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ENCAPSULATION OF SHORT-CHAIN BIOACTIVE PEPTIDES (BAPS) FOR GASTROINTESTINAL DELIVERY

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ABSTRACT

Bioactive peptides (BAPs) are protein fragments (generally 2-20 amino acids) that have potential benefits for human health and the prevention of diseases. The vast majority of BAPs that provide high bioactivities are presented in the form of short sequences (less than 10 or even 5 amino acids). However, gastrointestinal digestion was reducing the bioaccessibility of those short-chain BAPs. At the same time, stability in the gastrointestinal tract is crucial for developing BAPs as factional food, nutraceuticals, and food supplements. This proposed research aims to encapsulate short-chain BAPs using biopolymers to increase their bioaccessibility. Angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) inhibitors are used as short-chain BAPs models encapsulated into bio-polyelectrolyte complexes (PECs) chitosan-alginate. Precisely, the objectives of this research are characterizing the PECs-loaded short-chain BAPs with some experimental approaches. It includes analysis of biophysical properties such as particle size by dynamic light scattering (DLS), zeta potential by dynamic electrophoretic mobility, structure by small-angle X-ray scattering (SAXS), and morphology by electron and infrared microscopy. In addition, this research is also investigating the release and stability of encapsulated short-chain BAPs in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), as well as their in vitro digestion, permeability, and cytotoxicity.

I. GENERAL INTRODUCTION

Bioactive peptides (BAPs) are protein fragments composed of several amino acids (di, tri, tetra, or oligopeptides) and have the potential for preventing diseases and promoting human health. Over 60 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 (Wang et al., 2022, Lau and Dunn, 2018). Foods are the most promising source of BAPs. These BAPs are usually 2 to 20 amino acid residues long. They are obtained as a result of enzymatic hydrolysis (*in vitro*), microbial fermentation, and human gastrointestinal enzymes process (*in vivo*) (Sánchez and Vázquez, 2018). Studies of the origin, identification, isolation, and purification of BAPs and their mechanisms of action and application have increased significantly in the last decade. Food BAPs have been identified as having antihypertensive, antidiabetic, antioxidant, hypocholesterolemic, anti-inflammatory, and antimicrobial actions (Patil et al., 2022)

Considering food-derived BAPs as (antihypertensive) angiotensin-converting enzyme (ACE) inhibitors and (antidiabetic) dipeptidyl peptidase IV (DPP-IV) inhibitors, the vast majority of BAPs with high bioactivities are shorter than 10 amino acid residues. The "high" bioactivity is defined as an IC_{50} <10 μM for ACE inhibition and <50 μM for DPP-IV inhibition, where IC_{50} is the standard half-maximal inhibitory concentration. It is based on a preliminary review carried out on various food sources covering milk (Contreras et al., 2009, Lacroix and Li-Chan, 2014, Lacroix et al., 2016, Mao et al., 2007, Nongonierma et al., 2019, Nongonierma and FitzGerald, 2014, Shanmugam et al., 2021, Silveira et al., 2013, Uenishi et al., 2012, Yamada et al., 2015, Yan et al., 2019), fish (Byun and Kim, 2001, Fujita et al., 2000, Balti et al., 2015, Balti et al., 2010, Fahmi et al., 2004, Ghassem et al., 2012, Ghassem et al., 2011, Harnedy-Rothwell et al., 2020, Intarasirisawat et al., 2013, Lee et al., 2011, Li-Chan et al., 2012, Lin et al., 2019, Liu et al., 2016, Ngo et al., 2016, So et al., 2016, Wu et al., 2008a), animal based (Cao et al., 2020, Miguel et al., 2006, Miguel et al., 2008, Sangsawad et al., 2018a, Sangsawad et al., 2018b, Sangsawad et al., 2017), plants (Yang et al., 2003, Chen et al., 2013, Gu and Wu, 2013, Marczak et al., 2003, S Vallabha and Tiku, 2013, Wang et al., 2015, Wu et al., 2008b, Yang et al., 2011), microalgae (Lin et al., 2018, Lu et al., 2011, Montone et al., 2018, Xie et al., 2018) and mushrooms (Geng et al., 2016). These sources were investigated and reported as the source of high bioactivity BAPs. This preliminary review also shows that tripeptides and pentapeptides are dominant among ACE inhibitors, and tripeptides are supreme as DPP-IV inhibitor (Figure 1). These short sequences could be named short-chain bioactive peptides (BAPs). Skrzypczak et al. (2017) considered peptides of 2-10 amino acids as short-chain BAPs (Skrzypczak et al., 2017) while other workers only consider them as short-chain BAPs, those that contain 2-5 amino acid residues (Cerrato et al., 2022).

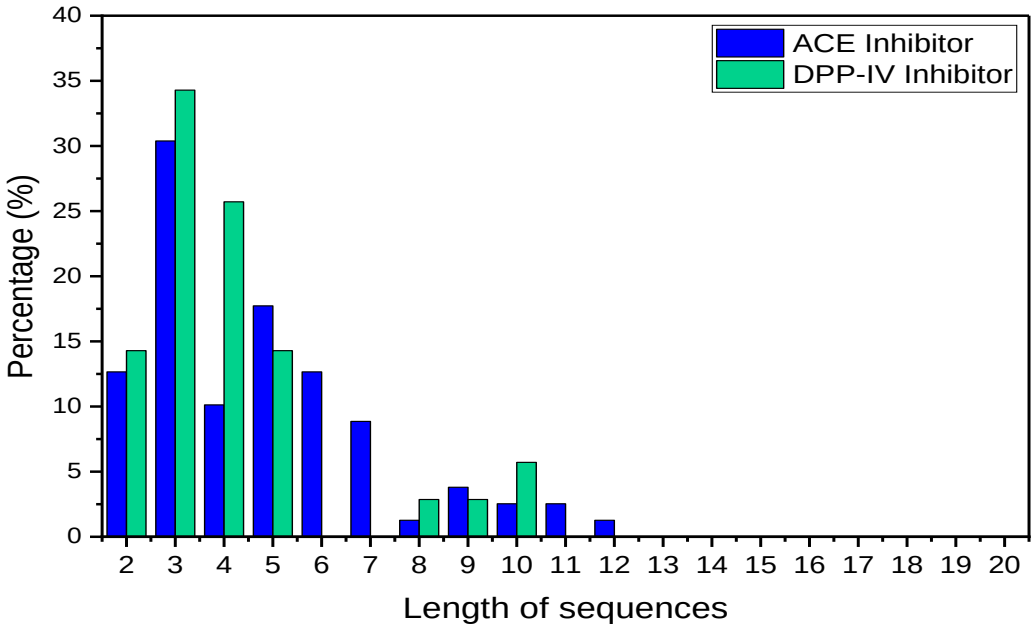

Figure 1. Percentage of food-derived BAPs based on high bioactivity

Gastrointestinal stability is a crucial aspect of BAPs for functional food, nutraceutical, and food supplement application. In these types of products, in the end, the BAPs have to be stable in gastrointestinal, bioaccessible, and bioavailable. However, gastrointestinal digestion was reducing the bioaccessibility of short-chain BAPs. It is because of digestive proteases and peptidases in the stomach, intestinal lumen, and intestinal brush border. Many short-chain BAPs reduced their bioactivities and lost their intact sequences after digestion (in vitro or in vivo studies) (Lacroix et al., 2017, Basirico et al., 2015, Fernández-Musoles et al., 2013, Miguel et al., 2008). While to provide their bioactivities, short-chain BAPs should be in the form of their intact sequences. In addition, most short-chain BAPs only absorbed in the form of di or tripeptides. Therefore, it is essential to enhance the bioaccessibility of short-chain BAPs by encapsulating, protecting, and delivering them to reach the intestinal layer in the whole structure and stable bioactivities. Figure 2 describes that encapsulation is a critical part of BAPs development.

