of food products during handling. *S. aureus* was selected as a representative food pathogen because milk can be easily contaminated by this gram-positive bacteria as a result of improper handling (Jin & Yamada, 2016; Salgado-Pabón & Tran, 2021). If the cold chain is not maintained, contamination can lead to enterotoxin production by this microorganism, eventually causing severe food poisoning (Kümmel et al., 2016). Samples were then incubated for 24 h at room temperature, to mimic this failure in maintaining the cold chain. Milk samples in this experiment contained free Nisin or Nisin:CMC PIC nanoparticles so that the final concentration of Nisin in each sample was 10 μg·mL^{-1}, twice the minimum inhibitory concentration in this food matrix (**Figure 8A**). This concentration is significantly below that used in dairy products (100-200 mg·kg^{-1}, *i.e.* ~ 100-200 μg·mL^{-1})(Jones et al., 2005) but allowed us to observe, in a laboratory setting, differences between the prepared formulations. In this experiment, when milk was contaminated with *S. aureus* at the time of sample preparation (**Figure 8B**, day 0) all Nisin containing samples showed an antimicrobial effect, and were able to reduce the number of colony forming units when compared to an untreated sample (Figure 8, ◯). As expected, samples containing free Nisin showed the highest antimicrobial activity, reducing the number of colony forming units of *S. aureus* in milk by almost 5 orders of magnitude (**Figure 8B**, ⊙). At this point, all Nisin:CMC PIC nanoparticles showed a weak antimicrobial effect, reducing the number of colony forming units by only 1-2.5 orders of magnitude. This difference in performance is not surprising, since over 75% of the Nisin is encapsulated in these Nisin:CMC PIC nanoparticles, and suggests that the particles are relatively stable in skimmed milk.

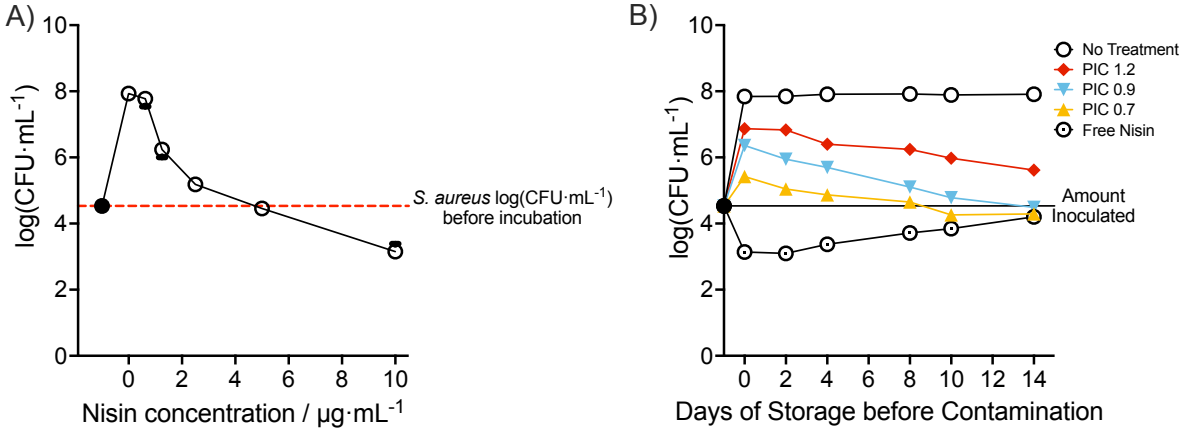


Figure 8 (A) Microbial counts for *S. aureus* in skimmed milk samples before (●) and after incubation (◯) at 25 °C for 24 h in the absence and presence of Nisin at different concentrations. Mean values shown. Error bars represent range. Data shown is the average of 2 biological replicates, each having 3 technical replicates. (B) Microbial counts for *S. aureus* in skimmed milk samples stored for different time periods at 4 °C in the absence (◯) and presence of Nisin (⊙), and Nisin:CMC PIC nanoparticles prepared with CMC with a degree of substitution of 0.7 (▲), 0.9 (▼) and 1.2 (◆) before artificial contamination with an overnight culture of *S. aureus*. Samples were incubated for 24 h at 25 °C following contamination with *S. aureus*. Nisin:CMC PIC nanoparticles prepared at 0.33 mg·mL^{-1} of CMC and 0.6 mg·mL^{-1} of Nisin, at pH 5.5. Data shown is the average of 3 biological replicates, each having 3 technical replicates. Mean values shown. Error bars represent range.

Since we wanted to mimic if contamination happened during transport or at the place of use, we repeated this experiment but spiking milk with *S. aureus* was delayed to 2, 4, 8, 10 and 14 days after the preparation of the samples. As expected, the efficacy of Nisin in preventing the growth of this pathogen was reduced with time and, after two weeks, it was only able to reduce the number of colony forming units by 4 orders of magnitude. Conversely, the efficacy of all the Nisin:CMC PIC nanoparticles increased with time. While **PIC1.2** showed a weak improvement, now reducing the number of colony forming units by over 2 orders of magnitude (**Figure 8B**, ◆), **PIC0.9** (**Figure 8B**, ▼) and **PIC0.7** (**Figure 8B**, ▲) were now showing the same antimicrobial activity of free Nisin (**Figure 8B**, ⊙). Remarkably, all these formulations were able to maintain the same number of colony forming units as those initially inoculated, thus preventing the proliferation of *S. aureus* in this food matrix. It should be noted that, as mentioned, the concentration of Nisin used in this experiment (10 $\mu g \cdot mL^{-1}$) was significantly below the higher limits allowed in diary products (100-200 $mg \cdot kg^{-1}$)(Jones et al., 2005) and, as such, the antimicrobial activity of these Nisin:CMC PIC nanoparticles could be improved by using higher concentrations in the food formulation.

4. Conclusions

In conclusion, we describe here the first application of CMC, an FDA-approved food additive in the GRAS category, for the encapsulation of Nisin, a natural food preservative currently used in food products such as dairy, deli meats, sweets or beverages. Taking advantage of Nisin's cationic nature, we have developed a simple method for the preparation of Nisin-loaded PIC nanoparticles. Our results suggest that the pH of the final solution together with the degree of substitution of CMC are critical parameters to optimize Nisin encapsulation efficiency. All the Nisin:CMC PIC nanoparticles showed sizes in the nanomolar range and a spherical morphology More importantly, we have demonstrated the ability of these Nisin:CMC PIC nanoparticles to display an antimicrobial effect against *B. subtilis*, a representative gram-positive bacteria commonly found in food products. Finally, we demonstrate that these particles can prolong the antimicrobial effect of Nisin in milk, a relevant food matrix, against *S. aureus*, the most common cause of foodborne diseases among gram positive bacteria. Our findings suggest that Nisin:CMC PIC nanoparticles could play an important role in enhancing the bioavailability of Nisin in food systems; underpinning novel Nisin-containing food formulations. Our efforts to optimize the antimicrobial effect of these nanoparticles, together with an investigation of their effect in other food matrices will be reported in due course.

5. Supplementary information

Fluorescence data of Nisin-fluorescamine adducts, additional DLS data, additional TEM images, and additional antimicrobial data for *B. subtilis*, can be obtained in the supporting information.

6. AUTHOR INFORMATION

6.1. Author Contributions

T.C. and P.F.-T. conceived the project, and designed Nisin:CMC PIC nanoparticle preparation and characterization. T.C., C.A., H.O. and T.M. designed the antimicrobial experiments. P.F.-T., H.O. and T.M. secured funding. T. C. performed all experiments. T.C. and P.F.-T. analyzed the data and wrote the paper, with all other authors contributing to the final version of the manuscript.

6.2. Conflicts of Interest

The authors declare no conflicts of interest.

7. ACKNOWLEDGMENTS

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Electronic Supplementary Information

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1. Fluorescence data for Nisin-Fluorescamine adducts

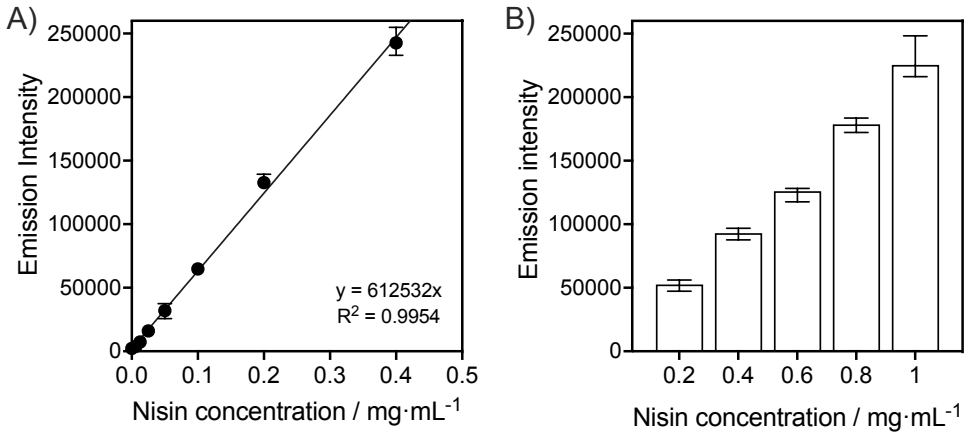


Figure S1 The standard curve of Free Nisin-Fluorescamine conjugates (left) and Emission Intensity of fluorescamine conjugates for Nisin:CMC PIC nanoparticles prepared with different Nisin concentrations of 0.2, 0.4, 0.6, 0.8,and 1 mg·mL^{-1} (right). Final concentration of CMC (DS 0.7) (0.33 mg·mL^{-1}). Final pH of the samples was 4.5. Mean values shown. Error bars represent range. Data shown is the average of 3 technical replicates.

2. Additional DLS data

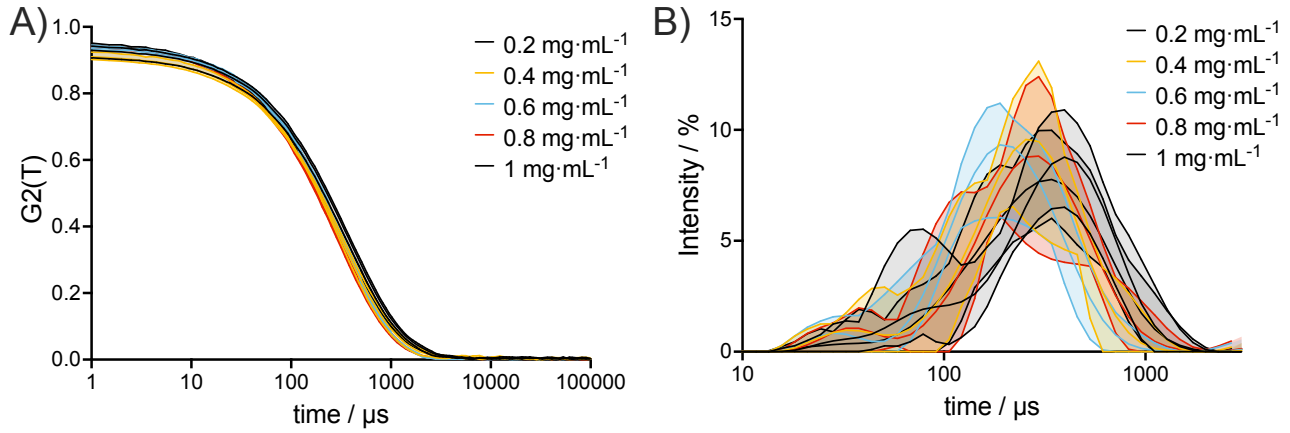


Figure S2 Autocorrelation function (ACF) curves (A) and representative size-intensity distributions (B) for Nisin:CMC PIC nanoparticles prepared at different concentrations of Nisin. Final concentration of CMC in each sample (0.33 mg·mL^{-1}). Final pH of the samples was 4.5. Mean values shown. Error bars represent range. Data shown is the average of 3 technical replicates.

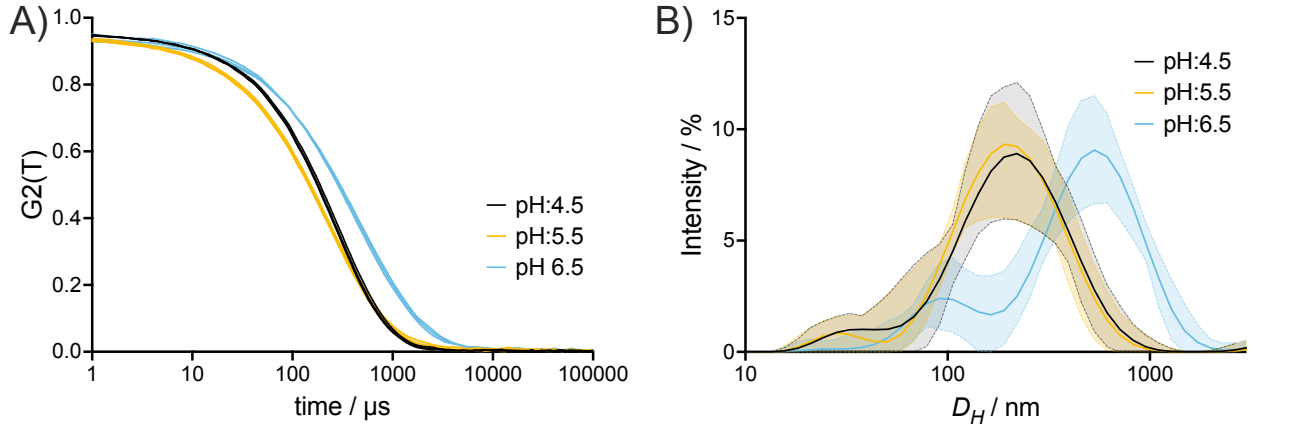


Figure S3 Autocorrelation function (ACF) curves (A) and representative size-intensity distributions (B) for Nisin:CMC PIC nanoparticles at different pHs. Final concentration of CMC (DS 0.7) in each sample (0.33 mg·mL^{-1}). Final concentration of Nisin in each sample (0.6 mg·mL^{-1}). Final pH of the samples was 5.5. Mean values shown. Error bars represent range. Data shown is the average of 3 technical replicates.

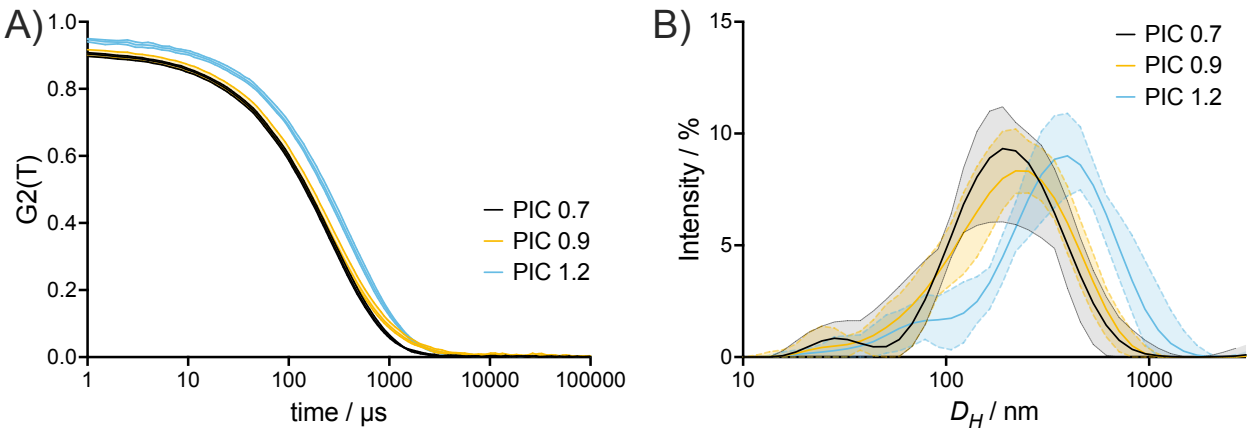


Figure S4 Autocorrelation function (ACF) curves (A) and representative size-intensity distributions (B) for Nisin:CMC PIC nanoparticles prepared with CMCs with different degree of substitution. Final concentration of CMC in each sample (0.33 mg·mL^{-1}). Final concentration of Nisin in each sample (0.6 mg·mL^{-1}). Final pH of the samples was 5.5. Mean values shown. Error bars represent range. Data shown is the average of 3 technical replicates.

3. Additional TEM Images

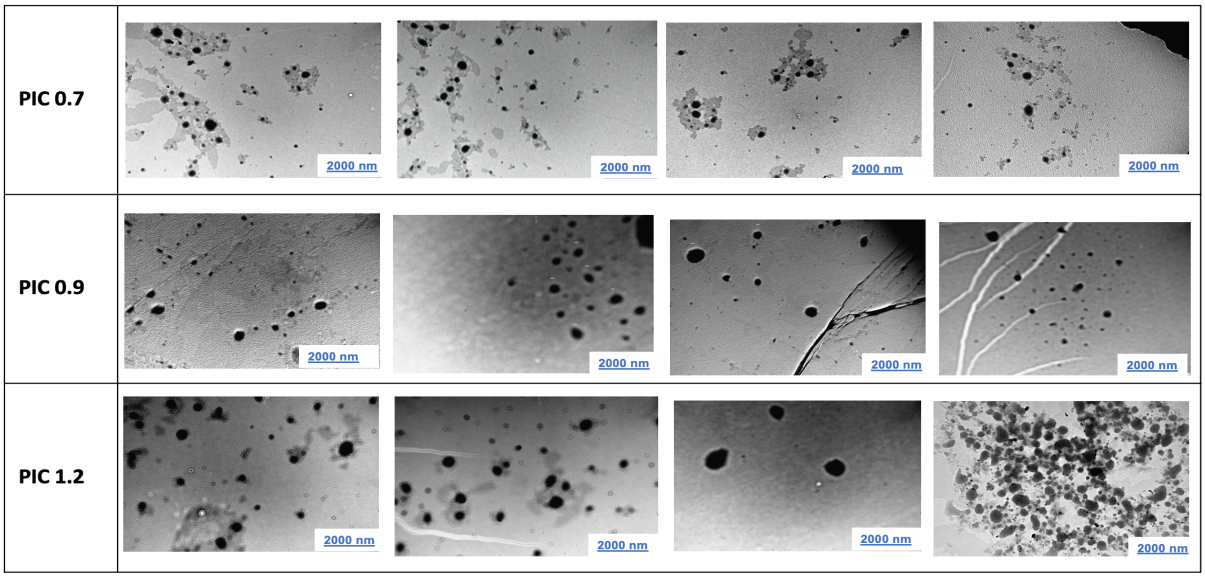


Figure S5 Representative TEM micrographs for Nisin:CMC PIC nanoparticles prepared with CMC with a degree of substitution of 0.7 (Top), 0.9 (Middle) and 1.2 (Bottom). Final concentration of CMC in each sample (0.33 mg·mL^{-1}). Final concentration of Nisin in each sample (0.6 mg·mL^{-1}). Final pH of the samples was 5.5.

Table S1 Size of Nisin:CMC PIC nanoparticles prepared with CMC with different degrees of substitution determined by DLS (D_H) and TEM (D_{TEM}). Mean values and standard deviation shown.

	D_H (nm)[a]	D_{TEM} (nm)[b]
PIC 0.7	244 ± 85	235 ± 36
PIC 0.9	244 ± 97	246 ± 39
PIC 1.2	388 ± 163	344 ± 53

[a] Standard deviation calculated for the only size population fitted by the Malverns's Zetasizer software. [b] Average diameter of 40 nanoparticles (for each Nisin:CMC PIC formulation) were measured twice (in their longest and shortest diameters) and standard deviation calculated from these 80 measurements.

4. Onset and Rate of Growth of *B. subtilis* cultures in the presence of Nisin:CMC PIC nanoparticles

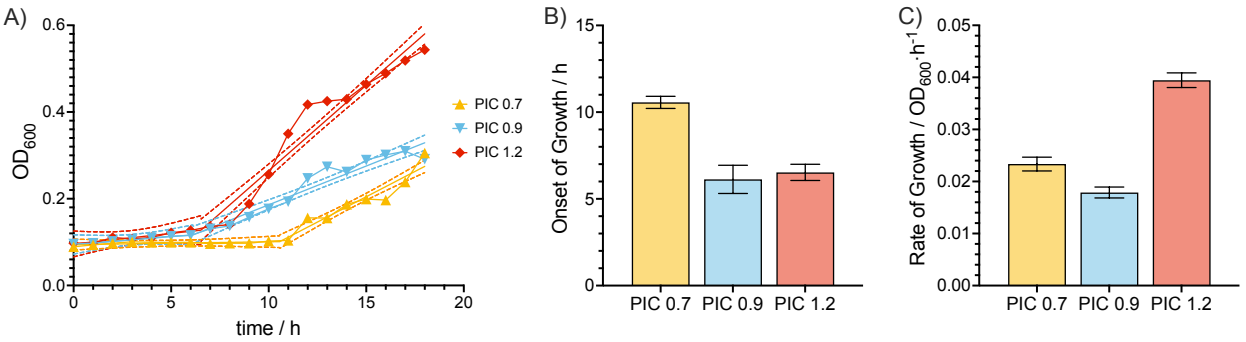


Figure S6 (A) Changes to the optical density at 600 nm of *B. subtilis* cultures in BHIB incubated in the presence of Nisin:CMC PIC nanoparticles prepared with CMC with a degree of substitution of 0.7 (▲), 0.9 (▼) and 1.2 (◆) and segmental line fitting. Final CMC concentration 0.82 mg·mL^{-1}. Data shown is the average of 3 technical replicates. Error bars for segmental line fitting shows the 99% conficence bands. (B) Onset of growth and (C) rate of growth for these *B. subtilis* cultures. Mean ± SEM of the fitting shown.

CHAPTER 3

Nisin:Hyaluronic acid PIC nanoparticles as a novel enzyme-responsive nisin carrier to prevent Staphylococcal food poisoning

Teyfik Celen, Christian Anumudu, Taghi Miri, Helen Onyeaka, and Paco Fernandez-Trillo

Format: Paper (Pending for submission)

Overview: Nisin:CMC PIC nanoparticles developed in Chapter 2 have provided a good example of a sustained antimicrobial release approach to enhance bioavailability of nisin in food systems. However, the amount of nisin released from such nanomaterials may not be sufficient in case of contamination, or the nisin released can interact with food components and ultimately lose its effectiveness before contamination. For the purpose of overcoming this potential problem, one of this thesis's important milestones was to develop a responsive nisin delivery system that either protects nisin during the shelf life of food products or releases nisin upon contamination of food products with specific pathogens.

In this chapter, we present a novel PIC nanoparticle system for the triggered release of nisin in the presence of *Staphylococcus aureus*, a severe foodborne pathogen. Here, we use Hyaluronic acid (HA) as an enzyme-degradable encapsulating material to prepare PIC nanoparticles loading with nisin. HA is a natural biopolymer degradable by hyaluronidase of *Staphylococcus aureus*. As such, we hypothesized that these nanoparticles can be cleaved by *S. aureus*, triggering the release of nisin and eventually, the proliferation of this pathogen can be inhibited. With this in mind, Nisin:HA PIC nanoparticles were prepared and characterised following the same methodology followed in Chapter 2.

The paper presented in this chapter includes the stability and release studies conducted at simulated conditions. Furthermore, the in vitro-antimicrobial activity of these nanoparticles was carried out using hyaluronidase producing *S. aureus* and non-hyaluronidase producing *Bacillus cereus*. Finally, the controlled contamination experiment designed and conducted in Chapter 2 was also carried out in this chapter to investigate the responsive antimicrobial activity of these nanoparticles in milk. Due their enzymatic cleavage upon contamination under simulated conditions, these nanoparticles displayed a better inhibitory effect against hyaluronidase-producing *S. aureus* as compared to *B. cereus* (control). To sum up, the first example of responsive nisin-carrier was developed, and their promising antimicrobial potential is presented in this chapter.

Contributions: T.C. and P.F.-T. conceived the project and designed the nanoparticle preparation and characterization. T.C., C.A, H.O. and T.M. designed the antimicrobial experiments. P.F.-T., H.O. and T.M. secured funding. T. C. performed all experiments. T.C. and P.F.-T. analysed the data. TC wrote the paper, with all other authors contributing to the final version of the manuscript.

Nisin-Hyaluronic acid PIC nanoparticles as a novel enzyme-responsive nisin carrier to prevent Staphylococcal food poisoning

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Abstract

The antimicrobial efficacy of nisin delivery systems could be more effective if its release could be triggered by the presence of undesired bacteria in food. To prepare Polyion Complex (PIC) nanoparticles loading with nisin, we use hyaluronic acid (HA) as an enzyme (i.e. hyaluronidase) degradable encapsulating polymer by *Staphylococcus aureus*. A straightforward electrostatic complexation method conducted at different polyelectrolyte concentrations allowed us to obtain spherical PIC nanoparticles loading with nisin, with polydispersity index (PDI): 0.3, size (DLS): 288 nm, encapsulation efficiency: 46%. These nanoparticles displayed good stability in the presence of simulated NaCl concentrations. In vitro dialyse experiment was carried out to monitor the release of nisin from PIC nanoparticles in the absence and presence of hyaluronidase. In vitro antimicrobial activity of Nisin:HA PIC nanoparticles showed a better inhibitory effect against hyaluronidase-producing S. aureus in comparison to *Bacillus cereus* (non-hyaluronidase-producing control). Finally, a controlled contamination experiment was performed to evaluate the antimicrobial activity of these nanoparticles, using milk as a representative food product. Our results suggest that these Nisin:HA PIC nanoparticles.

