



Cite this: *Food Funct.*, 2024, 15, 3959

# Encapsulation of short-chain bioactive peptides (BAPs) for gastrointestinal delivery: a review

Yoni Atma,<sup>a,b</sup> Brent S. Murray,<sup>a</sup> Amin Sadeghpour<sup>a</sup> and Francisco M. Goycoolea<sup>a,c</sup>

The majority of known peptides with high bioactivity (BAPs) such as antihypertensive, antidiabetic, anti-oxidant, hypocholesterolemic, anti-inflammatory and antimicrobial actions, are short-chain sequences of less than ten amino acids. These short-chain BAPs of varying natural and synthetic origin must be bioaccessible to be capable of being adsorbed systemically upon oral administration to show their full range of bioactivity. However, in general, *in vitro* and *in vivo* studies have shown that gastrointestinal digestion reduces BAPs bioactivity unless they are protected from degradation by encapsulation. This review gives a critical analysis of short-chain BAP encapsulation and performance with regard to the oral delivery route. In particular, it focuses on short-chain BAPs with antihypertensive and antidiabetic activity and encapsulation methods *via* nanoparticles and microparticles. Also addressed are the different wall materials used to form these particles and their associated payloads and release kinetics, along with the current challenges and a perspective of the future applications of these systems.

Received 30th September 2023,  
Accepted 24th March 2024

DOI: 10.1039/d3fo04195f

rsc.li/food-function

## 1 Introduction

Short-chain bioactive peptides (BAPs) are protein fragments composed of several amino acids (di-, tri-, tetra-, or oligopeptides) and have the potential to prevent diseases and promote human health. Over 60 therapeutic BAPs have been approved in the United States, Europe, and Japan. Over 170 are in active clinical development, and an additional 260 have been tested in human clinical trials.<sup>1,2</sup> Foods are the most promising source of BAPs and are usually 2 to 20 amino acid residues long. They are obtained as a result of chemical synthesis, *in vitro* enzymatic hydrolysis or microbial fermentation, or generated *in vivo* by human gastrointestinal enzymes.<sup>3</sup> Studies of the origin, identification, isolation, and purification of BAPs and their mechanisms of action and application have increased significantly in the last decade. *In vitro* and *in vivo* studies have shown that BAPs can display antihypertensive, antidiabetic, antioxidant, hypocholesterolemic, anti-inflammatory, and antimicrobial actions.<sup>4</sup> Antihypertensive and antidiabetic activities include demonstration of the capacity to inhibit angiotensin converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV). When used alongside synthetic drugs, the anti-

ACE and DPP-IV BAPs have also shown synergistic effect, further enhancing their therapeutic potential.<sup>5</sup>

A detailed analysis, in which by “high” bioactivity, we consider an IC<sub>50</sub> (standard half-maximal inhibitory concentration) <10 μM for ACE inhibition and <50 μM for DPP-IV inhibition, is shown in Fig. 1. This is based on a review of BAPs from various food sources such as milk,<sup>6–16</sup> fish,<sup>17–32</sup> animals,<sup>33–38</sup> plants,<sup>39–46</sup> microalgae,<sup>47–50</sup> and mushrooms.<sup>51</sup>

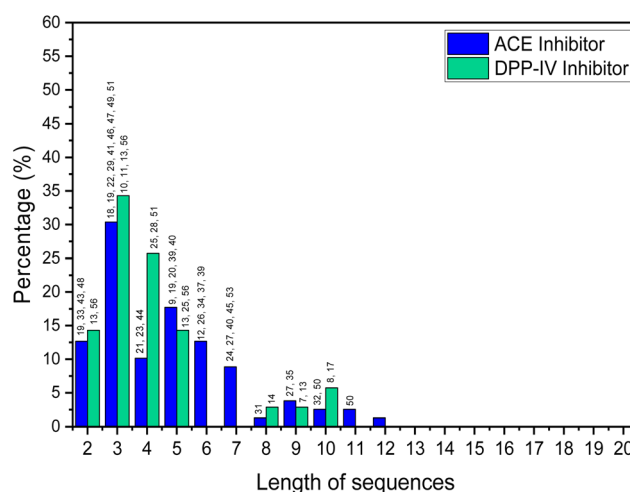


Fig. 1 Percentage of food-derived BAPs based on high bioactivities (as ACE and DPP-IV inhibitors).

<sup>a</sup>School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, United Kingdom. E-mail: f.m.goycoolea@leeds.ac.uk

<sup>b</sup>Department of Food Science and Technology, Universitas Trilogi, Jakarta, 12760, Indonesia

<sup>c</sup>Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain



The analysis shows that the greatest activity is displayed by BAPs of three amino acids. Skrzypczak *et al.*<sup>52</sup> considered peptides of 2–10 amino acids as short-chain BAPs, while other workers only consider those containing 2–4 amino acid residues as short-chain BAPs.<sup>53</sup> In general, peptide adsorption is most efficient for single amino acids or di- or tripeptides anyway.<sup>54,55</sup> Certainly, the vast majority of food-based BAPs that have high bioactivities seem to be shorter than 10 amino acids residues.

Several recent reviews published over the last five years have delved into the encapsulation of bioactive peptides. For instance, Mirzapour-Kouhdasht *et al.*'s<sup>56</sup> review focuses on enhancing the palatability of BAPs through encapsulation. Similarly, Berraquero-García *et al.*<sup>57</sup> discuss the encapsulation of BAPs using spray-drying and electrospraying techniques. Additionally, Aguilar-Toala *et al.*'s<sup>58</sup> provides an overview of strategies for encapsulating BAPs and presents a comprehensive analysis of the various methods for protection of the bioactivity and encapsulation of bioactive both proteins and peptides. They include a table summarizing encapsulation techniques for bioactive proteins and peptides. Furthermore, McClement's<sup>59</sup> review addresses nanoparticle and microparticle systems for encapsulating proteins and peptides, exploring challenges in oral delivery. These reviews, despite being comprehensive and timely, have kept the focus on proteins or protein hydrolysates (peptides) with a molecular weight ( $M_w$ ) exceeding  $\sim 3$  kDa. In the present review, the emphasis is on short BAPs, namely 10 to 50-fold smaller (2 to 20 amino acids) than the large therapeutic peptides and proteins which have been extensively studied and, in many cases, are available as approved drugs (generically known as "biologics"). BAPs have been discovered to exhibit antihypertensive, antidiabetic, anti-inflammatory, antimicrobial and antioxidant, among other activities. Features such as  $M_w$ , amino acid sequence, hydrophobicity and polarity, net charge, and ionisation state, dictate their association efficiency upon encapsulation in different type of carriers, as well as their release, bioaccessibility, and bioavailability. The case of low  $M_w$  and hydrophilic peptides entails unique challenges associated to their loading/encapsulation in different type of carriers, as further discussed in Section 4.1, but a simple case suffices to this well at the start. O'Neill *et al.*<sup>60</sup> encapsulated a dipeptide and a pentapeptide in the same material but showed that the dipeptide had 3 times lower encapsulation efficiency and 10 times higher release rate. While the amino acid tryptophan (W) exhibits an encapsulation efficiency approximately 1.25 to 1.5 times lower and releases approximately 1.3 times more compared to the dipeptide alanine-tryptophan (AW), both amino acid and dipeptide maintain a consistent trend in charge across varying pH levels. Thus the  $M_w$  of BAPs has profound effects, as further discussed in this review.

Gastrointestinal stability is a crucial prerequisite of BAPs with regard to their full realisation of their potential as ingredients in functional foods, nutraceuticals, and food supplements. However, chemical and enzymatic digestion *via* proteases in the stomach, intestinal lumen, and intestinal brush border leads to degradation of their intact sequences and overall decrease of their oral bioaccessibility and bioavailability,

as observed in both *in vitro* or *in vivo* studies.<sup>35,61–63</sup> To overcome these shortcomings, encapsulation helps to preserve their structural integrity until they reach the desired site of adsorption in the intestinal epithelium. Fig. 2 depicts the major features and layers of factors to account for BAPs and their encapsulation to develop innovative functional food ingredients and supplements. The breadth of this review is on critical analysis of the recently published literature on BAPs, while identifying existing gaps of knowledge and the key research aspects that underpin their potential innovative applications in foods.

## 2 Types of short-chain BAPs

Since 2010, only limited studies (*ca.* 13) have addressed BAP encapsulation for gastrointestinal delivery and our review therefore focuses on this time period. These were focused on di-, tri-, tetra- and up to undecapeptides of molecular weight ( $M_w$ ) ranging from 273.5 to 1493.7 g mol<sup>-1</sup>. Encapsulation of BAPs from varying sources with documented bioactivities such as antihypertensive, anti-inflammatory, antioxidant, antiproliferative, hypocholesterolemic, anxiolytic, anticancer and absorption permeation enhancers has been documented. Table 1 summarizes studies conducted on encapsulated BAPs of varying  $M_w$ , sequence, and their bioactivities.

The studies in Table 1 are based on both synthetic and naturally-derived BAPs. Most model studies have used purified synthetic compounds, but some of these were also obtained from food sources. Tripeptide IPP and hexapeptide RLSFNP are each derived from casein<sup>64</sup> and lactoglobulin,<sup>65</sup> respectively; tripeptide LKP was sourced from bonito fish,<sup>66</sup> and pentapeptide VLPVP was expressed in milk by engineered recombinant *E. coli*.<sup>67</sup> Tetrapeptide LQPE was obtained from milk casein hydrolysed using Neutrase®,<sup>68</sup> a commercialized brand mixture of proteases. Decapeptide YLGYLEQLLR ( $\alpha$ -casozepine) is obtained from whey protein hydrolysis by



Fig. 2 Multilevel pie chart illustrating some substantial aspects of the development of encapsulated BAPs as nutraceuticals, food supplements, and functional food ingredients.



**Table 1** Short-chain BAPs encapsulated for gastrointestinal delivery

Type BAPs	Sequence <sup>a</sup>	Molar mass (g mol <sup>-1</sup> )	Bioactivities <sup>b</sup>	Ref.
<b>Dipeptide</b>	AW	275.3	NR	75
	FW	351.4	NR	60 and 76
<b>Tripeptide</b>	IPP	352.4	Antihypertensive ACE inhibitor	77
	LKP	356.5	Antihypertensive ACE inhibitor	77
	ECG	307.3	Antioxidant	78
	KPV	342.4	Anti-inflammatory	79 and 80
	YKT	410.5	Anticancer	81
<b>Tetrapeptide</b>	LQPE	485.5	Hypocholesterole-mic	68
<b>Pentapeptide</b>	LWMRF	751.9	NR	60 and 76
	VLPVP	523.6	Antihypertensive ACE inhibitor	82
<b>Hexapeptide</b>	RLSFNP	732.8	Antihypertensive ACE inhibitor	83
<b>Octapeptide</b>	FCFWKTCT	1035.2	Antiproliferative	71
<b>Decapeptide</b>	YLGYLEQLLR	1267.4	Anxiolytic	84
<b>Undecapeptide</b>	GRKKRRQRRRP	1493.7	Permeation enhancer	85

<sup>a</sup> Amino acids single letter code key: A = alanine; C = cysteine; E = glutamic acid; F = phenylalanine; G = glycine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine. <sup>b</sup> NR = not reported.

bovine trypsin.<sup>69</sup> Glutathione ( $\gamma$ -glutamyl-cysteinyl glycine, GSH, or peptide ECG) is produced by bioprocessing with *Saccharomyces cerevisiae* and *Candida utilis*.<sup>70</sup> The tripeptide KPV is naturally produced in the human body, as alpha-melanocyte-stimulating hormone. Meanwhile, the octapeptide octreotide FCFWKTCT is a synthetic somatostatin with a cyclic structure.<sup>71</sup> In spite of the demonstrated potent bioactivity of many BAPs, their absolute bioavailability, when administered orally, is very low ( $\lesssim 1\%$ ) due to the degradation and fast elimination.<sup>72,73</sup> To improve their pharmacokinetics, colloidal carriers have been used.<sup>74</sup> Of note, only a relatively small amount of small peptides (<20) have been selected for encapsulation studies for gastrointestinal delivery. These span a wide diversity in terms of their biological sources, methods of production, size and structure, as discussed further on.

Tripeptides IPP and LKP, pentapeptide VLPVP, and hexapeptide RLSFNP have been reported to mediate antihypertensive activity *via* inhibition of angiotensin-converting enzyme (ACE).<sup>77,82,83</sup> Decapeptide YLGYLEQLLR ( $\alpha$ -casozepine) has anxiolytic activity.<sup>84</sup> Peptide ECG inhibits oxidative damage caused by free radicals from reactive oxygen species (ROS) circulating in the body.<sup>78</sup> Octapeptide FCFWKTCT (octreotide) has antiproliferative activity *via* inhibition of pancreatic exocrine and endocrine secretion.<sup>71</sup> In terms of their potential applications, even if the BAP does not need to circulate in the blood, in most cases, they must be absorbed systemically to display their bioactive effects fully. To this end, encapsulation is known to confer BAPs protection against degradation in the gastrointestinal environment, control their release in an intact form at the target sites, and promote their absorption, thus effectively, enhancing both their bioaccessibility and bioavailability. Other examples of encapsulated BAPs include hypocholesterolemic tetrapeptide LQPE and anti-inflammatory tripeptide KPV. LQPE demonstrated the ability to decrease micellar cholesterol solubility and affect the expression of proteins and enzymes related to cholesterol absorption in the small intestine epithelial cells.<sup>86</sup> This peptide must be released

in its intact form into the epithelial cells. KPV, in turn, has been reported as possessing anti-inflammatory activity *via* inhibition of proinflammatory cytokine synthesis and is naturally secreted into the colon. Thus, this tripeptide has been studied as a therapeutic peptide for treating inflammatory-related bowel diseases (IBD) in the large intestine.<sup>87,88</sup>

### 3 Types of particulate vehicles for encapsulation and their preparation

Colloidal nano- and microparticle carriers have been used to encapsulate therapeutic peptides and this has been comprehensively reviewed elsewhere.<sup>58,59,74</sup> The various approaches to associating the peptides include complexation, conjugation, crystallization, or self-assembly through chemical or physical additions.<sup>89</sup> Various types of particulate vehicles have been designed for encapsulation, protection and release of short-chain BAPs (hereafter simply referred to as 'BAPs'). In general, there are two main ways of loading the BAPs into these structures: (i) during the particle formation, sometimes referred to as *in situ* loading or the internal method and (ii) loading after particle formation, sometimes referred to as pro-loading or the external method. Method (i) typically involves mixing the BAP with particle ingredients in some way possibly influencing the final size and structure of the vehicle. The BAP may simply diffuse into the structure. Method (ii) typically involves placing a suspension of the preformed vehicles in some sort of solution of the BAPs. The latter may also cause some change in the size and structure of the vehicle if it is subject to swelling or shrinkage in the BAP solution. In fact, the size of the vehicle is commonly used to classify different types of delivery vehicles.