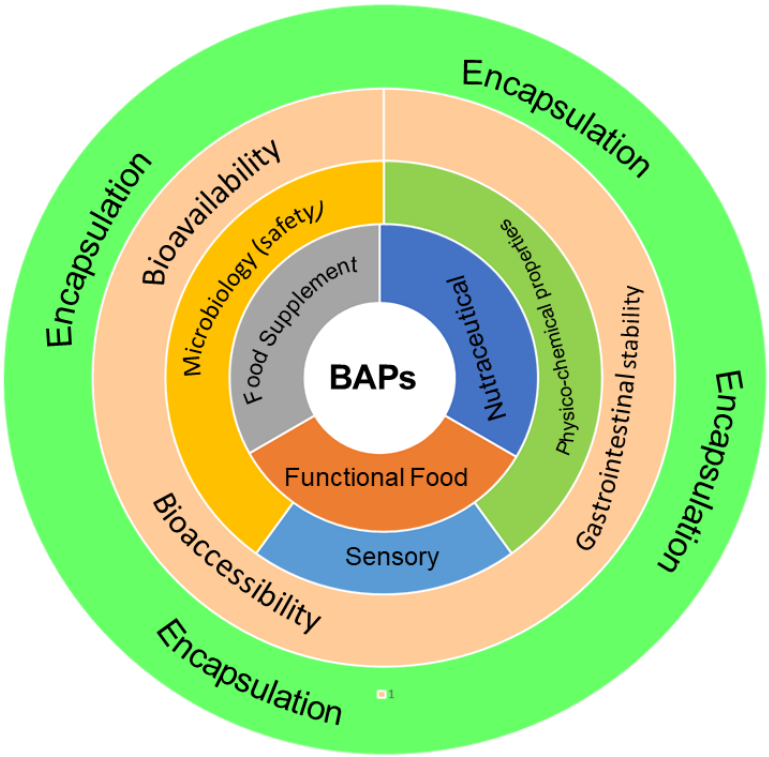

Figure 2. Multilevel pie chart describing some substantial aspects of developing BAPs as nutraceuticals, food supplements, and functional food

II. STATE OF THE ART

2.1 Types of Short-Chain BAPs

Since 2010, only limited studies (aprox. 13 studies) have been addressed short-chain BAPs encapsulation for gastrointestinal delivery. These are focused on di, tri, tetra and up to undecapeptide. All of those short-chain BAPs have molecular weight ranged 273.5 to 1493.7 g/mol. Encapsulated short-chain BAPs from varying sources have been documented with bioactivities such as antihypertensive, anti-inflammatory, antioxidant, antiproliferative, hypocholesterolemic, anxiolytic, anticancer, and absorption permeation enhancer. Table 1 presents types of short-chain BAPs, their sequences, bioactivities, and molecular weights.

The tripeptide IPP and hexapeptide RLSFNP are derived from casein (Nakamura et al., 1995) and lactoglobulin (Pan and Guo, 2010), respectively; tripeptide LKP from bonito fish (Fujita and Yoshikawa, 1999); and pentapeptide VLPVP is expressed in milk by engineered recombinant *E. coli* (Liu et al., 2007). Tetrapeptide LQPE is obtained from milk casein hydrolysed using the enzyme Neutrase®. The decapeptide YLGYLEQLLR or α-casozepine is obtained from whey protein hydrolysis. Glutathione (ɣ-glutamyl-cysteinyl glycine, GSH) or ECG is produced by bioprocessing technology using *Saccharomyces cerevisiae* and *Candida utilis* (Li et al., 2004). Tripeptide KPV is naturally produced in the body, found in the alpha-melanocyte-stimulating hormone. In turn, the octapeptide octreotide, FCFWKTCTA, is a synthetic somatostatin (Parmentier et al., 2011).

Tripeptides IPP and LKP, pentapeptide VLPVP, and hexapeptide RLSFNP have been reported with antihypertensive activity by inhibiting angiotensin-converting enzyme (ACE) (Danish et al., 2017, Huang et al., 2017, Zhang et al., 2019). Decapeptide YLGYLEQLLR (α-casozepine) has anxiolytic activity (Batista et al., 2021). Glutathione (ɣ-glutamyl-cysteinyl glycine, GSH) or ECG has antioxidant activity by inhibiting oxidative damage caused by free radicals from reactive oxygen species circulating in the body (Trapani et al., 2010). Octapeptide FCFWKTCTA has antiproliferative activity by inhibiting pancreatic exocrine and endocrine secretion (Parmentier et al., 2011). In terms of their potential application, even when not all these BAPs should reach blood circulation, in most cases, they must be absorbed systemically to fully display their bioactive effects. To this end, encapsulation is used to protect them from degradation in the gastrointestinal environment, control their release in intact form on the target sites, and promote their absorption, thus effectively, enhancing both their bioaccesibility and bioavailability. Other examples of encapsulated bioactive peptides include hypocholesterolemic tetrapeptide LQPE and anti-inflammatory tripeptide KPV. LQPE demonstrated the capacity to decrease micellar cholesterol solubility and affect the expression of protein and enzymes related to cholesterol absorption in the small intestinal epithelial cells (Jiang et al., 2020). This peptide must be released into epithelial cells in whole sequence length. In turn, KPV is an anti-inflammatory by inhibiting proinflammatory cytokine (molecule) synthesis and secretion in the colon. This type of tripeptide has been studied as a therapeutic peptide for treating inflammatory-related bowel diseases (IBD) in the large intestine (Dalmasso et al., 2008, Kannengiesser et al., 2008)

Table 1. Types of short-chain BAPs encapsulated for gastrointestinal delivery

Type BAPs	Sequence[1]	Molar mass (g/mol)	Bioactivities[2]	References
Dipeptide	AW	275.3	NR	(Stephansen et al., 2014)
	FW	351.4	NR	(O'Neill et al., 2015, O'Neill et al., 2015)
	IPP	352.4	Antihypertensive ACE Inhibitor	(Danish et al., 2017)
	LKP	356.5	Antihypertensive ACE Inhibitor	(Danish et al., 2017)
Tripeptide	ECG	307.3	Antioxidant	(Trapani et al., 2010)
	KPV	342.4	anti-inflammatory	(Laroui et al., 2010, Xiao et al., 2017)
	YKT	410.5	Anticancer	(Bicak et al., 2021)
Tetrapeptide	LQPE	485.5	Hypocholesterolemic	(Jiang et al., 2021b)
Pentapeptide	LWMRF	751.9	NR	(O'Neill et al., 2015, O'Neill et al., 2015)
	VLPVP	523.6	Antihypertensive ACE Inhibitor	(Huang et al., 2017)
Hexapeptide	RLSFNP	732.8	Antihypertensive ACE Inhibitor	(Zhang et al., 2019)
Octapeptide	FCFWKTCT	1035.2	Antiproliferative	(Parmentier et al., 2011)
Decapeptide	YLGYLEQLLR	1267.4	Anxiolytic	(Batista et al., 2021)
Undecapeptide	GRKKRRQRRRP	1493.7	Epithelial permeation enhancer	(Chen et al., 2017)

[1]*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.

[2]*NR=not reported*

III. TYPES OF VEHICLES AND METHODS OF ENCAPSULATION FOR GASTROINTESTINAL DELIVERY SHORT-CHAIN BAPS

3.1 Nanoencapsulation

- ***Nanoparticles (NPs)***

Nanoparticles (NPs) have been a central focus of research in drug delivery for more than four decades, and today their application and clinical translation are essential pillars of the nanomedicine field. Due to their size, composition and surface characteristics, they provide unique advantages for the administration of bioactive payloads that either exhibit toxicity, degradation or poor absorption. These include drugs that do not meet the Lipinski's rule of five (Lipinski et al., 2001) (ref) (*i.e*., less than five H donor groups, less than ten H acceptor groups, Mw < 500 Da, *logP* -octanol:water partition coefficient- < 5.0). NPs can be comprised by polysaccahrides such as chitosan (CS), alginate (ALG), hyaluronic acid, dextran, pectin and arabinogalactans and chemical derivatives of these, or by proteins such as zein, lysozyme, lactoferrin, gelatine, among other. These type of NPs constituted by natural biopolymers and macromolecules have been a focus of increasing attention, given the range of desirable properties they exhibit such as biocompatibility, biodegradability, stability, mucoactivity (mucoadhesion or mucodiffusion), and capacity to enhance the bioaccesibility and bioavailability of poorly absorbable payloads by promoting their epithelial absorption. Moreover, often these NPs can be produced under mild conditions, being particularly suitable to preserve the bioactivity and conformation of delicate biologics-type payloads such as hormones, peptides, antibodies, vaccines and nucleic acids.