Key words: Food antimicrobials; Nisin; Responsive antimicrobial delivery; PIC nanoparticles

1. Introduction

Staphylococcal food poisoning, an acute intoxication that develops following the consumption of food contaminated with enterotoxin produced by *Staphylococcus aureus,* is one of the top-four foodborne diseases in the world. Because *S. aureus* is a common commensal of the skin and mucosal membranes of humans, food products, such as meat, salad, milk and dairy products, can be easily contaminated with this pathogen as a result of improper handling, and it produces heat-stable enterotoxins in food (Argudín et al., 2010; Kadariya et al., 2014). Therefore, preventing the growth of *S. aureus* is crucial in case of contamination. In this regard, food antimicrobials are vital in controlling *S. aureus* growth and eventually inhibiting enterotoxin production in food. Nisin, a bacteriocin produced by *Lactococcus lactis*, is one of the most important FDA-approved food preservatives used in the food industry due to its broad inhibitory effect against gram-positive foodborne pathogens, including *S. aureus (Galanakis, 2019).* However, it has been commonly reported that the use of nisin in its free form leads to reduced antimicrobial activity due to its interaction with food components and its possible enzymatic degradation (Chollet et al., 2008; Khan & Oh, 2016). Consequently, various encapsulation strategies have been widely explored to protect nisin from its undesired interactions in food systems, including nanoliposomes (Boualem et al., 2013; Laridi et al., 2003), solid-lipid nanoparticles (Prombutara et al., 2012) and particularly chitosan-based ternary nanostructures (Alishahi, 2014; Bernela et al., 2014; Chopra et al., 2014; Niaz et al., 2018; Zohri et al., 2010).

Furthermore, in recent years, the use of food-grade anionic biopolymers such as pectin (Krivorotova et al., 2016), carboxymethyl cellulose and soluble soybean (Luo et al., 2019) has been described as a cheap and easy encapsulation methodology. This approach is simply based on the formation Polyion Complex (PIC) nanoparticles as a result of electrostatic interaction between positively charged peptide and negatively charged polymer (Insua, Wilkinson, et al., 2016). Nevertheless, in all these existing approaches, the antimicrobial activity of encapsulated nisin relies on non-specific antimicrobial release mechanisms such as passive diffusion, charge interactions, and swelling or slow breakdown of the encapsulating material, also called "passive release". As such, released nisin from these nanostructures into the uncontaminated food matrix may ultimately lose its effectiveness before contamination of food products with serious pathogens (i.e., *S. aureus*). Since food products can be contaminated anytime between production and consumption, the presence of a sufficient amount of nisin in the contaminated food is vital to inhibit the growth of foodborne pathogens and eventually prevent food intoxications such as Staphylococcal food poisoning. Therefore, a delivery system that can accelerate the release of nisin in response to food contamination is highly desirable.

In this regard, responsive nanocarriers have gained attention as an attractive way of accelerating the delivery of antimicrobial agents in response to specific stimuli such as pH, temperature, or enzymes. In particular, significant progress has been made to improve antibiotic delivery at the site of infection in response to toxins and enzymes released by pathogenic bacteria (Chen et al., 2018; Huang et al., 2022; Nazir et al., 2022; Yang et al., 2022). However, this approach has mainly been used in biomedical applications for delivering antibiotics, whereas it has not been applied for the delivery of food antimicrobials. Considering the intrinsic changes stemming from bacterial growth in food upon contamination, we anticipated that responsive antimicrobial delivery could be developed in food systems against foodborne pathogens. When considering *S. aureus* infections, it is well known that *S. aureus* is an opportunistic pathogens producing a range of virulence factors, including hemolysins, enterotoxins and extracellular enzymes. For instance, hyaluronidase, an enzyme produced by *S. aureus* and degrading hyaluronic acid, is an example of these exoenzymes allowing *S. aureus* to spread in tissues (Dovedytis et al., 2020; Hynes & Walton, 2000; Ibberson et al., 2014). Based on this specific hyaluronidase activity of *S. aureus* against HA, here we use hyaluronic acid to develop hyaluronidase-responsive PIC nanoparticles to accelerate nisin release in the presence of *S. aureus.* For this purpose, firstly, different nanoparticle formulations prepared simply through electrostatic complexation of Nisin and HA in a suitable buffer were evaluated in terms of size, polydispersity, charge and nisin-loading capacity. Then, we demonstrate that optimised Nisin:HA PIC nanoparticles are disassembled in the presence of hyaluronidase while they remain mostly stable in the presence of physiological triggers (i.e., NaCI). After that, in vitro antimicrobial evaluation of Nisin:HA PIC nanoparticles against hyaluronidase-producing *S. aureus* and non-hyaluronidase-producing foodborne pathogen *Bacillus cereus* was conducted. Finally, a controlled contamination experiment was performed to evaluate the antimicrobial effectiveness of our nanoparticles in the absence and presence of target pathogen *S. aureus* during the shelf life of food products, using milk as a representative food system. Our results suggest that Nisin:HA PIC nanoparticles exhibit responsive antimicrobial activity against *S. aureus* and thus effectively inhibit its proliferation in the contaminated milk samples. Therefore, they have promising potential to be the first example of the responsive antimicrobial delivery system in food.

2. Materials and Methods

2.1 Materials

Nisin (Ultrapure Nisin-≥%99 purity) was purchased from Handary S.A. (Brussels, Belgium). Hyaluronic acid sodium salt from *Streptococcus equi* (Mw: 1.5-1.8 x 10E6 Da), Hyaluronidase from bovine testes (400-1000 IU/mg solid), 4-(2- hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and Amicon Ultra centrifugal filter units (MWCO 100kDa) were purchased from Sigma-

Aldrich Co. Nylon 0.45 μm syringe filters were bought from Camlab Ltd. Float-a-Lyzer G2 (MWCO 100 kDa) dialysis membranes were purchased from Spectrum Laboratories. Fluorescamine was purchased from Acros Organics TM. Formvar Carbon support (300 mesh) TEM grids were purchased from Labtech International Ltd. All other chemicals used were of analytical grade. Tryptic Soy Broth (TSB) were purchased from Fisher Scientific (United Kingdom). UHT skimmed milk was bought from a local market in Birmingham, United Kingdom. *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* NCTC 11143 were kindly provided by Biochemical Engineering, University of Birmingham, United Kingdom.

2.2. Instrumentation

Dynamic light scattering (DLS) measurements were carried out in a Zetasizer Nano ZS (Malvern Instruments Ltd).All described samples in this chapter were analysed by using same conditions: Scattering angle- 173º, temperature= 25ºC, run duration= 60 s, fixed measurement position= 4.65 mm and automatic attenuation.The size distributions, Autocorrelation function (ACF) curves, and Polydispersity index (PDI) values were obtained from Malvern Zetasizer software 7.03. Zeta potentials were recorded 30 times at 140 V. A FLUOstar Omega (BMGLabtech Gmbh) microplate reader was used to measure fluorescamine reactions. TEM images of PIC nanoparticles were acquired on a Jeol 1400 Bio TEM.

2.3. Preparation of Nisin:HA PIC Nanoparticles

Nisin:HA PIC nanoparticles were prepared adopting a protocol previously reported by our group. Firstly, stock solutions of Nisin (0.5 mM, pH:4) and Hyaluronic acid (HA) (1mM, pH:7) were prepared in 5mM HEPES. Thereafter, ratios representing the proportion of amines in Nisin over carboxyl groups in HA, were prepared mixing stock solutions of nisin and HA. For instance, to prepare PIC particles at 3:1 [N: COOH] ratio (i.e. amines 3 times more than carboxyl groups), from stock solutions previously prepared in 5mM HEPES buffer and filtered through 0.45 um nylon syringe, drop-wise addition of 1 ml Nisin was applied to 1 ml HA under stirring. Finally, the pH of the reaction mixture was adjusted to 4.5, and the sample was incubated under stirring at 500rpm/rt. for 24 hours. The same protocol was followed to prepare PIC particles at other [N: COOH] ratios by changing the amount of nisin in samples.

2.4. Estimation of encapsulation efficiency (EE)

The loading efficiency of nisin in PIC nanoparticles was carried according to the percentage of available nisin nanoparticle samples to react with fluorescamine dye. To this end, firstly, a standard curve of nisin-fluorescamine conjugates was established by being reacted 0.125 ml of nisin solution at different concentrations (dissolved in 5mM HEPES) with 0.125 ml of fluorescamine dye (2mM) (dissolved in methanol) for 30 minutes and measuring fluorescence at 355 nm (excitation) and 460 +\- 10nm (emission) (Weigele et al., 1972). HEPES buffer was used as a blank reagent. Having established standard curve of nisin, PIC nanoparticle samples were analysed following same procedure (Fig. S1). Encapsulation efficiency was (%) estimated using (Equation 1)

$$\frac{[mg \cdot mL^{-1}]_{total\ Nisin} - [mg \cdot mL^{-1}]_{available\ Nisin}}{[mg \cdot mL^{-1}]_{total\ Nisin}} * 100 \qquad \textbf{Equation 1}$$

2.5. TEM of Nisin:HA PIC nanoparticles

For TEM analysis, 5uL of a suspension of PIC nanoparticles were deposited on the surface of the TEM grid and it was left to dry at ambient temperature covering from dust. Once dried, 5uL of a 1mg.mL^{-1} solution of phosphotungstic acid in distilled water were used to stain the nanoparticles. The excess staining solution was gently removed at the end of the staining period for 1 minute.

2.6. NaCl stability of Nisin HA PIC nanoparticles

To a sample of Nisin:HA PIC nanoparticles (0.5 ml) prepared at 3:1 [N:COOH] ratio as described above, 0.1 ml of a solution of NaCl in water was added individually to obtain total NaCl concentrations of 50, 100, 150, and 250 mM. The samples were incubated at ambient temperature and analysed by DLS after 1 h and 24 hours, respectively.

2.7. In-vitro release of nisin from Nisin:HA PIC in the presence of Hyaluronidase

2.5 ml of Nisin (1.02 mg/mL) and Nisin:HA PIC nanoparticles (Nisin equivalent of 1.02 mg/ml) were added to the dialysis membrane (M.W. cut off 100 kDa). Thereafter, 0.15 ml of hyaluronidase solution (1mg/ml) prepared in HEPES buffer was transferred to dialysis membranes containing PIC nanoparticles. They were dialysed against 12.5 ml HEPES buffer supplemented with NaCl to obtain final NaCl concentration of 100 mM at ambient temperature for 24 h. At predetermined time intervals, 1 ml of dialysate was withdrawn and replaced the 1 ml of fresh HEPES solution (with NaCI) to keep the total volume constant. Nisin content found in the collected dialysates was calculated by fluorescamine assay as described above. Briefly, 0.125 ml of collected dialysate was reacted with fluorescamine dye. Measured fluorescence of the mixture was normalised to the fluorescence of fluorescamine-nisin (0.06 mg.mL^{-1}), the total nisin concentration that was presumed to pass (%100) through the membrane. For enzyme-treated PIC nanoparticles, increases in emission intensity stemming from hyaluronidase-fluorescamine adducts were taken into account when calculating cumulative nisin release. Also, from the dialysis membranes containing enzyme-treated or non-enzyme-treated PIC nanoparticles, 0.5 ml of nanoparticle solution was analysed by DLS at different time points and placed back into the dialysis membrane.

2.8. Growth curves of *S.aureus* and *B. cereus* in the presence of Nisin:HA PIC nanoparticle

100 µl aliquots of *S. aureus* and *B. cereus* in TSB (OD600: 0.1) were individually loaded in the designated positions of a 96-well microplate. Then, firstly, to determine the minimum inhibitory concentration (MIC) of nisin against these two organisms, these bacterial cultures were mixed with 100 µl of nisin at different concentrations (in 5mM HEPES buffer pH 4.5). All samples were prepared in triplicate, with mean values and standard deviations reported. The microplate was incubated at 25^{o}C, and the OD600 was monitored every hour for 20 h. After incubation, having indicated the MIC value according to the growth curve of bacteria, the same protocol was followed using Nisin:HA PIC nanoparticles by adjusting total nisin concentration to MIC value of nisin against these organisms and its multiples, up to four times. At the end of the 20 h incubation, total growth inhibition (%) was calculated using (Equation 2). Since no growth was observed when *S. aureus* and *B. cereus* were treated with free Nisin, the data was normalised to give 100% inhibition for this condition.

$$Abs\left[\frac{OD_{600}\ untreated\ -\ OD_{600}\ sample}{OD_{600}\ untreated}\right] * 100 \qquad \textbf{Equation 2}$$

2.9. Antimicrobial activity of Nisin:HA PIC nanoparticles against *S. aureus* and *B. cereus* in skimmed milk

Nisin solutions at different concentrations in HEPES buffer were added to skimmed milk samples. OD600 of overnight cultures of *S. aureus* and *B. cereus* in TSB were diluted by phosphate buffered saline (PBS) to obtain final OD600 of 0.4. Then, milk samples containing nisin were artificially contaminated by *S. aureus* or *B. cereus*. Samples were incubated at 25°C for 24 h. After incubation, the enumeration of *S. aureus* and *B. cereus* was carried out by performing Miles and Misra technique (Mendez-Vilas, 2007). Also, initial bacteria concentration in contaminated milk samples was determined by enumerating these organisms without incubation. For each organism, the minimum inhibitory concentration (MIC) of nisin was determined according to the milk sample containing the least amount of nisin and in which no bacterial growth was observed. Having identified the MIC value of nisin to stop the growth of the exact amount of *S. aureus* and *B. cereus* in milk at 25°C for 24h at least, milk samples were firstly treated with free nisin or PIC nanoparticles at the double amount of MIC value and incubated at 4°C for 0, 2, 4, 8, 10, and 14 days. At the end of each incubation period, samples were artificially contaminated by an overnight culture of *S. aureus* and *B. cereus* (OD600: 0.4) and incubated at 25°C for 24 h. Finally, bacteria concentrations in Colony Forming Unit (CFU/ml) were calculated for each sample. All samples were prepared in triplicate.

3. Result and discussion

3.1. Preparation and Characterisation of Nisin:HA PIC nanoparticles

The main goal of this work is to prepare the first example of a responsive antimicrobial nisin delivery system against *S. aureus,* the main causative agent of Staphylococcal food poisoning. For this purpose, we selected PIC particles as an ideal platform for two main reasons. First, as aforementioned, due to the cationic nature of nisin, PIC nanoparticles are an easy and affordable platform that has already been used to protect nisin by delaying its release in the food system using food-grade anionic polymers (Krivorotova et al., 2016; Luo et al., 2019). Second, antimicrobial release kinetics from PIC nanoparticles can be easily adjusted using a sacrificial encapsulating polymer that a specific trigger can degrade (Insua, Liamas, et al., 2016). Depending on that, as an anionic encapsulating material, we choose Hyaluronic acid (HA), a natural polysaccharide allowed to be used in food products in the USA, EU, China, Japan, and Canada as a food supplement in recent years (FDA, 2020). Besides its anionic character, the main feature that makes HA appropriate for our system is that it is a degradable polymer by various species of *Streptococcus*, *Staphylococcus*, and *Clostridium*, including pathogens causing food intoxications such as *Staphylococcus aureus and Clostridium perfringens* (Hynes & Walton, 2000). Due to this its enzyme-degradable property, HA has been used as a component of various delivery systems developed for clinically relevant antiseptic drugs against *S. aureus* infections (Baier et al., 2013; Mohammed et al., 2022). Since either the use of hyaluronic acid or a responsive antimicrobial delivery approach has never been applied for the delivery of food antimicrobials, we focused on developing the first example of the responsive antimicrobial delivery system. Consequently, we anticipated that nisin-loaded PIC nanoparticles could be prepared by anionic HA. Thus, these nanoparticles could be cleaved by hyaluronidase in the presence of representative hyaluronidase-producing target pathogen *S. aureus* followed by the rapid release of nisin.

Taking these considerations into account, the first examples of Nisin:Hyaluronic acid PIC nanoparticles were prepared simply through the drop-wise addition of cationic nisin to anionic HA

under stirring in HEPES buffer. As the presence of a sufficient amount of charge is key to the formation of PIC particles, final pH of the samples was adjusted to 4.5. This is because nisin is fully protonated at this pH (Rollema et al., 1995). Also, the charge density of HA would not be significantly affected at pH 4.5 due to its pKa value, about 3 (Liao et al., 2005). Since we wanted to encapsulate cationic nisin, our initial attempt was to prepare PIC samples in excess of anionic charge ([N:COOH] < 1) and eventually obtain negatively charged nanoparticles. For this purpose, PIC samples were prepared through the drop-wise addition of nisin at different concentrations to HA solution (0.1mg/ml). As shown in Figure1A, when the mixtures were analysed by DLS after 24 hours-complexation period without further purification, it was observed that size distribution of the all complexes formed at non-stoichiometric ratios ([N:COOH] < 1) were interestingly big, close to micron level (**Figure 1A-**hollow bars**, Figure S1A**). Also, considering the fact that successful and efficient nano-delivery systems require the preparation of homogenous formulations, these samples were not acceptable due to their high polydispersity index (PDI) values above 0.4, which is the upper PDI limit of monodisperse or moderately polydisperse nanoparticle samples. (**Figure 1B**) (Bhattacharjee, 2016). Furthermore, the macroscopic appearance of PIC samples was fully transparent for all these samples, even at the stoichiometric mixing ratio (**Figure 1C**). This result was interesting because we expected to see the formation of unstable complexes resulting from the aggregation of uncharged complexes (Insua, Majok, et al., 2017; Lounis et al., 2016). All these results indicated that electrostatic activity in the samples prepared at [N:COOH] ≤ 1 ratios was insufficient to obtain PIC samples with desired size distribution and PDI.

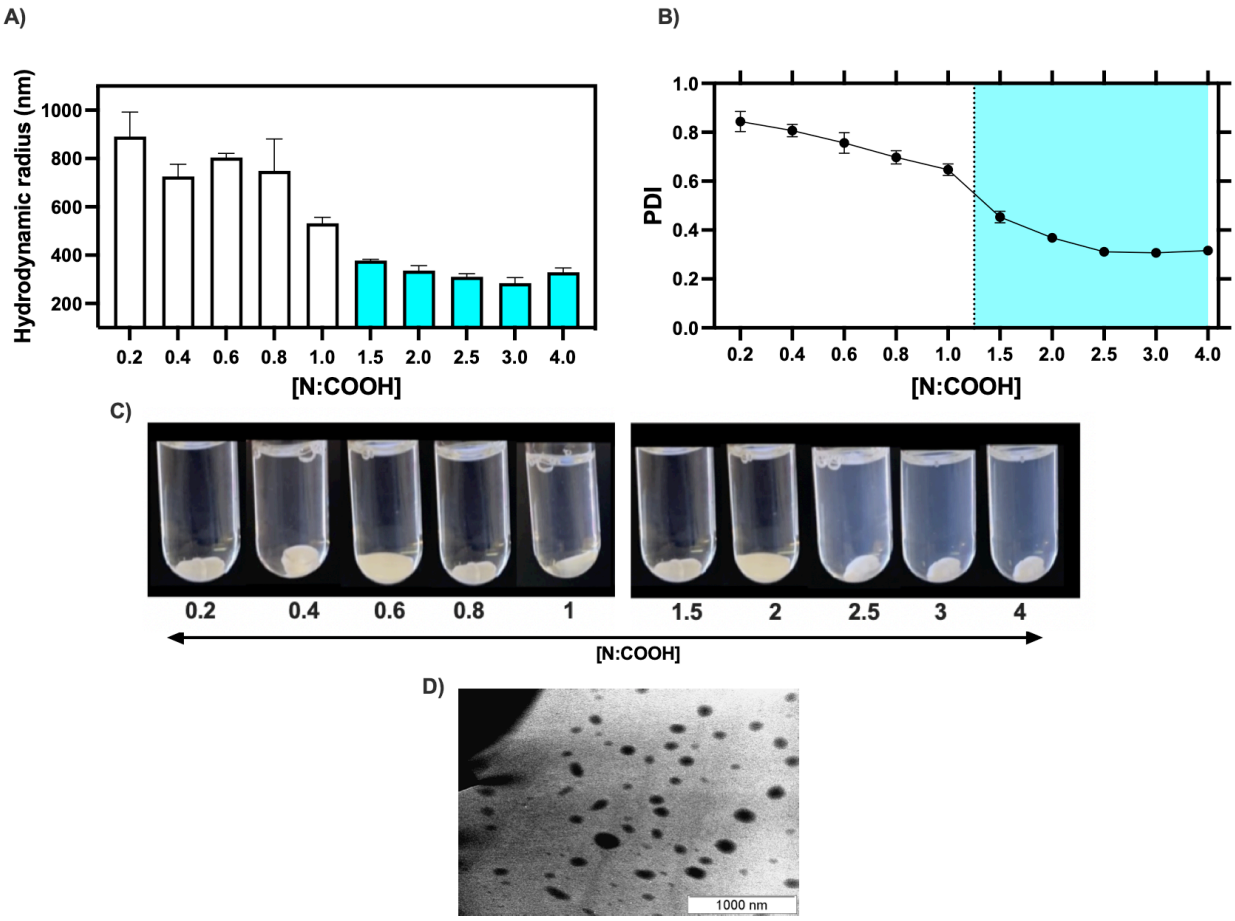


Figure 1 Avarage Hydrodynamic radius (A), Polydispersity index (PDI) values (B), and macroscopic appearance (C) of the Nisin:HA PIC nanoparticle samples prepared at different [N:COOH] ratios. Representative TEM micrograph for PIC nanoparticles prepared at 3:1 [N:COOH] ratio. Error bars represent range (n=3).

With this in mind, further Nisin:HA PIC samples were prepared at a broad range of [N:COOH] ratios, up to 4-fold excess of cationic nisin. As can be seen in **Figure 1A** (blue bars) and **Figure 1B**, the size and PDI values of all formulations prepared in excess of cationic nisin ([N:COOH] >1) showed notable changes and PIC samples with moderate polydispersity and smaller size distributions were obtained (**Figure S1B**). Among these, although the biggest size distributions were formed in the sample (1.5:1 [N:COOH]) containing the lowest nisin concentration (385nm), all PIC particles obtained from 2:1 to 4:1 ratio had very similar size distributions, with the smallest nanoparticles (288 nm) formed at 3:1 [N:COOH] ratio. Therefore, TEM was performed using PIC particles prepared at 3:1 ratio as a representative example of all five formulations characterised by DLS. As shown in **Fig. 1D and Figure S2**, PIC particles presented a spherical morphology. However, in agreement with the previous reports on this type of nanoparticles (Insua, Majok, et al., 2017), the sizes of nanoparticles calculated by TEM (218 nm) was smaller than Dh measured by DLS (288 nm). This size reduction observed by TEM could potentially stem from the drying step of sample preparation, which could lead to evaporation of the solvent in the highly hydrated nanomaterials and, eventually, PIC particles shrinking (Friedrich et al., 2010).

Having established the preparation of spherical Nisin:HA PIC nanoparticles with desired size and PDI in the samples prepared in excess of cationic nisin ([N:COOH] > 1), we wanted to further characterise these samples through their charges. Since nanoparticles with the same charge repel each other and maintain the colloidal stability of PIC solution by preventing aggregation, our initial assumption was that colloidally stable PIC samples ([N:COOH] > 1) were being stabilised by the excess of cationic Nisin. Contrary to this expectation, all Nisin:HA PIC nanoparticles were negatively charged (**Figure 2A**). Interestingly, the zeta potentials continuously increased (from -29.7 to -19.5) with increasing [N:COOH] ratios, indicating sequential absorption of nisin on HA chains. Since negatively charged nisin-loaded PIC nanoparticles would protect nisin from its undesired interactions with negatively charged proteins, such as casein in milk (Aasen et al., 2003), collected zeta potential values were a desired characteristic for our system. On the other hand, the formation of the negatively charged PIC nanoparticles in excess of cationic charge may be explained by the fact that interaction between Nisin and HA was not only via electrostatic but also via hydrophobic interactions and hydrogen bonds. Such arrangement could also correspond that not all nisin was being encapsulated by HA, and therefore we decided to further investigate this hypothesis.

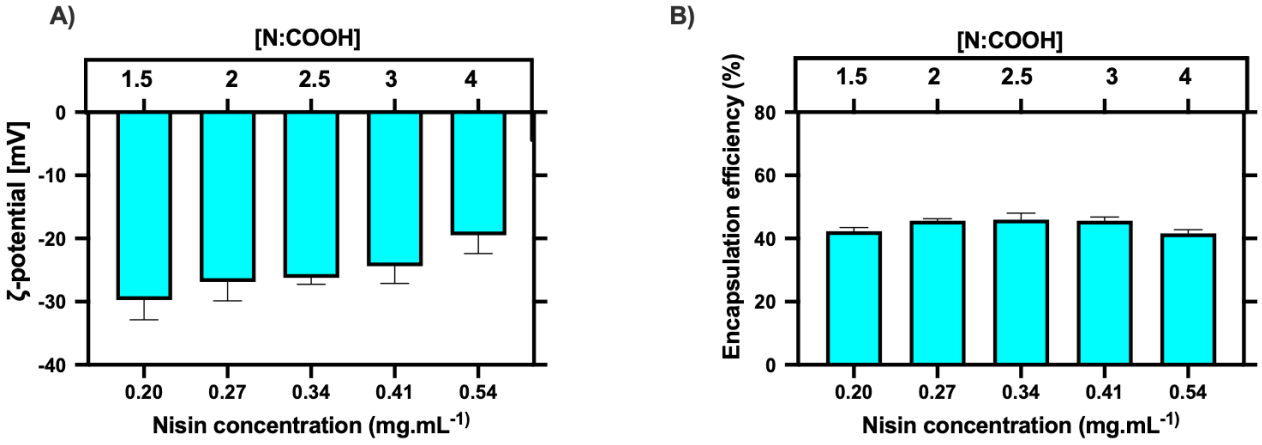


Figure 2 Zeta potential (A) and encapsulation efficiency (B) of Nisin:HA PIC nanoparticles prepared at different nisin concentrations. Final concentration of HA was equal in all samples (0.1 mg.mL^{-1}) Error bars represent range (n=3).