#### 3.1 Nanoencapsulation

**3.1.1 Nanoparticles (NPs).** Nanoparticles (NPs), *i.e.*, colloidal particles  $\lesssim 100$  nm have been a central focus of research in drug delivery for more than four decades. Today, their appli-



cation and clinical translation are essential pillars in the nanomedicine field. Due to their size, composition, the nature of their surfaces and a huge surface area to volume ratio, they provide unique advantages for the administration of bioactive payloads that exhibit either toxicity, degradation, or poor absorption. These include molecules that do not meet the Lipinski's rule of five (*i.e.*, less than five H-donor groups, less than ten H-acceptor groups,  $M_w < 500$  Da,  $\log P$  octanol water partition coefficient  $< 5.0$ ).<sup>90</sup> NPs can be comprised of polysaccharides such as chitosan (CS), alginate (ALG), hyaluronic acid, dextran, pectin, and arabinogalactans plus chemical derivatives of these, or by proteins such as zein, lysozyme, lactoferrin and gelatine, among others. NPs formed from natural biopolymers and macromolecules have been the focus of increasing attention, given the range of desirable properties they exhibit such as biocompatibility, biodegradability, stability and mucoactivity (mucoadhesion or mucodiffusion). In addition, they have the capacity to enhance the bioaccessibility and bioavailability of poorly absorbable payloads by promoting their epithelial absorption. Moreover, NPs based on these components can usually be produced under relatively mild conditions such as aqueous condition and room temperature, thus helping to preserve the bioactivity of delicate biological-type payloads such hormones, antibodies, vaccines, nucleic acids, as well as BAPs.<sup>91,92</sup>

In foods, NPs based on polysaccharides and proteins are known to occur and form during traditional cooking processes of both animal- and plant-based foods, that have been referred to as "incidental NPs".<sup>93</sup> In addition, polymeric NPs may be deliberately manufactured and added to foods as various colloidal dispersions.<sup>94</sup> NPs intended for oral delivery of BAPs have been largely prepared *via* two different protocols: (1) coacervation (ionic gelation),<sup>77,78,81,85</sup> and (2) double emulsion and solvent evaporation.<sup>79,80</sup> The preparation steps and main features of each of these methods are reviewed below.

With respect to the coacervation route, CS-based NP systems have been prepared from CS<sup>81</sup> plus: sodium tripolyphosphate (TPP),<sup>77</sup> cyclodextrin (CD),<sup>78</sup> and the synthetic methyl acrylate based copolymers termed Eudragit.<sup>85</sup> Under the double emulsion and solvent evaporation routes, CS/ALG<sup>79</sup> and hyaluronic acid-CS<sup>80</sup> NPs have been investigated.

Research conducted by Trapani *et al.*<sup>78</sup> addressed the encapsulation of glutathione (peptide ECG) into CS NPs and CS/CD NPs using two types of CD ( $\alpha$ -CD and sulphobutyl-ether- $\beta$ -CD – termed SBE- $\beta$ -CD). For the CS NPs, encapsulation was achieved by mixing ECG peptide and CS solutions then adding tripolyphosphate (TPP), to induce spontaneous NP formation *via* ionic cross-linking between the positive charges on the CS and the negative charges on the TPP. For encapsulation of tripeptides into CS/ $\alpha$ -CD or CS/SBE- $\beta$ -CD NPs, the tripeptide solution was first mixed with  $\alpha$ -CD or SBE- $\beta$ -CD solutions then CS solution added, with TPP added at the end. In some cases, NPs are formed and then further coated with another polymer. For example, Eudragit-coated CS NPs have been used to encapsulate a permeation enhancer undecapeptide alongside insulin (a 51 amino acid peptide). The undecapeptide was dissolved in

acetic acid before being mixed with CS and then TPP cross-linking agent was added to form undecapeptide-insulin-CS NPs. Eudragit (dissolved in ethanol) was then slowly injected into the loaded NP dispersion and the ethanol was then removed by evaporation to end up coating the NPs.<sup>85</sup> The antihypertensive tripeptides LKP and IPP have also been encapsulated into CS NP systems but a different order of addition of ingredients was used, *i.e.*, the peptides were mixed with TPP first and then CS was added.<sup>77</sup> These different orders of addition of ingredients (see Fig. 3) are likely to affect the size, structure, production yields and encapsulation efficiencies (EE) of the NPs.

Despite its important role in determining the properties of the formulation, the order of mixing of the components is often neglected. For instance, the size of CS-TPP NPs and the EE has been found to be sensitive to this. The strategic order of mixing might play a role in influencing the size of CS-TPP NPs and EE. Danish *et al.*<sup>77</sup> demonstrated that dissolving the peptide into the TPP solution first, followed by the addition of CS, creating smaller-sized NPs and higher EE compared to the sequence proposed by Trapani *et al.*<sup>78</sup> In the realm of CS-CD-TPP-NPs, no conclusive evidence seems to favour whether it is best to add first the ECG peptide into either CS or CD solution. Notably, despite SBE- $\beta$ -CD having a molecular weight ( $M_w$ ) twice as high as  $\alpha$ -CD, CS/SBE- $\beta$ -CD-TPP NPs exhibit a smaller size than both CS/ $\alpha$ -CD-TPP and CS-TPP NPs. Additionally, the EE of ECG peptide into CS/SBE- $\beta$ -CD-TPP NPs surpasses that of CS/ $\alpha$ -CD-TPP or CS-TPP NPs by a noteworthy around fivefold. In fact, the accepted principle in ionic gelation is that the mixing order depends on the charge of each molecule. Dissolving the peptide into the phase with a similar charge before introducing the counter charged phase is generally preferred to prevent premature interactions and aggregation driven by full charge compensation and neutralisation leading to loss of electrostatic colloidal stabilisation.

Moreover, the role of the biopolymer structural characteristics such as the degree of acetylation (DA) and pattern of acetylation (*e.g.* CS), degree of esterification (*e.g.* pectin), block composition (*e.g.* ALG), as well as the  $M_w$  and polydispersity ( $\mathcal{D}$ ) of the various biopolymer families, on the size, surface charge, morphology and peptides' EE (Section 4.2) of the different type of NPs, has been widely neglected.

Double emulsions and solvent evaporation have been used to encapsulate KPV into CS/ALG NPs and hyaluronic acid-CS NPs.<sup>79,80</sup> In fact, many hydrophilic drugs have been encapsulated *via* water-in-oil-in-water (w/o/w) emulsions where the middle non-aqueous phase is a mixture of polymer + solvent/oil mixture. The solvent is finally evaporated away to leave a hardened polymer layer encasing the inner aqueous phase and its bioactive payload. For BAPs, the polymer used is often poly(lactic-acid) (PLA) or poly(lactide-*co*-glycolide-acid) (PLGA) dissolved in dichloromethane and a final stabilizing layer polyvinyl alcohol (PVA) is used to stabilize the dispersion in water. Such systems are often freeze-dried and then re-dispersed in CS and/or ALG solutions before use. One general disadvantage of the double emulsion route is the lack of natural, biocompatible water-in-oil (w/o) emulsifiers.



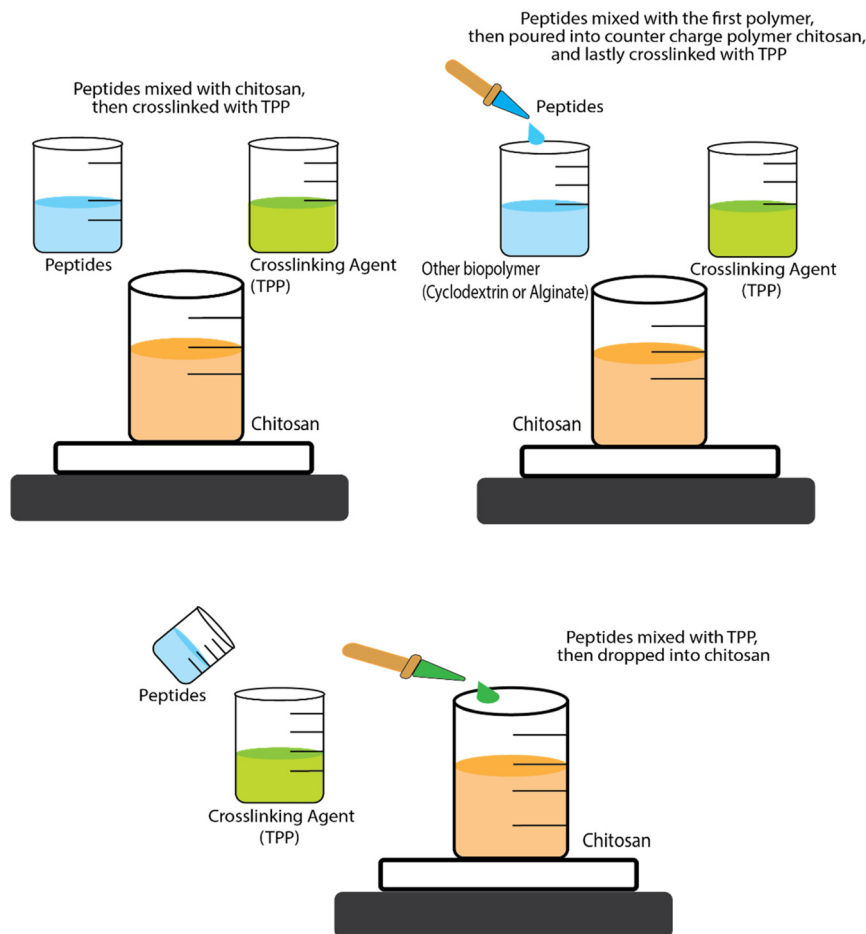


Fig. 3 Schematic representation of the three approaches for encapsulation BAPs by ionic gelation/coacervation.

Ultimately, the utilization of nanotechnology in food applications necessitates a careful consideration of potential risks to consumers' health, as emphasized by the European Food Safety Authority (EFSA) Committee. The EFSA guidance documents outline specific criteria for nano-specific risk assessment, particularly focusing on particles smaller than ~500 nm, with a distribution exceeding 10% below 250 nm.<sup>95,96</sup> Key elements in this assessment encompass the physicochemical properties, biokinetics, potential cellular uptake, physiological fate, and distribution of nanoparticles (NPs). The guidance underscores the importance of evaluating the persistence of particulate forms, such as solubility and dissolution rate, along with factors like dispersion, agglomeration, and biological relevance of particle size after reaching the gastrointestinal tract.<sup>97,98</sup> Notably, for food composed of natural polymers at the nanoscale, which undergo digestion and assimilation without translocation outside the gastrointestinal tract, and exhibit no adverse effects, a risk assessment similar to conventional food products is deemed appropriate.

**3.1.2 Liposomes.** Liposomes are vesicles formed by phospholipid bilayers that encase an aqueous cavity of significant volume compared to the volume of the bilayers. This type of vesicles mimic a cell and its cell membrane. They have been

used to deliver both hydrophilic and hydrophobic drugs and were the first 'nanomedicines' approved by the Food and Drug Administration (FDA), to deliver doxorubicin (Doxil®),<sup>99</sup> in 1995. Conventional methods of liposome preparation involve the dissolution of the lipids in an organic solvent followed by evaporation of the solvent and then rehydration with an aqueous medium. Slow hydration and mild agitation generally result in multilamellar vesicles (MLVs), while stronger agitation, often with sonication and extrusion, is used to create unilamellar liposomes.

In the case of BAPs, liposomes have been studied and loaded with hypocholesterolemic tetrapeptides, antihypertensive hexapeptides, and antiproliferative octapeptides. The BAP-loaded liposomes were further modified by encapsulating with *Lactobacillus* surface (S-layer) protein to modify their adhesion.<sup>68</sup> Alternatively, MLVs can be used to give extra protective layers to the payload.<sup>100</sup>

BAPs have been loaded into liposomes using both *in situ* and pro-loading methods.<sup>101</sup> *In situ* loading obviously follows the same steps for the liposome preparation but with the peptides present in the aqueous phase during liposome formation. In this way Zhang *et al.*<sup>83</sup> encapsulated RLSFNP in soy lecithin + cholesterol liposomes using ethyl ether as solvent,



while Jiang *et al.*<sup>68</sup> encapsulated the LQPE *via* 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine + cholesterol + octadecylamine liposomes, using trichloromethane as solvent.

Parmentier *et al.*<sup>71</sup> encapsulated the synthetic cyclic octreotide (Table 1) into tetraether lipid liposomes using a pre-loading approach. Egg phosphatidylcholine (EPC) dipalmitoyl phosphatidylcholine (DPPC), tetraether lipids from *Archaea* spp. and cholesterol were used as the lipid materials. After evaporation of the solvent and rehydration to MLVs, the latter were extruded through 400 and 200 nm filter membranes to create the (so-called) liposomes. Loading with octreotide was achieved by mixing with the liposome suspension, followed by freeze-drying and subsequent rehydration.

### 3.2 Microencapsulation

Microparticles (MPs) may be defined as particles that typically have average diameter sizes from one to several tens of  $\mu\text{m}$ . In other words, this class of particles is somewhat larger in size than NPs. Although these larger sizes may give rise to greater problems of sedimentation and colloidal stability, various types have been explored for BAP encapsulation.

**3.2.1 Microgel particles.** As encapsulation agents of BAPs, microgel particles have been produced by coacervation and membrane emulsification. CS, whey protein isolate (WPI), and sodium alginate (SA) are some of the most widely used polymer materials. A study conducted by Batista *et al.* (2021) encapsulated the anxiolytic decapeptide YLGYLEQLLR into guar-CS microgels. The microgels were prepared by dissolving CS and the peptide into acetic acid then cross-linking *via* TPP. The CS microgels were then immersed in guar gum (GG) solution to coat them.<sup>84</sup> Cold gelation has also been used to fabricate whey protein microgels encapsulating FW and LWMRF peptides. Pre-heated and cooled whey protein was mixed with calcium chloride ( $\text{CaCl}_2$ ) as a cross-linker and then immersed in a solution of the peptides for 24 h.<sup>60,76</sup>

Another method of producing microgel MPs is *via* membrane emulsification and extrusion.<sup>82</sup> These have been used to encapsulate the ACE inhibitor VLPVP in SA-coated O-carboxymethyl chitosan (O-CMCS) into a w/o emulsion. The SA and peptide in acetate formed the aqueous phase and paraffin oil containing petroleum ether and hexaglycerin penta ester surfactant formed the oil phase. After forcing the water phase to the oil phase through the pores in the membrane to form the first w/o emulsion, a second w/o emulsion containing  $\text{CaCl}_2$  was then formed and mixed with the first emulsion. The  $\text{CaCl}_2$  produced transfer of  $\text{Ca}^{2+}$  ions to cross-link the O-CMCS in the first emulsion and gel the water droplets. The peptide-containing microgel MPs obtained could then be washed and freeze dried. (The authors also tried extruding SA solution into  $\text{CaCl}_2$  *via* a thin nozzle then coating the particles with O-CMCS.) Fig. 3 and 4 illustrate the ionic gelation/coacervation and double emulsion/solvent evaporation approaches.