In foods, polysaccharide and protein NPs are also known to occur and form during the cooking processing of animal (*e.g*., pig bone soup) or plant (*e.g*., tea) foods in the form of "incidental nanoparticles" (Gao et al., 2021)(ref). They also occur as colloidal emulsions widespread in food and drinks.

Polymeric NPs are particles with diameter between 1 and 1000 nm (generally obtained in a range of 100-300 nm) (Zielinska et al., 2020). NPs intended for oral delivery of short-chain BAPs have been examined. Two different type of preparation protocols have been reported to this end, namely: 1) coacervation (ionic gelation) (Batista et al., 2021, Danish et al., 2017, Trapani et al., 2010)(ref) and 2) double emulsions and solvent evaporation (Xiao et al., 2017, Laroui et al., 2010)(ref). The preparation steps and main features of each are reviewed below, and particular attention to NPs comprised by chitosan (CS) and alginate (ALG) is on section 2.4.

Research conducted by Trapani et al. 2010 addressed the encapsulation of antioxidant tripeptide glutathione (GSH) into CS NPs and CS/cyclodextrin (CD) NPs comprising two types of CD (α-CD and sulphobutyl ether-β-cyclodextrin, SBE-β-CD). For the CS NPs, encapsulation started by mixing peptide solution with CS solution, and then NPs spontaneously formed upon adding tripolyphosphate (TPP) solution under stirring. While encapsulation of tripeptide into CS/α-CD or CS/SBE-β-CD NPs, it was by dissolving tripeptide solution into

each of α-CD and SBE-β-CD solution before mixing with CS solution. Then, NPs are formed by TPP crosslinking as mentioned above and isolated with centrifugation (Trapani et al., 2010). Eudragit-coated CS NPs have used to encapsulate a permeation enhancer undecapeptide (11 amino acids). This peptide was encapsulated with other bioactive protein, insulin (around 51 amino acid residue). The permeation enhancer undecapeptide dissolved with acetic acid before being mixed with CS. Then, a crosslinking agent, TPP, was added to form undecapeptide-insulin-CS NPs. The Eudragit (in ethanol solution) was injected slowly into loaded CS NPs, then stirred (~850 rpm) at room temperature for 12 h for ethanol evaporation (Chen et al., 2017). The antihypertensive tripeptide LKP and IPP were encapsulated with CS NP systems but with a slightly different procedure. The tripeptides were added into TPP solution, then dropwise into stirred CS solution. After that, the loaded CS NPs separated using centrifugation, and collected in the form of wet pellets, and finally freeze-dried (Danish et al., 2017).

Double emulsions and solvent evaporation applied to encapsulate anti-inflammatory tripeptide KPV into CS/ALG NPs and hyaluronic acid-CS NPs (Laroui et al., 2010, Xiao et al., 2017). Actually, most hydrophilic drugs have been encapsulated via water-in-oil-in-water (W/O/W) emulsion. This method involves the following general steps: i) incorporating internal aqueous phase into oil/organic phase/solvent followed by homogenizing to form W/O emulsion (first emulsion), ii) the W/O emulsion mixed with second aqueous phase as outer layer by appropriate stabilizer addition to form W/O/W emulsion, iii) evaporation of the organic solvent from dispersed phase leading to insolubility and hardening the polymer encapsulating core material. In the case of encapsulation short chain BAPs, the method involded preparing separately firstly, an internal aqueous phase (primary phase) comprising the tripeptide solution, and secondly, an organic phase containing poly(lactic-acid) (PLA) or poly(poly lactide-co-glycolide-acid) (PLGA) (dissolved in dichloromethane). W/O emulsion is formed by mixing these phases (i.e., dropping the primary aqueous phase into the organic phase) and followed by sonication. Then, the first W/O emulsion is poured into a second aqueous phase containing hydrophilic surfactant poly(vinyl alcohol) (PVA). The W/O/W double emulsion is formed after homogenizing process. The organic solvent then evaporated, and the obtained solid nanospheres re-suspended in water and centrifuged to remove excess PVA and freeze-dried. Finally, the freeze-dried W/O/W emulsion loaded with KPV tripeptide dispersed into CS and ALG solution (Laroui et al., 2010). Xiao et al., (2017) mixed depolymerized CS with PVA as a second aqueous phase to form CS-NPs by double emulsion process, then hyaluronic acid was conjugated to fabricate hyaluronic acid-CS NPs (Xiao et al., 2017).

- ***Liposomes***

Liposome is an aqueous-core vesicle coated by a phospholipid bilayer that mimics a cell membrane. They have been used to deliver both hydrophilic and hydrophobic drugs already for five decades. In the case of short-chain BAPs, liposomes have been studied and loaded with hypocholesterolemic tetrapeptide, antihypertensive hexapeptide, and antiproliferative octapeptide. The liposomes that have loaded BAPs were modified by encapsulating again with Lactobacillus S-layer protein or designed to become multilamellar vehicles (MLVs). Liposomes were loaded in those BAPs by two approaches, namely, during (in situ loading) and after preparation (pro-loading) nanocarriers (Ichikawa, 2008).

In situ loading of BAPs into liposomes was carried out in several steps. It includes organic and aqueous phases preparation, injection of aqueous phase into the organic phase, ultrasonication for mixing and homogenizing lipid phase, evaporation to remove the organic solvent and obtain dry lipid film liposomes, and then second ultrasonication to obtain homogeneous liposomes. Aqueous phase was prepared by dissolved BAPs into phosphate buffer saline (PBS). Organic phase, in general, is obtained by dissolving groups of phospholipids and cholesterol. A study conducted by Zhang et al. (2019) that encapsulated hexapeptide RLSFNP, dissolved soy lecithin and cholesterol into ethyl ether as an organic phase (Zhang et al., 2019) while Jiang et al. (2021) encapsulated the tetrapeptide LQPE, by dissolving it in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and octadecylamine into trichloromethane (Jiang et al., 2021b).

In pro-loading BAPs into a liposome, the aqueous phase is not prepared initially but after dried lipid film was obtained. Initially, only the organic phase was prepared. After organic phase solvent removed by evaporation, the dried lipid film formed, then isolated, or stored until further characterization. Parmentier et al. (2011) encapsulated octapeptide into tetraether lipid liposomes using a pro-loading approach. In this research, they used egg phosphatidylcholine (EPC), dipalmitoyl phosphatidylcholine (DPPC), tetraether lipid from *Archaea*, and cholesterol. All of these compounds dissolved in chloroform/methanol as an organic phase. They created liposome in the form of multilamellar vehicles (MLVs). The MLVs liposome froze in liquid nitrogen and stored until further steps. Next, the formed liposome was thawed, re-suspended, and extruded followed with gentle mixing into peptide solution. Liposome – peptide mix solution was added with teflon beads, freeze in liquid nitrogen, and lyophilized. Finally, the lyophilized liposome containing octapeptide was rehydrated (Parmentier et al., 2011).