In order to determine the encapsulation efficiency (EE) of nisin, Nisin:HA PIC nanoparticles were analysed by fluorescamine assay in terms of their available amine contents. In this fluorogenic assay, fluorescence intensity of free nisin samples were significantly higher than that of PIC particles at the same nisin concentrations **(Figure S3)**, suggesting the encapsulated nisin may have not reacted with fluorescamine dye in PIC particle solutions. Depending on this, a calibration curve was generated for the fluorescence intensity (460 nm) of fluorescamine- free nisin adducts to calculate encapsulated nisin in each PIC formulation prepared (Fig. S1). However, it should be noted that this method may underestimate EE because some of the fluorescamine dye may diffuse to the core of nanoparticles and react with encapsulated nisin. Despite this potential limitation, collected encapsulation efficiency values were very similar regardless of the [N:COOH] ratios, approximately 45-50% **(Figure 2B)**, indicating there was free nisin (~50%) in nanoparticle solution and eventually clarifying why all Nisin:HA PIC particles were negatively charged. Consequently, we obtained PIC nanoparticles loading with nisin, stabilized by the excess of HA chains. However, it should be noted that since unencapsulated antimicrobial nisin in PIC solution may lead to misinterpretation of further antimicrobial activity studies, it was successfully removed by an ultracentrifugation process ahead of the microbiology experiments **(Figure S4).**

3.2. Stability of Nisin:HA PIC nanoparticles at different NaCl concentrations

It is well established that PIC nanoparticles are generally disturbed in the presence of other electrolytes and charged species in the dilution media, which potentially leads to the decomplexation of the particles as a result of weakened electrostatic forces holding the particles together. The encapsulated drug can be released from PIC nanoparticles due to this usual decomposition, and this non-specific release mechanism is therefore preferred for antimicrobial delivery systems(Insua, Majok, et al., 2017; Insua, Zizmare, et al., 2017). However, since we wanted to achieve rapid nisin release in the presence of the target pathogen, a premature release (e.g. before contamination of food products) stemming from the rapid decomposition of unstable nanoparticles in food systems would not be a desirable release profile for our system (Boostani & Jafari, 2020). Considering that these nanoparticles might often be exposed to various electrolytes such as Na^+ and Cl^- in food systems, the preliminary stability of Nisin:HA PIC nanoparticles was assessed with respect to changes in size by DLS after 1 hour following NaCl addition(Fig. 4). The selected NaCl concentrations were in the range of approximate NaCl content of dairy products in which Nisin is commonly used, such as soft and semi-hard cheese (Dugat-Bony et al., 2016).

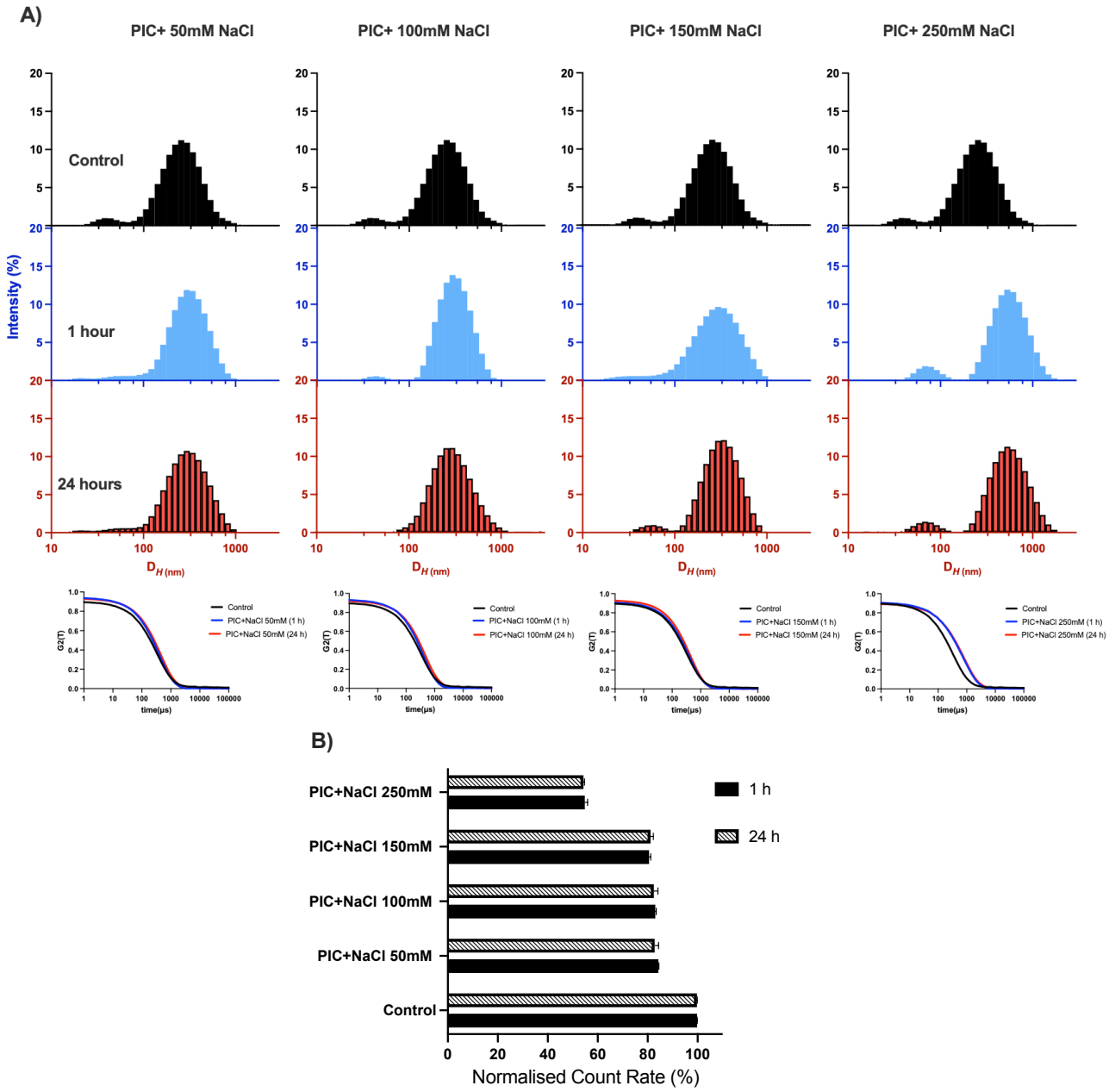


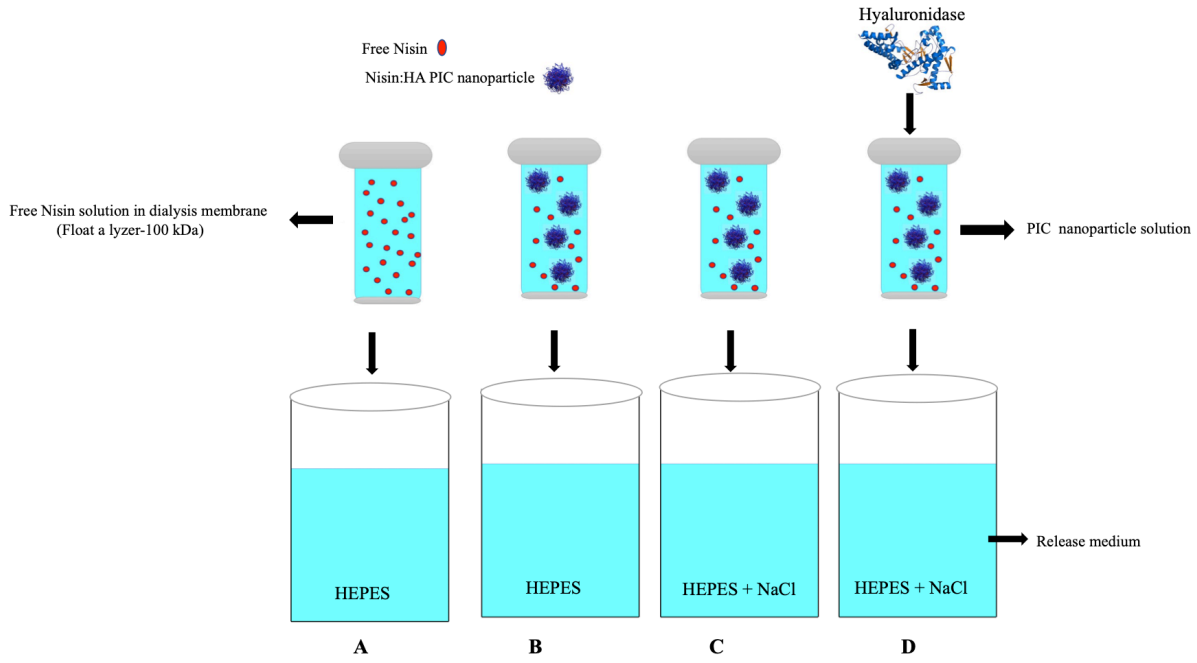
Figure 3 DLS Size-Intensity distributions, DLS correlograms (bottom) (A) and normalised DLS detection counts (%) (B) of Nisin:HA PIC nanoparticles prepared at 3:1 [N:COOH] ratio in the absence (control) and in the presence of different NaCl concentrations after incubation for 1 h and 24 h.

Notably, as shown in **Figure 3A** Nisin:HA PIC nanoparticles showed almost no changes in their autocorrelation functions (ACFs) and their size distributions in the presence of 50, 100, and 150mM NaCl. However, interestingly, approximately a 20% decrease was observed in the count rate regardless of the NaCl concentration (**Figure 3B**). This decrease in the count rate may have stemmed from various factors, such as the breakdown of the loose complexes in PIC nanoparticle solution or the formation of secondary complexes due to the aggregation of small complexes. Nevertheless, this change in the detection counts did not show a decreasing trend with time, suggesting that these nanoparticles remained stable. On the other hand, increasing NaCl concentration to 250mM caused PIC nanoparticles to swell, with an almost 2-fold increase in size. This is probably due to an increase in electrostatic repulsion as small electrolytes (Na^+ and Cl^-) shield the electrostatic attraction between the anionic HA and cationic nisin. In addition, a dramatic decrease of %45 was observed in the count

rate (**Figure 3B**), probably due to this osmotic swelling. However, all these stability profiles of Nisin:HA PIC nanoparticles at the end of the 1 hour did not exhibit a time dependent response when incubation period was extended to 24-h in the presence of NaCl regardless of the concentration. Consequently, despite a burst effect of NaCl on the count rate and size (at 250 mM NaCl), Nisin:HA PIC nanoparticles were able to remain stable in the presence of NaCl.

3.3. In vitro release of nisin from Nisin:HA PIC nanoparticles

Having identified that Nisin:HA PIC nanoparticles were able to remain stable in the presence of NaCl, we decided to investigate if the release of nisin from these nanoparticles can be triggered by hyaluronidase. For this purpose, we designed a release study by suspending a dialysis tubing containing PIC nanoparticles under different conditions to understand the absolute effect on hyaluronidase on nisin release. Firstly, free nisin solution at the same nisin concentration with PIC solution was also dialysed against HEPES buffer to make comparisons with PIC solution (**Scheme 1A**). As shown in Figure 4A (hollow sphere), 98% of total nisin in free nisin solution passed through the dialysis membrane in 24 h. For the dialysis of PIC nanoparticle solution, in this experiment, since we used Nisin:HA PIC nanoparticles without purification, an initial release study was conducted by immersing a dialysis tubing containing PIC nanoparticle solution in HEPES buffer **(Scheme 1B)**.This way, we wanted to monitor nisin release in the absence of any trigger (i.e. enzyme and NaCl). Interestingly, 48% of total nisin in PIC nanoparticle solution passed through the dialysis membrane after 8 h, and no time-dependent release profile was observed during further time points of dialysis. Moreover, when the PIC solution being dialysed was analysed by DLS, no change was observed in the count (**Figure 5B,** hollow bars) and the size of Nisin:HA PIC nanoparticles throughout the dialysis process (**Figure S4A1-B1**). Therefore, this observed 48% release could be directly attributed to the unencapsulated nisin in PIC solution because we used Nisin:HA PIC nanoparticles with the encapsulation efficiency of 46% without purification in this experiment. Consequently, nisin-loaded PIC nanoparticles were fully stable, and therefore no considerable nisin release was observed from PIC nanoparticles under these conditions, as expected.



Scheme 1 Dialysis of Free Nisin and Nisin:HA PIC solution under different conditions

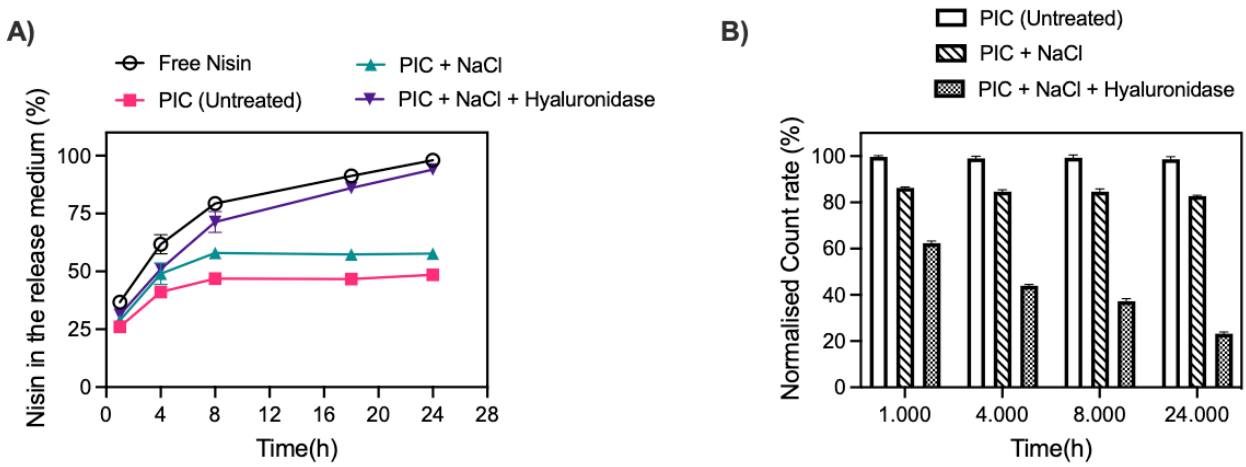


Figure 4 The nisin release profiles from free Nisin (blank circle) or Nisin:HA PIC nanoparticle (prepared at 3:1 [N:COOH] ratio) solution being dialysed in the absence or presence of hyaluronidase over time (1-24 hours) (A). DLS detection counts of Nisin:HA PIC nanoparticles being dialysed under different conditions over time (1-24 hours) (B)

We then decided to dialyse the PIC nanoparticle solution in the presence of osmotic pressure by adding NaCl (100 mM) to the release medium (**Scheme 1C**). Remarkably, in the first 8 h, 57% of total nisin in PIC nanoparticle solution passed through the dialysis membrane, 9% higher than that of untreated PIC nanoparticles. This 9 per cent increase in the release medium can be explained by the released nisin from PIC nanoparticles due to osmotic pressure. Interestingly, no further increase was detected in the nisin concentration collected dialysates after this point, suggesting that nisin-loaded PIC nanoparticles were stable, and therefore no detectable slow release occurred. In this regard, unchanged ACF curves and size distributions evidenced the stability of Nisin:HA PIC nanoparticles being dialysed under these conditions (**Figure S4B1-B2**). However, the count rate suddenly decreased by about 20% after 1 h (**Figure 4B**). This decrease in the count rate may be explained by the rapid disassembly of weak complexes with the effect of osmotic pressure, and eventually, this may have caused a nisin release observed in the first hours of dialysis.

In addition to osmotic pressure, when PIC nanoparticles were treated by hyaluronidase (**Scheme 1D**), the amount of nisin in the release medium remarkably increased to 72% after 8 h, 25% higher than that of non-enzyme-treated PIC solution being dialysed at the same conditions. In the following 16 h, nisin in the release medium continued to increase, and 93% of the total nisin in the PIC nanoparticle solution passed through the dialysis membrane at the end of the dialysis process. This result clearly indicates that the release of nisin from Nisin:HA PIC nanoparticles was triggered by hyaluronidase. On the other hand, the effect of hyaluronidase on these nanoparticles was monitored by DLS measurements of enzyme-treated PIC nanoparticles (**Figure S4C1-C2**). Significant changes were observed in ACF curves, size distributions, and a gradual decrease in detected counts (**Figure 4B**). Overall, these results proved that the Nisin:HA PIC nanoparticles could be cleaved by hyaluronidase, and thus, nisin release could be triggered.

3.4. Antimicrobial activity of Nisin:HA PIC nanoparticles in Tryptic Soy Broth

Having identified that nisin could be released from PIC nanoparticles in the presence of hyaluronidase, in vitro antimicrobial evaluation of these nanoparticles was done against *Staphylococcus aureus*, a hyaluronidase producing target foodborne pathogen. Additionally, *Bacillus*

cereus, a gram-positive foodborne pathogen, was used as a non-hyaluronidase-producing control organism (**Figure S6**). In this antimicrobial activity experiment, growth curves of *S. aureus* and *B. cereus* were monitored in the absence and presence of free nisin or Nisin:HA PIC nanoparticle samples at various nisin concentrations. This way, firstly, minimum inhibitory concentration (MIC) value of nisin was determined against these two bacteria. Then, their growth inhibition values were calculated as a percentage according to total changes in OD600 in the growth media treated by free nisin or PIC nanoparticles after 20 h incubation. Here, it should be noted that since we wanted to monitor the antimicrobial activity of nisin released from PIC nanoparticles only, unencapsulated nisin was removed by ultracentrifugation of PIC nanoparticles ahead of this antimicrobial activity experiment without significantly changing particle size (**Figure S5**).

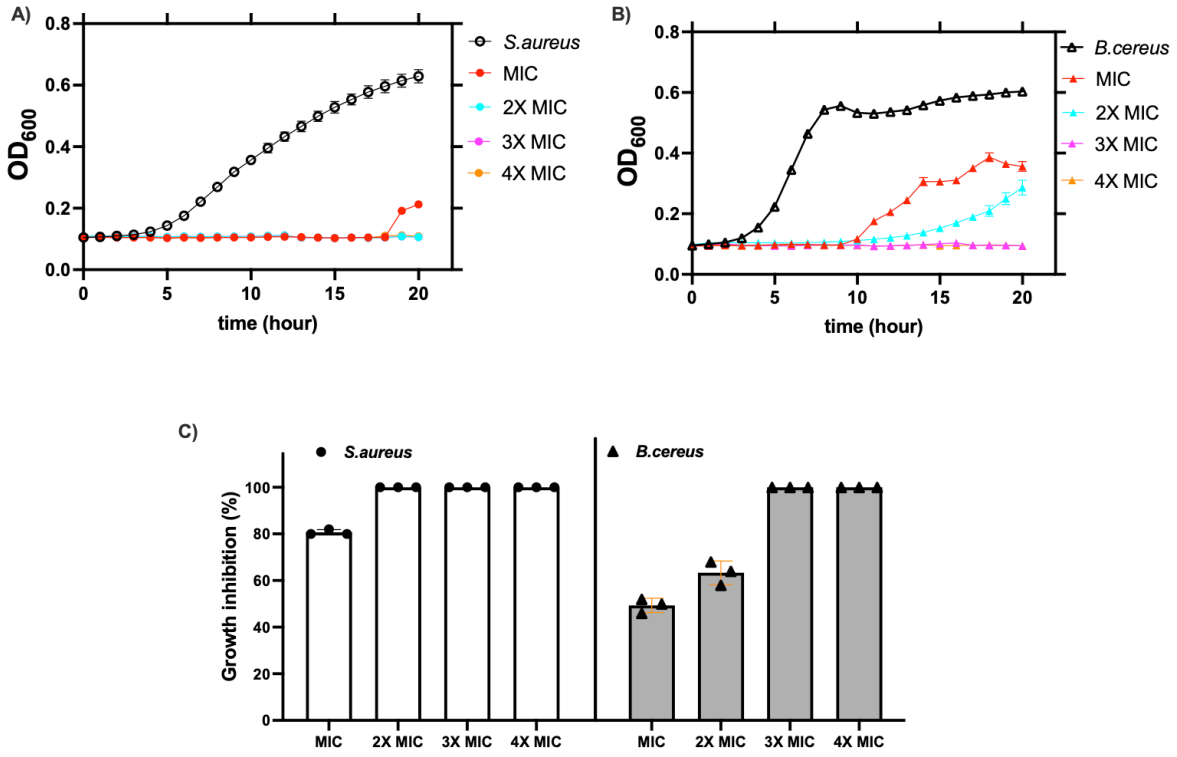


Figure 5 Growth curves of *S. aureus* and *B. cereus* cultures in TSB broth in the absence and presence of Nisin:HA PIC nanoparticles. B) Growth inhibition (%) caused by Nisin:HA PIC nanoparticles after 20 h incubation. Final concentration of Nisin in each sample was adjusted to the Minimum inhibitory concentration of Nisin and its multiples, up to 4X, against the related organism.

Growth curves of *S. aureus* and *B. cereus* in the absence and presence of Nisin:HA PIC nanoparticles are shown in **Figure 5A** and **Figure 5B**, respectively. Total nisin concentrations in PIC samples were adjusted to the MIC value of nisin (2.5 $\mu g.ml^{-1}$) and its multiples up to 4X. It is worth mentioning that Hyaluronic acid had no considerable inhibitory effect on the growth of *S. aureus* and *B. cereus* (**Figure S7**). Therefore, all observed growth inhibition was directly related to released nisin from Nisin:HA PIC nanoparticles. Interestingly, although *B. cereus* did not show hyaluronidase activity, Nisin:HA PIC nanoparticles exhibited an inhibitory effect against *B. cereus*. As shown in **Figure 5A**, when the total nisin concentration was adjusted to the MIC (10 $\mu g.ml^{-1}$) and double MIC (2X) (20 $\mu g.ml^{-1}$) value, Nisin:HA PIC nanoparticles caused a delay in the lag phase. However, they showed less growth inhibition than free nisin (%100) against *B. cereus*, 48% and 62%, respectively (**Figure 5C**). Total growth inhibition of *B. cereus* was obtained with PIC nanoparticles containing nisin at 3X (30 $\mu g.ml^{-1}$) and 4X MIC (40 $\mu g.ml^{-1}$). This limited antimicrobial activity of PIC

nanoparticles can be attributed to the burst release of nisin from the swelled nanoparticles or the disassembly of unstable complexes due to the osmotic effect in the growth media. On the other hand, PIC particles had a better inhibitory effect against *S. aureus* in the presence of nisin at MIC (2.5 $\mu g.ml^{-1}$) and double MIC (5 $\mu g.ml^{-1}$), with the growth inhibition values of 80% and 100% (**Figure 7C**). Consequently, Nisin:HA PIC nanoparticles showed better antimicrobial activity against hyaluronidase-producing *S. aureus,* proving their enzyme-responsive antimicrobial release properties.

3.5. Antimicrobial activity of Nisin:HA PIC nanoparticles in skimmed milk

Having identified that Nisin:HA PIC nanoparticles exhibited enzyme-responsive antimicrobial activity against *S. aureus* in a laboratory setting, we wanted to explore their antimicrobial activity in a real food system. For this purpose, milk was chosen as a relevant food system previously associated with staphylococcal food poisoning (Asao et al., 2003; Schmid et al., 2009). Like every other food product, milk can be easily contaminated with *S. aureus* as a result of poor hygiene practices. If the cold chain is not maintained, contamination can lead to enterotoxin production by this microorganism, eventually causing serious food intoxication. Considering the occurrence of this food poisoning, a controlled contamination experiment was designed to evaluate the responsive antimicrobial properties of Nisin:HA PIC nanoparticles against *S. aureus.* Also, as previously done, *Bacillus cereus* was employed as a control organism to evaluate the non-responsive antimicrobial activities of our nanoparticles in milk. For this purpose, sterile milk stored at 4°C in the presence of free nisin or Nisin:HA PIC nanoparticles was artificially contaminated by *S. aureus* or *B. cereus* at different times of storage period to mimic the potential contamination of food products during handling. After that, the contaminated milk was incubated for 24 h at room temperature to simulate the failure in maintaining the cold chain. Total nisin concentration in milk containing free nisin or Nisin:HA PIC nanoparticles was adjusted to double the MIC value of nisin against these two bacteria, 10 $\mu g.ml^{-1}$ for *S. aureus* and 20 $\mu g.ml^{-1}$ for *B. cereus* (**Fig S8).**

Upon contamination, total changes in *S. aureus* and *B. cereus* counts in milk samples at the end of the 24h incubation at 25°C are shown in **Figure 6**. No bacterial growth was observed in all milk samples containing free nisin during the first 6-day storage period. However, a decreasing trend in the nisin's antimicrobial efficacy was observed against these two pathogens during further storage periods, at 10 and 14 days. This is probably due to the interaction of nisin with milk components or the reduced stability of nisin at the pH of the milk (Ibarra-Sánchez et al., 2020). Remarkably, when compared to free nisin, Nisin:HA PIC nanoparticles were less effective against *B. cereus*, and therefore considerable bacterial growth was observed in all milk samples treated with PIC nanoparticles regardless of the storage period (**Figure 6A**). Moreover, no decreasing trend in *B. cereus* count was observed between day 0 and day 14. As previously observed in TSB broth, this limited antimicrobial activity against *B. cereus* can be attributed to the burst release of nisin from PIC nanoparticles in milk. These results indicated that Nisin:HA PIC nanoparticles were mostly stable in milk since this non-specific nisin release from PIC nanoparticles did not reach the MIC value of nisin against *B. cereus* despite the presence of double MIC in milk samples treated with Nisin:HA PIC nanoparticles.

