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1 **Comparative study of the structural and physicochemical properties of two food**
2 **derived antihypertensive tri-peptides, Isoleucine-Proline-Proline and Leucine-Lysine-**
3 **Proline encapsulated into a chitosan based nanoparticle system**

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26 **Abstract**

27 Food derived tri-peptides; Leucine-Lysine-Proline (LKP) and Isoleucine-Proline-Proline
28 (IPP) are angiotensin converting enzyme inhibitors and may have potential to attenuate
29 hypertension. The aim of this work was to understand the interactions of IPP and LKP when
30 formulated into a CL113 nanoparticle (NP) to help improve permeation. Our findings
31 indicate different IC_{50} values (LKP: $0.36 \pm 0.01 \mu\text{M}$ and IPP: $3.1 \pm 0.6 \mu\text{M}$) and encapsulation
32 efficiencies at different ratios of CL113: tripolyphosphate (LKP NPs: 65% at 6:1 and IPP
33 NPs: 43% at 4:1). Molecular modelling and circular dichroism showed different stable amino
34 side-chain-specific conformations for each peptide. IPP showed more steric hindrances to
35 intra-chain rotations, resulting in an unordered peptide structure, whereas LKP showed more
36 flexibility associated with a (disordered) β -strand-like conformer. *In-vitro* release kinetics
37 showed a slower release for LKP NPs in acidic pH compared to IPP NPs. In conclusion, LKP
38 NPs were found to have better binding compatibility with CL113.

39

40 Keywords: Leucine-Lysine-Proline (LKP); Isoleucine-Proline-Proline (IPP); bioactive
41 peptides; chitosan nanoparticles, ACE inhibitors; circular dichroism, *in-vitro* release kinetics

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48 **1. Introduction**

49 Isoleucine-Proline-Proline (IPP) and Leucine-Lysine-Proline (LKP) are both Angiotensin
50 Converting Enzyme (ACE) inhibitors, which have the potential to either maintain normal
51 blood pressure and prevent escalation of hypertension or be used for the treatment of mild or
52 pre-hypertension (Bütikofer, Meyer, Sieber, Walther, & Wechsler, 2008; Fujita &
53 Yoshikawa, 1999). ACE is responsible for the cleavage of a His-Leu dipeptide from
54 Angiotensin I (Ang I), converting it to Angiotensin II (Ang II), causing vasoconstriction,
55 increase in sympathetic tone, vasopressin release and aldosterone secretion (Maria et al.,
56 2016). ACE inhibitors block this conversion which consequently reducing the Ang II levels
57 and also inhibiting the catabolism of a vasodilatory peptide, bradykinin (Maria et al., 2016).
58 In recent years, bioactive peptides isolated from food have attracted market attention for their
59 physiological effects, but clinical applications are still limited due to variable or poor
60 permeation when administered orally. These limiting factors are due to a number of issues
61 such as insufficient gastric residence time, poor permeation across the intestinal epithelium
62 and/or solubility or chemical degradation within the gastrointestinal tract (GIT) due to low
63 pH, secretory and intracellular peptidases. The presence of other nutrients can also influence
64 absorption of the tri-peptides in the GIT (Jahan-Mihan, Luhovyy, Khoury, & Harvey
65 Anderson, 2011). Hypotensive effects of these tri-peptides have been demonstrated in the
66 Spontaneously Hypertensive Rat model (Fujita & Yoshikawa, 1999; Nakamura, Yamamoto,
67 Sakai, & Takano, 1995). Results from these studies indicated that both tripeptides do have a
68 pharmacodynamic effect when administered intravenously. However, to administer these
69 tripeptides by the oral route, their permeation needs to be enhanced. A number of techniques
70 have been previously investigated to help overcome such barriers, including carrier systems
71 made of lipids (Pandita, Kumar, Poonia, & Lather, 2014) or polysaccharides (Liu, Jiao,
72 Wang, Zhou, & Zhang, 2008) or the addition of inhibitors and enhancers (Gleeson, Heade,