**3.2.2 Microfibers.** Microfibers have also been utilized to encapsulate the dipeptide AW. To this end, fish sarcoplasmic protein (FSP) was dissolved in hexafluoro-2-propanol (HFIP),

AW added, then microfibers of the mixture formed by the electrospinning technique at room temperature.<sup>75</sup>

However, among the above techniques of encapsulation of BAPs especially by ionic gelation/coacervation and double emulsion solvent evaporation, there were no studies on gastrointestinal stability of the vehicle or encapsulant, both loaded and unloaded. In a study by Laroui *et al.*<sup>79</sup> BAPs were encapsulated within a double emulsion prepared *via* the solvent evaporation technique, after which the loaded NPs were embedded in a chitosan (CS)-alginate (ALG) hydrogel. But this group investigated the gastrointestinal stability of cross-linked CS-ALG hydrogel *before* loading with NPs. The CS-ALG mixed hydrogel, ionically cross-linked solution *via*  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$  ions, was assessed for its kinetics of swelling as a measure of its stability in simulated gastric fluid pH 1 to 3 (2 M buffer acetate,  $50 \mu\text{g mL}^{-1}$  pepsin,  $5 \mu\text{g mL}^{-1}$  trypsin) and intestinal fluid pH 4 until 6 (0.1 M buffer phosphate,  $5 \mu\text{g mL}^{-1}$  pepsin,  $30 \mu\text{g mL}^{-1}$  trypsin). As a result, they concluded that the composite CS-ALG hydrogel was stable at gastric pH 1, 2, and at 3 for 24 h, but shrank at pH 4 and finally collapsed to form a dispersion at pH 5 and 6 after 24 h.

Apart from encapsulation of BAPs, the CS-TPP nanoparticles produced through ionic gelation were assessed for stability in simulated salivary fluid at pH 6.8. Comparative analysis with ALG-NP and pectin-NP (Pec-NP) revealed CS-NP to be the least stable, evidenced by an increase in polydispersity index (PDI) and size (hydrodynamic diameter), accompanied by a reduction in zeta-potential. Prior to integration with artificial saliva, CS-NP exhibited a PDI below 0.2, escalating to 0.4 post-mixing and exceeding 0.6 after a 20-min incubation. The size of the CS-NPs continued to grow progressively after 40 minutes of incubation. Although the zeta-potential remained stable throughout the testing period (0 to 120 minutes), it decreased from +25 mV (pre-mixing) to below +10 mV (post-blending with artificial salivary fluid). These changes were attributed to an increase in pH, leading to CS deprotonation (*i.e.*  $\text{p}K_o \sim 6.1$ ), and the presence of sulphates, phosphates, and carbonates in the buffer solution, which could bind to chitosan's charged groups, causing particle bridging, reduced stability, and the formation of aggregates and precipitates.<sup>102</sup>

In one of our studies,<sup>103</sup> we explored the stability of CS-TPP-ALG NPs loaded with insulin using the ionic gelation method and its potential efficacy for intranasal delivery of insulin. Remarkably, the colloidal particles exhibited robust stability for an extended period, enduring up to 80 minutes of incubation in acetic buffer with a pH of 4.3. This was evidenced by the absence of any discernible alterations in their hydrodynamic radius, as determined *via* dynamic light scattering (DLS). At lower pH levels, CS undergoes increased protonation, fostering stronger ionic interactions between CS and its cross linkers such as TPP, as well as oppositely charged polyanionic macromolecules. The heightened protonation of the cationic CS contributes to the reinforcement of these ionic linkages. It is noteworthy that the stability of NPs formed *via*



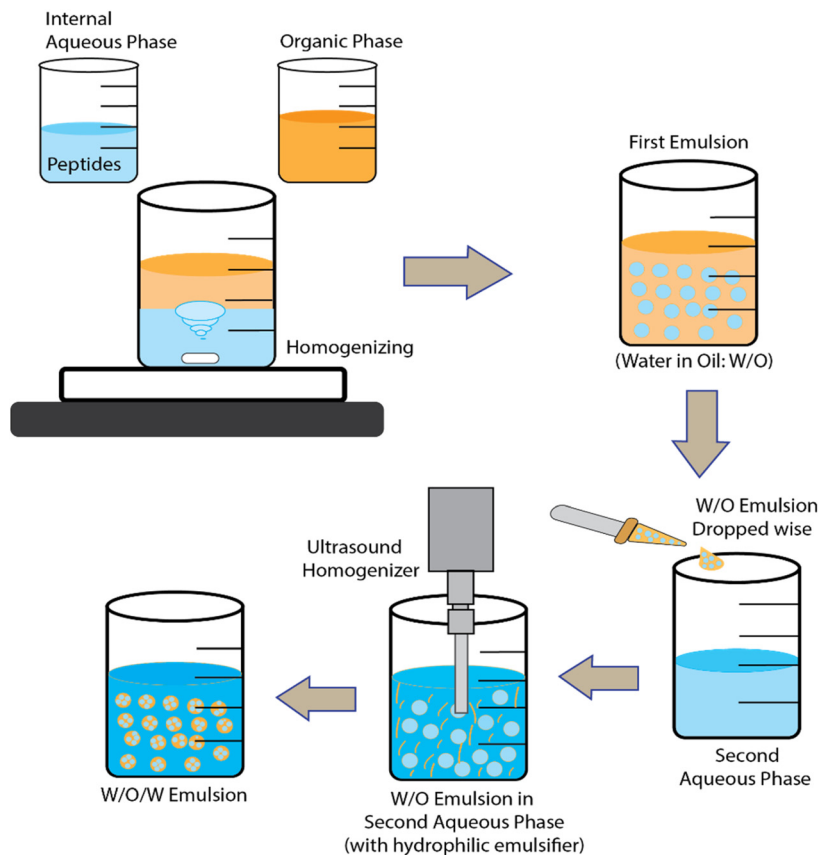


Fig. 4 Schematic representation of the methods for encapsulation BAPs via double emulsions and solvent evaporation.

ionic gelation, such as CS-TPP NPs, is not solely contingent on the ionic strength of the medium; rather, it is intricately influenced by the molar ratio between the involved molecules.

In relation to emulsion-based encapsulation systems, such as water-in-oil-in-water (w/o/w) double emulsions, in terms of water being the dispersed or continuous phase, and situated on the outermost part of the system, this technique shares similarities with the single oil-in-water (o/w) emulsion. The w/o/w encapsulation system is generally employed to address the inefficient encapsulation of highly hydrophilic bioactive compounds that tend to diffuse during formulation. The stability of this system in the gastrointestinal tract is influenced by the type and concentration of stabilizers used in the system.<sup>104</sup> Proteins and surfactants typically utilized as stabilizers for o/w emulsion formation have limitations due to the unwanted flocculation and coalescence of oil droplets, possibly resulting from pepsinolysis and the ionic environment during digestion.<sup>105</sup>

A study conducted by Torres *et al.*<sup>105</sup> analysed the stability of the o/w emulsion-based encapsulation system during *in vitro* gastric and intestinal digestion using whey protein isolate (WPI) as a stabilizer. In this study, the emulsion system was exposed to simulated gastric fluid (SGF) at pH 3 containing pepsin, simulating the fasted condition of the stomach. Subsequently, it was subjected to simulated intestinal fluid (SIF) at pH 6.8, consisting of bile salt and pancreatin solution.

The findings indicated that the emulsion experienced flocculation and coalescence in the SGF, resulting in a slightly more stable emulsion droplet distribution in the SIF, with sizes ranging between approximately 1 to 50  $\mu\text{m}$ , lasting longer than the emulsion mixed with SIF without *in vitro* gastric pre-digestion. In the latter case, the emulsion destabilized within 30 min (becoming very polydisperse) with a prominent peak in the size range 100 to 1000  $\mu\text{m}$ . During intestinal conditions, bile salts can potentially displace the WPI at the interface. (The size measurements in this study were obtained *via* confocal laser scanning microscopy – CLSM.)

## 4 Key characteristics of delivery vehicles

Table 2 lists various encapsulation vehicles, their sizes, methods of formation and encapsulation efficiency (EE) and release characteristics of various BAPs and Fig. 5 also illustrates the typical range of sizes and structures.

### 4.1 Association/entrapment efficiency (EE)

Generally, BAP association/entrapment/encapsulation efficiency (EE%) is defined as the % of BAPs added to a system that are successfully entrapped within the vehicles.<sup>68,77,78,81,83</sup>



**Table 2** Encapsulation agents, encapsulation methods, encapsulation efficiency (EE), sizes, and kinetic releases for short-chain BAPs

BAPs payload	Encapsulation wall materials	Encapsulation methods	Encapsulation efficiency (EE %)	Diameter (nm)	Release	Ref.
AW	FSP microfibers	Electrospinning	NR	100–700 and 1000	61.5% in SGF after 30 min, and max 71.8% in SIF after 15 min	75
FW	Whey microbeads	Cold set/ionic gelation	32–56		56% (6 h), ~80% ( $V_{\text{bead}}/V_{\text{aq}} < 0.2$ )	60 and 76
IPP	Chitosan NPs (CS: $M_w = 110$ kDa, DA = 14%)	Ionic gelation/coacervation	22.6–43.9	113–209	~75% in SGF (120 min); >90% in SIF (240 min). Over 60% released within the first 2 h in acidic simulated fluid	77
LKP	Chitosan NPs (CS: $M_w = 110$ kDa, DA = 14%)	Ionic gelation/coacervation	40.9–65.1	125–207	~75% in SGF (120 min); ~90% in SIF (240 min). Over 60% released within the first 2 h in acidic simulated fluid	77
ECG	Chitosan/CD NPs (CS: $M_w = 110$ kDa, DA = 14%)	Ionic gelation/coacervation	7.1–25.1	190–500	pH 1.2: CS/ $\alpha$ -CD, 100% (<200 min); CS/SBE- $\beta$ -CD, ~25% (<200 min). pH 6.8: CS/ $\alpha$ -CD, <60% (<200 min); CS/SBE- $\beta$ -CD, 100% (<100 min)	78
KPV	Chitosan/ALG NPs, and hyaluronic acid (HA)-chitosan/ALG NPs (CS: $M_w = \text{NR}$ , DA = NR; ALG: $M_w = \text{NR}$ , M/G = NR; HA: $M_w = 20$ kDa)	Double emulsions and solvent evaporation	NR	273–300	25% at pH 6.2 within 30 min. After 30 minutes, release kinetics is more linear about 35% within 30 h	79 and 80
YKT	Chitosan MPs (CS: $M_w = 50$ –190 kDa, DA = NR)	Ionic gelation/coacervation	35	~57	26% release at first 8 h, 34% (24 h), 43% (48 h), and 97% (264 h/11th day)	81
LQPE	Liposome coated SLP	Liposome	89	218.2	liposome-LQPE: 60% after 24 h, SLP-L-LQPE: 40% after 24 h	68
LWMRF	Whey microbeads	Cold set/ionic gelation	89–95.1	NR	<5% (6 h), ~10% ( $V_{\text{bead}}/V_{\text{aq}} < 0.2$ )	60 and 76
VLPVP	Carboxymethyl chitosan/Na-ALG microspheres (O-CMCS: $M_w = \text{NR}$ , DS = 35%; ALG: $M_w = \text{NR}$ , M/G = NR)	Membrane emulsification combined ionic gelation	64.6–87.8	5390–43 810	9.59% at pH 1.2 (2 h), and 87.63% at pH 6.8 (5 h)	82
RLSFNP	Liposome	Liposome	67.50	199	Diffuse through dialysis membrane: 75% (1 h), 98.4% (2 h). Cumulative release RLSFNP: 75% (12 h) and 94.92% (24 h).	83
FCFWKTCT	Tetraether lipid liposomes	Liposome	13	130–207	NR	71
YLGYLEQLLR	Guar gum Film-coated chitosan MPs (GG: $M_w = \text{NR}$ , CS: $M_w = 50$ –190 kDa, DA = 15–25%)	Ionic gelation/coacervation	86	1810	<2.5% (4 h)	84
GRKRRRQRRRP	Eudragit (ES)-coated chitosan NPs (CS: $M_w = 100$ kDa, DA = 5%)	Ionic gelation/coacervation	50	50.6	CS NPs: 85% (pH 1.2, 4 h), 40% (pH 6.8, 1 h). ES-coated CS NPs: 40% (pH 6.8, 1 h), 50% (pH 6.8, 4 h), 70% (pH 7.4, 1 h), almost completely released (pH 7.4, 4 h)	85

NR = not reported.

EE is therefore determined either by measuring the amount of peptide lost to the delivery vehicle from the original BAP solution, or by extracting the amount of BAP contained within the vehicles post loading, *e.g.*, by dialysis, centrifugation and dissolution of the vehicle. High performance liquid chromatography (HPLC) or reverse phase HPLC (RP-HPLC) is by far the most common method to assay for BAP content. It is often difficult to attain EE values close to 100%, whilst it is equally important the vehicle delivers a sufficiently high dose of bioactive at the target site.<sup>106</sup>

graph (HPLC) or reverse phase HPLC (RP-HPLC) is by far the most common method to assay for BAP content. It is often difficult to attain EE values close to 100%, whilst it is equally important the vehicle delivers a sufficiently high dose of bioactive at the target site.<sup>106</sup>





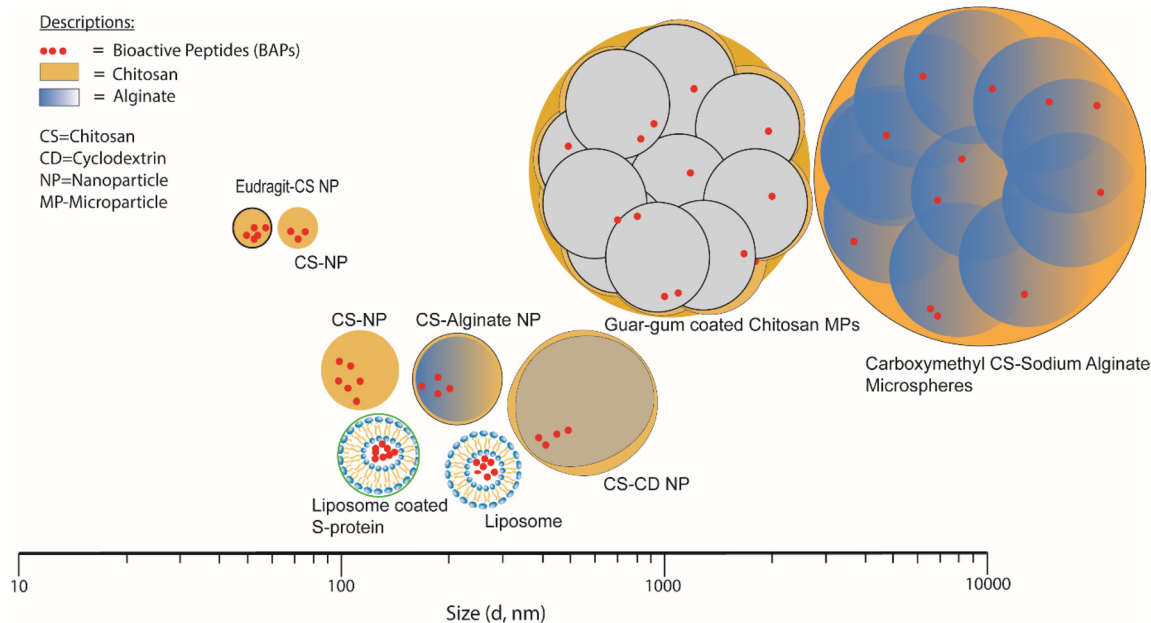


Fig. 5 Size distribution of NPs and MPs for short-chain BAPs encapsulation in biopolymer-based systems.