3.2 Microencapsulation

- ***Microgel Particles***

Microparticles are particles that have size range around 1 – 1000 μm in diameter (Lengyel et al., 2019). As encapsulation agents of short-chain BAPs, microgel particles have been produced by coacervation and membrane emulsification. CS, whey protein isolate (WPI), and sodium alginate (SA) were used as polymer materials. Slightly different with NPs formation, microgel particle fabrication via coacervation does not require application of ultrasound or sonication during encapsulation. A study was conducted by Batista et al. (2021) that encapsulated anxiolytic decapeptide YLGYLEQLLR into guar films-CS microparticles (MPs). The CS MPs prepared by dissolving chitosan into acetic acid, and followed decapeptide solution addition. Ionic gelation occurs through TPP addition as a counterion by interaction positive charge of CS with negative charge TPP. After TPP incorporate into the CS-peptide solution, MPs formed immediately under stirring conditions. Next, CS-MPs were collected and dropped into guar gam solution, then stirred until finely dispersed (Batista et al., 2021). Coacervation was also used to fabricate whey protein microbeads and then encapsulate two type of peptides, dipeptide FW and pentapeptide LWMRF. This whey protein microbeads were manufactured by dispensing pre-treatment WPI solution into a calcium chloride ($CaCl_2$) as a

crosslinker agent. Then the blank microbeads placed into peptide solution and stirred until 24 h (O'Neill et al., 2015, O'Neill et al., 2015).

Membrane emulsification was used to encapsulate ACE inhibitor pentapeptide VLPVP. The SA coated O-carboxymethyl chitosan (O-CMC) developed to encapsulate the peptide. SA (in acetate buffer) and peptide solution blended, then used as water phase. While the oil phase prepared by mixing paraffin, petroleum ether, and hexaglycerin penta ester. Membrane emulsification applied by pressing the water phase pass through the membrane (to control droplet sizes), then dropped into the oil phase to form W/O emulsion droplets. After that, another emulsion system ($CaCl_2$ solution dispersed into the oil phase, then sonicated) is incorporated into the first W/O emulsion while stirring. The microparticles were obtained, washed with petroleum ether and distilled water, subsequently, and followed by centrifugation. The microparticles coated with O-CMC solution and SA-O-CMC microspheres (containing ACE inhibitor peptide), were formed, washed, and finally freeze dried (Huang et al., 2017).

- ***Microfibers***

Microfibers have been utilized to encapsulate the dipeptide AW. Fish sarcoplasmic protein (FSP) was used as a polymer material. Initially, FSP was dissolved in hexafluoro-2-propanol (HFIP), and then a dipeptide added to the solution at a certain concentration. This solution added to the syringe and placed in the syringe pump. Syringe pump delivered FSP-peptide solution with conditional flow rate. Then FSP microfibers produced by electrospinning technique. In this study, electrospinning was conducted at room temperature, with the distance between the syringe tip and stainless steels collector plate was 15 cm (Stephansen et al., 2014).

Figure 6 and 7 below describe encapsulation procedure for short-chain BAPs by coacervation and double emulsions solvent evaporation approaches.

2.3 Chitosan and Alginate as Vehicles for Short Chain BAPs

Chitosan is an aminopolysaccharide obtained from chitin which is among the most abundant biopolymers in nature (along with cellulose). Chitosan is obtained at commercial scale by partial thermoalkaline N-deacetylation of chitin (Alonso and Goycoolea, 2008). Chitin is mostly found in the exoskeleton of crustaceans like crab and shrimp or their waste, and also found in molluscs, insects, and fungi. Production chitosan from these sources is inexpensive and easily (George and Abraham, 2006). Chitosan structure is described as a linear polysaccharide consisting of glucosamine (deacetylated units, D) and N-acetyl-glucosamine (acetylated units, A) units via β-(1→4) linkages (Figure 8). The molar fraction of A residues to the total (A+D), defines the degree of acetylation expressed as F_A. The distribution of A residues in chitosan, known as the pattern of acetylation, in chemically deacetylated chitosans is known to be random, but using enzymatic technology, it is possible to produced blockwise patterned chitosan (Wattjes et al., 2019) (ref). The other parameter that dictates chitosan's properties and applications is the molecular weight distribution. This is expressed by the weight average molecular weight (Mw), the number average molecular weight (Mn) and the polydispersity (Mw/Mn = $Đ$).

The positive charge of chitosan because they have amino groups (Potaś et al., 2020). Chitosan is insoluble in both water and organic solvents, however dissolve in aqueous solution

of acids because the existence of amine group at C2 positions of glucose rings (glucosamine units, D) which depend on degree of deacetylation (DD). Generally, DD of chitosan is about 70-95% and molecular weight between 10 and 1,000 kDa (Luo and Wang, 2014) . Figure 8 presents the structure of chitosan.

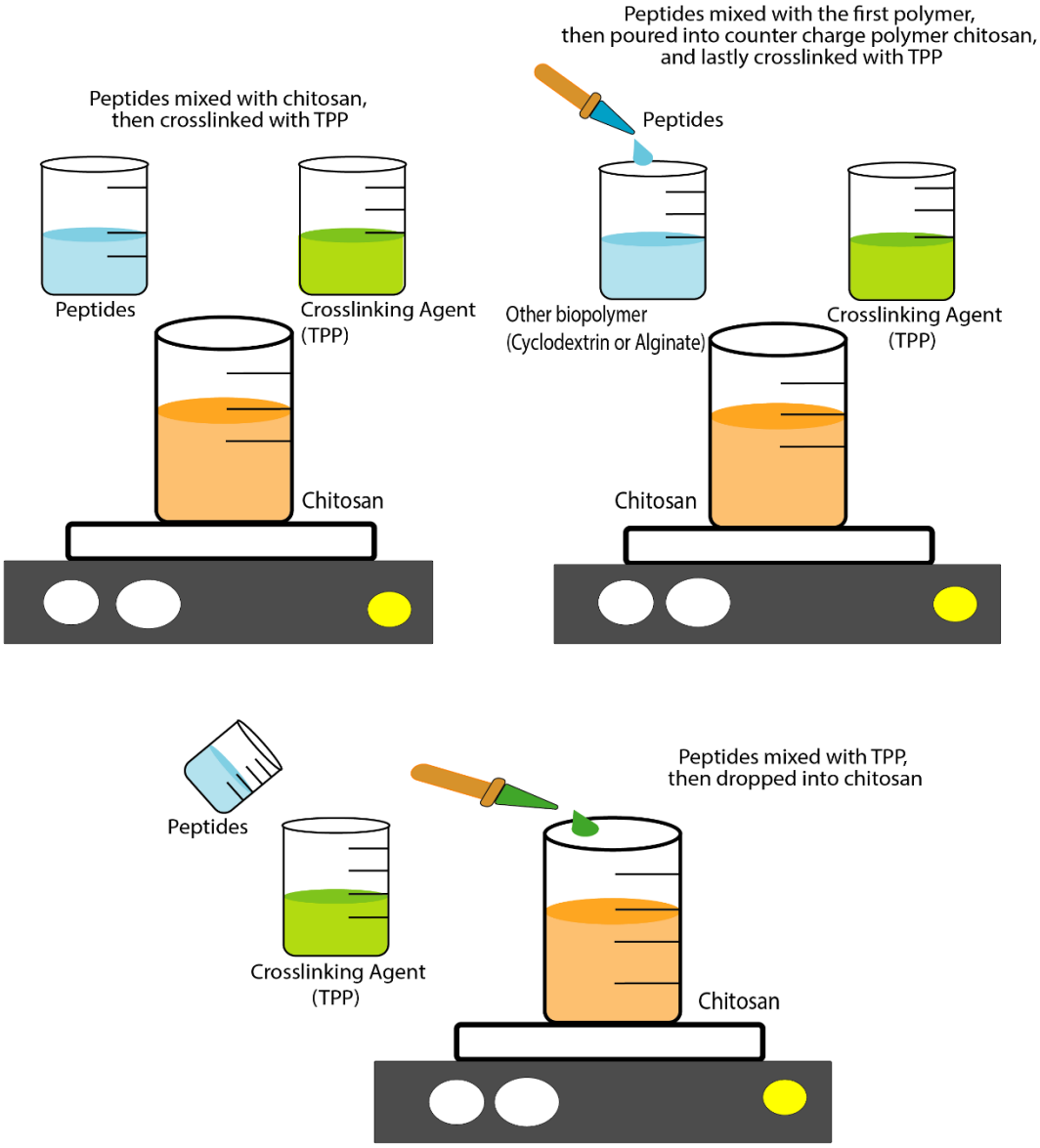

Figure 6. Three techniqiues for encapsulation short-chain BAPs by ionic gelation/coacervation.