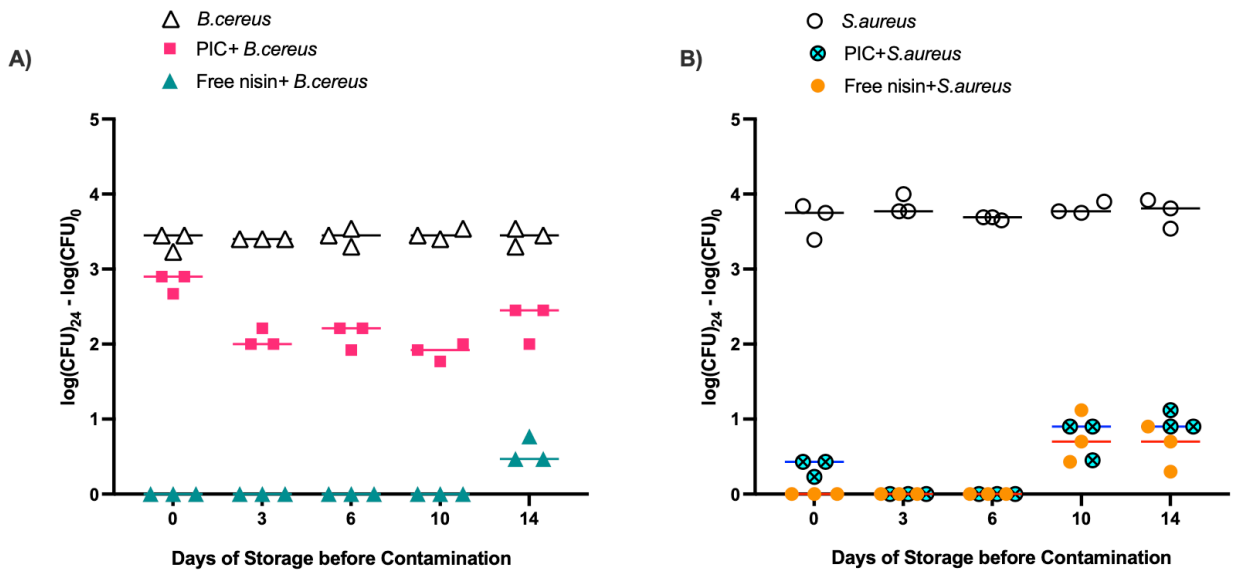


Figure 6 Changes in microbial counts for *B. cereus* (A) and *S. aureus* (B) in skimmed milk samples stored for different time periods at 4°C in the absence and presence of Nisin, and Nisin:HA PIC nanoparticles before artificial contamination with an overnight culture of *S. aureus* or *B. cereus.* Samples were incubated for 24 h at 25°C following contamination with *S. aureus* or *B. cereus.*

On the other hand, when we contaminated milk samples containing Nisin:HA PIC nanoparticles with *S. aureus, the* growth of this pathogen was totally inhibited during the first 6-day storage period (**Figure 6B**). Thus, Nisin:HA PIC nanoparticles displayed the same inhibitory effect with free nisin against *S. aureus*, while this was not the case for *B. cereus*. Taking into account that Nisin:HA PIC nanoparticles did not release a considerable amount of nisin before contamination, except for burst release, similar antimicrobial activity of PIC nanoparticles with nisin against *S. aureus* can be attributed to the enzymatic cleavage of our nanoparticles in the presence hyaluronidase-producing *S. aureus*, eventually allowing to the nisin release as expected. On the other hand, interestingly, about a 1-log increase in *S. aureus* counts was detected in milk samples stored with PIC particles for 14 days, similarly to nisin. This decrease in the antimicrobial activity of PIC nanoparticles could be explained by the inactivation of passively released nisin in milk during long storage periods. It should be noted that used nisin concentration in this experiment was limited to double MIC of *S. aureus (*10 $\mu g.ml^{-1}$), significantly lower than the permitted amount of nisin in dairy products (100-200 $mg.kg^{-1}$) (Jones et al., 2005). As such, *S. aureus* growth in milk during its extended storage periods could be more effectively inhibited by employing Nisin:HA PIC nanoparticles at higher nisin concentrations.

4. Conclusions

Here we presented the first example of enzyme responsive Nisin delivery system against *S. aureus*, the causative agent of staphylococcal food poisoning. Negatively charged, spherical Nisin-loaded PIC nanoparticles with moderate polydispersity were obtained following an easy methodology based on electrostatic complexation between high-Mw anionic hyaluronic acid and cationic Nisin. Nisin concentration in the reaction mixture has played a fundamental role in obtaining desirable PIC formulations. In vitro stability and release experiments demonstrated that Nisin:HA PIC nanoparticles were able to remain mostly stable in the presence of NaCl. At the same time, they could be rapidly degraded by hyaluronidase. We have shown that the antimicrobial effectiveness of Nisin:HA PIC nanoparticles was directly related to the enzymatic activity of the bacteria employed. In this regard, they were more effective against hyaluronidase-producing *S. aureus* and prevented this pathogen's

proliferation in both biological media and milk. They exhibited limited antimicrobial activity against non-hyaluronidase-producing *B. cereus* under the same conditions. Finally, the controlled contamination experiment has revealed that Nisin:HA PIC nanoparticles could protect nisin from its undesired interactions in the food matrix before contamination and release nisin in response to *S. aureus* contamination and subsequent growth in food. Overall, we believe that these enzyme-responsive Nisin:HA PIC nanoparticles have a promising potential to be the first example of responsive antimicrobial delivery system in food against *S. aureus*, and they could play an important role in preventing Staphylococcal food poisoning.

5. Supplementary information

Fluorescence data of Nisin-fluorescamine adducts, additional DLS data, additional TEM images, ultracentrifugation data, additional antimicrobial data for *S. aureus* and *B. cereus*, and hyaluronidase activity assay can be obtained in the supporting information.

6. AUTHOR INFORMATION

6.1. Author Contributions

T.C. and P.F.-T. conceived the project, and designed Nisin:CMC PIC nanoparticle preparation and characterization. T.C., C.A., H.O. and T.M. designed the antimicrobial experiments. P.F.-T., H.O. and T.M. secured funding. T. C. performed all experiments. T.C. and P.F.-T. analysed the data. TC wrote the paper, with all other authors contributing to the final version of the manuscript.

6.2. Conflicts of Interest

The authors declare no conflicts of interest.

7. Acknowledgements

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Electronic Supplementary Information

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1. DLS data: Preparation of Nisin:HA PIC samples at different [N:COOH] ratios

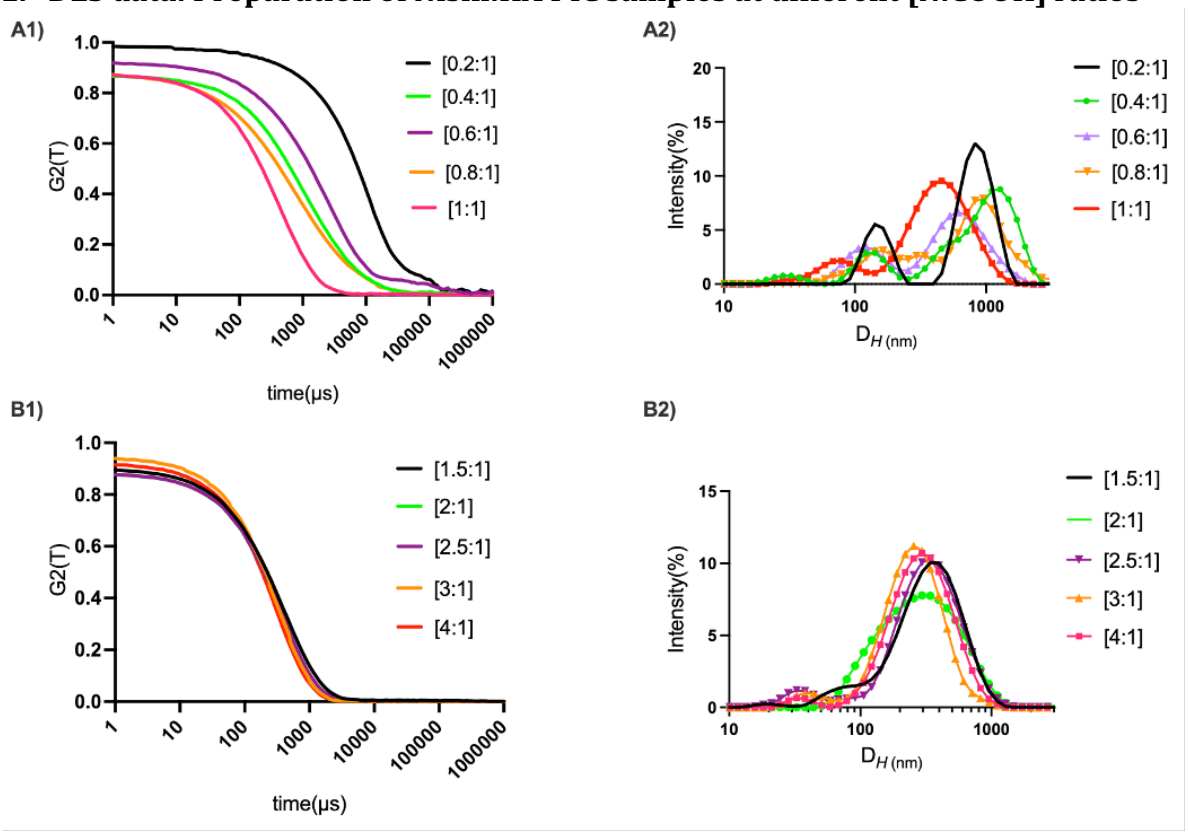
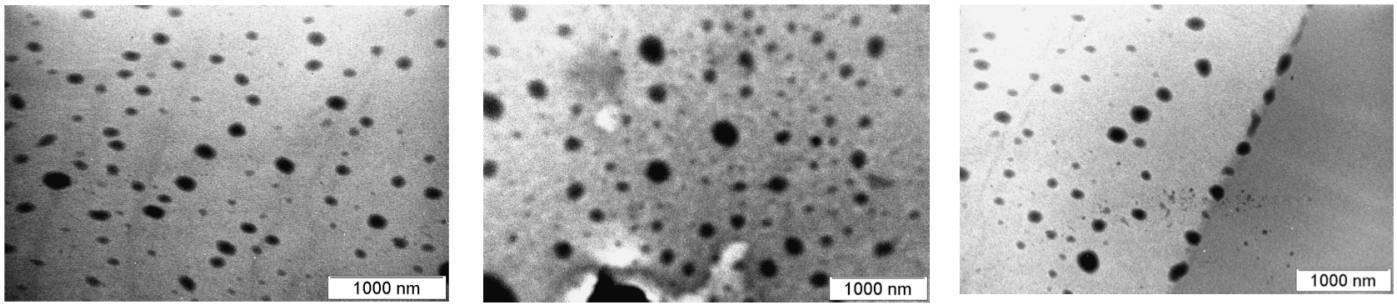


Figure S1 Autocorrelation function (ACF) curves (A1-B1) and intensity distributions of sizes for the Nisin:HA PIC nanoparticles samples prepared at different [N:COOH] ratios.

2. Additional TEM images of Nisin:HA PIC nanoparticles



$D_H \pm$ SD (nm)[a]	$D_{TEM} \pm$ SD (nm)[b]
288 ± 96	**218 ± 47**

[a] SD indicates the standart deviation found for the only size population fitted by the Malverns's Zetasizer software.
[b] Avarage diameter of 70 nanoparticles were measured twice (in their longest and shortest diameters) and SD was calculated from a total of 140 measurements.

3. Fluorescence data for Nisin-Fluorescamine adducts

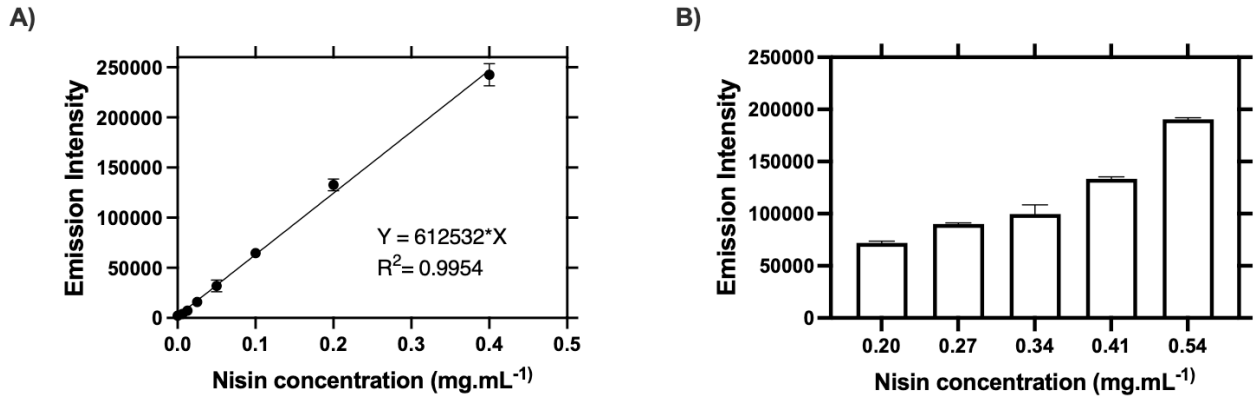


Figure S3 The standart curve of Free Nisin-Fluorescamine conjugates (left) and Emission Intensity of fluorescamine conjugates for Nisin:HA PIC nanoparticles prepared with different Nisin concentrations (right).

4. DLS data: Dialysis of Nisin:HA PIC nanoparticles under different conditions

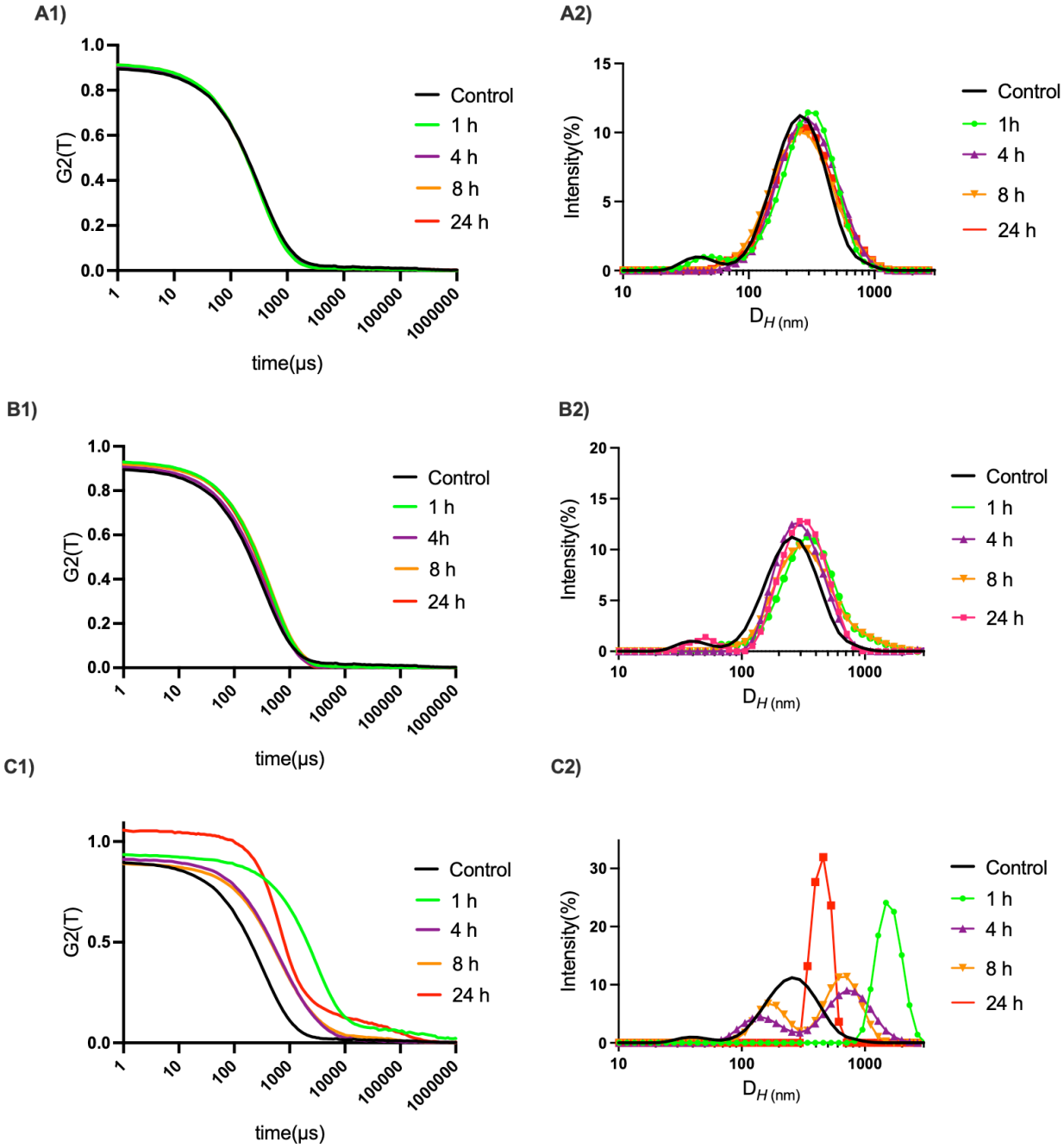
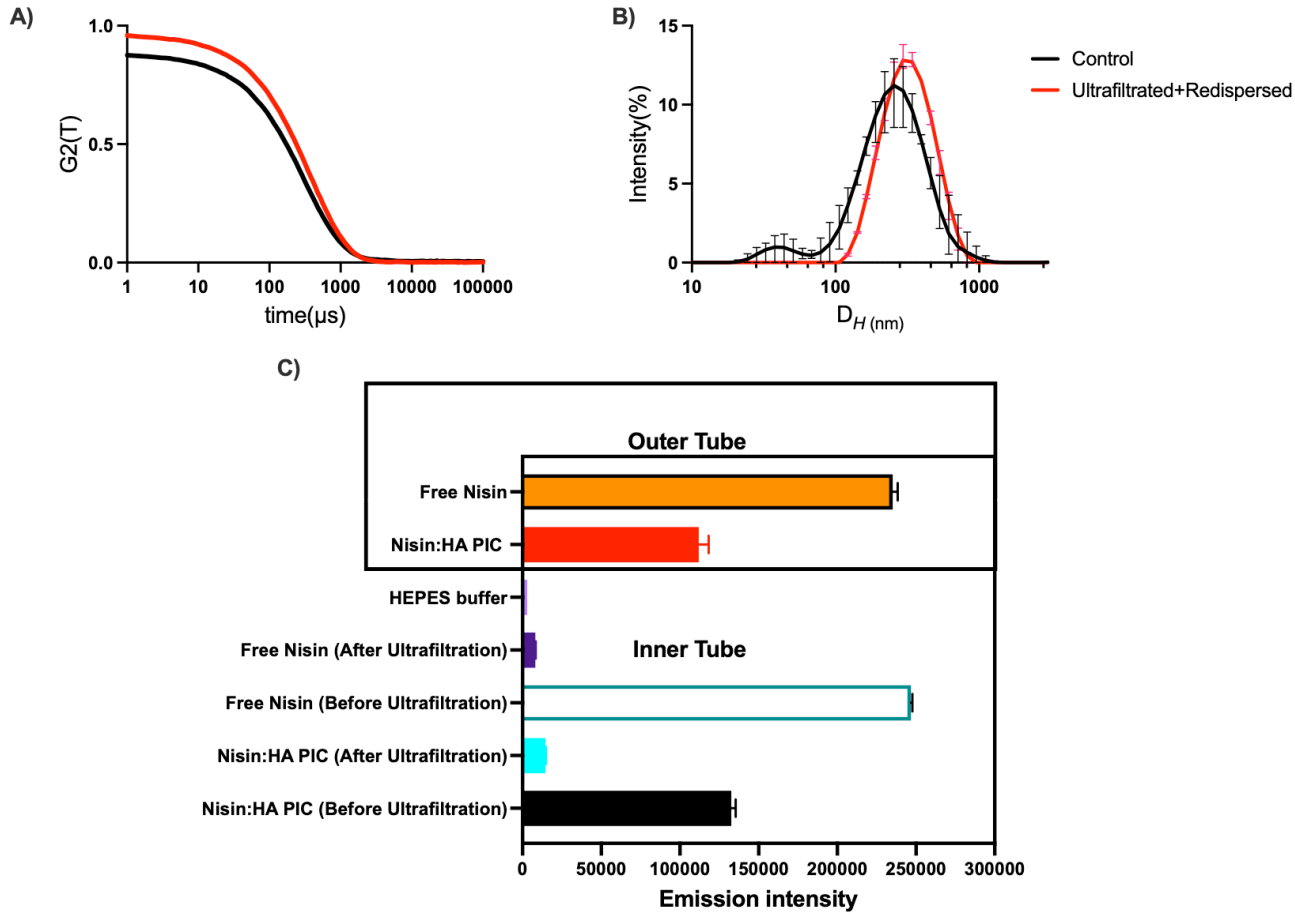


Figure S4 Representative autocorrelation function (ACF) curves and intensity distributions of sizes for the Nisin:HA PIC nanoparticles being dialysed in the absence NaCl and hyaluronidase (untreated PIC) (A1-A2), in the presence only only NaCl (B1-B2), and in the presence of NaCl and hyaluronidase (C1-C2) over time (1-24 hours)

5. Removal of Unencapsulated Nisin from Nisin:HA PIC nanoparticle solution

2ml of free Nisin (0.41mg/ml) or PIC nanoparticle solution prepared at 3:1 [N:COOH] (nisin equivalent of 0.41 mg/ml) ratio was centrifuged at 3500 x g at 20°C for 10 minutes using ultra centrifugal filter units (Amicon-MWCO-100kDa). Internal solution was redispersed in 2ml of HEPES buffer at pH 4.5 and analysed by DLS and fluorescamine assay. The content of unencapsulated nisin in the supernatant (in the outer tube) was calculated using fluorescamine assay using calibration curve of Nisin:Fluorescamine conjugates.
(**Figure S3A**).



	Free Nisin solution	Nisin:HA PIC solution
Nisin in the outer tube (mg/ml)	0.396	0.188
Total Nisin removed from inner tube (%)	**96.58%**	**45.85%**

Figure S5 Representative autocorrelation function (ACF) curves (A) and intensity distributions of sizes (B) for the Nisin:HA PIC nanoparticles before (black) and after (red) of removing unencapsulated nisin from PIC solution using ultracentrifugation process. Emission Intensity of fluorescamine conjugates for free Nisin and Nisin:HA PIC solution before and after ultracentrifugation process.

6. Qualitative Hyaluronidase activity of *S. aureus* and *B. cereus*

To investigate if *S. aureus* and *B. cereus* used in microbiology experiments are capable of degrade hyaluronic acid, their hyalurinidase activities was assessed by adopting a simple plate assay (Patil & Chaudhari, 2017). Briefly, Tryptic soy broth (TSB) was supplemented with agarose as a solidifying agent (1%), and this medium was sterilised and cooled to 50ºC. Filter-sterilised Hyaluronic acid solution was aseptically added to this cooled medium to obtain final HA concentration of 0.4 mg/ml. This medium was added to petri dish to allow it to solidify. Then, 100ul of the supernatant of overnight cultures of *S. aureus* and *B. cereus* in TSB supplemented with HA (0.4 mg/ml) was transferred to the holes aseptically dug on the HA containing agar plates. Finally, plates flooded with Grams iodine (for 1 minute) were photographed at the end of the incubation period at 37ºC for 24

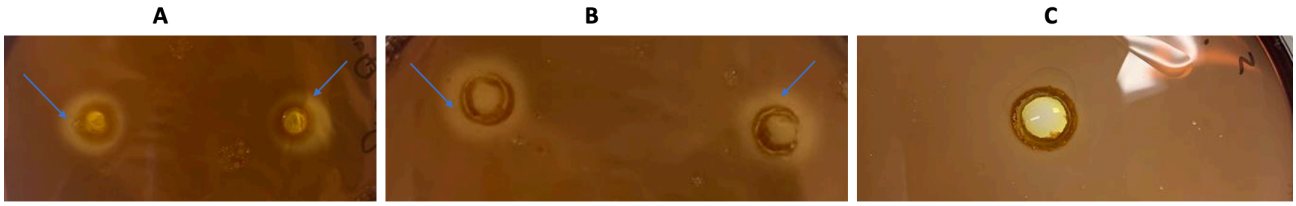


Figure S6 Plate assay for the detection of microbial hyaluronidase activity of commercial hyaluronidase (control) *S. aureus* (B) and *B. cereus* (C). Light-colored areas (shown by arrows) around the holes indicate HA degradation.

7. Growth curves of *S.aureus* and *B.cereus* in TSB treated by free nisin or HA

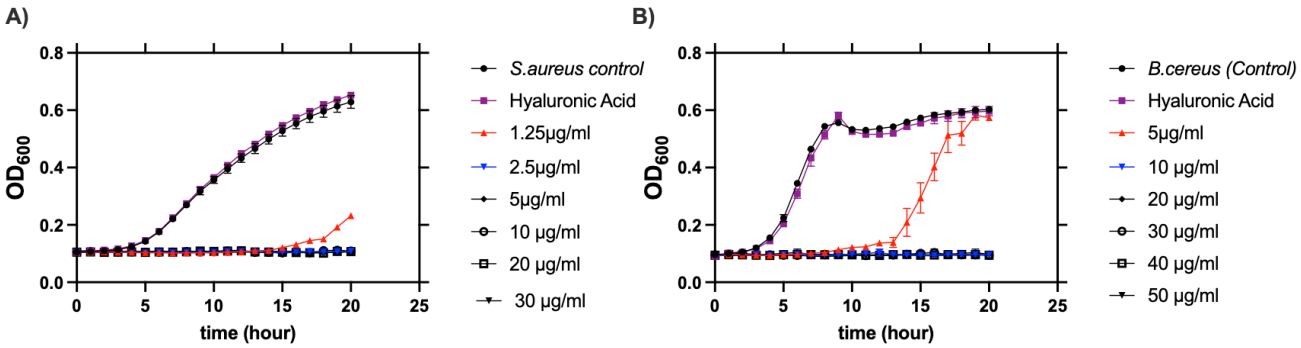


Figure S7 Changes to the optical density at 600 nm of *S. aureus* (A) and *B. cereus* (B) cultures in TSB in the absence (control) and presence of Hyaluronic acid in HEPES (50ug/ml, pH:4.5) or free Nisin at different concentration prepared in HEPES (pH= 4.5).