As mentioned earlier, loading BAPs in nano- and microparticle carriers deserves special considerations and challenges to identify the most optimal carrier system. Indeed, the EE is influenced by  $M_w$ , amino acid sequence, solubility, hydrophobicity, polarizability, net charge and ionisation state of the peptide, as it has been reviewed in a few comprehensive reviews focused on therapeutic peptides.<sup>74,107</sup> On the one hand, during their formulation into matrix-type systems such as nanoprecipitates obtained by solvent displacement, they are not retained and are released into the aqueous phase. On the other, when loaded into self-assembled electrostatic polyelectrolyte complexes, the low  $M_w$  BAP interacting with one of the polyelectrolyte components is ejected from the system, given that the low charge density and lack of cooperativity of the BAP is outcompeted by the polyelectrolyte bearing the same charge. The loading efficiency of low  $M_w$  BAPs is bound to be determined by similar phenomena to those that dictate the association of small drug molecules. Previous studies in model drugs of low  $M_w$  (242 to 479 Da) have shown that the hydrophobicity and polarizability, dictated their association efficiency. The more hydrophilic compound with lowest polarizability showed the highest tendency to be displaced by chitosan and associated with efficiencies as low as 12%.<sup>108</sup> As shown in Table 2, the BAPs associated in various types of carrier formulations, with efficiencies varying over a wide range 7 to 95%, thus reflecting the complexity of factors at play.

As already described, a relatively wide range of delivery vehicles has been developed, but no single BAP has been tested for EE and release across a wide range of different vehicles: at most, a maximum of two,<sup>68,85</sup> and rarely three<sup>78</sup> different types of vehicle seem to have been tested for the same BAP. At the same time, each type of vehicle has mostly

only been tested with one BAP and occasionally two different BAPs.<sup>77</sup> The most optimal type of carrier for a given low  $M_w$  peptide must be identified considering the intrinsic characteristics of both the peptide and the carrier components. This makes it hard to compare the efficiency of different systems and the effects that the BAP itself may have on the loaded vehicle size and structure. In almost all cases HPLC or RPHPLC have been used to assay for the BAP content to determine EE. One exception is KPV loading into w/o/w emulsions, where fluorescence spectroscopy was used.<sup>79</sup> In general, *in situ* methods of loading different peptides into different types of vehicle seem to have been more widespread but EE values seem to be lower – exceptions being YLGYLEQLLR into CS + TPP + GG microparticles (86%)<sup>84</sup> and LQPE into liposomes (89.0%).<sup>68</sup> YLGYLEQLLR will have both a positive and negative charges at neutral pH whereas LQPE has one negative charge.

Nanoparticles formed by CS-TPP systems, where three different tripeptide BAPs (IPP, LKP and YKT) were encapsulated *in situ*, had EE values ranging between 23 and 65%, depending upon the TPP:CS ratio;<sup>77,81</sup> the same vehicle gave only EE = 7% for encapsulation of ECG.<sup>78</sup> The reasons for these differences with different peptides are not clear, although it should be noted that LKP and YKT will both have a positive charge at neutral pH whereas ECG will have a negative charge. Using the slightly different CS +  $\alpha$ -CD + TPP and CS +  $\beta$ -CD + TPP systems<sup>78</sup> for encapsulation of ECG, EE values were 6 and 25%, respectively. The BAP was mixed first with the CD before adding the CS, so that the difference here probably relates to the greater affinity of ECG for  $\beta$ -CD. Using the higher  $M_w$  GRKKRRQRRRP and the same general CS + TPP method<sup>84</sup> EE = 51%, *i.e.*, higher than most of the other CS + TPP systems tested, so this might point to higher retention of higher  $M_w$



peptides using the *in situ* method, but varying peptide hydrophobicity and charge must also be taken into account – GRKKRRQRRRP has a relatively high number of eight positive charges at neutral pH. The addition of Eudragit polymer to the last system, after the initial encapsulation, resulted in no change in EE.

Turning to liposomal nanoparticles where EE has been fully quantified,<sup>68</sup> *in situ* encapsulation gave EE values of 89%, for liposomes uncoated and coated with SLP. It seems obvious that dissolution of all the ingredients in a solvent which is then evaporated off before re-hydration will force intimate contact between the BAP and the encapsulating ingredients, so leading to high EE values. (However, this does not necessarily mean that retention will be high on rehydration – see next section that discusses the BAPs release.) NPs formed *via* the double emulsion and solvent evaporation route<sup>79</sup> similarly gave high EE values of 70 to 71%. On the other hand, pre-loaded microgels have also been shown to give relatively high EE values. For example, WPI microbeads pre-loaded with FW and LWMRF using different volume ratios of microbead particles to BAP solution<sup>60,109</sup> gave EE values up to 95.1%. EE increased as the ratio increased and at the same ratio the maximum EEs obtained were 56.3% for FW and 95.1% for LWMRF, the latter having a positive charge. SAL + O-CMC beads formed by the membrane emulsification method<sup>82</sup> gave EE as high as 87.8% for VLPVP loading, though here loading was *in situ*. Interestingly, attempts to form the same types of particle by extrusion through ‘encapsulator’ nozzles and needles gave lower values of *in situ* EE (maximum values of 65 and 35%, respectively), pointing to the superiority of the emulsification method for this system.

#### 4.2 Size, surface properties and shape (morphology)

Morphology, size, surface charge and shape of NPs and MPs are other key aspects can affect EE and release, as well as the mechanisms of interaction with biological interfaces such as mucosa, epithelia, skin and therefore cellular uptake and intracellular trafficking. These aspects can be controlled to some extent by the overall composition and method of preparation.

In terms of size, it is known that only lipid NPs of size ~30 nm are able to cross the cell membrane by passive diffusion.<sup>110</sup> Larger particles rely on active, energy-dependent processes.<sup>111</sup> The main mechanism of cellular particle uptake in eukaryotic cells is endocytosis, which is commonly receptor-mediated pinocytosis and phagocytosis. In general, nanoparticles  $\leq 100$  nm can be taken up pinocytosis, whilst ~150–200 nm particles are internalised by receptor-mediated endocytosis. Particles ranging from ~250 nm to 3  $\mu$ m are taken up by phagocytic mechanisms.<sup>112</sup> Other studies have shown that particles have to be under ~200 nm in order to diffuse easily through the through the intestinal mucus layers to the epithelial cells.<sup>113,114</sup> In terms of shape, rod-like NPs with a higher aspect ratio were shown to be more efficient in penetrating into human cell line (HeLa) and colorectal adenocarcinoma-2 (Caco-2) cells.<sup>115</sup>

Examples of NPs and MPs that have been more thoroughly investigated in terms of their size distribution include Eudragit-coated CS NPs and CS NPs, whose mean diameters were reported as 51 and 57 nm, respectively<sup>81,85</sup> whilst the largest size reported for this type of NP was 500 nm, for CS/CD NPs.<sup>78</sup> It is found that in the context of producing CS-based NPs consistently, it is advisable to consider manipulating the initial concentration and the solvent environment as controllable factors.<sup>116</sup> The liposomes loaded with BAPs have been reported as having average diameters ranging from ~130 to 218 nm.<sup>68,71,83</sup> Guar film-coated CS MPs loaded with BAPs were ~1.8  $\mu$ m in diameter<sup>84</sup> and carboxymethylated gum/sodium ALG microspheres loaded with BAPs varied in size from ~5.4 to 43.8  $\mu$ m.<sup>82</sup> Excluding carboxymethylated gum/sodium ALG microspheres characterised by laser diffraction using equipment such as a Mastersizer (Malvern Panalytical Ltd) to measure size distribution, all the vehicle sizes mentioned were measured by DLS, more suitable for particles of smaller size (<1  $\mu$ m), to determine the distribution of the hydrodynamic radius ( $R_h$ ). DLS relies on the measurement of the diffusion coefficient of particles under Brownian motion in a solution in which small particles move rapidly, while bigger particles move slowly. This method is usually applied for particles less than 10  $\mu$ m. By assuming the particles shapes are spherical or round, the apparent particle diameter is inversely related to the coefficient of diffusion of particles according to the Stokes–Einstein equation.<sup>117,118</sup> Therefore, temperature and viscosity of buffer are critical to obtain appropriate results.<sup>119</sup> Study conducted by Luque-Alcaraz *et al.*<sup>92</sup> on CS NPs *via* nanoprecipitation found that the  $R_h$  depended on the suspension medium for measurement (methanol, water, or acetone). Furthermore, polydispersity aspects are limiting factors that affect the accuracy and precision of this method.<sup>120</sup>

As already mentioned, NP and MP surface properties are also known to significantly impact the interaction with the biological interfaces and, therefore cellular uptake.<sup>121</sup> Detailed topology of soft surfaces and particle shape can be characterised for fixed or frozen samples *via* transmission electron microscopy (TEM) and cryo-scanning electron microscopy (SEM),<sup>122</sup> provided one is aware of the artefacts that these techniques can introduce.<sup>81</sup> Some workers<sup>68,78,79,83</sup> have found fine correspondence between the results of DLS and such electron microscopy (EM) techniques. However, it should be noted that DLS averages the distribution as intensity (even though mathematically, can also provide volume or number distributions), and assumes all particles are spherical, whereas in analysing EM images, a number average is obtained, as well as information on particle shape. In principle, atomic force microscopy (AFM) can examine the surface of native particles in an aqueous environment, subject to the limitations of particle immobilisation on a solid surface and the softness of the surface structure *versus* the AFM cantilever stiffness.<sup>123,124</sup> Small-angle X-ray scattering (SAXS) and neutron scattering (SANS) can reveal the external internal structure of particles,<sup>125</sup> also hydrodynamic thickness of NPs MPs layers can be investi-



gated<sup>118</sup> related to stability and release where these aspects are discussed hereafter. Surface charge is most commonly inferred from electrophoretic mobility and zeta potential measurements.

NPs and MPs loaded with BAPs have been reported to exhibit a regular spherical morphology for liposomes coated with SLP, sodium ALG-O-CMCS microspheres and Eudragit-coated CS NPs.<sup>68,82,85</sup> Huang *et al.*<sup>82</sup> studied VLPVP-loaded sodium ALG-O-CMCS microspheres produced *via* membrane emulsification. They noticed that surface of the microspheres was rough but became smoother as the membrane pore size increased to 5  $\mu\text{m}$  or higher.

#### 4.3 Stability, release, absorption/permeability, and bioactivity of encapsulated BAPs

The bioaccessibility of BAPs, reflecting the proportion of peptides released from their delivery system during digestion for absorption in the gastrointestinal tract to manifest their bioactivities, underscores the critical importance of both BAPs' stability and the efficacy of the encapsulating agent. By enhancing their stability and bioaccessibility, encapsulation also aims to increase their bioavailability, since many studies have found that the biological activity of non-encapsulated BAPs is significantly reduced after gastrointestinal digestion and *in vivo* analysis.<sup>126–128</sup> In the pharmaceutical sector, the target is to increase the bioavailability of therapeutic peptides to 30–50%.<sup>129</sup> This section addresses the intricate interplay surrounding BAPs and their bioaccessibility within the gastrointestinal milieu. Table 2 and Fig. 6 summarise the various encapsulation methods that have been sought to enhance the bioaccessibility of BAPs in the gastrointestinal tract.

**Stability.** The stability of delivery vehicles for BAPs has been investigated during simulated gastrointestinal digestion. FSP microfibers were found to degrade slowly in simulated gastric

fluid (SGF) containing 0.0032% (w/v) pepsin after 24 h, and it was compared to SGF containing a high amount of pepsin (0.32%, w/v) that seems to be completely degraded. Around 50% of same microfibers had an altered structure under simulated intestinal fluid (SIF) conditions containing a low activity (0.01%, w/v) of pancreatin after 24 h, while at a high level (1% w/v pancreatin) the degradation became 100% as well.<sup>75</sup> For LQPE loaded into liposomes covered with and without a coating of SLP, the % of intact peptides were 74.5% and 55%, respectively, after incubation in artificial intestinal juice for 3 h. After incubation in artificial gastric juice, the % of intact peptides were 68% and 50%, respectively. The non-encapsulated tetrapeptide was completely degraded after only 1 h in either artificial intestinal or gastric juice.<sup>68</sup> The findings of this study indicate that encapsulation of this pentapeptide can enhance its gastrointestinal bioaccessibility by over 50%. Such studies illustrate the value of such encapsulation, but also the need for more systematic studies of gastrointestinal digestion under controlled and comparable conditions.

**Release.** When it comes to trying to understand the different release characteristics of the different vehicles containing the same or different BAPs, the situation is even more fraught with difficulty than trying to rationalize the different EE values (see above). Sometimes the release medium is just water, which is not very relevant to the fate of the vehicle during gastrointestinal digestion. Although the majority of release studies have used SGF and/or SIF conditions to examine % release, unfortunately these conditions tend to vary across all studies-different pH, times, different enzymes and other digestive compounds, varying in their concentration and/or specific activity. Even though human digestive enzymes used may be inactive against many of the encapsulating materials (the polysaccharide gums, for example), others are active (*e.g.*, against lipids, proteins and starch-based molecules) so it would be wise to try



Fig. 6 The effect of encapsulation on peptide digestion.



and standardize conditions as far as possible in future.<sup>130,131</sup> For quite a few of the encapsulation systems already discussed, release studies have not even been reported. The most common method of measuring release is to seal the loaded vehicles with a solution of the digestive enzymes or other release media in a low  $M_w$  dialysis bag placed in water then measure the BAPs diffusing out of the bag. Most studies show a high % release after several hours of SGF and/or SIF conditions for all types of vehicles studied: nanoparticles, liposomes and microgels. In many cases the extended times to achieve full release are not representative of typical human digestion conditions. Release is usually increased by SGF then also SIF, in some cases dramatically so, for example VLPVP in SAL + O-CMC microgels<sup>82</sup> and ECG in CS + CD + TPP systems.<sup>78</sup> The inclusion of an extra layer of Eudragit polymer on CS + TPP nanoparticles loaded with GRKKRRQRRRP seemed to give some slowing of release at low pH<sup>84</sup> and coating LQPE-loaded liposomes with SLP also seems to slow down release to some extent. Less permeable more inert layers on the outside of the delivery vehicle might be expected to do this. Unfortunately, KPV-loaded PVA nanoparticles coated with and without extra layers of CS and ALG were only subjected to digestive conditions without the extra layers.<sup>79</sup>

Other notable results include VLPVP encapsulated within O-carboxymethyl CS/SA microspheres, where only 9.6% was released after 2 h at pH 1.2 but 87.6% after 5 h at pH 6.8,<sup>82</sup> indicating a system potentially highly suited to gastric protection but intestinal release. Guar film-coated CS MPs also successfully protected YLGYLEQLLR for 4 h in artificial saliva (pH 6.8), giving <2.5% release after 4 h.<sup>84</sup> However, all these results also indicate the need for a consistent approach where systems are studied under the same set of physiological conditions.