73 Ryan, & Brayden, 2015; Maher, Mrsny, & Brayden, 2016). Chitosan in particular, a partially
74 deacetylated polymer consisting of a β - (1, 4)-linked-D-glucosamine residue with amine
75 groups, has been applied successfully in many areas of research (Al-Naamani, Dobretsov, &
76 Dutta, 2016; Koppolu et al., 2014; S. M. Ryan et al., 2013; Sarvaiya & Agrawal, 2015).
77 Chitosan is a good example of a polysaccharide carrier which has been widely used in
78 bioactive encapsulation in the form of nanoparticles (NPs), providing muco-adhesiveness, the
79 ability to open epithelial tight junctions and pH-dependant swelling behaviour (Garcia-
80 Fuentes & Alonso, 2012). Chitosan is positively charged under acidic conditions, to an extent
81 which depends on the degree of deacetylation (DD), the percentage of primary amino groups
82 in the polymer backbone and molecular weight. An increase in DD allows the ionisation of
83 the chitosan, which increases the viscosity, resulting in an extended conformation with more
84 charge repulsion and a more flexible chain (Kumirska et al., 2010; Vozza, Khalid, Byrne,
85 Ryan, & Frias, 2016). For encapsulation purposes, chitosan at high DD is usually selected to
86 allow more possibilities for the bioactive to interact (i.e. increasing molecular mobility)
87 (Szymańska & Winnicka, 2015). Nanoparticle based drug delivery systems have been widely
88 used in the area of encapsulation, using different carrier systems (Vozza et al., 2016). Studies
89 have shown the potential of controlling NP size and surface properties and the resultant
90 formulations have provided site-specific action of the drug at the optimal therapeutic rate and
91 dose (Yun, Cho, & Park, 2013a).

92 Ionotropic gelation is a physical process used to formulate chitosan nanoparticles using a
93 crosslinker (counterion), tripolyphosphate (TPP). Although the ionotropic process is simple,
94 without the use of organic solvents, it is important to ensure that the intrinsic properties of a
95 peptide are maintained before and after any formulation process occurs. Depending on the
96 formulation and the bioactive, particles with different properties can be obtained. Chitosan
97 nanoparticles for the formulation of peptides such as nisin (Bernela, Kaur, Chopra, & Thakur,

98 2014; Khan & Oh, 2015), insulin (Mukhopadhyay, Mishra, Rana, & Kundu, 2012), Z-
99 DEVD-FMK (Aktaş et al., 2005), calcitonin (S. M. Ryan et al., 2013) and dermaseptin
100 (Medeiros, Joanitti, & Silva, 2014) were formulated and their bioactivity was assessed after
101 the formulation processes have taken place. Sarmiento et al. (2007) investigated the possible
102 interactions and changes of secondary structure of insulin when encapsulated into a chitosan
103 nanoparticle using FT-IR and circular dichroism, they found that the intrinsic property of
104 insulin was maintained after encapsulation. The interaction of chitosan with the encapsulated
105 bioactive is an important aspect which can predict the suitability of future formulations and
106 may help provide a more systematic approach to future bioactive encapsulation.
107 In this study, the preformulation and formulation steps were assessed for IPP and LKP
108 encapsulated into chitosan NPs. The different characteristic properties of IPP, LKP and the
109 loaded nanoparticles were investigated with the aim of establishing a better fundamental
110 understanding of any physicochemical changes of the bioactive before and after the
111 formulation process.

112 **2. Materials and Methods**

113 Both peptides, LKP (Mw 356.47, purity = 97% according to the manufacturer's
114 specifications) and IPP (Mw 325.41, purity = 97% according to the manufacturer's
115 specifications) were synthesised by ChinaPeptides Co. Ltd, (Shanghai, China). CL113 (Mw =
116 110 kDa, deacetylation degree = 86% according to manufacturer's specifications) was
117 obtained from Pronova Biopolymer (Norway). CellTitre 96® AQueous One Solution Cell
118 Proliferation Assay was supplied by Promega (Madison, USA). Caco-2, heterogeneous
119 human epithelial colorectal adenocarcinoma cells were obtained from European Collection of
120 Cell Cultures (Salisbury, UK). HepG2, human liver cancer cells were obtained from
121 American Type Culture Collection. Ultrapure water was used for all experiments and was
122 obtained from a Milli-Q water purification system (Millipore Corporation, USA). TPP

123 (sodium tripolyphosphate), Angiotensin-I converting enzyme (from rabbit lung), captopril, N-
124 α -hippuryl-L-histidyl-L-leucine hydrate salt (HHL) and all other reagents were purchased
125 from Sigma Aldrich, Ireland.