8. MIC value of Free Nisin against *S. aureus* and *B. cereus* in skimmed milk

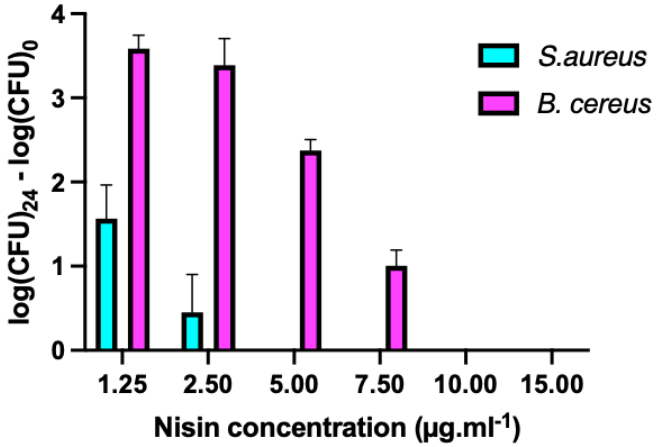


Fig S8 Changes in microbial counts for *S. aureus* and *B. cereus* in skimmed milk samples containing free nisin at different concentrations at the end of the 24 h incubation at 25°C. Error bars represent SD. (n=3)

CHAPTER 4

Nisin-loaded thermoresponsive Polyion Complex (PIC) nanoparticles as a novel antimicrobial delivery system in food

Teyfik Celen, Sameh El-Sayed, Taghi Miri, Helen Onyeaka and Paco Fernandez-Trillo

Format: Paper (Pending for submission)

Overview: In addition to the enzyme-responsive nisin carrier presented in Chapter 2, the other milestone of this thesis was to deliver nisin in food systems in response to abnormal temperature increases in food to prevent possible temperature-related rapid microbial growth. To this end, in this chapter, we attempt to encapsulate nisin in PIC nanoparticles using a thermosensitive anionic polymer. As such, unlike the polysaccharide-based biopolymers employed so far in this thesis, here we use synthetic poly(VPGVG-OH) as a thermosensitive encapsulating agent to prepare PIC nanoparticles. poly(VPGVG-OH) is a type of elastin-based side chain polymer (EBPs), and a chemically accessible variant of the famous elastin-like polypeptide (ELP), poly(VPGVG) exhibiting lower critical solution behaviour (LCST). Using this anionic polymer, nisin-loaded PIC nanoparticles (Nisin:poly(VPGVG-OH PIC) were prepared and characterised following the same methods in previous chapters.

The paper presented in this chapter covers comparative thermoresponsive studies (DLS, zeta potential, fluorescamine assay, and antimicrobial activity) between Nisin:poly(VPGVG-OH) and Nisin:PSS (control) PIC nanoparticles. Nisin:poly(VPGVG-OH) PIC nanoparticles displayed reversible thermoresponsive behaviour, resulting in a rapid increase in nisin accessibility at 40^{o}C and above. In-vitro antimicrobial activity studies supported this observed thermoresponsive activity, and their inhibitory effect against *Bacillus subtilis* increased as a function of temperature. Finally, we

observed that these nanoparticles showed promising encapsulation performance to protect nisin from its degradation in a model meat system during storage at refrigeration temperature, while they showed better antimicrobial activity than free nisin at simulated temperature abuse against *Clostridium perfringens*, a severe foodborne pathogen capable of rapidly proliferating above 40ºC. Overall, although these nanoparticles' synthetic character may limit its practical application in the food industry, these nanoparticles could play an effective role in enhancing the antimicrobial efficacy of nisin and preventing temperature-related microbial food safety and quality problems.

Contributions: T.C. and P.F.-T. conceived the project, and designed the nanoparticle preparation and characterisation. T.C.,, H.O. and T.M. designed the antimicrobial experiments. P.F.-T., H.O. and T.M. secured funding. T. C. performed all experiments. T.C. and P.F.-T. analysed the data. TC wrote the paper, with all other authors contributing to the final version of the manuscript.

Experimental contribution: SE carried out synthesis and characterisation steps towards the synthesis of poly(VPGVG-OH), the synthetic polymer employed in PIC nanoparticle preparation in this chapter.

Nisin-loaded thermoresponsive Polyion Complex (PIC) nanoparticles as a novel antimicrobial delivery system in food

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Abstract

Here we present a novel approach to deliver antimicrobial nisin in response to abnormal temperature increases in food systems. Nisin-loaded Polyion Complex (PIC) nanoparticles were prepared with an elastin-based side chain polymer, poly(VPGVG-OH), following a straightforward methodology based on electrostatic complexation. Nanoparticle samples prepared at different nisin concentrations allowed us to optimise particle size (from 175 to 329 nm), zeta potential (from -12.5 to -34), and encapsulation efficiency (from 54% to 68%). Also, stability profiles of these nanoparticles under simulated conditions showed formulation-dependent character. Reversible thermoresponsive behaviours of Nisin:poly(VPGVG) PIC nanoparticles were detected by DLS, zeta potential measurements, and fluorescamine assay. Antimicrobial activity studies confirmed that these nanoparticles have the potential to show a rapid inhibitory effect against food spoilage (i.e. *B. subtilis*) and foodborne pathogen (i.e. *C. perfringens*) at 40°C and above. Our results suggest that these Nisin:poly(VPGVG-OH) PIC nanoparticles could protect nisin from its undesired interactions in food systems during the storage of food products at safe temperatures (i.e. 4°C), while they could prevent temperature-related rapid microbial growth in food products.

Keywords: Nisin; Food antimicrobials; Responsive antimicrobial delivery; Polyion Complex Nanoparticles

1. Introduction

Nanoparticles are an attractive way to deliver of antimicrobial agents. In particular, due to their unique features, the first generation of antimicrobial carriers has been used to enhance the effectiveness of existing antibiotics in biomedical applications to address the antimicrobial resistance problem. For example, they can potentially prolong systemic circulation time and antibiotic half-time, release antibiotics in a sustained manner, eventually reduce systemic side effects and decrease the drug doses needed (Canaparo et al., 2019). However, over the past decade, recent developments in clinically relevant antimicrobial delivery applications have emerged novel antimicrobial delivery approaches. In this regard, stimuli-responsive nanoparticles releasing encapsulated cargo in a dynamic way in response to either a specific pathogen or a physical stimulation have been introduced as an effective way of improving antimicrobial-treatment efficacy (Huang et al., 2022; Nazir et al., 2022).

Thermoresponsive nanoparticles are an example of such delivery systems. Particularly, in clinical applications, since they are able to release of drugs in response to local hyperthermia, they have played fundamental role in increasing local drug concentration at the site of infection (Amin et al., 2020; Meyer et al., 2001). To develop thermoresponsive nanocarriers, thermosensitive polymers have been introduced to nanocarrier systems due to their interesting features resulting from changes in the temperature, such as changes in their conformation, solubility, and hydrophilic/hydrophobic balance. For instance, polymers exhibiting lower critical solution temperature (LCST) phenomenon by

undergoing reversible phase transition at a critical temperature have been frequently employed to place their thermoresponsive behaviour to nanocarriers (Bordat et al., 2019). However, although traditional (non-responsive) antimicrobial delivery systems have been employed in various platforms, this effective drug delivery approach has remained limited with biomedical applications, and no attempts have been paid for responsive antimicrobial delivery in alternative platforms, such as food systems (Fu et al., 2016).

In food systems, nanoparticle-based delivery systems have been employed to enhance the antimicrobial efficacy of food preservatives. For instance, nisin, the most famous natural food additive approved by FDA (Ibarra-Sánchez et al., 2020), has been encapsulated in nanoliposomes (Boualem et al., 2013; Laridi et al., 2003) and polysaccharide-based polymeric nanoparticles (Bernela et al., 2014; Gruskiene et al., 2021; Krivorotova et al., 2016; Luo et al., 2019) to minimise its undesired interactions with food components. However, in all these existing nisin-delivery systems, the antimicrobial activity of encapsulated nisin relies on non-specific, slow-release mechanisms such as passive diffusion, charge interactions, and swelling or slow breakdown of encapsulating matrix (Wu et al., 2016). Nisin is therefore released from these types of nanostructures in a sustained manner in food, including food products not contaminated and adequately stored (i.e. at safe temperatures). Consequently, it may lose its effectiveness during the storage of food products, leading to microbial food safety problems and eventually decreasing the shelf life of food products. Therefore, there is a need for a delivery system that can respond to abnormal changes in food, resulting in possible food safety and quality problems.

In this respect, we focused on using temperature as an external stimulus to develop a novel antimicrobial delivery system in food. It is well known that there is a strong relationship between microbial food safety problems and the storage temperature of food products (Wu et al., 2016). Spores of critical foodborne pathogens, such as *Bacillus cereus* and *Clostridium perfringens*, can rapidly germinate in food products kept at unsafe temperatures and not adequately cooled. For instance, *C. perfringens* rapidly grows between 43^{o}C and 47^{o}C in food, where generation time may be as short as 8 min (Griffiths & Schraft, 2017; Márquez-González et al., 2012). Therefore, we hypothesised that a rapid increase in the availability of nisin in food as a function of temperature could play an important role in preventing temperature-related microbial growth.

In recent years, Polyion Complex (PIC) nanoparticles, formed as a result of electrostatic interaction between cationic peptide and anionic polymer, have been reported as a safe and cost-effective encapsulation methodology (Insua, Majok, et al., 2017). Therefore, since PIC nanoparticles would be one of the most suitable platform to cationic nisin's encapsulation, here we use an anionic thermoresponsive polymer, poly(VPGVG-OH) (where V = valine, P = proline, and G = glycine) to prepare nisin-loaded PIC nanoparticles. This is a type of elastin-based side-chain polymers (EBPs), with pendant VPGVG peptide sequences as readily accessible elastin-like peptide (ELP) analogues displaying LCST behaviour (Fernández-Trillo et al., 2007, 2008). Due to their thermosensitive properties, ELPs have been used as a useful component in various drug delivery applications (MacEwan & Chilkoti, 2014; McDaniel et al., 2010; Varanko et al., 2020). Numerous studies on the biological composition of ELPs have revealed their biocompatibility and biodegradation. Importantly, the effectiveness of the biomaterials based on complex coacervation between oppositely charged ELP and polyelectrolytes have been previously reported (Janorkar et al., 2008). Consequently, considering the similar thermosensitive behaviour of EBPs with ELPs, we anticipated that encapsulated nisin by an EBP (poly(VPGVG-OH) could rapidly become available and show antimicrobial activity against a possible microbial growth above the transition temperature of this polymer.

In the present work, the first example of thermosensitive PIC nanoparticles were prepared simply through electrostatic complexation of cationic Nisin and anionic poly(VPGVG-OH). The synthesised PIC nanoparticles were characterised in terms of size, morphology, charge, and encapsulation efficiency. Then, the stability of different formulations was evaluated in the simulated conditions. We then demonstrated the thermosensitive properties of nisin-loaded PIC nanoparticles through further DLS, zeta potential, and fluorescence measurements by comparing them to Nisin: polystyrene sulfonate (PSS) PIC nanoparticles, non-thermoresponsive control. After that, in-vitro thermoresponsive activity of these nanoparticles was verified against *B. subtilis*, gram-positive food-spoilage organism. Finally, an antimicrobial activity experiment was designed to evaluate the role of Nisin:poly(VPGVG-OH) PIC nanoparticles in prolonging nisin's antimicrobial efficacy against *Clostridium perfringens* during the storage of a model food system (i.e. meat). Our results suggest that Nisin:poly(VPGVG-OH) PIC nanoparticles have a promising potential in the protection of nisin at low temperatures while they considerably increase the accessibility of nisin at critical temperatures associated with rapid microbial growth.

1. Materials and Methods

1.1. Materials

Nisin (Ultrapure Nisin-≥%99 purity) was purchased from Handary S.A. (Brussels, Belgium). Nylon 0.45 μm syringe filters were bought from Camlab Ltd. Fluorescamine was purchased from Acros Organics TM. All other chemicals used were of analytical grade. 4-(2- hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and Glutathione (Certified Reference Material-PHR1359) were purchased from Sigma-Aldrich Co. Formvar Carbon support (300 mesh) TEM grids were purchased from Labtech International Ltd. Nutrient Broth (NB) and Brain Heart Infusion Broth (BHIB) were purchased from Fisher Scientific (United Kingdom). *Clostridium perfringens* NCTC 8238 and *Bacillus subtilis* NCIMB 3610 were kindly provided by Biochemical Engineering, University of Birmingham, United Kingdom.

1.2. Instrumentation

Dynamic light scattering (DLS) measurements were carried out in a Zetasizer Nano ZS (Malvern Instruments Ltd). All described samples in this chapter were analysed by using same conditions: Scattering angle- 173^{o}, temperature= 25 ^{o}C, run duration= 60 s, fixed measurement position= 4.65 mm and automatic attenuation. The size distributions, Autocorrelation function (ACF) curves, and Polydispersity index (PDI) values were obtained from Malvern Zetasizer software 7.03. Zeta potentials were recorded 30 times at 140 V. A FLUOstar Omega (BMGLabtech Gmbh) microplate reader was used to measure fluorescamine reactions. TEM images of PIC nanoparticles were acquired on a Jeol 1400 Bio TEM.

1.3. Preparation of Nisin:poly(VPGVG-OH) PIC nanoparticles

The stock solution of poly(VPGVG-OH) previously synthesised and characterised (**Fig S1-S3**) was prepared dissolving in HEPES buffer (pH:8) and stored at 4^{o}C until usage. PIC nanoparticles were prepared by adopting a protocol previously reported by our group (Insua, Liamas, et al., 2016). Firstly, stock solutions of Nisin (0.5 mM, pH:4) and poly(VPGVG-OH) (1mM, pH:8) were prepared in 5mM HEPES. Thereafter, ratios representing the proportion of amines in Nisin over carboxyl groups in poly(VPGVG-OH), were prepared mixing stock solutions of nisin and poly(VPGVG-OH). For instance, to prepare PIC particles at 1.5:1 [N:COOH] ratio (i.e. amines 1.5 times more than carboxyl groups), from stock solutions previously prepared in 5mM HEPES buffer and filtered through 0.45 um nylon syringe, drop-wise addition of 1 ml Nisin was applied to 2 ml of

poly(VPGVG-OH) under stirring. Finally, the pH of the reaction mixture was adjusted to 5.5, and the sample were incubated under stirring at 500rpm/rt. for 24 hours. The same protocol was followed to prepare PIC particles at other [N:COOH] ratios by changing the amount of Nisin in samples.

1.4. Estimation of Encapsulation Efficiency (EE)

The loading efficiency of nisin in PIC nanoparticles was carried according to the percentage of available nisin nanoparticle samples to react with fluorescamine dye. To this end, firstly, a standard curve of nisin-fluorescamine conjugates were established by being reacted 0.125 ml of nisin solution at different concentrations (dissolved in 5mM HEPES) with 0.125 ml of fluorescamine dye (2mM) (dissolved in methanol) for 30 minutes and measuring fluorescence at 355 nm (excitation) and 460 +\- 10nm (emission) (Weigele et al., 1972). HEPES buffer was used as blank reagent. Having established standard curve of nisin, PIC nanoparticle samples were analysed following the same procedure (**Fig. S1**). Encapsulation efficiency was (%) estimated using (**Equation 1**)

$$\frac{[mg \cdot mL^{-1}]_{total\ Nisin} - [mg \cdot mL^{-1}]_{available\ Nisin}}{[mg \cdot mL^{-1}]_{total\ Nisin}} * 100 \qquad \textbf{Equation 1}$$

1.5. TEM of Nisin:poly(VPGVG-OH) PIC nanoparticles

For TEM analysis, 5uL of a suspension of PIC nanoparticles prepared at 0.75:1 [N:COOH] ratio were deposited on the surface of the TEM grid and it was left to dry at ambient temperature by covering from dust. Once dried, 5uL of a 1mg.mL^{-1} solution of phosphotungstic acid in distilled water were used to stain the nanoparticles. The excess of staining solution was gently removed at the end of the staining period for 1 minute.

1.6. Monitoring Stability of Nisin:poly(VPGVG-OH) PIC in the presence of NaCl

To a sample of Nisin:poly(VPGVG-OH) PIC nanoparticles (0.5 ml) prepared at 0.75:1 [N:COOH] ratio as described above, 0.1 ml of a solution of NaCl in water was added individually to obtain total NaCl concentrations of 75, 150, and, and 300 mM. Then samples were incubated for 1 h at ambient temperature and analysed by DLS.

1.7. Thermoresponsive studies 1: DLS and Zeta potential measurements

0.5 ml of Nisin:poly(VPGVG-OH) PIC sample (prepared at 0.75:1 [N:COOH]) was incubated at 20, 30, 40, and 50^{o}C for 20 min, and analysed by DLS and zeta potential measurements at related temperatures. Finally, the PIC solution was left to cool at room temperature and analysed again.

1.8. Thermoresponsive studies 2: Fluorescamine assay

0,15 ml of Nisin (0,2mg/ml) and Nisin:poly(VPGVG-OH) PIC solution (nisin equivalent: 0.2mg/ml) were added to 96-well black-walled microplate and incubated at 20, 30, 40, and 50^{o}C for 20 min. After each incubation, each sample was and mixed with 0,15 ml of fluorescamine solution (2mM in methanol). The microplate was incubated under orbital shaking for 15 minutes at related temperature. Finally, fluorescence was measured by exciting at 355 nm and reading the emission at 460 +/- 10 nm. Finally, the same experiment was repeated for the PIC solution cooled from 50^{o}C to 20^{o}C.

1.9. Antimicrobial evaluation of PIC nanoparticles against *Bacillus subtilis*

100 µl aliquots of *B. subtilis in* nutrient broth ($OD6_{00}$: 0.1) were loaded in the designated positions of a 96-well microplate. Then, to determine the minimum inhibitory concentration (MIC) of Nisin for *B. subtilis*, these bacterial cultures were mixed with 100 µl of Nisin (in 5mM HEPES buffer-pH:5.5). Two microplates were prepared, they were individually incubated at 30°C and 40°C, and the OD_{600} was monitored every hour for 18 h. Thereafter, the same protocol was followed using 100 µl of free Nisin and PIC nanoparticles at double MIC value. All samples were prepared in triplicate, with mean values and standard deviations reported. Also, at the end of the incubation, total growth inhibition values (%) was calculated for each sample using the formula (**Equation 2**)

$$Abs\left[\frac{OD_{600}\ untreated\ -\ OD_{600}\ sample}{OD_{600}\ untreated}\right] * 100 \qquad \textbf{Equation 2}$$

1.10. Antimicrobial evaluation of PIC nanoparticles against *C. perfringens* in the absence or presence of Glutathione

0.5 ml of Free Nisin solutions prepared at different concentrations (in HEPES-pH:5.5), and 0,4 ml of HEPES buffer (pH:5.5) was transferred to 4 ml of sterile Brain Heart Infusion broth (BHIB, pH:5.5). To determine the minimum inhibitory concentration (MIC) of Nisin against *Clostridium perfringens*, 100 µl aliquots of *C. perfringens (*OD600: 0.1-in BHIB*)* were aseptically transferred to these samples, and the samples were individually incubated at 25°C and 45°C for 24 h. Having identified the MIC value of Nisin, 0.5 ml of free Nisin and PIC nanoparticle solution at the double amount of MIC value was transferred to 4.4 ml of sterile BHIB with or without Glutathione (total glutathione concentration: 0.2 $mg.mL^{-1}$). These samples were incubated at 4°C for 2 days. After incubation, they were artificially contaminated by 100 µl of the overnight culture of *C. perfringens* (OD_{600}:0.1 in BHIB), and individually incubated at 25°C and 45°C for 24 h. Finally, OD_{600} of the samples were recorded at the end of the incubation, and total growth inhibition values were calculated as a percentage using the formula described above (**Equation 2**).

3. Result and Discussion

3.1. Preparation and Characterisation of Nisin:poly(VPGVG-OH) PIC nanoparticles

This work aimed to develop a novel encapsulation system capable of delivering antimicrobial nisin in response to temperature changes in food systems. For this purpose, we selected PIC nanoparticles as an ideal platform for two main reasons. First, due to the cationic nature of nisin, PIC nanoparticles are easy and affordable materials that has already been used to protect nisin by delaying its release in the food system using food-grade anionic polymers. Second, drug release kinetics can be adjusted depending on the characteristics of the polymer used as a coating agent. Depending on these, we anticipated thermoresponsive PIC nanoparticles loaded with nisin could be prepared using a thermosensitive anionic polymer; hence thermoresponsive antimicrobial activity could be achieved at desired temperature ranges depending on the properties of the selected polymer. On this basis, we selected poly(VPGVG) undergoing a transition from soluble to insoluble in aqueous solutions at a

lower critical solution temperature (LCST). The transition temperature of poly(VPGVG) is generally between 27°C and 40°C (Li et al., 2018; Rodríguez-Cabello et al., 2009) depending on the various factor such as ionic strength and Mw of the polymer. This temperature range is suitable for the rapid growth of pathogenic foodborne pathogens such as *Clostridium perfringens.* Consequently, owing to potential structural changes on Nisin:poly(VPGVG) PIC nanoparticles above the transition temperature, encapsulated nisin in PIC nanoparticles may rapidly become available and stop the growth of undesired bacteria in food.

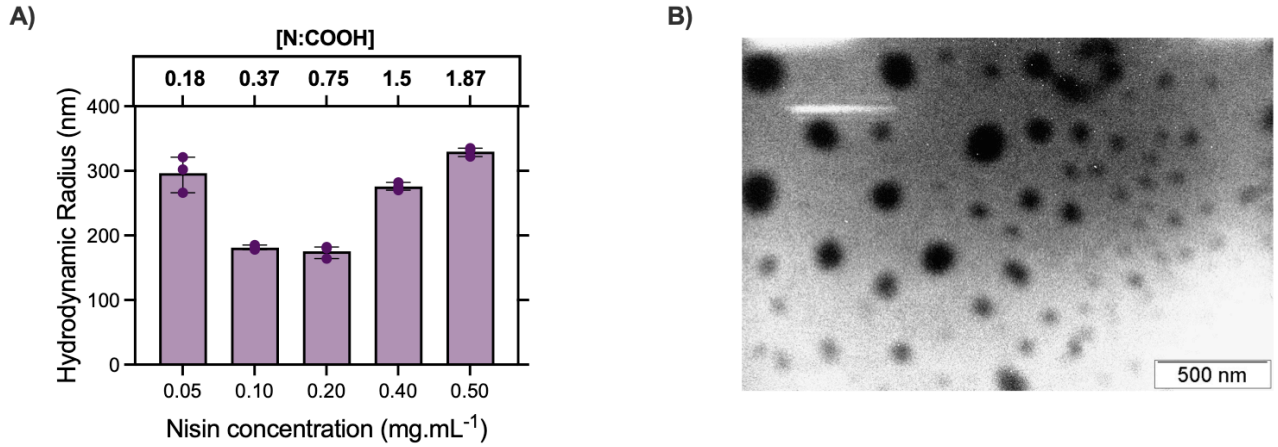


Figure 1 The size of Nisin:poly(VPGVG-OH) PIC nanoparticles prepared at different nisin concentrations (A) and representative TEM image for PIC nanoparticles prepared at 0.75 [N:COOH] ratio. Final concentration of poly(VPGVG-OH) was equal in all samples (0.21 mg.mL^{-1}) Data shown is the average of 3 technical replicates.

With these considerations in mind, our initial effort was to bring this functional polypeptide in negatively charged character to prepare PIC nanoparticles loaded with cationic nisin. Therefore, firstly, we synthesised an anionic, elastin-based side-chain polymer (EBS) poly(VPGVG-OH) by the free radical polymerisation of (Ma-VPGVG-OH), a methacrylate derivative of (VPGVG)**(Fig S1-S2).** Then the first example of Nisin:poly(VPGVG-OH) PIC nanoparticles was prepared by adding different amounts of a solution of Nisin at pH 4 to a solution of poly(VPGVG-OH) at pH 7. To maximise electrostatic interaction in the samples, the pH of the final solution was adjusted to pH 5.5 at which nisin is fully protonated (Rollema et al., 1995) while carboxyl group in the poly(VPGVG-OH) structure is mostly deprotonated.

As shown in **Fig 1A**, all PIC nanoparticles formed at different Nisin concentrations were at the nanometric scale (**Fig S3**). However, there was no clear correlation between the amount of Nisin added and the size of the nanoparticles. When the lowest nisin concentration was used (0.05 mg.mL^{-1}), the size of formed nanoparticles was 296 nm, while smaller size distributions were obtained at higher nisin concentrations of 0.1, 0.2 mg.mL^{-1}, 181 and 174 nm, respectively. The formation of smaller complexes with increasing nisin concentrations could be explained by sufficient electrostatic interactions in the presence of a more cationic charge, leading to obtain tighter PIC nanoparticles. With the amount of nisin further increased to 0.4 and 0.5 mg.mL^{-1}, particle size then became bigger, 275 and 328 nm, respectively. This observed increase in size is probably due to electrostatic repulsion between cationic moieties in Nisin. Further size and morphology characterisation nanoparticles was carried out by TEM measurements using the PIC nanoparticles prepared at 0.75:1 [N:COOH] as a representative formulation of Nisin:poly(VPGVG-OH) PIC nanoparticles (**Fig 1B and Fig S5**). PIC

nanoparticles presented a spherical morphology. However, the sizes of nanoparticles calculated by TEM (113 nm) were smaller than Dh measured by DLS (174 nm). This size reduction observed by TEM could stem from the drying step of sample preparation, which could lead to evaporation of the solvent in the highly hydrated nanomaterials and, eventually, PIC particles shrinking (Friedrich et al., 2010).

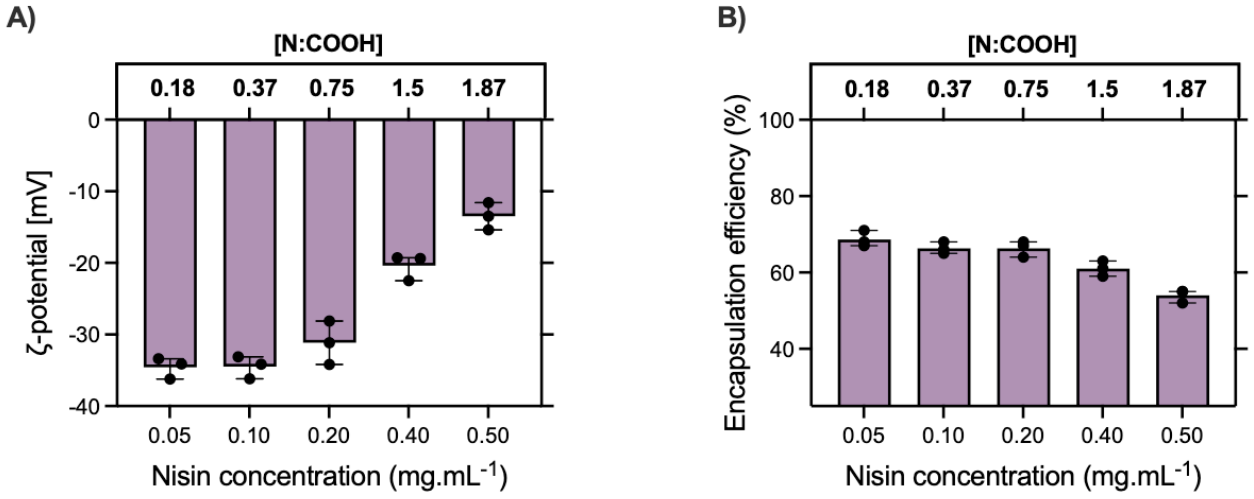


Figure 2 The effect of Nisin concentration in the sample on the ζ-potential (A) and encapsulation efficiency of Nisin:poly(VPGVG-OH) PIC nanoparticles (B). Data shown is the average of 3 technical replicates.