Yet an alternative approach has focused on the release of small peptides of varying  $M_w$  (3.4 to 20 kDa) conjugated in poly(ethylene glycol) diacrylate (PEGDA)-peptide hydrogels.<sup>132</sup> To this end, the degradation by matrix metalloproteinase-2 (MMP-2) proteases to drive the release of peptides was harnessed. It was found that the peptides  $M_w$  had a direct effect on the hydrogel mesh size that itself influenced the penetration of the MMP-2 protease into the gel, and the optimal release of the bioactive peptide was for the PEGDA-10 kDa. Further research is needed to investigate these phenomena in matrix-type nano- and microparticles where the peptide is loaded, not as a polymer conjugate, but by virtue of other interactions, where the peptide release is mediated by the degradation of the particles by proteases, such as protein-poly-saccharide systems.

Based on the published contradicting and inconsistent results, we cannot conclude firmly about roles of the BAPs sequences, type of encapsulation agents, and physiological medium to their release kinetic and behaviour. Only further systematic studies using identical standardised protocols will help to elucidate in greater detail the role of each these factors.

**Absorption.** Encapsulation can improve the absorption of peptides. Permeability of the proximal segment of frog duodenum to ECG encapsulated in CS/ $\alpha$ -CD and CS/SBE- $\beta$ -CD MPs

was increased by a factor of 1.4 and 1.2, respectively, compared to the non-encapsulated tripeptide. In the distal segment, the permeability increased by 1.3 (for CS/ $\alpha$ -CD) and 1.8 (for CS/SBE- $\beta$ -CD).<sup>78</sup> CS/SA NPs loaded with KPV interacting with membranes of Caco-2 cells have been studied by fluorescence microscopy and TEM.<sup>79</sup> It was observed that surface functionalization of these NPs with hyaluronic acid (HA) promoted cellular uptake of the NPs, markedly higher than NPs without HA.<sup>80</sup> Also, using a Caco-2 cell model, it was shown that the transepithelial transport of LQPE and RLSFNP increased.<sup>68</sup>

The apparent permeability coefficient of liposomal RLSFNP liposome was at least 2× higher than free RLSFNP.<sup>83</sup> Similarly, the permeability of YLGYLEQLLR loaded into CS MPs across a human adenocarcinoma Caco-2/HT29 colorectal cell line was 4-fold higher than the free decapeptide.<sup>84</sup> The results of this study are consistent with the notion that encapsulation under different strategies contributes to enhance the bioavailability of BAPs. More mechanistic studies are needed to elucidate the optimal encapsulation systems for given BAPs, so as to identify the most optimal systems to enhance their absorption.

**Bioactivity.** The activity of IPP as an ACE inhibitor after nanoencapsulation and release was 84% and 82%, respectively. ACE activity of LKP after CS-NPs encapsulation was 85%; this tripeptide retained 83% of its activity post-incubating in SIF solution.<sup>77</sup> Microencapsulation of VLPVP into SA-O-carboxymethyl CS-MPs did not significantly reduce the activity against ACE: the native pentapeptide had 48.2% inhibitory activity, whereas the encapsulated pentapeptide showed inhibitory activity of around 45.2%.<sup>82</sup> Thus, there is important evidence that the appropriate choice of encapsulating vehicle will not affect BAP bioactivity once it has been delivered, although very few studies have followed in detail the whole process of encapsulation, stability, release and bioactivity from start to finish.

The assessment of the bioactivity of the tripeptides LKP and IPP subsequent to encapsulation involved measuring the loaded-NPs capacity to inhibit angiotensin-converting enzyme (ACE) during the conversion of the substrate hippuryl-L-histidyl-L-leucine (HHL) to the product hippuric acid (HPA). This methodology entailed quantifying the total HPA concentration in the reaction solution *via* HPLC. A diminished concentration of HPA in the reaction solution signified elevated ACE inhibitory activity. In turn, the assessment of LKP and IPP activity post-release entailed sonication of the loaded-NPs, followed by centrifugation. The quantification of BAPs in the supernatant was conducted using HPLC, and the BAPs were subsequently introduced into a reaction solution containing ACE enzyme and HHL. The total HA content in the reaction solution was analysed using HPLC-UV/Vis.<sup>77</sup>

In the study conducted by Huang *et al.*<sup>82</sup> encapsulating antihypertensive BAPs into microspheres, freeze-dried loaded microspheres were added into borate buffer, followed by sonication for one hour. The quantification of peptide release was achieved through HPLC, and ACE inhibitory activity was determined using spectrophotometry, following the method outlined by Cushman and Cheung (1971).<sup>133</sup>



#### 4.4 Cytotoxicity

Establishing any cytotoxicity of NPs/MPs is of paramount if particular encapsulating vehicles are to be used in practice. Caco-2, HT29-MTX, and IEC6 cell lines have been used to mimic gastrointestinal cells, and HepG2 cells were applied to mimic liver exposure. There are three commonly used assays to determine the cell viability of NPs/MPs (with or without loaded BAPs), namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), cell counting kit-8 (CCK-8), and water-soluble tetrazolium salt (WST-1) assay.

CS-NPs loaded with IPP and LKP (at 1–10 mM) showed no effects on cell viability (Caco-2 and HepG2).<sup>77</sup> Similarly, KPV encapsulated into (HPA)-CS/SA NPs did not affect cellular death rates of both Caco2-BBE and IEC6 cell lines over 72–80 h exposure.<sup>79,80</sup> Membrane integrity of Caco-2 cells was not significantly affected after treatment with Eudragit-CS NPs loaded with GRKKRRQRRRP.<sup>85</sup> Liposome loaded hexapeptide RLSFNP also displayed no cytotoxicity effect on Caco-2 cells.<sup>83</sup>

MPs loaded with decapeptide YLGYLEQLLR showed no cytotoxicity effects either, with a cell viability of about 75–99% after incubation with 0.1–5  $\mu\text{g mL}^{-1}$  of peptides on MPs solution.<sup>84</sup> Thus there seems very little evidence that these types of biopolymer-based NPs and MPs themselves have strong cytotoxic effects, pointing again to the advantage of basing delivery vehicles based (mostly) on food-derived materials.

## 5 Summary – gastrointestinal delivery vehicles for BAPs: challenges and opportunities

Overall, BAP release from the various NPs and MPs studied so far seems to be in the range 60–75% in simulated gastric fluid (SGF) but higher in (*e.g.*, 90%) in simulated intestinal fluid (SIF). Obviously, these rates could be advantageous or disadvantageous depending on the actual desired site of action, but a general positive aspect is that most vehicles seem to give sustained (not burst) release in both SGF and SIF, *e.g.*, typically 2 h in SGF and 4 h in SIF for full release. Food in the human digestive system needs around 2–6 h for full digestion and adsorption. Since more than half of encapsulated BAPs are released under gastric conditions, this might give a chance for pepsin to hydrolyse them and change their original amino sequence before reaching intestinal brush border. Pepsin is an enzyme that hydrolyses all peptide bonds between aromatic amino acid residues.<sup>134</sup> Moreover, achieving even 90% release of BAPs in the intestine does not guarantee their successful arrival at target sites, because they encounter further challenges from other proteases such as trypsin, chymotrypsin, carboxypeptidase A, elastase and carboxypeptidase B. Trypsin predominantly cleaves peptide bonds involving the carboxyl groups of arginine (R) or lysine (K).<sup>135</sup> Chymotrypsin targets peptide bonds formed by the carboxyl groups of Tyr (Y), Phe

(F), Trp (W), and Leu (L).<sup>136</sup> Carboxypeptidase A selectively cleaves C-terminal peptides, with increased hydrolysis rates observed when the side-chain R is aromatic or branched aliphatic, such as +Phe (F), +Tyr (Y), +Trp (W), +Leu (L), or +Ile (I).<sup>137</sup> Furthermore, additional proteases are secreted by the brush border of the small intestine, including aminopeptidases and dipeptidases,<sup>54</sup> potentially hydrolysing BAPs further. Based on this review, CS and CS-ALG NPs seem to give a prolonged release of BAPs under gastrointestinal conditions (see Table 2) no matter whether or not the BAPs encapsulated might be susceptible to any of the above enzymes. This biopolymer combination is therefore not particularly disrupted by human gastric and intestinal enzymes and of course inherently is not susceptible to protease action. One of core concept of encapsulation for food application is protecting bioactives during gastric digestion and release them on intestine at certain specific rate (fast/sustained/control) to maximize bioavailability.<sup>138</sup>

A second critical challenge is creating encapsulation vehicles in the appropriate size range. The pore size of the mucus layer covering the epithelial cells in the intestine is ~150–200 nm, so sizes smaller than this are required to enable vehicles to freely penetrate this layer. It seems challenging to create biopolymer-based vehicles that have diameters less than ~200 nm that possess at least two or three biopolymer layers surrounding the BAPs, which of course is partly a reflection of the dimensions of the biopolymer molecules themselves. On the other hand, partly synthetic Eudragit-CS NPs can have sizes less than 60 nm,<sup>85</sup> whereas purely biopolymer-based NPs seem to have the advantage of low toxicity. Thus, a balance may have to be reached between the use of synthetic *versus* natural biopolymers with regard to controlling and minimizing NP size and toxicity. NPs that are able to reach the intestine and penetrate through the epithelial cell membrane must undergo the nano-specific risk assessment stages as discussed earlier. Mucosa-adhesive carriers, such as CS, ALG, or their composite on the vehicle surfaces, not only augment the absorption and delivery of particles within the mucosa but also present attributes of biodegradability and minimal toxicity.<sup>103,139</sup>

There seems to have been a limited number of studies that have fully characterized BAP stability during gastrointestinal simulation fluids and the consequent effects on the bioavailability and bioactivity of any encapsulated BAPs, so that much more work is required in this area. In particular, attention needs to be paid to the internal structure of the vehicles and the location of the BAPs, plus the effects that this has on BAP bioactivity. In this respect, there are opportunities for further exploration of structures based on more than two biopolymer types, for instance, a third biopolymer (or other) type of coating layer. For example, possible ALG/CS/ALG or CS/ALG/CS combinations. Another potentially promising scenario is using a lipid to protect the BAPs inside the biopolymer layers.<sup>59</sup> This creates steric hindrance so that the peptides are prevented from diffusing into the outer aqueous biopolymer layers.



Most of previous studies used buffers during *in vitro* gastrointestinal simulation without added proteases nor other attributes (e.g., bile salts, lipases) to mimic human gastrointestinal digestion. Only a few studies have applied *in vivo* models. However, given the elevated costs of *in vivo* digestion, *in vitro* approaches are preferred. However, they are complicated to set up and recapture the physiological context. Numerous studies have documented the use of *in vitro* static and dynamic digestion models. For examples, Chen *et al.*<sup>140</sup> developed a simple model device to imitate dynamic physiological processes within gastrointestinal tract *in vitro*. They examined the influence of gastric juice ratio, pH, fluid flow, and pepsin concentration on peanut digestion. By this model, the parameters that influence gastric digestion like digestion time, enzyme

concentration, food to gastric juice ratio, shearing and hydrodynamic flow were controllable and adjustable easily. Koza *et al.*<sup>141</sup> developed the Gastric Digestion Simulator (GDS) for *in vitro* gastric digestion, focusing on replicating peristalsis effects. This device observes food disintegration and gastric content changes. Using two tofu types as food models, it unravelled stomach digestion mechanisms. Egger *et al.*<sup>130</sup> aimed to standardize *in vitro* simulation protocols, emphasizing enzyme presence. *In vitro* models, despite human studies being gold standards, offer advantages like speed, cost-efficiency, ethics, and scalability, especially for screening. They replicate oral, gastric, small intestinal phases, and sometimes large intestinal fermentation, controlling factors like enzymes, pH, duration, and salts.<sup>131</sup> Some computer models simulate digestion dyna-