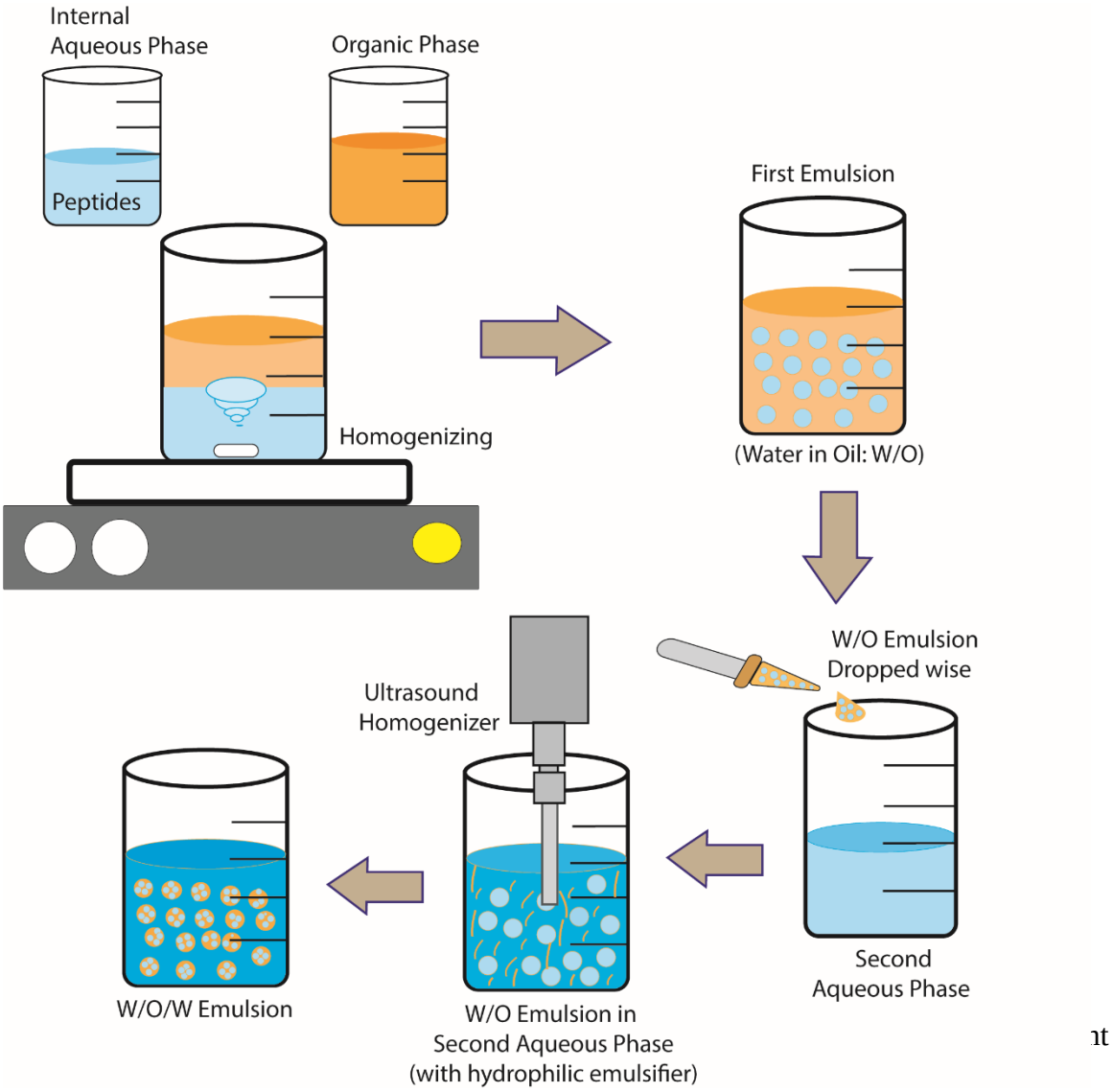

Figure 7. Method for encapsulation short-chain BAPs by double emulsions and solvent evaporation

Chitosan has been widely used in food and pharmaceutical industries. Chitosan has superior properties as an encapsulation agent including for oral or gastrointestinal delivery. It is because they are biodegradable, biocompatible, mucoadhesive, permeation enhancer, and easy to modify. Chitosan increases the bioaccessibility and bioavailability of encapsulated protein and peptides during gastrointestinal delivery because the mucoadhesive ability makes they can penetrate into intestinal mucus layer pores. Chitosan is also enable to open tight junction and absorbed into paracellular route of intestinal epithelium (Sonaje et al., 2012). In addition, chitosan has positive charge below pH 6.5, and many natural polymers have negative (polyanionic) charge in this pH range. So that it is simply to creating polyelectrolyte complex

(PEC) system with them by electrostatic interaction. For instance, encounter charge alginate have been composited with chitosan and proven improve the chitosan stability at various pH (Potaś et al., 2020).

Alginate is an anionic polysaccharide that commonly extracted from brown seaweeds. Polyanionic charge of alginate provide by their carboxyl groups. Alginate is unbranched biopolymers consisting (1→4) linked α-guluronic acid (G) block and β-mannuronic acid (M) block (Goycoolea et al., 2009). The sequences of polymer are homosequences (M blocks and G blocks) interspersed with heterosequences (G-M blocks) (Figure 9) with molecular weight between 60 and 700 kDa. Alginate is water soluble that enable to form gel whether by decreasing pH and adding multivalent cations. Their mannuronic and guluronic (M/G) ratio and molecular weight affect physical properties of alginate (Cazorla-Luna et al., 2021). Alginate has been used extensively in food industries and approved as GRAS (Generally Recognized as Safe) group by FDA (George and Abraham, 2006). Nowadays, alginate is biopolymer that mostly developed for biomaterial applications.

In term of encapsulation agent for gastrointestinal delivery, alginate offers many advantages. It is including their mucoadhesive characteristic, low toxicity, biocompatible, unhydrolyzed by most enzymes in gastrointestinal tract. Furthermore, polyanionic charge of alginate supporting their ability to encapsulate cationic BAPs. In addition, anionic charge of alginate also make them suitable counter ion for cationic biopolymer such as chitosan. Polyelectrolyte complex alginate, chitosan, and peptides are promising system for gastrointestinal delivery. Polycationic and polyanionic interaction chitosan and alginate enable them to create multiple layer of complex delivery. Some studies proven that crosslinking alginate and chitosan complex were reduce porosity of alginate so that preventing peptide leaching, diffusion, and reducing peptide release in gastric juice (George and Abraham, 2006, Luo and Wang, 2014, Potaś et al., 2020).

Chitosan has been studied to encapsulate short chain BAPs in sole and composite form with alginate. Chitosan NPs used to encapsulate IPP, LKP, ECG, YKT with ionic/coacervation method (Danish et al., 2017, Trapani et al., 2010, Bicak et al., 2021). Chitosan/Alginate have studied to encapsulate KPV by double emulsion solvent evaporation method (Xiao et al., 2017) and to encapsulate VLPVP by membrane emulsification combined ionic gelation (Huang et al., 2017).

IV. KEY CHARACTERISTICS OF DELIVERY VEHICLES FOR SHORT CHAIN BAPS

4.1 Association/Entrapment Efficiency (EE)

BAPs' association/entrapment/encapsulation efficiency (EE) is the detected amount of BAPs which successfully entrapped into encapsulation agent. EE is usually less than their initial added concentration. EE of BAPs is calculated based on attention initial BAPs added into formulation subtracted with free BAPs that not entrapped, then divided by (again) initial concentration, and finally multiplied with 100%. The percentage efficiency is better as higher as possible or close to 100%. The percentage EE is important because describing concentration BAPs in carrier agents before further processing like food incorporation, nutraceutical, and drug formulations until they are administrated in to the body circulation system and reach target sites. It is recommended BAPs have sufficient concentration on their target sites to provide bioactivity (Park et al., 2019).