It is worth mentioning that further nisin increase in PIC mixtures resulted in macroscopically unstable/flocculated samples **(Fig S3C),** suggesting that all negative charge being displayed by poly(VPGVG-OH) was neutralised, and non-charged PIC complexes were formed and eventually flocculated. This result indicated that all colloidally stable PIC nanoparticles discussed above were negatively charged, and zeta potential measurements evidenced this. As shown in **Fig 2A**, all nanoparticles were negatively charged while the zeta potentials continuously increased with increasing nisin concentrations, indicating sequential absorption of nisin on poly(VPGVG-OH). Since charged corona could protect cationic Nisin from its undesired interactions with negatively charged components in food, these negatively charged nanoparticles obtained were a desired outcome. Nevertheless, considering the [N:COOH] ratio of the PIC samples prepared, negative zeta potential values were surprising for the samples prepared at 1.5 and 1.8.7 [N:COOH] ratio, in excess of cationic Nisin. This is probably because not all nisin was being encapsulated in PIC samples.

In order to determine the encapsulation efficiency (EE) of nisin, PIC nanoparticles were analysed by fluorescamine assay. In this fluorogenic assay, hypothesizing that unencapsulated nisin would directly react with fluorescamine dye in PIC particle solutions, samples were analysed according to their fluorescence intensity (460 nm) following their incubations with this dye. A calibration curve previously generated for the fluorescence intensity (460 nm) of fluorescamine-free nisin adducts was used to calculate unencapsulated nisin in each PIC solution. **(Fig. S4)**. However, it should be noted that this method may underestimate EE because some of the fluorescamine dye may diffuse to the core of nanoparticles and react with encapsulated nisin. Despite this potential limitation, encapsulation efficiency values varied from 68% to 55% **(Fig 2B)**, tending to decrease at higher nisin concentrations. Consequently, there was free nisin in all nanoparticle solutions prepared, clarifying why all Nisin:HA PIC particles were negatively charged in the samples prepared 1.5 and 1.87 [N:COOH] ratios. Overall, we obtained 5 different PIC nanoparticle samples stabilised by the excess

poly(VPGVG-OH) chains and capable of encapsulating nisin. These nanoparticles were used in further stability and responsivity experiments without purification.

3.2. Stability of Nisin:poly(VPGVG-OH) PIC nanoparticles in the presence of NaCl

The electrostatic forces that keep PIC nanoparticles together can be disturbed by small electrolytes in the dilution media (i.e. food), which can potentially result in swelling and, ultimately breakdown of these nanoparticles. Thus, encapsulated drugs can be rapidly released from PIC nanoparticles due to this usual decomposition (Insua, Wilkinson, et al., 2016; Insua, Zizmare, et al., 2017). In this work, since our priority is to increase the bioavailability of nisin depending on the temperature changes in food, a premature release occurring in the presence of small electrolytes commonly found in food, such as Na^+ and Cl^- would not be desirable for our system. With this in mind, in order to assess the tolerance of Nisin:poly(VPGVG-OH) PIC nanoparticles to NaCl, changes in the particle size were monitored following 1-hour incubation in the presence of NaCl. Total NaCl concentrations in the samples were adjusted to 75, 150, and 300mM, considering the approximate NaCl content of food products in which nisin is applied, such as cheese (El-Bakry, 2011) and processed meat (Sparks et al., 2018).

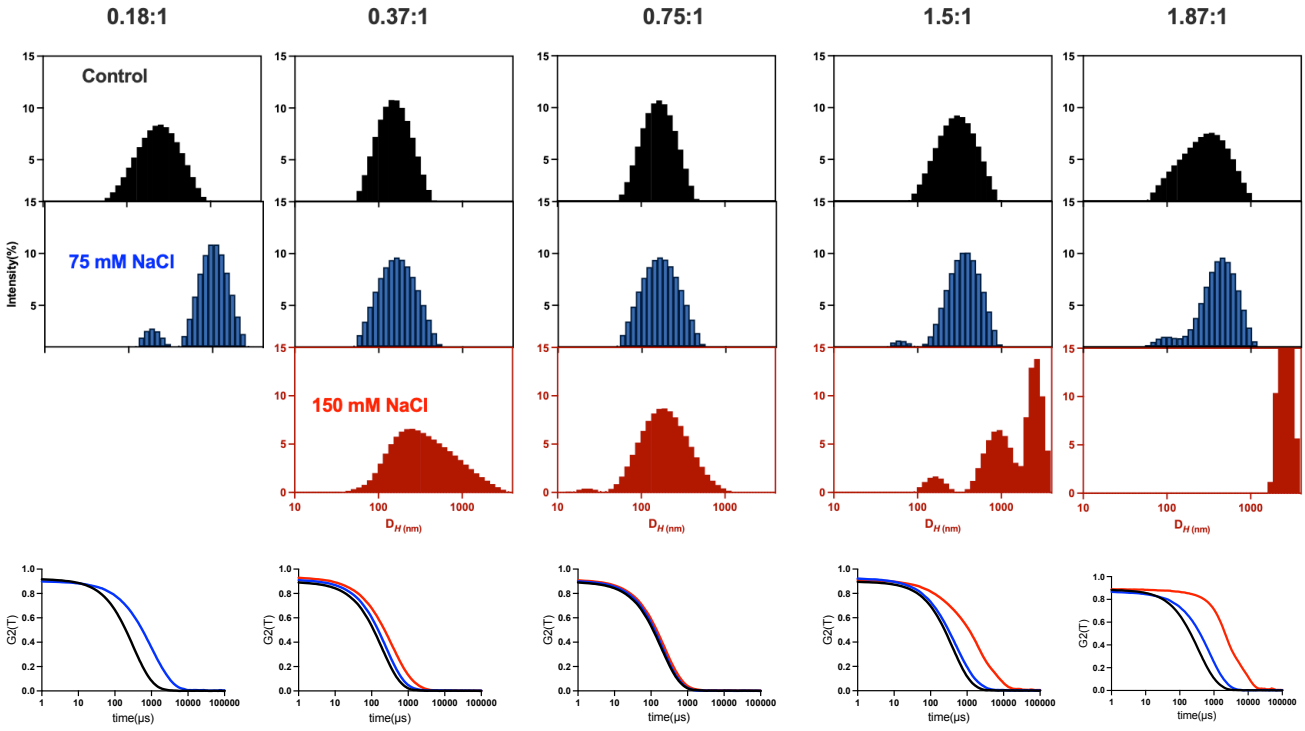


Figure 3 DLS Size-Intensity distributions and DLS correlograms (bottom) of Nisin:poly(VPGVG-OH) PIC nanoparticles prepared at different [N:COOH] ratios in the absence (control) and in the presence of NaCl concentrations of 75 and 150 mM after incubation for 1 h. No DLS measurement was conducted for the macroscopically flocculated PIC nanoparticle samples.

As shown in **Fig 3**, in the presence of NaCl of 75 mM, the size of PIC nanoparticles prepared at the 0.18 [N:COOH] ratio remarkably increased and micron size-complex was detected by DLS, suggesting electrostatic attraction was weaker, and therefore particles rapidly swelled. Interestingly, when we analysed other formulations under the same conditions, there was a clear correlation between [N:COOH] ratio of the samples and particle stability. In this regard, PIC nanoparticles prepared at [N:COOH] < 1 (0.37:1 and 0.75:1) showed unchanged size distributions, while the size

of PIC formulations at [N:COOH] > 1 (1.5:1 and 1.87:1) doubled. Notably, when NaCl concentration was increased to 150 mM, only the formulation prepared at 0.75 [N:COOH] ratio remained stable, a 3-fold increase was observed in the size of PIC particles prepared 0.37 [N:COOH] ratio. On the other hand the size of particles prepared at 1.5 and 1.87 [N:COOH] significantly increased. Since the zeta potential was lower in these samples **(Fig 2A)** , the current negative charge on particle corona may be fully screened at this Na^+ concentration. Consequently, the micron-size complex observed in these formulations can be attributed to the inter-particle aggregation of uncharged complexes (Lounis et al., 2016). As evidence of this hypothesis, when NaCl concentration raised to 300mM, all formulations macroscopically flocculated**,** indicating inter-particle aggregation of uncharged complexes. Therefore, we determined that 300 mM NaCl is the upper limit for the stability of Nisin:poly(VPGVG-OH) PIC nanoparticles. Consequently, due to its desirable stability profile at the NaCl concentrations of 75 mM and 150 mM, we choose the formulation prepared 0.75:1 [N:COOH] ratio to be employed in further thermoresponsive studies and antimicrobial activity experiments.

3.3. Thermoresponsive properties of Nisin:poly(VPGVG-OH) PIC nanoparticles

The use of thermosensitive polymers exhibiting LCST behaviour, i.e. increase in hydrophobicity at higher temperature, is one of the most common way of developing stimuli-responsive nanostructures for controlled drug delivery. Above the transition temperature of such polymers, drug release can be triggered as a result of structural changes (e.g. shrinkage) in this type of nanocarriers (Karimi et al., 2016; Le et al., 2022; Reis et al., 2019). With this in mind, having established that nisin could be encapsulated by using poly(VPGVG-OH) possessing LCST, further DLS, zeta, and fluorescence measurements (fluorescamine assay) were conducted at various temperatures verify thermoresponsive properties of Nisin:poly(VPGVG-OH) PIC nanoparticles (prepared at 0.75:1 [N:COOH]). Additionally, to make comparisons with Nisin:poly(VPGVG-OH) PIC nanoparticles during these thermoresponsive studies, as a non-thermosensitive control Nisin:poly styrene sulfonate (PSS) PIC nanoparticles were previously prepared at the same nisin concentration (0.2 $mg.ml^{-1}$)**.**

Firstly, when the temperature was increased from 20ºC to 30ºC, no change was observed in the hydrodynamic radius (D_h) of Nisin:poly(VPGVG-OH) PIC nanoparticles **(Fig 4A)**. Interestingly, increasing the temperature to 40ºC and 50ºC, respectively, caused a slight decrease in Dh, while polydispersity index of these nanoparticles considerably decreased **(Fig 4B)**, and therefore a narrower size distribution was observed **(Fig S6).** Furthermore, detected counts by DLS were higher for Nisin:poly(VPGVG-OH) PIC samples at 40ºC compared to lower temperatures applied **(Fig 4C)**. Since the temperature did not cause such changes in Nisin:PSS PIC nanoparticles, they can be explained by increased hydrophobicity of poly(VPGVG-OH) chains in the PIC structure, resulting in the uniform and denser nanoparticles scattering more light. Changes in the zeta potential were also monitored to further investigate this behaviour between 20ºC and 50ºC **(Fig 4D)**. Notably, when the temperature was raised from 30ºC to 40ºC, the zeta potential of Nisin:poly(VPGVG-OH) PIC nanoparticles sharply increased from -31 to -9. Since the negatively-charged nanoparticle corona is formed by the excess of poly(VPGVG-OH) chains, this increase in zeta potential (decrease in the absolute value) is probably due to collapsed particle corona upon heating (**Scheme 1).** On the other hand, PDI, count rate, and zeta potential values which are close to the initial values were obtained DLS and zeta potential measurements made upon cooling of Nisin:poly(VPGVG-OH) PIC nanoparticles from 50ºC to 20ºC. Overall, these findings clearly indicated the reversible thermoresponsive behaviour of Nisin:poly(VPGVG-OH) PIC nanoparticles.

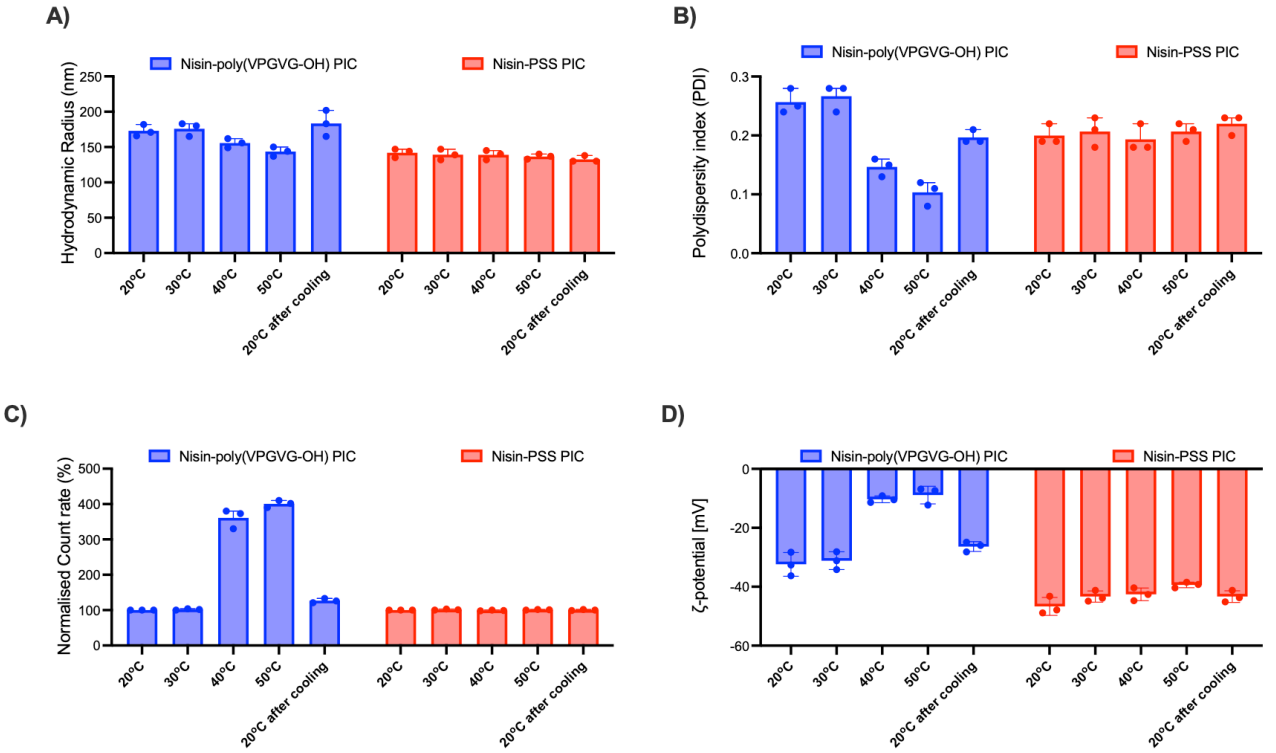


Figure 4 The effect of temperature on the size (A), Polydispersity Index (B), Count rate (C), and zeta potential (D) of Nisin poly(VPGVG-OH) and Nisin:PSS PIC nanoparticles. Data shown is the average of 3 technical replicates.

Having observed the temperature-based changes in the structural characteristics of poly(VPGVG-OH) PIC nanoparticles, a fluorescamine assay was conducted to investigate whether temperature could affect the accessibility of nisin encapsulated in the PIC nanoparticles. The same methodology was previously used to estimate unencapsulated nisin in Nisin:poly(VPGVG-OH) PIC nanoparticle samples. In these additional fluorogenic assays, Nisin:poly(VPGVG) and Nisin:PSS PIC samples reacted with fluorescamine dye immediately after incubating at different temperatures. Then, accessible nisin in PIC samples was calculated as a percentage for each temperature by normalising the emission intensity of PIC samples to that of free nisin-fluorescamine adducts (%100) **(Figure S6).** As expected, when the temperature was increased from 20° to 30°, no change was observed in the accessible nisin amount in the Nisin:poly(VPGVG-OH) PIC samples **(Fig 5)**. However, with increasing the temperature from 30°C to 40°C and 50°C, accessible nisin significantly increased from 35% to 77% and 80% respectively. This is clearly due to structural changes in nanoparticle structure at this temperature range, making encapsulated nisin accessible by fluorescamine dye. Moreover, when PIC nanoparticles incubated at 50°C was rapidly cooled to 20°C, accessible nisin decreased to 42%, indicating reversibility of thermoresponsive behaviour previously evidenced by DLS and zeta potential measurements above. On the other hand, as expected, temperature applications did not affect the accessibility of nisin in the Nisin:PSS PIC nanoparticle system. Consequently, it was verified that Nisin:poly(VPGVG-OH) PIC nanoparticles exhibited thermoresponsive activity, resulting in a rapid increase in nisin availability at 40°C (**Scheme 1**).

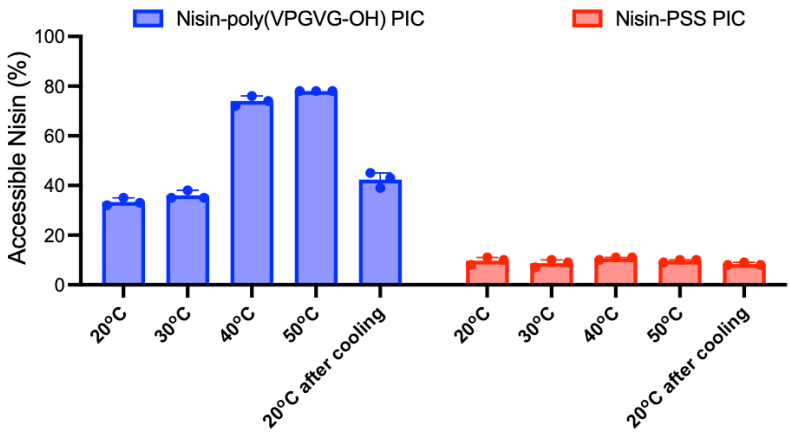
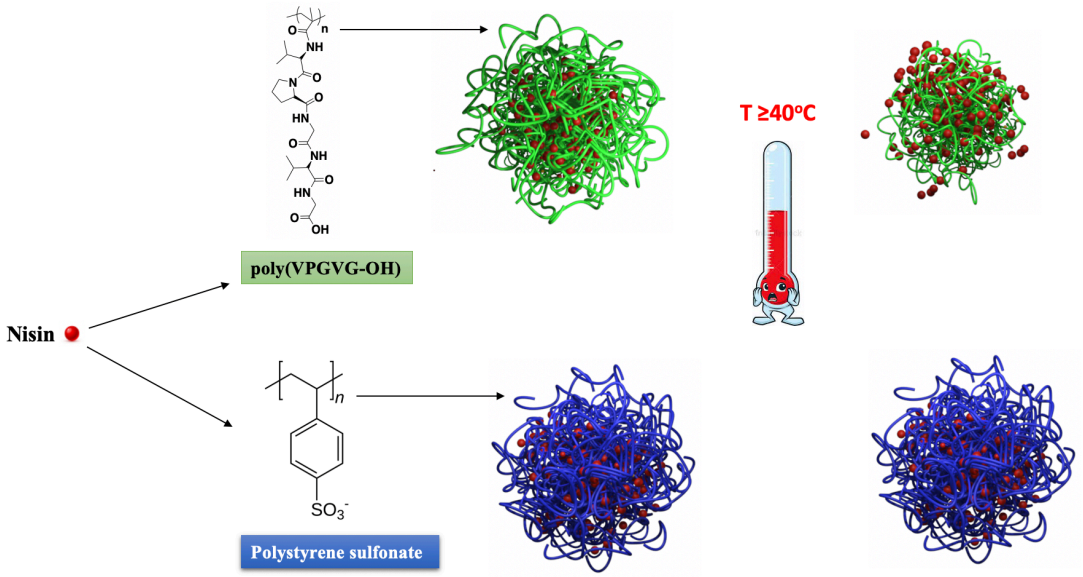


Fig 5 The effect of temperature on the accessible Nisin (%) in the Nisin:poly(VPGVG-OH) and Nisin:PSS PIC nanoparticle samples. Data shown is the average of 3 technical replicates.



Scheme 1 The effect of temperature on the structure of Nisin:poly(VPGVG) and Nisin:PSS PIC nanoparticles

3.4. The effect of temperature on the antimicrobial activity of Nisin:poly(VPGVG-OH) PIC nanoparticles

Having established the thermosensitive behaviour of Nisin:poly(VPGVG-OH) PIC nanoparticles in HEPES buffer, we then decided to investigate whether antimicrobial activity of these nanoparticles shows a temperature-dependent tendency in biological settings against *Bacillus subtilis*, a representative gram-positive bacteria. This ubiquitous microorganism can form heat-resistant endospores, and therefore can be commonly found in heat-processed food products such as pasteurised milk. *B. subtilis* usually rapidly grows above 30°C, and thus shows proteolytic activity affecting product acceptance and product shelf-life, if the cold chain is not maintained (Heyndrickx & Scheldeman, 2002). Therefore, *B. subtilis* was chosen as a relevant organism to evaluate the potential role of our nanoparticles in preventing temperature-related microbial food spoilage. For this purpose, we designed an experiment to demonstrate the antimicrobial effectiveness of Nisin:poly(VPGVG-OH) PIC nanoparticles at two different temperatures at which we previously

verified and not verified the thermoresponsive behaviour of these nanoparticles, 40ºC and 30ºC, respectively. Additionally, Nisin:PSS PIC nanoparticles were employed as non-thermoresponsive control. In this experiment, the optical density (at 600 nm) of *B. subtilis* was monitored as an indicator of microbial growth in the absence and presence of different PIC nanoparticle formulations and their components.

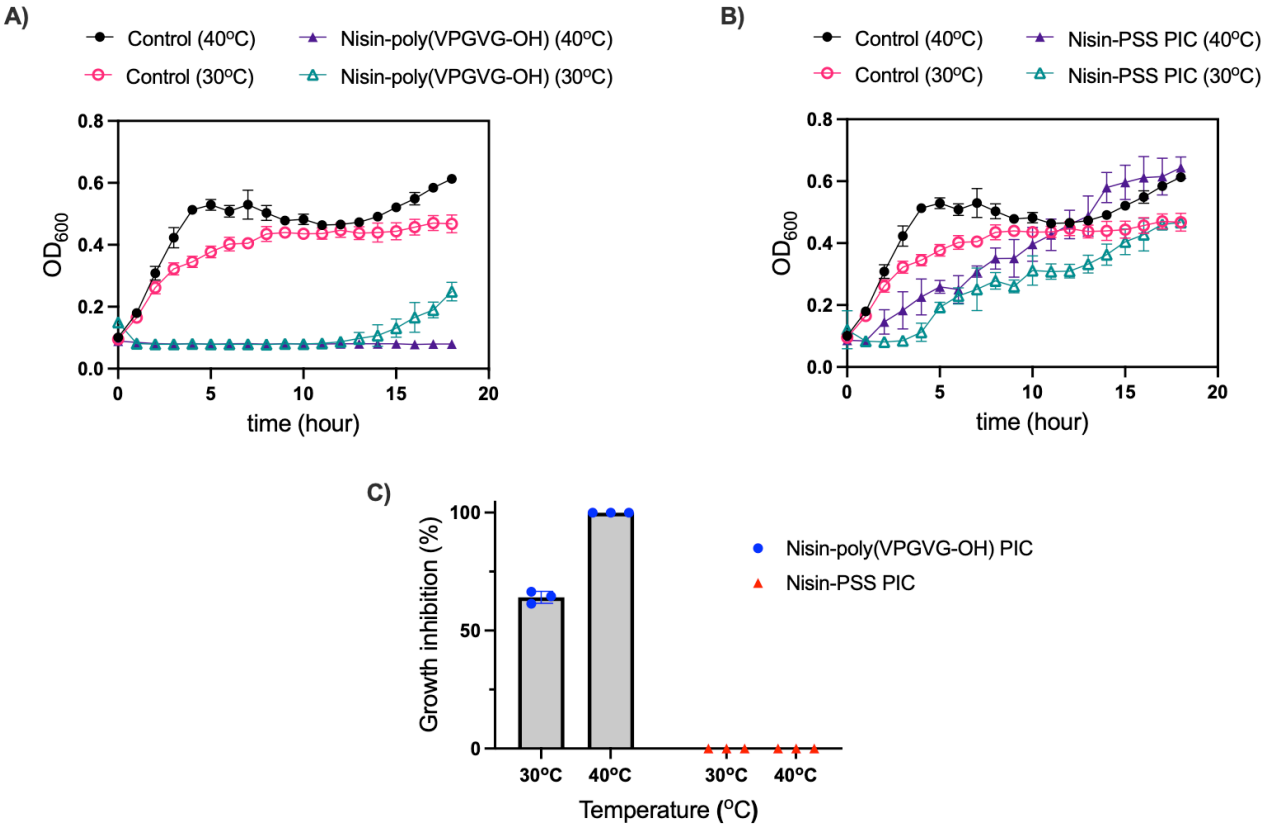


Fig 6 Changes to the optical density at 600 nm of *B. subtilis* cultures in Nutrient Broth incubated at 30ºC and 40ºC in the absence (control) and presence of Nisin:poly(VPGVG-OH) PIC nanoparticles (A) and Nisin:PSS PIC nanoparticles (B). Changes in the growth inhibition (%) caused by PIC nanoparticles as a function of temperature (C)

The total nisin concentration in the growth media treated by free nisin or PIC nanoparticles was twice the minimum inhibitory concentration (10 ug.ml^{-1}) of this food additive towards this microorganism **(Fig S7).** In other words, there was sufficient antimicrobial nisin to inhibit all bacterial growth in all samples, except for the samples treated by polymers only. Therefore, not surprisingly, the growth of *B. subtilis* was not inhibited by polymers, both poly(VPGVG-OH) and PSS. **(Fig S7)** . As also expected, free Nisin was able to show complete inhibition of growth. Interestingly, Nisin:PSS PIC nanoparticles did not cause growth inhibition at the end of the 18 h incubation regardless of the temperature **(Fig 6B-6C)**, suggesting that nisin was still being properly encapsulated under these conditions and therefore not accessible by bacterial receptors. They delayed the bacterial growth for 2 h at 40ºC and 4 h at 30ºC but we believe that this limited antimicrobial activity was due to unencapsulated nisin this PIC nanoparticle samples as well as the nisin passively released from these nanoparticles (i.e. burst release). Consequently, Nisin:PSS PIC nanoparticles did not exhibit a thermoresponsive antimicrobial activity against *B. subtilis,* as expected.