126 2.1 Molecular Modelling

127 Molecular models were built for chitosan, IPP and LKP using Chem3D Pro software (version
128 12.0.2.1076 developed by ChembridgeSoft corporation). Refinement of the structures by
129 minimisation of conformational energy was performed using MM2 (Molecular Mechanics)
130 force field (Burkert & Allinger, 1982). The exact structure of chitosan was translated from
131 SMILES (chemicalize.org, 2016) to Chem3D Pro software.

132 2.2 Conformational analysis

133 Circular dichroism was performed using a Jasco J-810 Spectropolarimeter with a 150 W Xe
134 arc lamp and an operating range from 163 to 900 nm. This measured the absorption
135 difference between left and right circularly polarized light for an electronic transition. A
136 quartz cell with a light-path length of 0.5 cm was used for all measurements. IPP and LKP
137 were measured in water and chitosan in pH3 acetate buffer solution to mimic formulation
138 conditions.

139 2.3 LKP and IPP-loaded NPs formulation and physicochemical characterisation

140 A mixture amount design was produced using two variable parameters (CL113 and TPP)
141 composed of 5 experiments (table 1).

142 **Table 1** Mixture amount design of LKP and IPP nanoparticles in which the concentrations of
143 CL113 and TPP were the variable parameters in the model

Formulation	Ratio (CL113:TPP)	CL113 (mg/mL)	TPP (mg/mL)	Peptide (μ g/mL)
A	8:1	1.64	0.21	100
B	6:1	1.58	0.27	100

C	5:1	1.52	0.33	100
D	4:1	1.45	0.40	100
E	3:1	1.39	0.46	100

144 Preliminary studies showed concentrations within the range of 1.3-1.6 mg/mL chitosan and
145 0.2-0.5 mg/mL TPP produced unloaded nanoparticles with the best physicochemical
146 characteristics in terms of size 100-500 nm (des Rieux, Fievez, Garinot, Schneider, & Pr at,
147 2006), polydispersity (PDI) <0.4 (Abdel-Hafez, Hathout, & Sammour, 2014a) and zeta
148 potential (ZP) >30 mV (Lakshmi & Kumar, 2010) for an oral drug delivery system.
149 Therefore, these concentration ranges were selected for this study.

150 Stock solutions of 10 mg/mL CLL113 and TPP were prepared. CL113 was dispersed in
151 acetate buffer (pH 3) and TPP in 0.01 M sodium hydroxide solution. The stock solutions of
152 CL113 and TPP were diluted to different concentration ratios following a third order
153 polynomial mixture amount design (table 1). A fixed concentration of 0.1 mg/mL peptide
154 was added to the diluted TPP solutions. The TPP-peptide solution was added dropwise to the
155 CL113 solution while stirring. NPs were separated using ultrafiltration-centrifugation
156 (Centriplus YM-30, MWCO of 30kDa, Millipore, USA). The volume of the solution in the
157 filtrate vial was measured and the filtrate was assayed for the amount of LKP or IPP by
158 Reverse Phase High Performance Liquid Chromatography (RP-HPLC). The wet pellet was
159 re-suspended in purified water and immediately characterised using a range of physico-
160 chemical techniques. The nanoparticle size and electrophoretic mobility measurements were
161 performed using folded capillary cells in a Nanosizer ZS fitted with a 633 nm laser (Malvern
162 Instruments Ltd.). Each analysis was carried out at 25°C with the equilibration time set to 2
163 min. The experimental design and data analysis was performed using Minitab 17 software
164 (Minitab Inc, USA). The regression coefficients and responses for IPP and LKP were
165 analysed and compared.