**Table 3** Other important features on encapsulation of BAPs

BAPs payloads	Encapsulation materials	Remarks on essential characteristics	Ref.
AW	FSP microfibers	<i>Stability</i> : microfiber degraded slowly by low activity SGF until 24 h (compared to after 30 min with higher amount of pepsin); microfiber has low activity in SIF, only 50% (after 24 h) (compared 100% in the higher amount of pancreatin)	75
IPP	Chitosan nanoparticles	<i>Cytotoxicity</i> : IPP loaded CS NPs were tested on Caco-2 and HepG2 cell lines, and no significant decrease of cell viability was found compared to negative control analysed by MTS viability assay. <i>Bioactivity</i> : 84% (after encapsulation); 82% (controlled release)	77
LKP	Chitosan nanoparticles	<i>Cytotoxicity</i> : LKP loaded CS NPs were tested on Caco-2 and HepG2 cell lines, and no significant decrease of cell viability was found compared to negative control analysed by MTS viability assay. <i>Bioactivity</i> : 85% (after encapsulation); 83% (controlled release)	77
ECG	Chitosan/CD nanoparticles	<i>Absorption/Permeability</i> : CS/ $\alpha$ -CD: 1.42 times higher (on proximal segment) and 1.3 times higher (on distal segment). CS/SBE- $\beta$ -CD: 1.2 times higher (on proximal segment) and 1.8 higher (on distal segment). All of these compared to unencapsulated tripeptide ECG	78
KPV	Chitosan/ALG nanoparticles, and hyaluronic acid (HA)-chitosan/ALG nanoparticles	<i>Absorption/Permeability</i> : qualitative data shown that surface functionalization with HA can promote the cellular uptake efficiency of NPs by targeting cells. <i>Bioactivity</i> : HA-KPV-CS NPs can enhance the healing of the wounded colonic epithelial layer. <i>Cytotoxicity</i> : concentration 1 mg mL <sup>-1</sup> do not cause cellular death of Caco2-BBE and IEC6 cell line over 72–80 h. Cell treated with HA-KPV-NPs had significantly lower TNF- $\alpha$ mRNA expression levels compared with those treated with KPV-NPs	79 and 80
YKT	Chitosan nanoparticles	<i>Cytotoxicity</i> : viability (control). 100%. (1) 0.1–2.5 mg mL <sup>-1</sup> : 62.72–79% (after 24 h); (2) 0.1–1.0 mg mL <sup>-1</sup> : 34–38% (after 48 h).	81
LQPE	Liposome coated Lactobacillus S-layer protein (SLP)	<i>Absorption/Permeability</i> : all of the transshipment significantly increased. Compared L-LQPE, L-LQPE coated by SLP significantly increases the concentration of LQPE on the Basolateral side with initial concentration of 2 and 6 mmol L <sup>-1</sup> . <i>Stability</i> : LQPE Residue. Intestinal juice: 74.5% (SLP-L-LQPE), 55% (L-QPE). Gastric juice: 68% (SLP-L-LQPE), 50% (L-LQPE).	86
VLPVP	Carboxymethylated gum/sodium ALG microspheres	<i>Bioactivity</i> : 48.2% (before encapsulation); 45.2% (after encapsulation)	82
RLSFNP	Liposome	<i>Absorption/Permeability</i> : both RLSFNP and RKSFNP liposomes exhibited red fluorescence, indicating cellular uptake. The transport of the hexapeptide RLSFNP in liposome increased significantly in comparison to the peptide alone. <i>Cytotoxicity</i> : MTT Results indicated that the RLSFNP display no cytotoxicity in Caco-2 Cells	83
FCFWKTCT	Tetraether lipid liposomes	<i>Bioavailability</i> : >750 pg mL <sup>-1</sup> in plasma, after 100 $\mu$ g dosage	71
YLGYLEQLLR	Guar film-coated chitosan MPs	<i>Absorption/Permeability</i> : MPs loaded peptide in Caco-2/HT29-MTX: permeability >70% (120 min) and ~50% (60 min). Peptide alone <60% (120 min) and <30% (60 min). <i>Cytotoxicity</i> : cell viability 75–99% (MTT assay) (0–5 $\mu$ g mL <sup>-1</sup> )	84
GRKRRRQRRRP	Eudragit (ET)-coated chitosan nanoparticles	<i>Cytotoxicity</i> : membrane integrity of Caco-2 was not destroyed after treatment with ES-Tat-cNPs and ET-cNPs. There were not toxic toward Caco-2 cell using CCK8 assay (100% of cell viability after treatment)	85



mically,<sup>142</sup> but many remain static.<sup>143</sup> These approaches can investigate bioactive compounds (*e.g.*, BAPs) in particles or functional food models.

All in all, much further work is required in order to optimize encapsulation vehicles for BAPs that provide them with adequate protection during gastrointestinal digestion yet, provide adequate release characteristics that ensure the required activity of the BAPs (Table 3).

## Abbreviations

ACE	Angiotensin converting enzyme
AFM	Atomic force microscopy
ALG	Alginate
AP	Apical
BAPs	Bioactive peptides
BL	Basolateral
CCK-8	Cell counting kit-8
CD	Cyclodextrin
CS	Chitosan
DA	Degree of acetylation
DLS	Dynamic light scattering
DPP-IV	Dipeptidyl peptidase IV
DPPC	Dipalmitoyl phosphatidylcholine
EE	Encapsulation efficiency
EFSA	European Food Safety Authority
EPC	Egg phosphatidylcholine
ET	Eudragit
GDS	Gastric digestion simulator
GG	Guar gum
HA	Hyaluronic acid
HHL	Hippuryl-L-histidyl-L-leucine
HPA	Hippuric acid
MLVs	Multilamellar vesicles
MPs	Microparticles
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NPs	Nanoparticles
O-CMCS	O-carboxymethyl chitosan
o/w	Oil-in-water emulsion
PDI	Polydispersity index
PEGDA	Poly(ethylene glycol) diacrylate
PLA	Poly(lactic acid)
PLGA	Poly(lactide-co-glycolic acid)
PVA	Polyvinyl alcohol
SA	Sodium alginate
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
SBE- $\beta$ -CD	Sulphobutyl ether- $\beta$ -cyclodextrin
SEM	Scanning electron microscopy
SLP	Lactobacillus S-layer protein
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid

TEM	Transmission electron microscopy
TPP	Tripolyphosphate
w/o	Water-in-oil emulsion
WPI	Whey protein isolate
WST-1	Water-soluble tetrazolium

## Author contributions

YA: conceptualization, writing – original draft, visualization; BS: conceptualization, writing – review and editing, supervision; AS: writing – review and editing, supervision; FG: conceptualization, writing – review and editing, supervision.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

The authors are grateful for the funding provided by the Center for Higher Education Funding (BPPT), the Ministry of Education, Culture, Research and Technology of the Republic of Indonesia, Ref. Number 1145/PLPP.1/BPI.LG/III/2023 and Indonesian Endowment Fund for Education (LPDP) in the form of a full scholarship for the PhD study of Yoni Atma.

## References

- 1 L. Wang, N. Wang, W. Zhang, X. Cheng, Z. Yan, G. Shao, X. Wang, R. Wang and C. Fu, Therapeutic peptides: current applications and future directions, *Signal Transduction Targeted Ther.*, 2022, **7**, 48.
- 2 J. L. Lau and M. K. Dunn, Therapeutic peptides: historical perspectives, current development trends, and future directions, *Bioorg. Med. Chem.*, 2018, **26**, 2700–2707.
- 3 P. J. Patil, M. Usman, C. Zhang, A. Mehmood, M. Zhou, C. Teng and X. Li, An updated review on food-derived bioactive peptides: focus on the regulatory requirements, safety, and bioavailability, *Compr. Rev. Food Sci. Food Saf.*, 2022, **21**, 1732–1776.
- 4 L. Jia, L. Wang, C. Liu, Y. Liang and Q. Lin, Bioactive peptides from foods: production, function, and application, *Food Funct.*, 2021, **12**, 7108–7125.
- 5 R. Han, J. Maycock, B. S. Murray and C. Boesch, Identification of angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory peptides derived from oilseed proteins using two integrated bioinformatic approaches, *Food Res. Int.*, 2019, **115**, 283–291.
- 6 M. d. M. Contreras, R. Carrón, M. J. Montero, M. Ramos and I. Recio, Novel casein-derived peptides with antihypertensive activity, *Int. Dairy J.*, 2009, **19**, 566–573.



- 7 I. M. Lacroix and E. C. Li-Chan, Isolation and characterization of peptides with dipeptidyl peptidase-IV inhibitory activity from pepsin-treated bovine whey proteins, *Peptides*, 2014, **54**, 39–48.
- 8 I. M. E. Lacroix, G. Meng, I. W. Y. Cheung and E. C. Y. Li-Chan, Do whey protein-derived peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities?, *J. Funct. Foods*, 2016, **21**, 87–96.
- 9 X.-Y. Mao, J.-R. Ni, W.-L. Sun, P.-P. Hao and L. Fan, Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides, *Food Chem.*, 2007, **103**, 1282–1287.
- 10 A. B. Nongonierma, C. Cadamuro, A. Le Gouic, P. Mudgil, S. Maqsood and R. J. FitzGerald, Dipeptidyl peptidase IV (DPP-IV) inhibitory properties of a camel whey protein enriched hydrolysate preparation, *Food Chem.*, 2019, **279**, 70–79.
- 11 A. B. Nongonierma and R. J. FitzGerald, Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis, *Food Chem.*, 2014, **145**, 845–852.
- 12 V. P. Shanmugam, S. Kapila, T. S. Kemgang, S. Reddi, R. Kapila, S. Muthukumar and D. Rajesh, Isolation and characterization of angiotensin converting enzyme inhibitory peptide from buffalo casein, *Int. J. Pept. Res. Ther.*, 2021, **27**, 1481–1491.
- 13 S. T. Silveira, D. Martinez-Maqueda, I. Recio and B. Hernandez-Ledesma, Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in beta-lactoglobulin, *Food Chem.*, 2013, **141**, 1072–1077.
- 14 H. Uenishi, T. Kabuki, Y. Seto, A. Serizawa and H. Nakajima, Isolation and identification of casein-derived dipeptidyl-peptidase 4 (DPP-4)-inhibitory peptide LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats, *Int. Dairy J.*, 2012, **22**, 24–30.
- 15 A. Yamada, T. Sakurai, D. Ochi, E. Mitsuyama, K. Yamauchi and F. Abe, Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro, *Food Chem.*, 2015, **172**, 441–446.
- 16 J. Yan, J. Zhao, R. Yang and W. Zhao, Bioactive peptides with antidiabetic properties: a review, *Int. J. Food Sci. Technol.*, 2019, **54**, 1909–1919.
- 17 H. G. Byun and S. K. Kim, Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin, *Process Biochem.*, 2001, **36**, 1155–1162.
- 18 H. Fujita, K. Yokoyama and M. Yoshikawa, Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins, *J. Food Sci.*, 2000, **65**, 564–569.
- 19 R. Balti, A. Bougateg, A. Sila, D. Guillochon, P. Dhulster and N. Nedjar-Arroume, Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats, *Food Chem.*, 2015, **170**, 519–525.
- 20 R. Balti, N. Nedjar-Arroume, A. Bougateg, D. Guillochon and M. Nasri, Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases, *Food Res. Int.*, 2010, **43**, 1136–1143.
- 21 A. Fahmi, S. Morimura, H. C. Guo, T. Shigematsu, K. Kida and Y. Uemura, Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales, *Process Biochem.*, 2004, **39**, 1195–1200.
- 22 M. Ghassem, K. Arihara and A. S. Babji, Isolation, purification and characterisation of angiotensin I-converting enzyme-inhibitory peptides derived from catfish (*Clarias batrachus*) muscle protein thermolysin hydrolysates, *Int. J. Food Sci. Technol.*, 2012, **47**, 2444–2451.
- 23 M. Ghassem, K. Arihara, A. S. Babji, M. Said and S. Ibrahim, Purification and identification of ACE inhibitory peptides from Haruan (*Channa striatus*) myofibrillar protein hydrolysate using HPLC–ESI-TOF MS/MS, *Food Chem.*, 2011, **129**, 1770–1777.
- 24 P. A. Harnedy-Rothwell, C. M. McLaughlin, M. B. O'Keeffe, A. V. Le Gouic, P. J. Allsopp, E. M. McSorley, S. Sharkey, J. Whooley, B. McGovern, F. P. M. O'Harte and R. J. FitzGerald, Identification and characterisation of peptides from a boarfish (*Capros aper*) protein hydrolysate displaying in vitro dipeptidyl peptidase-IV (DPP-IV) inhibitory and insulinotropic activity, *Food Res. Int.*, 2020, **131**, 108989.
- 25 R. Intarasirisawat, S. Benjakul, J. Wu and W. Visessanguan, Isolation of antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack (*Katsuwana pelamis*) roe, *J. Funct. Foods*, 2013, **5**, 1854–1862.
- 26 J. K. Lee, J.-K. Jeon and H.-G. Byun, Effect of angiotensin I converting enzyme inhibitory peptide purified from skate skin hydrolysate, *Food Chem.*, 2011, **125**, 495–499.
- 27 E. C. Y. Li-Chan, S. Hunag, C. L. Jao, K. P. Ho and K. C. Hsu, Peptides derived from atlantic salmon skin gelatin as dipeptidyl-peptidase IV inhibitors, *J. Agric. Food Chem.*, 2012, **60**, 973–978.
- 28 Y. H. Lin, C. A. Chen, J. S. Tsai and G. W. Chen, Preparation and identification of novel antihypertensive peptides from the in vitro gastrointestinal digestion of marine cobia skin hydrolysates, *Nutrients*, 2019, **11**, 1351.
- 29 X. Liu, M. Zhang, Y. Shi, R. Qiao, W. Tang and Z. Sun, Production of the angiotensin I converting enzyme inhibitory peptides and isolation of four novel peptides from jellyfish (*Rhopilema esculentum*) protein hydrolysate, *J. Sci. Food Agric.*, 2016, **96**, 3240–3248.
- 30 D.-H. Ngo, T.-S. Vo, B. Ryu and S.-K. Kim, Angiotensin- I-converting enzyme (ACE) inhibitory peptides from Pacific cod skin gelatin using ultrafiltration membranes, *Process Biochem.*, 2016, **51**, 1622–1628.
- 31 P. B. So, P. Rubio, S. Lirio, A. P. Macabeo, H. Y. Huang, M. J. Corpuz and O. B. Villaflores, In vitro angiotensin I converting enzyme inhibition by a peptide isolated from *Chiropsalmus quadrigatus* Haeckel (box jellyfish) venom hydrolysate, *Toxicon*, 2016, **119**, 77–83.