Interestingly, when compared to Nisin:PSS PIC nanoparticles, Nisin:poly(VPGVG-OH) PIC nanoparticles considerably delayed the bacterial growth for 13 h, and caused growth inhibition of 66% after 18 h at 30°C. inhibition **(Fig 6A-6C)**. Since, we did not expect a thermoresponsive antimicrobial activity at 30°C, this observed antimicrobial activity can be attributed to two different reasons. First, Nisin:poly(VPGVG-OH) PIC samples contains more unencapsulated nisin at the beginning because their encapsulation efficiency (%67) is lower than that of Nisin:PSS PIC nanoparticles (96%). Second, the stability of Nisin:poly(VPGVG-OH) PIC in the growth medium may be weaker as compared to Nisin:PSS PIC nanoparticles; hence, more nisin may have passively released from these less stable PIC nanoparticles and caused growth inhibition. On the other hand, the same nanoparticles caused complete growth inhibition (100%) at 40°C **(Fig 6A-6C)**, suggesting more nisin become accessible to bind to *B. subtilis* with the temperature increase from 30°C to 40°C. Overall, together with the thermosensitive behaviour of these nanoparticles previously verified at the same temperature, these results clearly proved that Nisin:poly(VPGVG-OH) PIC nanoparticles could show thermoresponsive antimicrobial activity.

3.6. The effect of PIC nanoparticles on the antimicrobial effectiveness of nisin against C. perfringens in a simulated food system

Similar to all encapsulation systems developed for Nisin, one of the main goals of this study was to protect Nisin from its undesired interactions with food components. Now that we obtained PIC nanoparticles capable of rapidly increasing the bioavailability of antimicrobial nisin at 40°C, we designed an experiment to explore the role of our nanoparticles in the protection of Nisin's antimicrobial effectiveness in food (**Scheme 2**). For this purpose, free nisin and PIC nanoparticle solutions were exposed to glutathione (GSH), a meat component decreasing nisin's antimicrobial effectiveness in meat products (Rose et al., 1999; Stergiou et al., 2006). In this way, we wanted to evaluate the antimicrobial activity of free and encapsulated nisin following their incubations in the absence and presence of glutathione. In this experiment, brain heart infusion broth (BHIB, pH:5.5) supplemented with GSH was employed as a type of model meat medium. Total GSH concentration was adjusted to its approximate concentration in beef (0.2 mg. g^{-1}) (Kulczyński et al., 2019). Samples were incubated at 4°C, representative of the storage temperature of meat products (**Scheme 2- 1**) To monitor the antimicrobial activity of these samples, *C. perfringens* was employed as a representative gram-positive pathogen commonly linked with meat-related food poisoning (Yibar et al., 2018). Since the optimal growth temperature for this pathogen is 43°C-47°C (Márquez-González et al., 2012), meat products holding this unsafe temperature range are considered the main cause of *C. perfringens* infection. Therefore, these samples were artificially contaminated with this pathogen (**Scheme 2- 2)** and incubated at 25°C and 45°C (**Scheme 2- 3, 4**). After the incubation period of 24 h, microbial growth inhibiton in the samples were calculated as a percentage according to changes in the OD600 of samples. Additionally, the same experimental procedure was conducted using Nisin:PSS PIC nanoparticles as a non-thermoresponsive control.

The total nisin concentration in the samples was twice the minimum inhibitory concentration of this food additive towards this microorganism (15 µg.ml^{-1}-**Fig S8**). Therefore, as expected, no *C. perfringens* growth was observed in the samples treated with free nisin in the absence of glutathione (**Fig 7)**. However, this total growth inhibition considerably decreased to 53% at 45°C and 58% at 25°C upon glutathione treatment due to the inactivation of antimicrobial Nisin (**Scheme 2A**). Compared to free nisin, Nisin:poly(VPGVG-OH) PIC nanoparticles showed lower antimicrobial activity at 25°C in the absence of glutathione, suggesting that encapsulated nisin was inaccessible. Also, due to the non-thermoresponsive activity of Nisin:poly(VPGVG-OH) PIC nanoparticles at 25°C, the growth inhibition observed at this temperature can be attributed to unencapsulated nisin in

PIC nanoparticle solution and the nisin passively released from PIC nanoparticles during storage period. As such, although PIC nanoparticles were not disturbed by glutathione (**Fig S5**), this free nisin in PIC solution was inactivated by glutathione and eventually resulted in a decrease in growth inhibition from 52% to 33% at 25°C. Remarkably, compared to antimicrobial activity at 25°C, antimicrobial activity of this Nisin:poly(VPGVG-OH) PIC nanoparticles was higher at 45°C in both the absence and presence of glutathione, with the growth inhibition values of 83% and 76%, respectively (**Scheme 1B**). On the other hand, we did not observe such increase in the antimicrobial activity for Nisin:PSS PIC nanoparticles (**Scheme 1C**). Consequently, these results clearly indicate Nisin:poly(VPGVG-OH) PIC nanoparticles limited the release of Nisin from PIC nanoparticles at refrigeration temperature, and thus Nisin's antimicrobial efficacy was not adversely affected by GSH during storage. In this way, they displayed relatively more effective antimicrobial activity than free Nisin and non-responsive Nisin:PSS PIC nanoparticles in case of abnormal increases in the temperature (above 40°C).

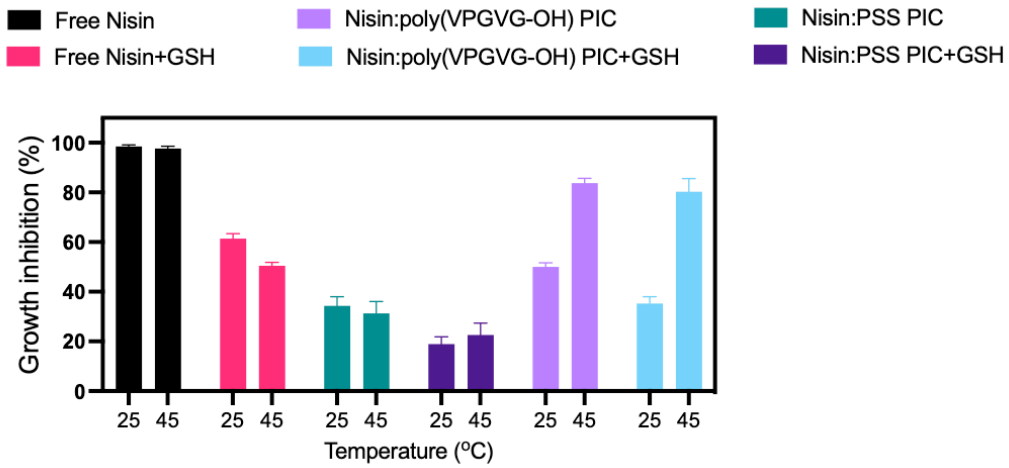
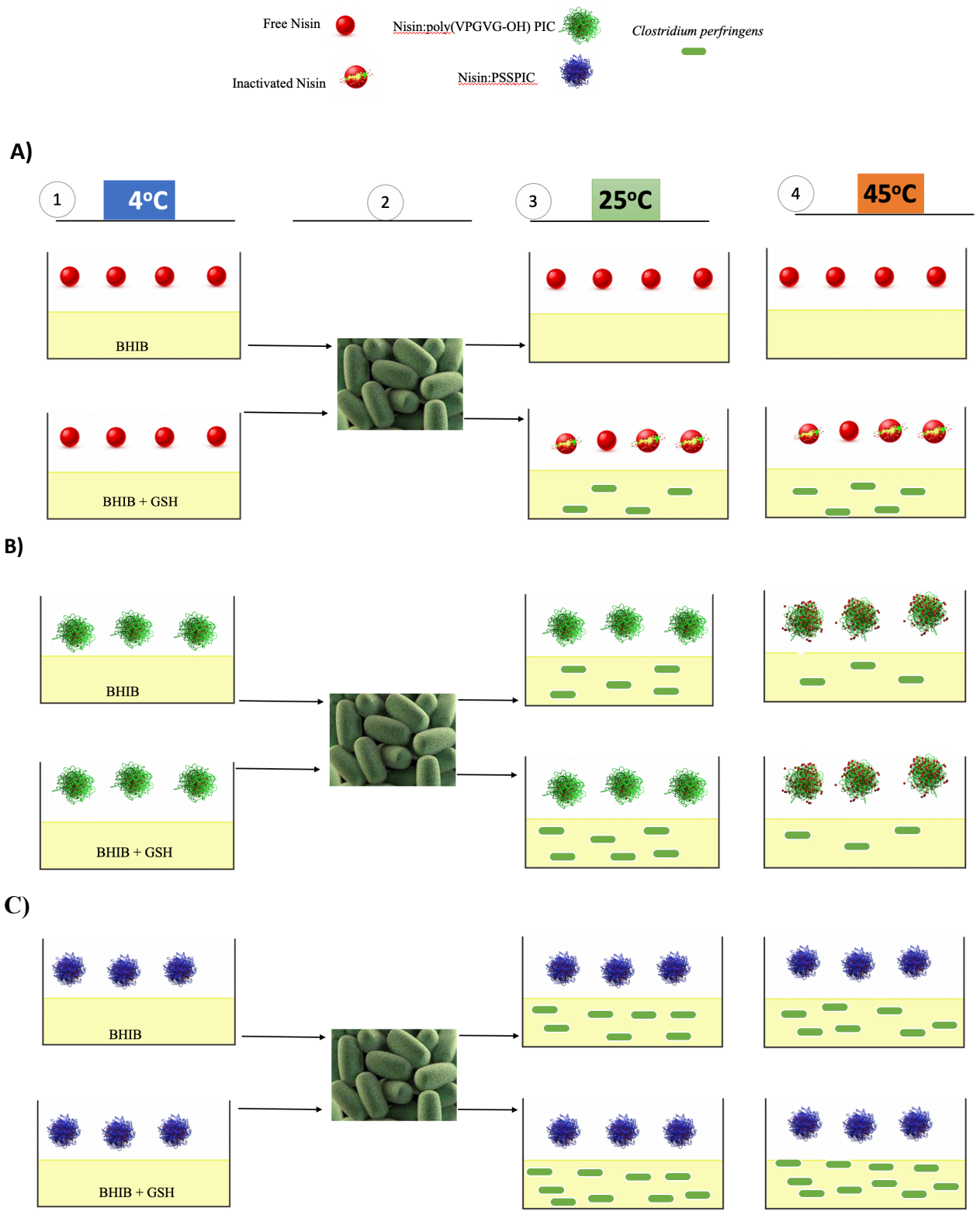


Fig 7 Growth inhibition (%) caused by Free Nisin, Nisin:poly(VPGVG-OH) PIC, and Nisin:PSS nanoparticles following their incubations in BHIB in the absence and presence of glutathione at 4°C. At the end of the incubation of 2 days, samples were artificially contaminated by *C. perfringens,* and individually incubated at 25°C and 45°C. Error bars represent SD. (n=3)



Scheme 2 Incubation of free nisin and PIC nanoparticles in BHIB at different conditions. Incubation of free Nisin (A), PSS PIC (B), and poly(VPGVG-OH) PIC (C) in the absence (top) or presence (bottom) of GSH for 2 days before contamination (1), artificial contamination of the samples by *C. perfringens* (2), representative appearances of the samples reflecting bacterial growth following incubation period of 24 h at 25°C (3) *and* 45°C (4).

4. Conclusions

Here we presented the first example of thermoresponsive PIC nanoparticles for delivering Nisin, an important antimicrobial used as a food additive. Using a thermosensitive elastin-based side-chain polymer (EBP), poly(VPGVG-OH), spherical PIC nanoparticles capable of encapsulating Nisin were obtained. Our results suggest that Nisin:poly(VPGVG-OH) PIC nanoparticles have a reversible thermoresponsive property, confirmed by directly temperature related changes in PDI, count rate and zeta potentials of these nanoparticles at 40ºC. We demonstrated that this thermoresponsive behaviour resulted in a rapid increase in accessibility of Nisin at 40ºC. This behaviour was supported by antimicrobial activity experiments. Firstly, we have demonstrated the in-vitro antimicrobial activity these nanoparticles could be enhanced at 40ºC as compared to lower temperatures (30ºC) against *B. subtilis*. Finally, we have demonstrated that these nanoparticles played an important role in protecting the antimicrobial effectiveness of Nisin during storage of a model meat system at refrigeration temperature (4ºC), they displayed better inhibitory effect against *C. perfringens* than free nisin upon contamination and temperature abuse during storage (45ºC). Overall, our findings suggest that these nanoparticles can potentially prevent temperature-related microbial food safety problems in food at 40ºC and above. We also believe that this study could underpin the development of novel responsive antimicrobial delivery systems in food. Our efforts to achieve a responsive antimicrobial activity at lower abusive temperatures (e.g. room temperature) will be reported in due course.

5. Supplementary information

Preparation and characterisation of poly(VPGVG-OH), Fluorescence data of Nisin-fluorescamine adducts, additional DLS data, additional TEM images, and additional antimicrobial data for *B. subtilis* and *Clostridium perfingens* can be obtained in the supporting information.

6. AUTHOR INFORMATION

6.1. Author Contributions

T.C. and P.F.-T. conceived the project, and designed Nisin:CMC PIC nanoparticle preparation and characterisation. T.C.,, H.O. and T.M. designed the antimicrobial experiments. P.F.-T., H.O. and T.M. secured funding. SE synthesised and characterised poly(VPGVG-OH). T. C. performed all experiments. T.C. and P.F.-T. analysed the data. TC wrote the paper, with all other authors contributing to the final version of the manuscript.

6.2. Conflicts of Interest

The authors declare no conflicts of interest.

7. Acknowledgements

P.F.-T. thanks the University of Birmingham for the John Evans Fellowship and the Spanish Ministerio de Educación, Cultura y Deporte for a Beatriz Galindo Award [BEAGAL18/00142]. T.C. thanks The Ministry of National Education, Republic of Turkey for a PhD scholarship.

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Electronic Supplementary Information

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1. Synthesis and Characterisation of poly(VPGVG-OH)

1.1. Materials

Fmoc-protected-L-amino acids, H-Gly-chlorotrityl chloride resin were purchased from Novabiochem. Piperidine and N,N-diisopropylethylamine (DIPEA), 2,2,2-Trifluoroethanol (TFE), were bought from Alfa Aesar®. N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) was purchased from Carbosynth Ltd. Dialysis membranes (molecular weight cut off 1 KDa) were purchased from Spectrum® Laboratories.

1.2. Instrumentation

A FLUOstar Omega (BMGLabtech Gmbh) microplate reader was used to measure fluorescamine reactions. NMR data was acquired on a Bruker Avance III operating at 300 MHz and fitted with a 5 mm DUL probe (1H/13C). Reversed- phase (RP) HPLC analysis was run through a Kinetex® C18-EVO column (Phenomenex®): 5 µm, 100 Å, 250 x 4.60 mm. A gradient from 5 to 60% of (CH3CN + 0.05% TFA) in (H2O + 0.05% TFA) in 40 min was used at 1 mL/min. The column

was maintained at 35°C and UV-VIS detection was set at 215 nm. GPC was recorded on a Shimadzu Prominence LC-20A, fitted with Shodex Asaphipak GF-510 HQ (300 × 7.5 mm, 5 μm) and GF-310 HQ (300 × 7.5 mm, 5 μm) columns in series, and equipped with a Thermo Fisher Refractomax 521 detector. GPC analysis was carried out using DPBS buffer as eluent at 35 °C and a flow rate of 1 mL/min. Molecular weights were calculated based on a standard calibration method.

1.3. Solid phase synthesis of Maa-VPGVG-OH

Commercial H-Gly-chlorotrityl chloride resin (1000 mg, 0.58 mmol) was swollen in 10 mL of DMF solid-phase cartridge and left for 30 minutes in a vertical shaker. After removing the DMF, washing with fresh DMF (10 ml), a previously prepared solution of 5 ml Fmoc-Valine-OH (2.8 eq in 10 ml in DMF, 1.74 mmol), followed by 5 ml of HBTU (2.8 eq in 10 ml DMF, 1.62 mmol) and DIPEA (2.8 eq, 1.62 mmol) were added to the resin. The reaction mixture was shaken for 45 minutes at room temperature, then washed with 10 ml of fresh DMF and a negative chloranil test indicated the reaction was complete. N-terminal Fmoc protecting group was removed using 10 mL of piperidine in DMF (20%, v/v) for 10 minutes. A positive chloranil test confirmed the removal of the Fmoc group, and the previous steps were repeated to couple all amino acids in the sequence. After coupling of the last amino acid, the Fmoc group was removed and the terminal amine in the peptide was capped with 5 mL of Methacrylic acid (6 eq in 10 ml DMF, 3.48 mmol), HBTU and DIPEA as in the previous steps. Then, the resin was thoroughly washed with Et2O, let to dry for 10 min and the peptide was cleaved from the resin using 10 mL of TFE solution (2ml of TFE in 6ml of AcOH and 2 ml of DCM) for 1 hour. Later the cleaved solution was obtained in a round bottom flask, was concentrated under argon and precipitated in chilled Et2O: Hexane (3:1, v/v) under stirring. Finally, the peptide pellets were obtained by centrifuges at 10000 rpm for 10 minutes and freeze-dried from water over night to obtained white solid. Purity was determined by HPLC.

1.4. Characterisation of Maa-VPGVG-OH

Maa-VPGVG-OH (210 mg, 95% yield)
1H NMR (300 MHz, DMSO-d6) δ 8.35 – 8.15 (m, 2H), 7.83 (d, J = 8.2 Hz, 1H), 7.64 (d, J = 9.0 Hz, 1H), 5.71 (s, 1H), 5.36 (s, 1H), 4.31 (d, J = 9.1 Hz, 2H), 4.20 (d, J = 8.0 Hz, 1H), 3.95 – 3.78 (m, 2H), 3.73 (d, J = 5.7 Hz, 5H), 3.65 – 3.55 (m, 3H), 2.15 – 1.87 (m, 4H), 1.85 (s, 3H), 1.07 – 0.66 (m, 12H).
13C NMR (101 MHz, DMSO) δ 172.47, 171.66, 171.58, 171.56, 170.63, 169.11, 168.10, 139.79, 120.22, 60.08, 58.11, 56.78, 47.69, 42.55, 41.16, 40.60, 40.55, 40.39, 40.34, 40.14, 39.93, 39.72, 39.51, 39.30, 31.33, 31.08, 30.21, 29.73, 24.99, 21.53, 19.57, 19.32, 19.19, 19.12, 18.58, 18.52, 18.34.

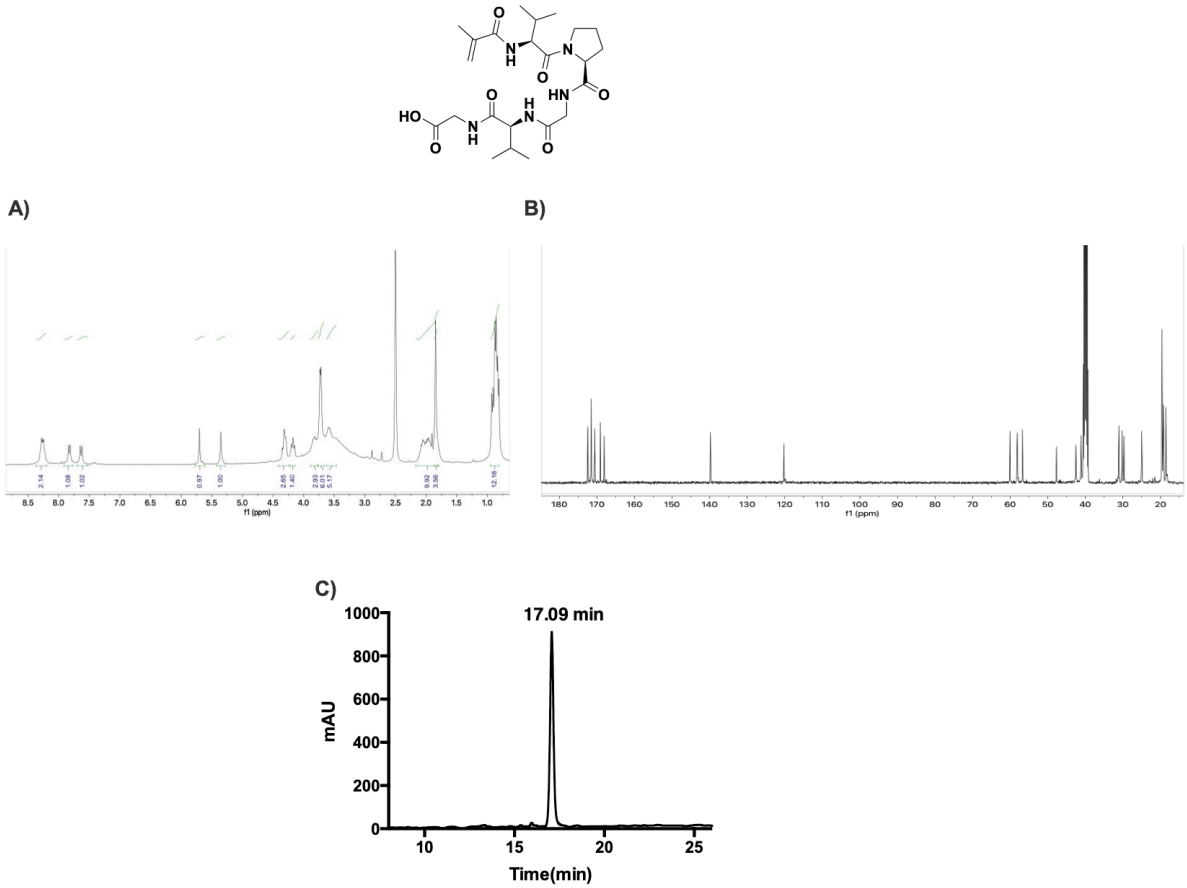


Figure S1 1HNMR (300 MHz, DMSO-d6) (A), 13HNMR (400 MHz, DMSO-d6) (B) spectra, and RP-HPLC chromatogram of (Maa-VPGVG-OH) (C).

1.5. Synthesis of poly(VPGVG-OH)

Peptide (Maa-VPGVG-OH) (200 mg, 0.4 mmol), 4,4'-azobis (4-cyanovaleric acid) (ACVA) (2.3 mg, 0.01 mmol) were dissolved in water/DMSO (400 mL, 1:1 v/v). This solution was degassed under argon for 20 minutes and then heated at 70 °C under stirring for 20 hours in an oil bath. After this time, the reaction tube was opened to the air to decrease temperature and the crude was transferred to dialysis membranes (1 KD) against distilled water for 3 days. After was freeze-dried and a crystalline white solid was obtained (90.0 mg, 21% yield). 1H NMR (300 MHz, DMSO-d6) δ 8.35 – 8.15 (m, 2H), 7.83 (d, J = 8.2 Hz, 1H), 7.64 (d, J = 9.0 Hz, 1H), 5.71 (s, 0.01H), 5.36 (s, 0.01H), 4.31 (d, J = 9.1 Hz, 2H), 4.20 (d, J = 8.0 Hz, 1H), 3.95 – 3.78 (m, 2H), 3.73 (d, J = 5.7 Hz, 5H), 3.65 – 3.55 (m, 3H), 2.15 – 1.87 (m, 4H), 1.85 (s, 3H), 1.07 – 0.66 (m, 12H). From GPC, Mn 32182.