166 2.4 Determination of encapsulation efficiency peptide-loaded nanoparticles

167 The encapsulation efficiency (EE %) of the peptides in the NPs was calculated by the indirect
168 method (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997). The supernatant was assayed
169 for the content of LKP or IPP by RP-HPLC. The RP-HPLC analysis was performed on a
170 Waters 1525 pump (Waters, Milford, Massachusetts) with a Photo Diode Array detector 2487
171 (Waters) using a Luna C18 column (5 μm , 250 mm x 4.6 mm, Phenomenex). Analytes were
172 detected at the wavelength of $\lambda_{\text{max}} = 220 \text{ nm}$ (LKP) and 215 nm (IPP). For LKP; the column
173 was eluted at a flow rate of 1 mL min^{-1} with an isocratic system (15% Acetonitrile, 0.05%
174 TFA in 84.95% water) and 10 μL injection volume. For IPP; the column was eluted at a flow
175 rate of 0.8 mL min^{-1} with an isocratic system (20% Acetonitrile, 0.05% TFA in 79.95%
176 water) and 5 μL injection volume. The EE % was calculated using the following equation:

$$177 \text{ EE \%} = \frac{(\text{Total amount Peptide} - \text{free amount Peptide in supernatant})}{\text{Total amount of Peptide}} \times 100 \quad (1)$$

178 2.5 Comparison of ACE inhibition for peptides and nanoparticles

179 ACE inhibition was measured for IPP and LKP over the concentration range 0.001-50 μM ,
180 using an ACE inhibition assay with HHL as substrate (Henda et al., 2013; Lahogue, Réhel,
181 Taupin, Haras, & Allaume, 2010). Briefly, HHL (5 mM) was dissolved in pH 8.3 buffer (0.1
182 M borate buffer in 0.3 M NaCl). 100 μL substrate solution and 25 μL inhibitor (peptides or
183 peptide loaded NPs) were incubated for 10 min at 37°C. 10 μL ACE solution (100 mU/mL)
184 were then added, incubated for 30 min and terminated using 100 μL of 1 M HCL. HPLC was
185 performed using a C8 column (2.7 μm , 3.0 x 100 mm, Agilent Technologies UK & Ireland
186 Ltd) at a wavelength of 228 nm. An isocratic method was used at a flow rate of 0.4 mL.min^{-1} ,
187 25% Acetonitrile, 0.1% TFA in 74.9% water for 5 min. Captopril was used as the control.
188 The % of ACE activity was calculated:

$$189 \text{ ACE inhibition \%} = 1 - \left(\frac{A^{\text{inhibitor}}}{A^{\text{blank}}} \right) * 100 \quad (2)$$

190 where $A^{\text{inhibitor}}$ and A^{blank} are the peak areas of HA (product of HHL) and negative control.
191 The IC_{50} of the inhibitor was determined using the Hill Slope equation (Prism 5, GrapPad
192 Software Inc., USA). The ACE inhibition assay was also performed on the released peptide
193 from the loaded NP at the last sample point (720 min SIF). Loaded NPs (19.5 mg in 10 mL
194 water) were subjected to sonication for 2 min at 40% amplitude (Branson Ultrasonics;
195 Ultrasonic processor VCX-750W, Wilmington, North Carolina, USA). Peptides were then
196 isolated using centrifugation and the supernatant was quantified using HPLC, and diluted to
197 10 μM to investigate any change of inhibition activity compared to the native peptides using
198 HHL substrate.