- 32 H. Wu, H.-L. He, X.-L. Chen, C.-Y. Sun, Y.-Z. Zhang and B.-C. Zhou, Purification and identification of novel angiotensin-I-converting enzyme inhibitory peptides from shark meat hydrolysate, *Process Biochem.*, 2008, **43**, 457–461.
- 33 S. Cao, Y. Wang, Y. Hao, W. Zhang and G. Zhou, Antihypertensive effects in vitro and in vivo of novel angiotensin-converting enzyme inhibitory peptides from bovine bone gelatin hydrolysate, *J. Agric. Food Chem.*, 2020, **68**, 759–768.
- 34 M. Miguel, M. A. Aleixandre, M. Ramos and R. Lopez-Fandino, Effect of simulated gastrointestinal digestion on the antihypertensive properties of ACE-inhibitory peptides derived from ovalbumin, *J. Agric. Food Chem.*, 2006, **54**, 726–731.
- 35 M. Miguel, A. Davalos, M. A. Manso, G. de la Pena, M. A. Lasuncion and R. Lopez-Fandino, Transepithelial transport across Caco-2 cell monolayers of antihypertensive egg-derived peptides. PepT1-mediated flux of Tyr-Pro-Ile, *Mol. Nutr. Food Res.*, 2008, **52**, 1507–1513.
- 36 P. Sangsawad, K. Choowongkamon, D. D. Kitts, X.-M. Chen, E. C. Y. Li-Chan and J. Yongsawatdigul, Transepithelial transport and structural changes of chicken angiotensin I-converting enzyme (ACE) inhibitory peptides through Caco-2 cell monolayers, *J. Funct. Foods*, 2018, **45**, 401–408.
- 37 P. Sangsawad, S. Roytrakul, K. Choowongkamon, D. D. Kitts, X. M. Chen, G. Meng, E. C. Y. Li-Chan and J. Yongsawatdigul, Transepithelial transport across Caco-2 cell monolayers of angiotensin converting enzyme (ACE) inhibitory peptides derived from simulated in vitro gastrointestinal digestion of cooked chicken muscles, *Food Chem.*, 2018, **251**, 77–85.
- 38 P. Sangsawad, S. Roytrakul and J. Yongsawatdigul, Angiotensin converting enzyme (ACE) inhibitory peptides derived from the simulated in vitro gastrointestinal digestion of cooked chicken breast, *J. Funct. Foods*, 2017, **29**, 77–83.
- 39 Y. Yang, E. D. Marczak, M. Yokoo, H. Usui and M. Yoshikawa, Isolation and antihypertensive effect of angiotensin I-converting Enzyme (ACE) inhibitory peptides from spinach rubisco, *J. Agric. Food Chem.*, 2003, **51**, 4897–4902.
- 40 J. Chen, S. Liu, R. Ye, G. Cai, B. Ji and Y. Wu, Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: purification and characterization, *J. Funct. Foods*, 2013, **5**, 1684–1692.
- 41 Y. Gu and J. Wu, LC-MS/MS coupled with QSAR modeling in characterising of angiotensin I-converting enzyme inhibitory peptides from soybean proteins, *Food Chem.*, 2013, **141**, 2682–2690.
- 42 E. D. Marczak, H. Usui, H. Fujita, Y. Yang, M. Yokoo, A. W. Lipkowski and M. Yoshikawa, New antihypertensive peptides isolated from rapeseed, *Peptides*, 2003, **24**, 791–798.
- 43 V. S. Vallabha and P. K. Tikku, Antihypertensive peptides derived from soy protein by fermentation, *Int. J. Pept. Res. Ther.*, 2013, **20**, 161–168.
- 44 F.-J. Wang, X.-Y. Yin, J. M. Regenstein and J.-Z. Wang, Separation and purification of angiotensin-I-converting enzyme (ACE) inhibitory peptides from walnuts (*Juglans regia* L.) meal, *Eur. Food Res. Technol.*, 2015, **242**, 911–918.
- 45 J. Wu, R. E. Aluko and A. D. Muir, Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal, *Food Chem.*, 2008, **111**, 942–950.
- 46 R. Yang, Y. Zou, N. Yu and Z. Gu, Accumulation and identification of angiotensin-converting enzyme inhibitory peptides from wheat germ, *J. Agric. Food Chem.*, 2011, **59**, 3598–3605.
- 47 Y. H. Lin, G. W. Chen, C. H. Yeh, H. Song and J. S. Tsai, Purification and identification of angiotensin I-converting enzyme inhibitory peptides and the antihypertensive effect of *Chlorella sorokiniana*, protein hydrolysates, *Nutrients*, 2018, **10**, 1397.
- 48 J. Lu, Y. Sawano, T. Miyakawa, Y. L. Xue, M. Y. Cai, Y. Egashira, D. F. Ren and M. Tanokura, One-week antihypertensive effect of Ile-Gln-Pro in spontaneously hypertensive rats, *J. Agric. Food Chem.*, 2011, **59**, 559–563.
- 49 C. M. Montone, A. L. Capriotti, C. Cavaliere, G. La Barbera, S. Piovesana, R. Zenezini Chiozzi and A. Lagana, Peptidomic strategy for purification and identification of potential ACE-inhibitory and antioxidant peptides in *Tetrademus obliquus* microalgae, *Anal. Bioanal. Chem.*, 2018, **410**, 3573–3586.
- 50 J. Xie, X. Chen, J. Wu, Y. Zhang, Y. Zhou, L. Zhang, Y. J. Tang and D. Wei, Antihypertensive effects, molecular docking study, and isothermal titration calorimetry assay of angiotensin I-converting enzyme inhibitory peptides from *Chlorella vulgaris*, *J. Agric. Food Chem.*, 2018, **66**, 1359–1368.
- 51 X. Geng, G. Tian, W. Zhang, Y. Zhao, L. Zhao, H. Wang and T. B. Ng, A *Tricholoma matsutake* peptide with angiotensin converting enzyme inhibitory and antioxidative activities and antihypertensive effects in spontaneously hypertensive rats, *Sci. Rep.*, 2016, **6**, 24130.
- 52 K. Skrzypczak, W. Gustaw, D. Szwajgier, E. Fornal and A. Wasko, kappa-Casein as a source of short-chain bioactive peptides generated by *Lactobacillus helveticus*, *J. Food Sci. Technol.*, 2017, **54**, 3679–3688.
- 53 A. Cerrato, S. E. Aita, A. L. Capriotti, C. Cavaliere, A. M. I. Montone, C. M. Montone and A. Lagana, Investigating the short peptidome profile of italian dry-cured ham at different processing times by high-resolution mass spectrometry and chemometrics, *Int. J. Mol. Sci.*, 2022, **23**, 3193.
- 54 Y. D. Bhutia and V. Ganapathy, in *Physiology of the Gastrointestinal Tract*, ed. H. M. Said, Academic Press, United Kingdom, 2018, vol. 6, ch. 47, pp. 1063–1086.
- 55 W. Shen and T. Matsui, Intestinal absorption of small peptides: a review, *Int. J. Food Sci. Technol.*, 2019, **54**, 1942–1948.
- 56 A. Mirzapour-Kouhdasht, D. J. McClements, M. S. Taghizadeh, A. Niazi and M. Garcia-Vaquero,



- Strategies for oral delivery of bioactive peptides with focus on debittering and masking, *NPJ Sci. Food*, 2023, 7, 22.
- 57 C. Berraquero-García, R. Perez-Galvez, F. J. Espejo-Carpio, A. Guadix, E. M. Guadix and P. J. Garcia-Moreno, Encapsulation of bioactive peptides by spray-drying and electrospraying, *Foods*, 2023, 12, 2005.
- 58 J. E. Aguilar-Toala, D. Quintanar-Guerrero, A. M. Liceaga and M. L. Zambrano-Zaragoza, Encapsulation of bioactive peptides: a strategy to improve the stability, protect the nutraceutical bioactivity and support their food applications, *RSC Adv.*, 2022, 12, 6449–6458.
- 59 D. J. McClements, Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and microparticle systems: a review, *Adv. Colloid Interface*, 2018, 253, 1–22.
- 60 G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan, Kinetics of immobilisation and release of tryptophan, riboflavin and peptides from whey protein microbeads, *Food Chem.*, 2015, 180, 150–155.
- 61 I. M. E. Lacroix, X. M. Chen, D. D. Kitts and E. C. Y. Li-Chan, Investigation into the bioavailability of milk protein-derived peptides with dipeptidyl-peptidase IV inhibitory activity using Caco-2 cell monolayers, *Food Funct.*, 2017, 8, 701–709.
- 62 L. Basirico, E. Catalani, P. Morera, S. Cattaneo, M. Stuknyte, U. Bernabucci, I. De Noni and A. Nardone, Release of angiotensin converting enzyme-inhibitor peptides during in vitro gastrointestinal digestion of Parmigiano Reggiano PDO cheese and their absorption through an in vitro model of intestinal epithelium, *J. Dairy Sci.*, 2015, 98, 7595–7601.
- 63 R. Fernández-Musoles, J. B. Salom, M. Castelló-Ruiz, M. D. M. Contreras, I. Recio and P. Manzanares, Bioavailability of antihypertensive lactoferricin B-derived peptides: transepithelial transport and resistance to intestinal and plasma peptidases, *Int. Dairy J.*, 2013, 32, 169–174.
- 64 Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki and T. Takano, Purification and characterization of angiotensin i-converting enzyme inhibitors from sour milk, *J. Dairy Sci.*, 1995, 78, 777–783.
- 65 D. Pan and Y. Guo, Optimization of sour milk fermentation for the production of ACE-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate, *Int. Dairy J.*, 2010, 20, 472–479.
- 66 H. Fujita and M. Yoshikawa, LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein, *Immunopharmacology*, 1999, 44, 123–127.
- 67 D. Liu, H. Sun, L. Zhang, S. Li and Z. Qin, High-Level expression of milk-derived antihypertensive peptide in *Escherichia coli* and its bioactivity, *J. Agric. Food Chem.*, 2007, 55, 5109–5112.
- 68 X. X. Jiang, D. D. Pan, M. X. Tao, T. Zhang, X. Q. Zeng, Z. Wu and Y. X. Guo, New nanocarrier system for liposomes coated with *Lactobacillus acidophilus* S-layer protein to improve Leu-Gln-Pro-Glu absorption through the intestinal epithelium, *J. Agric. Food Chem.*, 2021, 69, 7593–7602.
- 69 L. Miclo, E. Perrin, A. Driou, V. Papadopoulos, N. Boujrad, R. Vanderesse, J. F. Boudier, D. Desor, G. Linden and J. L. Gaillard, Characterization of alpha-casozepine, a tryptic peptide from bovine alpha(s1)-casein with benzodiazepine-like activity, *FASEB J.*, 2001, 15, 1780–1782.
- 70 Y. Li, G. Wei and J. Chen, Glutathione: a review on biotechnological production, *Appl. Microbiol. Biotechnol.*, 2004, 66, 233–242.
- 71 J. Parmentier, B. Thewes, F. Gropp and G. Fricker, Oral peptide delivery by tetraether lipid liposomes, *Int. J. Pharm.*, 2011, 415, 150–157.
- 72 X. H. Zhou and A. L. W. Po, Peptide and protein drugs: II. non-parenteral routes of delivery, *Int. J. Pharm.*, 1991, 75, 117–130.
- 73 A. Fjellestad-Paulsen, P. Hoglund, S. Lundin and O. Paulsen, Pharmacokinetics of 1-deamino-8-D-arginine vasopressin after various routes of administration in healthy volunteers, *Clin. Endocrinol.*, 1993, 38, 177–182.
- 74 S. Martins, B. Sarmiento, D. C. Ferreira and E. B. Souto, Lipid-based colloidal carriers for peptide and protein delivery – liposomes versus lipid nanoparticles, *Int. J. Nanomed.*, 2007, 2, 595–607.
- 75 K. Stephansen, I. S. Chronakis and F. Jessen, Bioactive electrospun fish sarcoplasmic proteins as a drug delivery system, *Colloids Surf., B*, 2014, 122, 158–165.
- 76 G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan, Whey microbeads as a matrix for the encapsulation and immobilisation of riboflavin and peptides, *Food Chem.*, 2015, 160, 45–52.
- 77 M. K. Danish, G. Voza, H. J. Byrne, J. M. Frias and S. M. Ryan, Comparative study of the structural and physicochemical properties of two food derived antihypertensive tri-peptides, Isoleucine-Proline-Proline and Leucine-Lysine-Proline encapsulated into a chitosan based nanoparticle system, *Innovative Food Sci. Emerging*, 2017, 44, 139–148.
- 78 A. Trapani, A. Lopodota, M. Franco, N. Cioffi, E. Ieva, M. Garcia-Fuentes and M. J. Alonso, A comparative study of chitosan and chitosan/cyclodextrin nanoparticles as potential carriers for the oral delivery of small peptides, *Eur. J. Pharm. Biopharm.*, 2010, 75, 26–32.
- 79 H. Laroui, G. Dalmasso, H. T. Nguyen, Y. Yan, S. V. Sitaraman and D. Merlin, Drug-loaded nanoparticles targeted to the colon with polysaccharide hydrogel reduce colitis in a mouse model, *Gastroenterology*, 2010, 138, 843–853.
- 80 B. Xiao, Z. Xu, E. Viennois, Y. Zhang, Z. Zhang, M. Zhang, M. K. Han, Y. Kang and D. Merlin, Orally targeted delivery of tripeptide KPV via hyaluronic acid-functionalized nanoparticles efficiently alleviates ulcerative colitis, *Mol. Ther.*, 2017, 25, 1628–1640.
- 81 B. Bicak, Y. Budama-Kilinc, S. Kecel-Gunduz, T. Zorlud and G. Akman, Peptide based nano-drug candidate for cancer treatment: preparation, characterization, in vitro