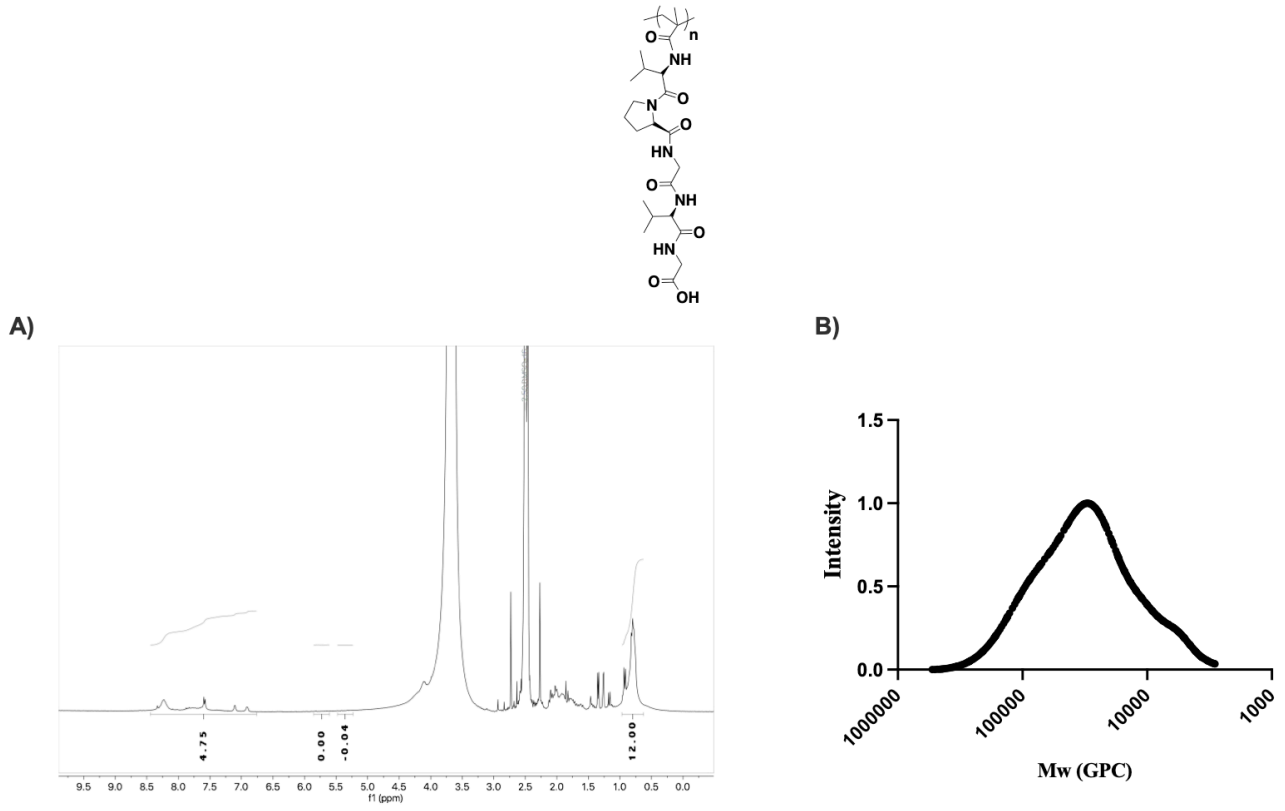


Figure S2 [1]HNMR (300MHz, DMSO-d6) spectra (A) and GPC chromatogram (B) of poly(-VPGVG-OH)

2. Additional DLS data-Characterisation of PIC nanoparticles

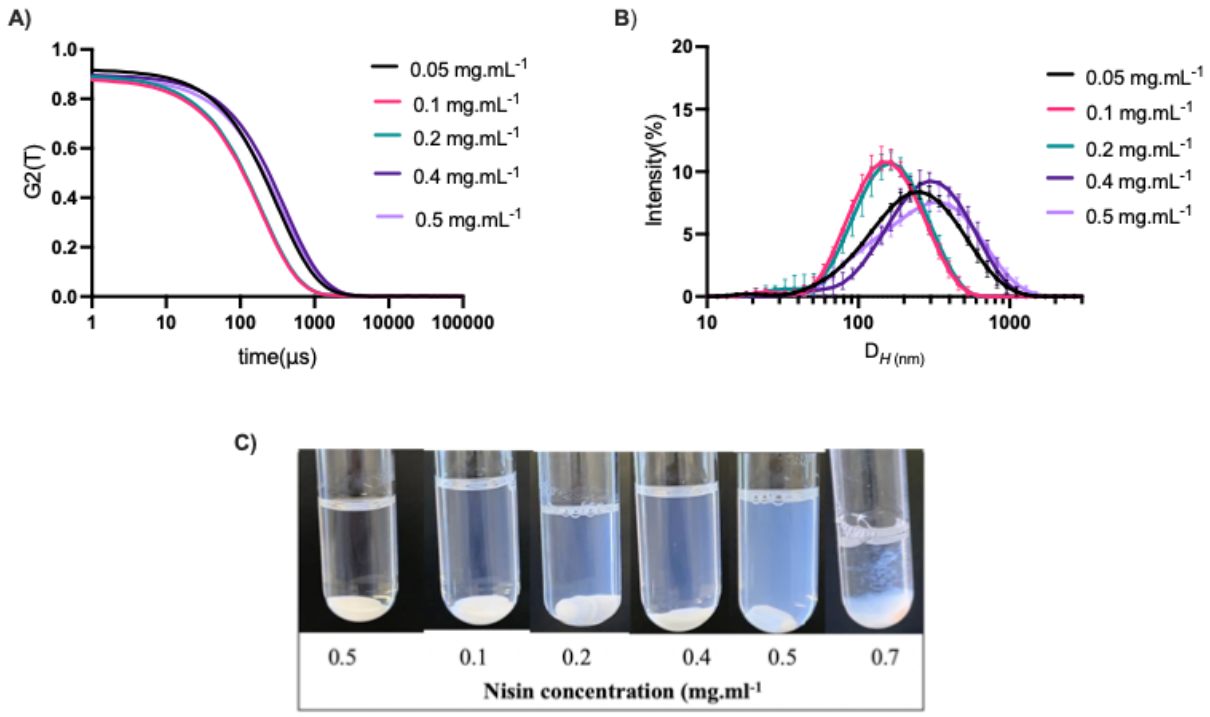


Figure S3 Autocorrelation function (ACF) curves (A), representative size-intensity distribution (B), and macroscopic appearance Nisin:poly(VPGVG-OH) PIC nanoparticles prepared at different concentrations of Nisin. Final concentrations of poly(VPGVG-OH) in each sample was 0.21 mg.mL^{-1}. Error bars represent range. Data shown is the average of 3 technical replicates.

3. Additional DLS data: The effect of temperature on PIC nanoparticles

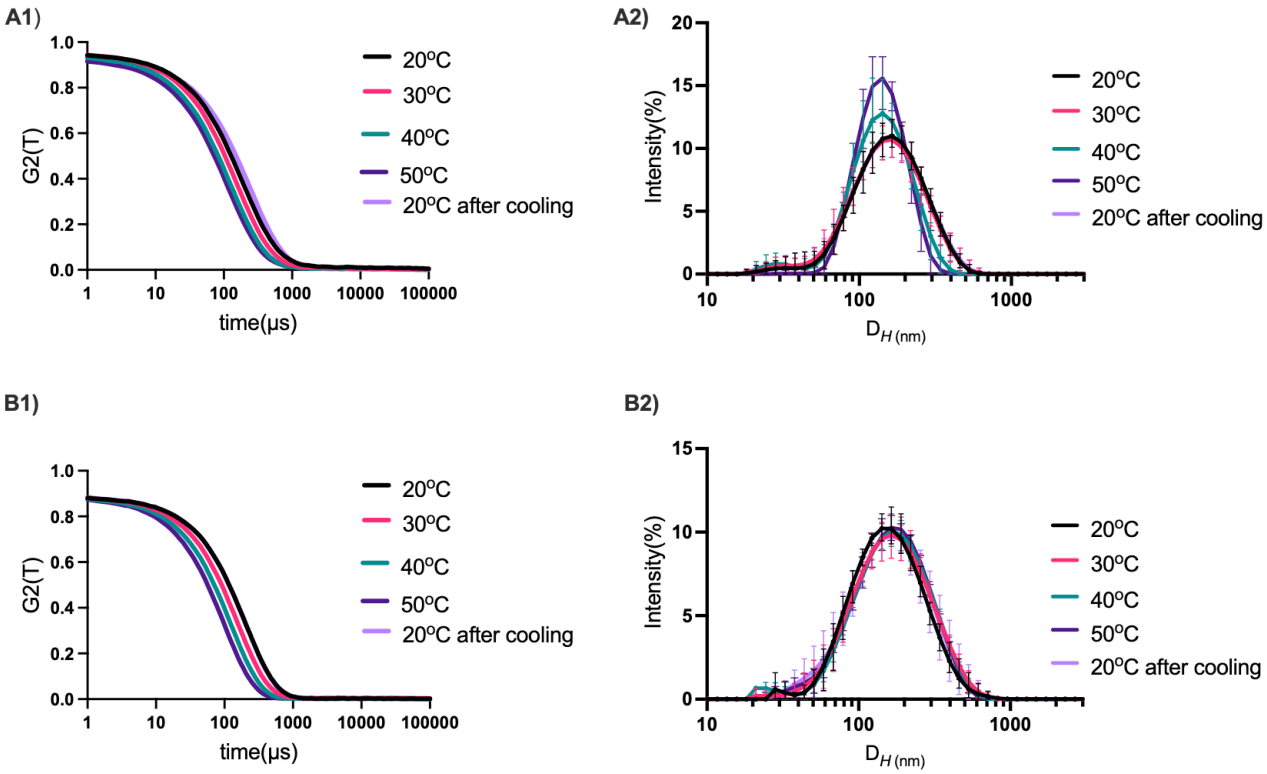


Figure S4 Changes in the autocorrelation function and size-intensity distributions of Nisin:poly(VPGVG-OH) (A1-A2) and Nisin:PSS PIC nanoparticles as a function of temperature. Error bars represent range. Data is the average of 3 technical replicates.

4. Additional DLS data: The effect of glutathione on PIC nanoparticles

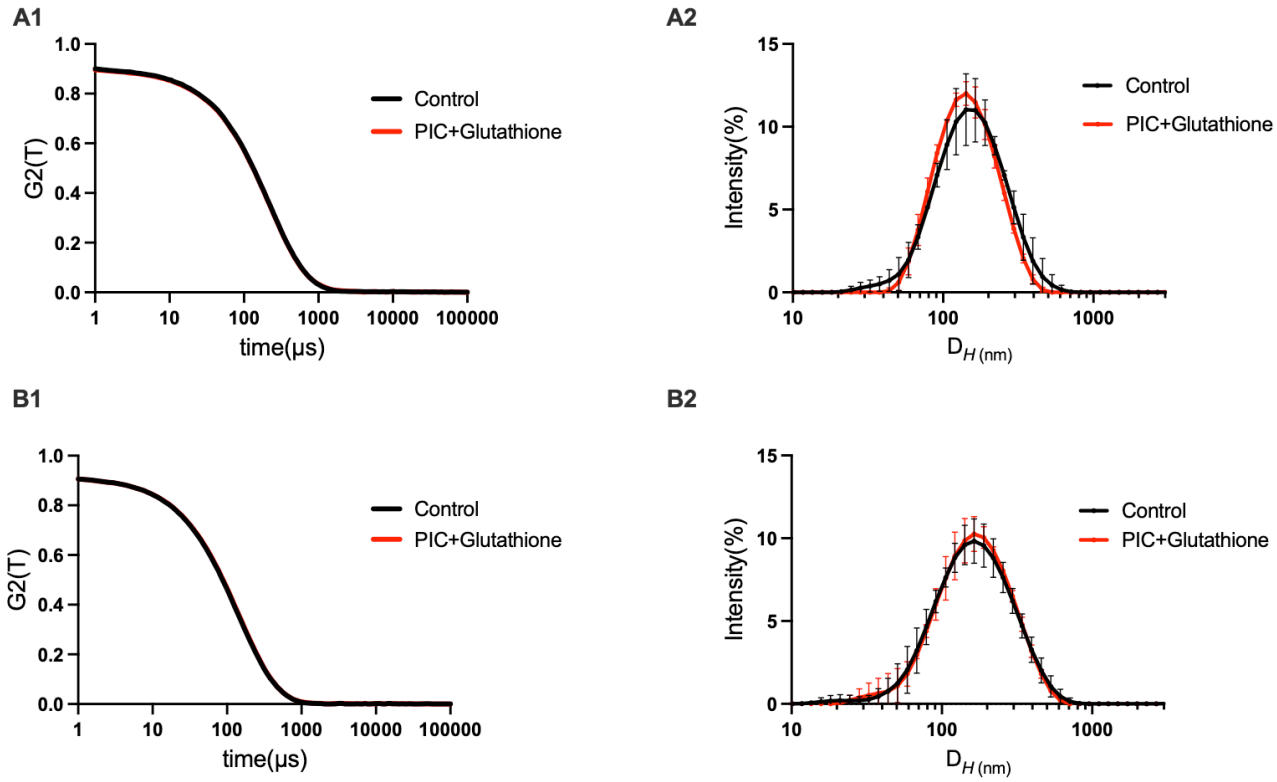
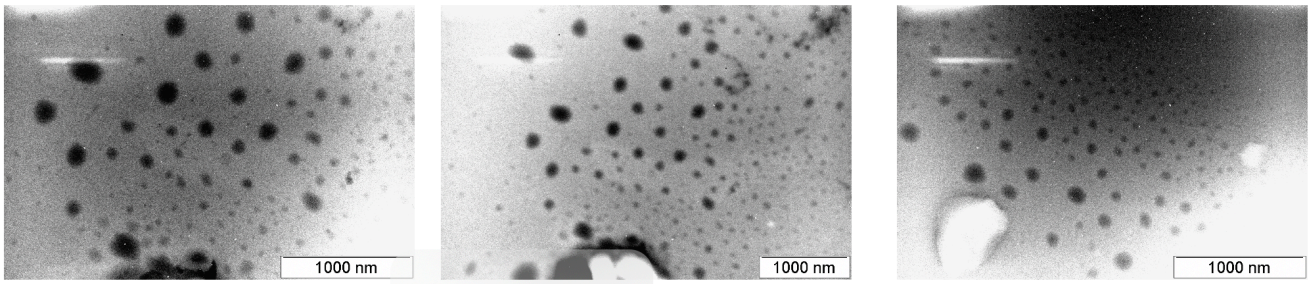


Figure S5 Representative autocorrelation function and size-intensity distributions for the Nisin:poly(VPGVG-OH) (A1-A2) and Nisin:PSS (B1-B2) PIC nanoparticles before (control) and after incubation with Glutathione.

5. Additional TEM images



$D_H \pm$ SD (nm)[a]	$D_{TEM} \pm$ SD (nm)[b]
174 ± 72	**113 ± 41**

[a] SD indicates the standart deviation found for the only size population fitted by the Malverns's Zetasizer software.
[b] Avarage diameter of 60 nanoparticles were measured twice (in their longest and shortest diameters) and SD was calculated from a total of 120 measurements.

6. Fluorescence data for Nisin-Fluorescamine adducts

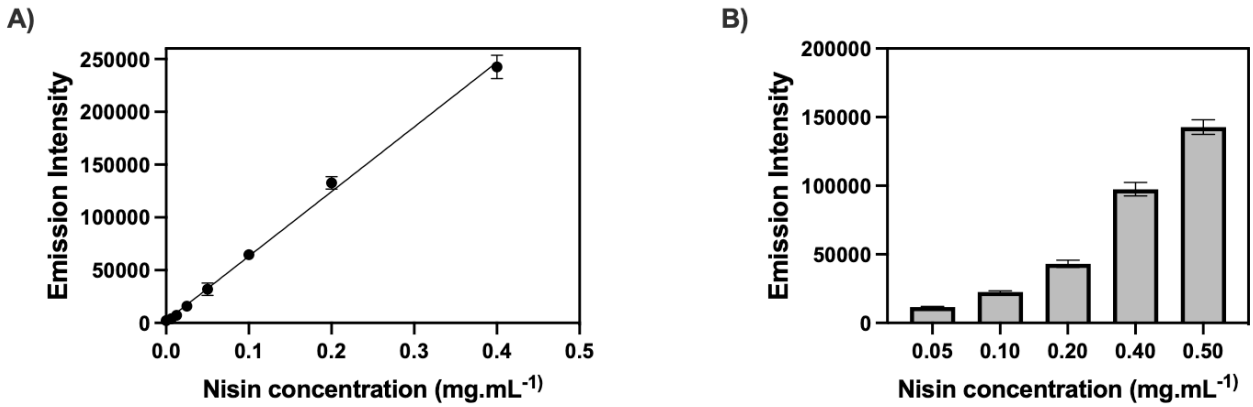


Figure S6 The standart curve of Free Nisin-Fluorescamine conjugates (left) and Emission Intensity of fluorescamine conjugates for Nisin:poly(VPGVG-OH) PIC nanoparticles prepared with different Nisin concentrations (right).

7. Growth curve of *Bacillus subtilis* in the absence and presence of free Nisin and polymers

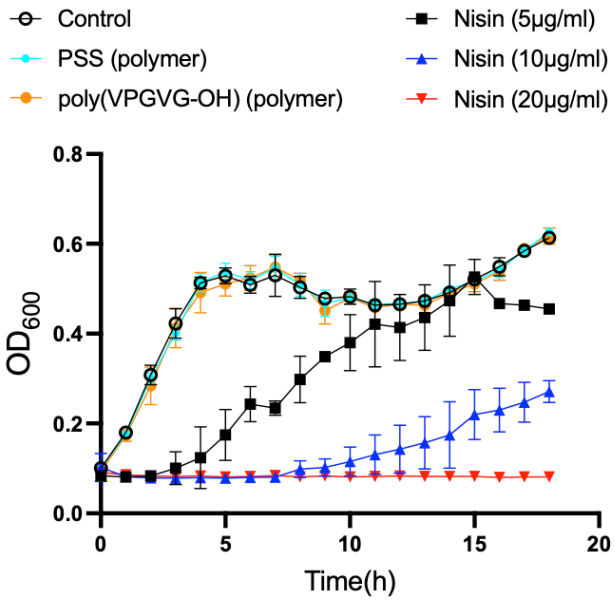


Figure S7 Changes to the optical density at 600 nm of *B. subtilis* cultures in nutrient broth incubated in the absence (control) and presence of components employed in PIC nanoparticle preparation.

8. Minimum Inhibitory Concentration (MIC) of Nisin against *C. perfringens* in BHIB

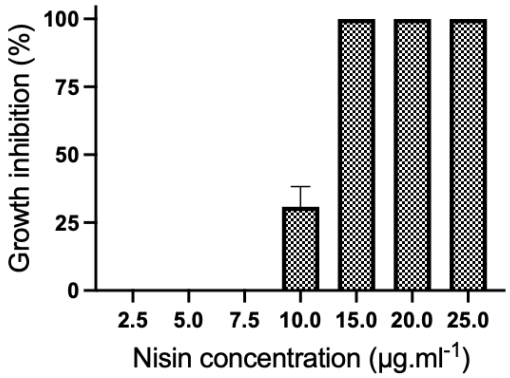


Figure S8 Total inhibition (%) in the growth of *C. perfringens* at the end of the incubation (24h at 45ºC) in brain heart infusion broth (BHIB) supplemented with free nisin at different concentrations. Error bars represent SD. (n=3)

CHAPTER 5

Conclusions and Future Works

The overall goal of this thesis was to improve the antimicrobial effectiveness of nisin in food. Since using nisin in its free form is the main factor decreasing its antimicrobial efficacy, encapsulation of nisin was the most crucial step to minimize its undesired interactions with food components while enhancing its stability and bioavailability. PIC nanoparticles were chosen as the most suitable nanoencapsulation platform because they could be prepared from commercially available, cheap biopolymers following a straightforward methodology. More importantly, this platform allowed to adjust the antimicrobial activity of PIC nanoparticles by exploiting the type of anionic polymer employed as encapsulating component.

The first effort presented in this thesis, (Chapter 2-Nisin:Carboxymethylcellulose (CMC) PIC nanoparticles), has provided a new, simple, cost-effective, food-grade example of a nisin delivery system exhibiting a promising antimicrobial potential for the prolonged food preservation for milk. One of the key findings derived from these systems is that the pH-dependent charge density of nisin is a critical factor that needs to be taken into account while adjusting the size and encapsulation efficiency of PIC nanoparticles. Low pH values (4.5 and 5.5) were optimal for better electrostatic complexation of nisin with CMC, thereby decreasing size while increasing encapsulation efficiency. More importantly, the degree of substitution of CMC is another critical parameter allowing to simply obtain nanoparticles with higher encapsulation efficiency and using the CMC with higher DS possessing more negative charge led to increasing encapsulation efficiency of nisin. Although, in vitro antimicrobial activity of these nanoparticles was initially lower than free nisin, they demonstrated promising potential to prolong nisin's antimicrobial efficacy due to their sustained

release property during the storage of milk. As such, investigation of long-term antimicrobial effect of such nanoparticles will be crucial to understand their definite antimicrobial performance in real food systems, and therefore should not be limited with rapid in-vitro methods.

When compared to sustained delivery systems, although, enzyme-responsive antimicrobial carriers for nisin could be more effective against specific foodborne pathogens, their accessibility and affordability may be challenging due to fact that there is a limited number of anionic biopolymers degraded by food-related bacteria. In this regard, hyaluronic acid (HA) was one of the most suitable anionic commercially available biopolymers that can be acceptable for food applications and degraded by the hyaluronidase of *S. aureus.* As such, PIC nanoparticles prepared from this polymer were cleaved upon enzymatic activity of this bacteria. Consequently, this polymer allowed us to access an attractive food preservation method against this severe foodborne pathogen (Chapter 3).

Another important learning derived from this study is that the stability of such responsive nanoparticles in food products is of great importance to increase bioavailability of nisin before contamination of food products with target organism. Since Nisin:HA PIC nanoparticles displayed promising (i.e intermediate) stability in the microbiological growth media and milk, they showed limited antimicrobial activity against *B. cereus* (non-hyaluronidase foodborne pathogen). Under the same conditions, these nanoparticles showed much better antimicrobial activity against *S. aureus.* As such, slow antimicrobial release or limited burst release (i.e. passive release) should not be regarded as an disadvantage of responsive PIC nanoparticles.

The final step of this thesis was to develop the first example thermoresponsive antimicrobial nisin delivery system (Chapter 4); however, this attempt was more challenging because of the requirement for synthetic thermosensitive components, making accessibility and the cost higher. Therefore, an anionic synthetic polymer, poly(VPGVG-OH), was employed for the first time to encapsulate nisin.

Reversible thermoresponsive behaviour of Nisin:poly(VPGVG-OH) PIC nanoparticles (at 40^{o}C and above) was confirmed due to considerable changes in the polydispersity index and zeta potentials as a function of temperature. More importantly, upon heating, increases in the accessibility of nisin was one of the most important parameters confirming temperature-related structural changes in these nanoparticles. Since thermoresponsive PIC nanoparticles are not extensively studied for cationic antimicrobial delivery, including in clinical and biomedical applications, methods, experiments and observations in this study could contribute to the development of novel thermoresponsive antimicrobial delivery systems in various platforms.

In terms of food applications, similar to enzyme-responsive PIC nanoparticles, stability is an important parameter to avoid a premature release and thus increase the retention of nisin in these thermoresponsive PIC nanoparticles before a temperature abuse. Therefore, these types of particles should display good stability during storage of food products at low temperatures. Nisin:poly(VPGVG-OH) PIC nanoparticles showed passive antimicrobial activity at 25^{o}C and 30^{o}C due to their intermediate stability profiles, below their thermoresponsive domain (40^{o}C). However, this inherent passive antimicrobial release mechanism may be an acceptable and beneficial property for thermoresponsive nanoparticles developed for food application considering that the most of foodborne pathogens and spoilage organism can grow at low temperatures, even some of which can grow at refrigeration temperature. Overall, due to enhanced inhibitory effect of Nisin:poly(VPGVG-OH) PIC nanoparticles as a function temperature (at 40^{o}C and above), this thesis has presented a novel antimicrobial delivery approach that can open a new page to overcome temperature-related microbiological food safety and quality problems.

In conclusion, this thesis has provided not only novel nisin delivery systems to improve its performance in food systems for food applications, but also new and promising examples that could

underpin the development of PIC-based novel antimicrobial delivery systems in various platforms.

Future Works

- Since either nisin or all anionic polymers employed in Chapter 2 and Chapter 4 do not have antimicrobial activity against Gram-negative foodborne pathogens, antimicrobial activities of all nanoparticles prepared in this thesis were evaluated against only gram-positive foodborne pathogens and spoilage organisms. As such, future efforts should focus on integrating additional antimicrobial components into these nanoparticles to inhibit the growth of gram-negative foodborne pathogens and spoilage organisms, thereby achieving widespread antimicrobial activity in food systems. For instance, as a less explored food-grade antimicrobial agent, epsilon-poly-L-lysine (ε-PL), may be one of the most appropriate components to encapsulate nisin due to its cationic nature as well as its inhibitory effect against gram-negative pathogens.[1] Using cationic nisin, ε-PL, and an anionic polymer , novel ternary-type PIC nanoparticles could be prepared. In addition to PIC nanoparticles, these components could be employed to access novel encapsulation systems which have never been applied for antimicrobial delivery in food systems. For instance, as an alternative electrostatically driven platform, Layer-by-Layer nanocapsules[2] may be a suitable to combine nisin and ε-PL in one system.

- Antimicrobial activity of Nisin:CMC (Chapter 1) and Nisin:HA PIC (Chapter 2) prepared at pH 5.5 and 4.5, respectively, were evaluated in skimmed milk (pH:6.5-6.6). Considering that these nanoparticles exhibited a passive release profile in milk during storage, the high pH of milk may have triggered the release of nisin from these particles. This release profile may be a result of increased solubility of encapsulating polymers at higher pH values,causing swelling of PIC nanoparticles and eventually the release of nisin. To investigate this phenomenon, pH-related changes on the particle size should be monitored after diluting

PIC samples at different pH values. Additionally, the effect of pH in the dilution media on the nisin release profiles of these PIC nanoparticles should be investigated through a dialysis experiment conducted at various pH (e.g. from pH 4 to 7) relevant to food systems in which nisin is frequently used, such as cheese, yoghurt and canned products.[3]

- Since *C. perfringens* is another gram-positive foodborne pathogen capable of degrading hyaluronic acid,[4] it could be an additional candidate to evaluate the responsive-antimicrobial activity of Nisin-HA PIC nanoparticles using a relevant food product associated with this pathogen. For instance, since meat products are generally associated with *C. perfringens* contamination,[5] further antimicrobial activity evaluation of Nisin-HA PIC nanoparticles against this pathogen should be carried out in meat samples.

- Since temperature-related food safety problems can frequently occur in food products left at room temperature, preparation of new thermoresponsive polymers should focus on using polymers with lower transition temperatures to encapsulate nisin to achieve enhanced antimicrobial activity at these critical temperatures (e.g. 25-30^{o}C). For instance, considering that the phase transition temperature of elastin-based side-chain polymers (EBP) like poly(VPGVG-OH) used in chapter 3 is strongly dependent on the chain length of polymer, use of EBPs with higher molecular weight may allow the development of PIC nanoparticles exhibiting phase transition lower temperatures. Furthermore, since EBPs are pH-sensitive polymers due to the presence of carboxylic acid groups on their C-terminus of the pentapeptide side chains, the thermoresponsive behaviour of Nisin-poly(VPGVG-OH) PIC nanoparticles may be varied depending on the pH (i.e. the increase in the pH may lead to decrease in transition temperature).[6] Therefore, the use of Nisin-poly(VPGVG-OH) PIC nanoparticles in more acidic food systems may lead to enhanced antimicrobial activity at lower temperature values.

- Since the possible toxicity and biodegradability of poly(VPGVG-OH) were not evaluated in Chapter 4, future works should focus on investigating the applicability of these types of synthetic polymers in food products. Particularly, due to the elastin-based structure of poly(VPGVG-OH), it should be investigated whether Nisin-poly(VPGVG-OH) PIC nanoparticles could be degraded by human pancreatic enzymes (i.e. elastase). Since these nanoparticles will be offered for consumption with food, in-vitro degradability experiments should be carried out simulating human stomach conditions (i.e. at 37°C, pH: 1.5-2). Finally, the toxicological assessment of poly(VPGVG-OH) should be done following "Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food" (a.k.a the Redbook).[7]

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