The EE short-chain BAPs by microgel particles was reaching 95% (using whey microbead). Loading dipeptide (FW) and pentapeptide (LWMRF) into whey microbead showed EE of 56.3% and 95.1%, respectively. These BAPs were loaded into whey microbead by diffusion method for 80 min (O'Neill et al., 2015). When blank whey microbeads were placed into BAPs solution at 0.2% ratio volume microbeads: volume BAPs solution (Vbead/Vaq) for 24 h, the EE dipeptide and pentapeptide were 32% and 89%, respectively (O'Neill et al., 2015). EE of decapeptide YLGYLEQLLR into guar film-coated chitosan microparticles was 86% (Batista et al., 2021). In addition, the EE of pentapeptide VLPVP in carboxymethylated gum/sodium alginate microspheres was 64.6-87.8% (Huang et al., 2017).

EE of tripeptide IPP, LKP, and YKT into CS NPs were 22.6-43.9%, 40.9-65.1%, and 35%, respectively (Danish et al., 2017, Bicak et al., 2021). In a study conducted by Trapani et al. (2010), the EE of tripeptide ECG into CS/Cyclodextrin NPs was 7.1-25.1% (Trapani et al., 2010). The undecapeptide GRKKRRQRRRP loaded into Eudragit-coated CS NPs had EE of around 50% (Chen et al., 2017). In addition, liposome is another nanocarrier that has been investigated for short-chain BAPs encapsulation. EE of octapeptide (FCFWKTCT), hexapeptide (RLSFNP), and tetrapeptide (LQPE) into liposomes was about 13%, 67.5%, and 89%, respectively (Parmentier et al., 2011, Zhang et al., 2019, Jiang et al., 2021b). The higher EE for tetrapeptide because they applied *Lactobacillus* S-layer protein outside of the liposome (two layers) (Jiang et al., 2021b).

4.2 Morphology: Sizes and Shapes

Morphology of nano and microparticles covers their size, shape, and composition. These characteristics are essential, especially for carriers with a high possibility contact with living organisms' biological surfaces (like membranes). In terms of size nanocarrier, many studies have shown that nanoparticles under 200 nm in size effectively diffuse through the intestinal mucus layers (Yun et al., 2013). Pearson et al. (2016) added that the pore size of mucin is 100-200 nm so only nanocarriers less than 200 nm potentially pass through the mucin barrier and reach the small intestinal epithelium fastly (Pearson et al., 2016). In terms of shape, the rod-like NPs and with a higher aspect ratio, were more efficient in penetrating Hela and

Caco-2 cells (Salatin et al., 2015). In terms composition, carrier agents with more natural components have less toxicity level.

Measuring sizes of NPs and MPs loaded BAPs has been done using dynamic light scattering (DLS). DLS, based on the study of a scattered light beam on interaction with matters, then provides information about the sizes of particles. The size of vehicles that encapsulate short-chain BAPs varies depending on their types. NPs have diameter sizes around 50.6 – 500 nm. The smallest NPs derived from Eudragit-coated CS NPs and CS NPs whose diameter sizes were 50.6 and 57 nm, respectively (Chen et al., 2017, Bicak et al., 2021) while the biggest NPs in size for encapsulating BAPs were CS/cyclodextrin nanoparticles around 500 nm (Trapani et al., 2010). Nanoliposomes loaded short-chain BAPs have diameter ranged 130-218.2 nm (Parmentier et al., 2011, Zhang et al., 2019, Jiang et al., 2021b). Biggest liposome is caused by additional protein on the outer layer (Jiang et al., 2021b). Then, guar film-coated CS microparticles loaded with short-chain BAPs have 1.81 µm in diameter (Batista et al., 2021). Moreover, the diameter carboxymethylated gum/sodium alginate microspheres loaded BAPs was 5.39 - 43.81 µm (Huang et al., 2017). Figure 10 summarizes the size distribution of vehicles for encapsulation short-chain BAPs.

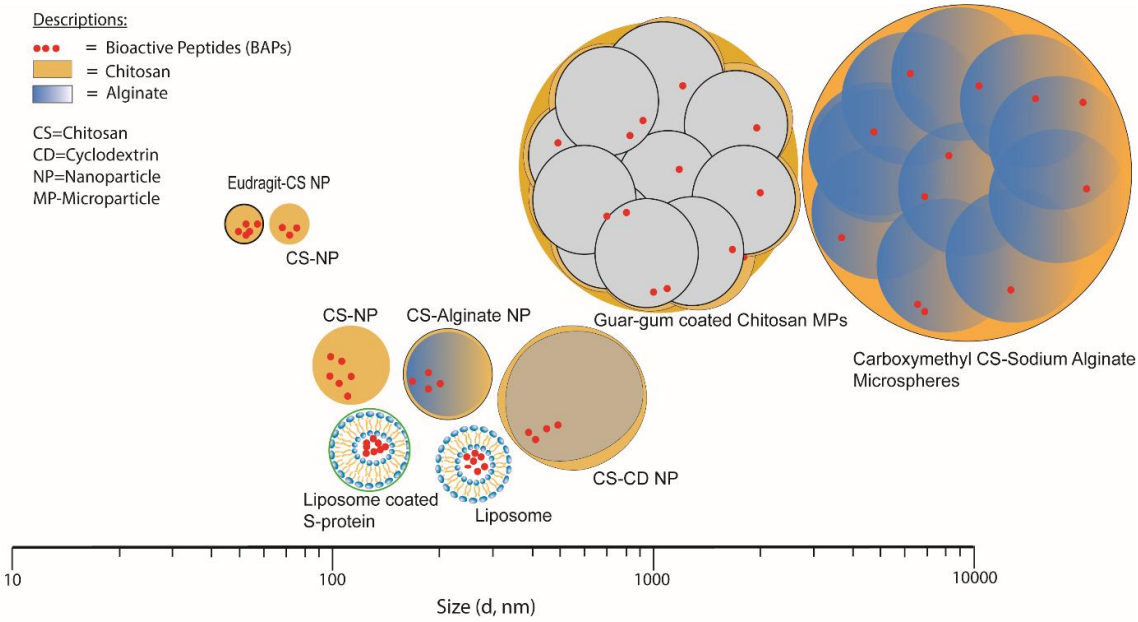

Figure 8. Size distribution of NPs and MPs for short-chain BAPs encapsulations.

Regarding the shape, mostly NPs and MPs loaded BAPs were regular spherical whether in liposome coated *Lactobacillus* S-layer protein, sodium alginate-O-carboxymethyl CS microspheres, or Eudragit-coated CS NPs (Jiang et al., 2021b, Chen et al., 2017, Huang et al., 2017). The shape was investigated with transmission electron microscopy (TEM) and scanning electron microscopy (SEM). A study conducted by Huang et al. (2017) toward micrograph of pentapeptide VLPVP-loaded sodium alginate-O-carboxymethyl CS microspheres noticed that

surface of microspheres was rough; however, the surface became smoother as membrane pore size increased to 5 μm or higher (Huang et al., 2017). In addition, CS NPs loaded tripeptide YKT has a solid and dense structure (Bicak et al., 2021).

4.3 Stability, Release, Absorption/Permeability, and Bioactivity of Encapsulated Short-Chain BAPs

Entrapment peptides aim to increase their bioavailability by improving their stability and bioaccessibility. Many studies have found that biological activity of BAPs reduced significantly after simulated gastrointestinal digestion and in vivo analysis. It is mainly caused by harsh gastrointestinal condition, such as low pH on gastric juice, proteolytic enzymes in gastrointestinal tract and cytoplasmic epithelium cells, and low transportability intact form peptides via transporter systems from the small intestine lumen into the blood, especially for tetrapeptide or longer sequences. For the latter, it is known peptides are only absorbed in the form of amino acids, dipeptides, and tripeptides (Bhutia and Ganapathy, 2018, Shen and Matsui, 2019).