- and in silico evaluation, *J. Mol. Struct.*, 2021, **1240**, 130573.
- 82 G. Q. Huang, J. X. Xiao, L. Q. Hao and J. Yang, Microencapsulation of an angiotensin i-converting enzyme inhibitory peptide VLPVP by membrane emulsification, *Food Bioprocess Technol.*, 2017, **10**, 2005–2012.
- 83 T. Zhang, M. Su, X. X. Jiang, Y. Q. Xue, J. X. Zhang, X. Q. Zeng, Z. Wu, Y. X. Guo and D. D. Pan, Transepithelial transport route and liposome encapsulation of milk-derived ACE-inhibitory peptide Arg-Leu-Ser-Phe-Asn-Pro, *J. Agric. Food Chem.*, 2019, **67**, 5544–5551.
- 84 P. Batista, P. M. Castro, A. R. Madureira, B. Sarmiento and M. Pintado, Preparation, characterization and evaluation of guar films impregnated with relaxing peptide loaded into chitosan microparticles, *Appl. Sci.*, 2021, **11**, 9849.
- 85 S. X. Chen, F. Guo, T. T. Deng, S. Q. Zhu, W. Y. Liu, H. J. Zhong, H. Yu, R. Luo and Z. Y. Deng, Eudragit S100-coated chitosan nanoparticles co-loading Tat for enhanced oral colon absorption of insulin, *AAPS PharmSciTech*, 2017, **18**, 1277–1287.
- 86 X. Jiang, D. Pan, T. Zhang, C. Liu, J. Zhang, M. Su, Z. Wu, X. Zeng, Y. Sun and Y. Guo, Novel milk casein-derived peptides decrease cholesterol micellar solubility and cholesterol intestinal absorption in Caco-2 cells, *J. Dairy Sci.*, 2020, **103**, 3924–3936.
- 87 G. Dalmaso, L. Charrier-Hisamuddin, H. T. Nguyen, Y. Yan, S. Sitaraman and D. Merlin, PepT1-mediated tripeptide KPV uptake reduces intestinal inflammation, *Gastroenterology*, 2008, **134**, 166–178.
- 88 K. Kannengiesser, C. Maaser, J. Heidemann, A. Luegering, M. Ross, T. Brzoska, M. Bohm, T. A. Luger, W. Domschke and T. Kucharzik, Melanocortin-derived tripeptide KPV has anti-inflammatory potential in murine models of inflammatory bowel disease, *Inflammatory Bowel Dis.*, 2008, **14**, 324–331.
- 89 N. Kommineni, V. G. S. Sainaga Jyothi, A. Butreddy, S. Raju, T. Shapira, W. Khan, P. Angsantikul and A. J. Domb, SNAC for enhanced oral bioavailability: an updated review, *Pharm. Res.*, 2023, **40**, 633–650.
- 90 C. A. Lipinski, F. Lombardo, B. W. Dominy and P. J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Adv. Drug Delivery Rev.*, 2001, **46**, 3–26.
- 91 F. J. Caro-Leon, W. Arguelles-Monal, E. Carvajal-Millan, Y. L. Lopez-Franco, F. M. Goycoolea-Valencia, J. San Roman Del Barrio and J. Lizardi-Mendoza, Production and characterization of supercritical CO(2) dried chitosan nanoparticles as novel carrier device, *Carbohydr. Polym.*, 2018, **198**, 556–562.
- 92 A. G. Luque-Alcaraz, J. Lizardi-Mendoza, F. M. Goycoolea, I. Higuera-Ciapara and W. Argüelles-Monal, Preparation of chitosan nanoparticles by nanoprecipitation and their ability as a drug nanocarrier, *RSC Adv.*, 2016, **6**, 59250–59256.
- 93 G. Gao, H. Wang, J. Zhou, P. Rao, L. Ke, J. J. Lin, B. Sun Pan, Y. Zhang and Q. Wang, Isolation and characterization of bioactive proteoglycan-lipid nanoparticles from freshwater clam (*Corbicula fluminea* Muller) soup, *J. Agric. Food Chem.*, 2021, **69**, 1610–1618.
- 94 A. Zielinska, F. Carreiro, A. M. Oliveira, A. Neves, B. Pires, D. N. Venkatesh, A. Durazzo, M. Lucarini, P. Eder, A. M. Silva, A. Santini and E. B. Souto, Polymeric nanoparticles: production, characterization, toxicology and ecotoxicology, *Molecules*, 2020, **25**, 3731.
- 95 S. More, V. Bampidis, D. Benford, C. Bragard, T. Halldorsson, A. Hernandez-Jerez, S. H. Bennekou, K. Koutsoumanis, C. Lambre, K. Machera, H. Naegeli, S. Nielsen, J. Schlatter, D. Schrenk, V. Silano, D. Turck, M. Younes, J. Castenmiller, Q. Chaudhry, F. Cubadda, R. Franz, D. Gott, J. Mast, A. Mortensen, A. G. Oomen, S. Weigel, E. Barthelemy, A. Rincon, J. Tarazona and R. Schoonjans, Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles, *EFSA J.*, 2021, **19**, 6769.
- 96 EFSA, Outcome of the public consultation on the draft guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health, *EFSA Supporting Publ.*, 2018, **15**, 1430E, DOI: [10.2903/sp.efsa.2018.EN-1430](https://doi.org/10.2903/sp.efsa.2018.EN-1430).
- 97 R. Schoonjans, J. Castenmiller, Q. Chaudhry, F. Cubadda, T. Daskaleros, R. Franz, D. Gott, J. Mast, A. Mortensen, A. G. Oomen, H. Rauscher, S. Weigel, M. C. Astuto, I. Cattaneo, E. Barthelemy, A. Rincon and J. Tarazona, Regulatory safety assessment of nanoparticles for the food chain in Europe, *Trends Food Sci. Technol.*, 2023, **134**, 98–111.
- 98 M. Xavier, I. A. Parente, P. M. Rodrigues, M. A. Cerqueira, L. Pastrana and C. Gonçalves, Safety and fate of nanomaterials in food: The role of in vitro tests, *Trends Food Sci. Technol.*, 2021, **109**, 593–607.
- 99 R. K. Thapa and J. O. Kim, Nanomedicine-based commercial formulations: current developments and future prospects, *J. Pharm. Invest.*, 2023, **53**, 19–33.
- 100 P. Uhl, G. Bajraktari-Sylejmani, D. Witzigmann, C. Bay, S. Zimmermann, J. Burhenne, J. Weiss, W. E. Haefeli and M. Sauter, A nanocarrier approach for oral peptide delivery: evaluation of cell-penetrating-peptide-modified liposomal formulations in dogs, *Adv. Ther.*, 2023, **2300021**, 1–12.
- 101 H. Ichikawa, in *Nanoparticle Technology Handbook*, ed. M. Hosokawa, K. Nogi, M. Naito and T. Yokoyama, Elsevier Science, 2008, pp. 442–450.
- 102 S. Pistone, F. M. Goycoolea, A. Young, G. Smistad and M. Hiorth, Formulation of polysaccharide-based nanoparticles for local administration into the oral cavity, *Eur. J. Pharm. Sci.*, 2017, **96**, 381–389.
- 103 F. M. Goycoolea-Valencia, G. Lollo, C. Remunan-Lopez, F. Quaglia and M. J. Alonso, Chitosan-Alginate Blended Nanoparticles as Carriers for the Transmucosal Delivery of Macromolecules, *Biomacromolecules*, 2009, **10**, 1736–1743.



- 104 M. Iqbal, N. Zafar, H. Fessi and A. Elaissari, Double emulsion solvent evaporation techniques used for drug encapsulation, *Int. J. Pharm.*, 2015, **496**, 173–190.
- 105 O. Torres, B. S. Murray and A. Sarkar, Overcoming in vitro gastric destabilisation of emulsion droplets using emulsion microgel particles for targeted intestinal release of fatty acids, *Food Hydrocolloids*, 2019, **89**, 523–533.
- 106 H. Park, D. H. Ha, E. S. Ha, J. S. Kim, M. S. Kim and S. J. Hwang, Effect of stabilizers on encapsulation efficiency and release behavior of exenatide-loaded PLGA microsphere prepared by the W/O/W solvent evaporation method, *Pharmaceutics*, 2019, **11**, 627.
- 107 T. D. Brown, K. A. Whitehead and S. Mitragotri, Materials for oral delivery of proteins and peptides, *Nat. Rev. Mater.*, 2019, **5**, 127–148.
- 108 J. P. Fuenzalida, M. E. Flores, I. Moniz, M. Feijoo, F. Goycoolea, H. Nishide and I. Moreno-Villoslada, Immobilization of hydrophilic low molecular-weight molecules in nanoparticles of chitosan/poly(sodium 4-styrenesulfonate) assisted by aromatic-aromatic interactions, *J. Phys. Chem. B*, 2014, **118**, 9782–9791.
- 109 G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan, Whey microbeads as a matrix for the encapsulation and immobilisation of riboflavin and peptides, *Food Chem.*, 2015, **160**, 45–52.
- 110 K. Kettler, K. Veltman, D. van de Meent, A. van Wezel and A. J. Hendriks, Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type, *Environ. Toxicol. Chem.*, 2014, **33**, 481–492.
- 111 C. Yang, J. Uertz, D. Yohan and B. D. Chithrani, Peptide modified gold nanoparticles for improved cellular uptake, nuclear transport, and intracellular retention, *Nanoscale*, 2014, **6**, 12026–12033.
- 112 P. Foroozandeh and A. A. Aziz, Insight into cellular uptake and intracellular trafficking of nanoparticles, *Nanoscale Res. Lett.*, 2018, **13**, 339.
- 113 Y. Yun, Y. W. Cho and K. Park, Nanoparticles for oral delivery: targeted nanoparticles with peptidic ligands for oral protein delivery, *Adv. Drug Delivery Rev.*, 2013, **65**, 822–832.
- 114 J. P. Pearson, P. I. Chater and M. D. Wilcox, The properties of the mucus barrier, a unique gel—how can nanoparticles cross it?, *Ther. Delivery*, 2016, **7**, 229–244.
- 115 S. Salatin, S. M. Dizaj and A. Y. Khosroushahi, Effect of the surface modification, size, and shape on cellular uptake of nanoparticles, *Cell Biol. Int.*, 2015, **39**, 881–890.
- 116 S. Sreekumar, F. M. Goycoolea, B. M. Moerschbacher and G. R. Rivera-Rodriguez, Parameters influencing the size of chitosan-TPP nano- and microparticles, *Sci. Rep.*, 2018, **8**, 4695.
- 117 L. Pravinata, M. Akhtar, P. J. Bentley, T. Mahatnirunkul and B. S. Murray, Preparation of alginate microgels in a simple one step process via the Leeds Jet Homogenizer, *Food Hydrocolloids*, 2016, **61**, 77–84.
- 118 E. Seyrek, J. Hierrezuelo, A. Sadeghpour, I. Szilagyi and M. Borkovec, Molecular mass dependence of adsorbed amount and hydrodynamic thickness of polyelectrolyte layers, *Phys. Chem. Chem. Phys.*, 2011, **13**, 12716–12719.
- 119 A. O'Connell, F. M. Goycoolea, A. Gulotta, P. Holmqvist, P. Schuetz and J. Mattsson, The structure and dynamics of locust bean gum in aqueous solution, *Food Hydrocolloids*, 2023, **138**, 108446.
- 120 N. I. Anaraki, A. Sadeghpour, K. Iranshahi, C. Toncelli, U. Cendrowska, F. Stellacci, A. Dommann, P. Wick and A. Neels, New approach for time-resolved and dynamic investigations on nanoparticles agglomeration, *Nano Res.*, 2020, **13**, 2847–2856.
- 121 I. M. Adjei, B. Sharma and V. Labhassetwar, Nanoparticles: cellular uptake and cytotoxicity, *Adv. Exp. Med. Biol.*, 2014, **811**, 73–91.
- 122 M. C. Okeudo-Cogan, B. S. Murray, R. Ettelaie, S. D. Connell, S. J. Radford, S. Micklethwaite and A. Sarkar, Understanding the microstructure of a functional meat analogue: Demystifying interactions between fungal hyphae and egg white protein, *Food Hydrocolloids*, 2023, **140**, 108606.
- 123 A. R. Mackie, F. M. Goycoolea, B. Menchicchi, C. M. Caramella, F. Saporito, S. Lee, K. Stephansen, I. S. Chronakis, M. Hiorth, M. Adamczak, M. Waldner, H. M. Nielsen and L. Marcelloni, Innovative methods and applications in mucoadhesion research, *Macromol. Biosci.*, 2017, **17**, 1600534.
- 124 J. Chu, P. Metcalfe, H. V. Linford, S. Zhao, F. M. Goycoolea, S. Chen, X. Ye, M. Holmes and C. Orfila, Short-time acoustic and hydrodynamic cavitation improves dispersibility and functionality of pectin-rich biopolymers from citrus waste, *J. Cleaner Prod.*, 2022, **330**, 129789.
- 125 D. Sanver, A. Sadeghpour, M. Rappolt, F. Di Meo and P. Trouillas, Structure and dynamics of dioleoyl-phosphatidylcholine bilayers under the influence of quercetin and rutin, *Langmuir*, 2020, **36**, 11776–11786.
- 126 T. Zhao, L. Zheng, Q. Zhang, S. Wang, Q. Zhao, G. Su and M. Zhao, Stability towards the gastrointestinal simulated digestion and bioactivity of PAYCS and its digestive product PAY with cognitive improving properties, *Food Funct.*, 2019, **10**, 2439–2449.
- 127 W. Liao, H. Fan, P. Liu and J. Wu, Identification of angiotensin converting enzyme 2 (ACE2) up-regulating peptides from pea protein hydrolysate, *J. Funct. Foods*, 2019, **60**, 103395.
- 128 M. Mirzaei, S. Mirdamadi, M. Safavi and N. Soleymanzadeh, The stability of antioxidant and ACE-inhibitory peptides as influenced by peptide sequences, *LWT - Food*, 2020, **130**, 109710.
- 129 J. Shaji and V. Patole, Protein and peptide drug delivery: oral approaches, *Indian J. Pharm. Sci.*, 2008, **70**, 269–277.
- 130 L. Egger, O. Ménard, C. Delgado-Andrade, P. Alvito, R. Assunção, S. Balance, R. Barberá, A. Brodkorb, T. Cattenoz, A. Clemente, I. Comi, D. Dupont, G. Garcia-Llatas, M. J. Lagarda, S. Le Feunteun, L. JanssenDuijghuijsen, S. Karakaya, U. Lesmes, A. R. Mackie, C. Martins, A. Meynier, B. Miralles, B. S. Murray, A. Pihlanto, G. Picariello,



- C. N. Santos, S. Simsek, I. Recio, N. Rigby, L.-E. Rioux, H. Stoffers, A. Tavares, L. Tavares, S. Turgeon, E. K. Ulleberg, G. E. Vegarud, G. Vergères and R. Portmann, The harmonized INFOGEST in vitro digestion method: From knowledge to action, *Food Res. Int.*, 2016, **88**, 217–225.
- 131 M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Menard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. Wickham, W. Weitschies and A. Brodtkorb, A standardised static in vitro digestion method suitable for food - an international consensus, *Food Funct.*, 2014, **5**, 1113–1124.
- 132 A. E. Ross, M. Y. Tang and R. A. Gemeinhart, Effects of molecular weight and loading on matrix metalloproteinase-2 mediated release from poly(ethylene glycol) diacrylate hydrogels, *AAPS J.*, 2012, **14**, 482–490.
- 133 D. W. Cushman and H. S. Cheung, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung, *Biochem. Pharmacol.*, 1971, **20**, 1637–1648.
- 134 E. Schnaith, Determination of the pepsin activity in human gastric juice, using defined oligopeptides as substrates, *Clin. Biochem.*, 1989, **22**, 91–98.
- 135 R. J. Simpson, Fragmentation of Protein Using Trypsin, *Cold Spring Harb. Protoc.*, 2006, **2006**(5), DOI: [10.1101/pdb.prot4550](https://doi.org/10.1101/pdb.prot4550).
- 136 L. Graff, L. Szilagyí and I. Venekei, in *Handbook of Proteolytic Enzymes*, ed. Barrett A., N. Rawlings and J. Woessner, Elsevier Ltd, Oxford, UK, 3rd edn, 2013.
- 137 D. S. Auld, in *Handbook of Proteolytic Enzymes*, ed. A. Barrett, J. Woessner and N. Rawlings, Elsevier, Academic Press, Oxford, UK, 2004.
- 138 B. S. Murray, R. Ettelaie, A. Sarkar, A. R. Mackie and E. Dickinson, The perfect hydrocolloid stabilizer: Imagination versus reality, *Food Hydrocolloids*, 2021, **117**, 106696.
- 139 V. S. Goncalves, P. Gurikov, J. Poejo, A. A. Matias, S. Heinrich, C. M. Duarte and I. Smirnova, Alginate-based hybrid aerogel microparticles for mucosal drug delivery, *Eur. J. Pharm. Biopharm.*, 2016, **107**, 160–170.
- 140 J. Chen, V. Gaikwad, M. Holmes, B. Murray, M. Povey, Y. Wang and Y. Zhang, Development of a simple model device for in vitro gastric digestion investigation, *Food Funct.*, 2011, **2**, 174–182.
- 141 H. Kozu, Y. Nakata, M. Nakajima, M. A. Neves, K. Uemura, S. Sato, I. Kobayashi and S. Ichikawa, Development of a human gastric digestion simulator equipped with peristalsis function for the direct observation and analysis of the food digestion process, *Food Sci. Technol. Res.*, 2014, **20**, 225–233.
- 142 M. Minekus, P. Marteau, R. Havenaar and J. H. J. Huis in't Veld, A multicompartamental dynamic computer-controlled model simulating the stomach and small intestine, *ATLA, Altern. Lab. Anim.*, 1995, **23**, 197–209.
- 143 S. Zhang, B. S. Murray, M. Holmes, R. Ettelaie and A. Sarkar, Gastrointestinal fate and fatty acid release of pickering emulsions stabilized by mixtures of plant protein microgels + cellulose particles: an in vitro static digestion study, *Food Biophys.*, 2022, **18**, 120–132.