Stability. Stability delivery vehicles for short-chain BAPs was investigated during simulated gastrointestinal digestion. Fish sarcoplasmic protein (FSP) microfiber was degraded slowly by low activity 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). This microfiber was degraded 50% under simulated intestinal fluid (SIF) containing low activity of pancreatin (0.01%, w/v) after 24 h, while compared to a high concentration of pancreatin (1%, w/v), the degradation became 100% (Stephansen et al., 2014). The amount of hypocholesterolemic tetrapeptide LQPE coated into liposome-*Lactobacillus* S-layer protein, and only liposome (one lipid bilayer) remained 74.5% and 55%, respectively after incubated on artificial intestinal juice for 3 h. While after incubated into artificial gastric juice, the amount was 68% and 50%, respectively. However, the unencapsulated tetrapeptide was almost completely degraded after incubation for 1 h, whether on artificial intestinal or gastric juice (Jiang et al., 2021b).

Release. Short-chain BAPs are released during gastrointestinal simulation in almost all types of reviewed carrier agents. The release kinetic was diminished on composited or hybrid wall materials. Tripeptides IPP and LKP loaded into CS NPs have been released reaching 90% in simulated intestinal fluid (SIF) after 240 minutes incubation and approximately 75% after 120 minutes incubation in simulated gastric fluid (SGF). In acidic simulation fluids, these peptides released more than 60% within two hours of incubation (Danish et al., 2017). Another tripeptide, YKT, loaded into CS NPs was released 26%, 34%, 43%, and 97% after 8, 24, 48, and 264 h incubation respectively. The release kinetic was getting lower in CS-Alginate NPs; for example, tripeptide KPV encapsulated into CS/Alginate NPs was released about 25% at pH 6.2 within 30 min, then after 30 minutes, release kinetics was linear on 35% until 30 h (Laroui et al., 2010, Xiao et al., 2017). On the contrary, tripeptide ECG loaded into CS/CD NPs released 100% and ~25%, respectively, after 200 min incubation at pH 1.2 and at pH 6.8. The tripeptide released about 60% from CS/α-CD before 200 min and 100% from CS/SBE-β-CD at <100 min (Trapani et al., 2010). In addition, tetrapeptide LQPE loaded into liposome and liposome coated with *Lactobacillus acidophilus* S-layer protein was released around 60% and 40%, respectively

after 24 h incubation (pH 7.2) (Jiang et al., 2021b). Hexapeptide RLSFNP loaded into a liposome, and after dialysis, the peptide released around 75% in 1 h and 98.4% in 2 h (Zhang et al., 2019). For the MPs, the maximum release of peptides was around 87.6%, and the minimum release was <2.5%. Dipeptide AW loaded into fish protein sarcoplasmic microfiber was released 61.5% after incubated on SGF and released 71.8% after incubated on SIF (Stephansen et al., 2014). Dipeptide FW loaded into whey microbeads released 56% after 6 h incubation, and at volume microbeads: volume peptide (Vbead: Vaq) <0.2 released around 80% (O'Neill et al., 2015, O'Neill et al., 2015). The pentapeptide VLPVP encapsulated with O-carboxymethyl CS/SA microspheres released 9.59% (pH 1.2, 2 h), and 87.63% (pH 6.8, 5 h) (Huang et al., 2017). Guar film-coated CS MPs successfully protected decapeptide YLGYLEQLLR by only <2.5% released after 4 h. For the latter, the study was only using artificial saliva (Batista et al., 2021).

The BAPs release was typically measured by incubating vehicles-loaded BAPs in a physiological solution containing enzymes or without enzymes. It is to represent gastric and intestinal fluids. Then the mixture solution placed into dialysis bag, clumped, and dipped into buffer. In certain period of times, buffer solution withdrawn and the BAPs concentration identified by using HPLC-UV detector or spectrophotometer assay (Batista et al., 2021, Zhang et al., 2019, Danish et al., 2017). Some studies were not using dialysis bag, but withdrawn solution on physiological directly, centrifuged, and finally analysis peptide concentration in supernatant (Huang et al., 2017, Stephansen et al., 2014).

Absorption. Permeability and absorption of peptides have been increased after encapsulation. Permeability of encapsulated tripeptide ECG with CS/α-CD and CS/SBE-β-CD on the proximal segment of frog duodenum was increased by 1.42 and 1.2 times higher, respectively, than tripeptide alone. On the distal segment, the permeability increased by 1.3 (CS/α-CD) and 1.8 (CS/SBE-β-CD), higher than tripeptide alone (Trapani et al., 2010). CS/SA NPs loaded tripeptide KPV was interact with membrane cell of Caco-2 monolayer by transmission electron microscopy (TEM) analysis (Laroui et al., 2010), and surface functionalization of these NPs with hyaluronic acid (HA) promoted cellular uptake efficiency CS/SA NPs noticed based on fluorescence intensity, markedly higher on treated cell lines than NPs without HA (Xiao et al., 2017). Liposomes also significantly improved transepithelial transport of tetrapeptide LQPE and hexapeptide RLSFNP compared with peptides alone. It was investigated on Caco-2 cell monolayer when series concentration of LQPE was incubating on the cell model. With 2 mmol/L of incubated concentrations, so uncoated peptide on basolateral (BL) solution was 0 mg/mL, and peptide coated liposome was 0.01-0.02 mg/mL. Then with initial concentration 3 and 6 mmol/L, so the concentration on basolateral (BL) for uncoated peptide was 0.02 and 0.04 mg/mL respectively, while peptide coated liposome was >0.04 mg/mL and 0.08-0.12 mg/mL respectively (Jiang et al., 2021b). The apparent permeability coefficient (Papp) of RLSFNP liposome increased more than two times compared with free RLSFNP (Zhang et al., 2019). Papp value of CS microparticles loaded decapeptide was four times higher than free decapeptide which investigated across human adenocarcinoma colorectal cell line of Caco-2/HT29 cell layers (Batista et al., 2021).

The bioactivity of short-chain BAPs after encapsulation and after release need to quantify. In vitro inhibitory activity was measured based on the ability BAPs to inhibit ACE (angiotensin-converting enzyme) by converting the substrate of hippuryl-L-histidyl-L-leucine (HHL) to become hippuric acid (HA). The concentration of HA is quantified using a spectrophotometric assay. The activity of IPP as an ACE inhibitor after nanoencapsulation and release was 84% and 82%, respectively. Activity LKP against ACE after CS-NPs encapsulation was 85%; this tripeptide retained the activity on 83% post incubation in SIF (Danish et al., 2017) Microencapsulation of pentapeptide into SA-O-carboxymethyl CS-MPs did not significantly reduce the activity against ACE, where native pentapeptide has 48.2% inhibitory activity. In comparison, pentapeptide encapsulated into MPs showed inhibitory activity around 45.2% (Huang et al., 2017).

4.4 Cytotoxicity

Cytotoxicity of NPs/MPs loaded BAPs was predominantly identified by in vitro cell viability approach. Caco-2, HT29-MTX, and IEC6 cell lines have been used to mimic gastrointestinal cells, and HepG2 cells are applied to mimic liver exposure. There were three assay techniques that applied to measuring cell viability on the existence of NPs/MPs loaded BAPs including 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 tripeptide IPP and LKP with concentrations (1 mM – 10 mM) showed similar cell line viability (Caco-2 and HepG2) compared to untreated cell lines. The cell viability for free tripeptides, unloaded CS NPs, and CS NPs loaded IPP/LKP was around 100%, while cell viability treated with Triton X-100 was lower than 50% (Danish et al., 2017). Concentration 1 mg/mL tripeptide coated into hyaluronic acid (HA)-CS/SA NPs did not make cellular death of both Caco2-BBE and IEC6 cell lines over a 72-80 h exposure (Laroui et al., 2010, Xiao et al., 2017). Membrane integrity of Caco-2 was not destroyed after treatment with Eudragit-cNPs loaded undecapeptide. The cell viability after treatment (2 h incubation) was 100%, which means no toxicity toward Caco-2 cells (Chen et al., 2017). Liposome loaded hexapeptide RLSFNP displayed no cytotoxicity effect on Caco-2 cells (Zhang et al., 2019). MPs loaded decapeptide YLGYLEQLLR showed no cytotoxicity effect also, which is represented by cell viability of 75-99% after incubation on 0.1 µg/mL–5 µg/mL of peptides on MPs solution (Batista et al., 2021).

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

BAPs Sequences	Encapsulation Materials	Encapsulation Methods	Encapsulation efficiency (EE)	Size	Release/Kinetic	References
AW	Fish sarcoplasmic protein (FSP) microfibres	Electrospinning	NR	100-700 nm and 1000 nm	61.5% in SGF after 30 min, and max 71.8% in SIF after 15 min	(Stephansen et al., 2014)
FW	Whey microbeads	Ionic gelation/Coacervation	32-56.3%		56% (6 h), ~80% (Vbead/Vaq <0.2)	(O'Neill et al., 2015, O'Neill et al., 2015)
IPP	Chitosan nanoparticles	Ionic gelation/Coacervation	22.6-43.9%;	113-209 nm	~75% in SGF (120 min); > 90% in SIF (240 min). Over 60% released within the first 2 h in acidic simulated fluid	(Danish et al., 2017)
LKP	Chitosan nanoparticles	Ionic gelation/Coacervation	40.9-65.1%	125-207 nm	~75% in SGF (120 min); ~90% in SIF (240 min). Over 60% released within the first 2 h in acidic simulated fluid	(Danish et al., 2017)
ECG	Chitosan/cyclodextrin nanoparticles	Ionic gelation/Coacervation	7.1-25.1%	190-500 nm	pH 1.2: CS/α-CD, 100% (< 200 min); CS/SBE-β-CD, ~25% (<200 min). pH 6.8: CS/α-CD, <60% (< 200 min); CS/SBE-β-	(Trapani et al., 2010)

					CD, 100% (<100 min)	
KPV	Chitosan/alginate nanoparticles, and hyaluronic acid (HA)-chitosan/alginate nanoparticles	Double Emulsions and Solvent Evaporation	NR	273 - 300 nm	25% at pH 6.2 within 30 min. After 30 minutes, release kinetics is more linier about 35% within 30 h	(Laroui et al., 2010, Xiao et al., 2017)
YKT	Chitosan nanoparticles	Ionic gelation/Coacervation	35%	~ 57 nm	26% release at first 8 h, 34% (24 h), 43% (48 h), and 97% (264h/11th day)	(Bicak et al., 2021)
LQPE	Liposome coated Lactobacillus S-layer protein	Liposome	89%	218.2 nm	liposome-LQPE: 60% after 24 h, SLP-L-LQPE: 40% after 24 h	(Jiang et al., 2021b)
LWMRF	Whey microbeads	Ionic gelation/Coacervation	89-95.1%		<5% (6 h), ~10% (Vbead/Vaq <0.2)	(O'Neill et al., 2015, O'Neill et al., 2015)
VLPVP	Carboxymethyl chitosan/sodium alginate microspheres	Membrane emulsification combined ionic gelation	64.6-87.8%	5.39 - 43.81 µm	9.59% at pH 1.2 (2 h), and 87.63% at pH 6.8 (5 h)	(Huang et al., 2017)
RLSFNP	Liposome	Liposome	67.50%	199 nm	Diffuse through dialysis membrane: 75% (1 h), 98.4% (2h). Commulative release RLSFNP: 75% (12 h) and 94.92% (24 h).	(Zhang et al., 2019)

FCFWKTCT	Tetraether lipid liposomes	Liposome	13%	130-207 nm	NR	(Parmentier et al., 2011)
YLGYLEQLLR	Guar Film-coated Chitosan Microparticles	Ionic gelation/Coacervation	86%	1.81 µm	<2.5% (4 h)	(Batista et al., 2021)
GRKKRRQRRRP	Eudragit (ES)-coated Chitosan Nanoparticles	Ionic gelation/Coacervation	50%	50.6 nm	CS NPs: 85% (pH 1.2, 4h), 40% (pH6.8, 1 h). ES-coated CS NPs: 40% (pH 6.8, 1h), 50% (pH 6.8, 4h), 70% (pH 7.4, 1h), almost completely released (pH 7.4, 4 h)	(Chen et al., 2017)

Table 3. Other important characteristics on encapsulation short-chain BAPs

BAPs Sequences	Encapsulation Materials	Other essential characteristics	References
AW	Fish sarcoplasmic protein (FSP) microfibers	*Stability*: 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)	(Stephansen et al., 2014)
IPP	Chitosan nanoparticles	*Citotoxicity*: 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. *Bioactivity*: 84% (after encapsulation); 82% (controlled release)	(Danish et al., 2017)
LKP	Chitosan nanoparticles	*Citotoxicity*: 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. *Bioactivity*: 85% (After encapsulation); 83% (controlled release)	(Danish et al., 2017)
ECG	Chitosan/cyclodextrin nanoparticles	*Absorption/Permeability*: CS/α-CD: 1.42 times higher (on proximal segment) and 1.3 times higher (on distal segment). CS/SBE-β-CD: 1.2 times higher (on proximal segment) and 1.8 higher (on distal segment). All of these compared to unencapsulated tripeptide ECG	(Trapani et al., 2010)
KPV	Chitosan/alginate nanoparticles, and hyaluronic acid (HA)-chitosan/alginate nanoparticles	*Absorption/Permeability*: Qualitative data shown that surface functionalization with HA can promote the cellular uptake efficiency of NPs by targeting cells. *Bioactivity*: HA-KPV-CS NPs can enhance the healing of the wounded colonic epithelial layer. *Cytotoxicity*: Concentration 1 mg/mL 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-a mRNA expression levels compared with those treated with KPV-NPs	(Xiao et al., 2017, Laroui et al., 2010)

YKT	Chitosan nanoparticles	*Cytotoxicity*: Viability (Control). 100%. 1) 0.1-2.5 mg/mL: 62.72-79% (after 24 h); 2) 0.1-1.0 mg/mL: 34-38% (after 48 h).	(Bicak et al., 2021)
LQPE	Liposome coated Lactobacillus S-layer protein (SLP)	*Absorption/Permeability*: All of the transhipment 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. *Stability*: LQPE Residue. Intestinal juice: 74.5% (SLP-L-LQPE), 55% (L-QPE). 68% (SLP-L-LQPE), 50% (L-LQPE)	(Jiang et al., 2020)
VLPVP	Carboxymethylated gum/sodium alginate microspheres	*Bioactivity*: 48.2% (before encapsulation); 45.2% (after encapsulation)	(Huang et al., 2017)
RLSFNP	Liposome	*Absorption/Permeability*: 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. *Cytotoxicity*: MTT Results indicated that the RLSFNP display no cytotoxicity in Caco-2 Cells	(Zhang et al., 2019)
FCFWKTCT	Tetraether lipid liposomes	*Bioavailability:* > 750 pg/mL in plasma, after 100 µg dosage	(Parmentier et al., 2011)
YLGYLEQLLR	Guar Film-coated Chitosan Microparticles	*Absorption/Permeability*: 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). *Cytotoxicity:* Cell viability 75-99% (MTT assay) (0-5 µg/mL)	(Batista et al., 2021)
GRKKRRQRRRP	Eudragit (ES)-coated Chitosan Nanoparticles	*Cytotoxicity:* Membrane integrity of Caco-2 was not destroyed after treatment with ES-Tat-cNPs and ES-cNPs. There were not toxic toward Caco-2 cell using CCK8 assay (100% of cell viability after treatment)	(Chen et al., 2017)

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