199 2.6 Cytotoxicity assessment of LKP and IPP by MTS

200 Caco-2 (passage 24-26) and HepG2 (passage 32-34) cell lines were seeded at a cell density of
201 2×10^4 cells/well and cultured on 96 well plates in DMEM and EMEM respectively,
202 supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin
203 and 1% non-essential amino acids, and incubated at 37°C in a humidified incubator with 5%
204 CO_2 and 95% O_2 . Caco-2 cells were exposed to IPP, LKP, unloaded NPs and IPP and LKP
205 loaded nanoparticles (in serum free media) for 4 h (Neves, Martins, Segundo, & Reis, 2016)
206 to mimic intestinal exposure and HepG2 cells for 72 h (Brayden, Gleeson, & Walsh, 2014) to
207 mimic liver exposure at concentrations (1, 5 and 10 mM for IPP/LKP and NPs). 0.05 %
208 Triton X-100™ (detergent) was used as a positive control. After exposure, treatments were
209 removed and replaced with MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carbo
210 xymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Optical density (OD) was measured at
211 490 nm. Each value presented was normalised against untreated control and calculated from
212 three separate experiments, each of which included six replicates. Data was analysed using
213 one-way ANOVA with Dunnett's multiple comparison test.

214 2.7 *In-vitro* controlled release analysis of peptides

215 The release of the peptide from chitosan NPs at the optimal ratio of CL113:TPP (ratio 6:1
216 (LKP), ratio 4:1 (IPP)) was studied by a dialysis method, 2 h in simulated gastric fluid (SGF)
217 followed by 4 h in simulated intestinal fluid (SIF). Freeze dried NPs in 2.5% trehalose,
218 equivalent to 10 mL formulation, were placed in a cellulose dialysis bag (cutoff molecular
219 weight 10 kDa, Spectra-Por[®] Float-A-Lyzer[®] G2). The dialysis bag was placed into the
220 receptor compartment containing the dissolution medium (40 mL), which was set at 100 rpm
221 and maintained at 37°C using a thermostatic shaker. This procedure was performed without
222 enzymes in accordance with British Pharmacopoeia, 2016, but with the exception of using a
223 dialysis membrane. SGF was composed of 0.1 M HCL and SIF was the buffering stage
224 composed of 1 volume of 0.2 M trisodium phosphate dodecahydrate and 3 volumes of 0.1 M
225 HCL (adjusted to pH 6.8), without enzymes (British Pharmacopoeia, 2016) . Samples were
226 withdrawn at different time intervals; 1 mL of sample fluid was removed at each time point
227 and replaced with 1 mL simulated buffer. The samples were measured using RP-HPLC
228 (method 5.2.2).

$$229 \quad \text{cumulative release \%} = \frac{\text{Peptide release}}{\text{Peptide initial}} * 100 \quad (3)$$

230 where peptide (LKP/IPP) release and initial represents the concentration of peptide
231 (LKP/IPP) release and the amount of peptide (LKP/IPP) initially loaded into the NPs,
232 respectively.

233 2.7.1 Release kinetics

234 The release kinetics of the peptide formulation under the SGF and SIF sequential controlled
235 release experiments were fitted using diffusive models derived from swellable systems
236 (Siepmann & Peppas, 2011).

237 For the SGF:

$$238 \quad \frac{M_t}{M_\infty} = ks_1 * (\sqrt{time}) + ks_2 * time \quad (4)$$

239 where M_t is the diffused mass at a given time, M_∞ is the asymptotic diffused mass at infinite
240 time, k_{s1} and k_{s2} are diffusive and relaxation rate constants.

241 For the SIF:

$$242 \frac{M_t}{M_\infty} - \frac{M_{120}}{M_\infty} = k_{i1} * (\sqrt{time - 120}) + k_{i2} * (time - 120) \quad (5)$$

243 where M_{120} is the predicted diffused mass at the time of changing from SGF to SIF (120
244 min), k_{i1} and k_{i2} are diffusive and relaxation rate constants.

245 The model building process involved:

- 246 1. A model with k_{i1} , k_{i2} , k_{s1} and k_{s2} for each peptide was built using nonlinear regression and
247 all the available data.
- 248 2. A set of candidate reduced models were produced by following two model reduction
249 strategies:
 - 250 a. If any of the parameters was not significantly different between peptides, a new model
251 with common parameters for both LKP and IPP was fitted.
 - 252 b. If the parameters k_{i2} , or k_{s2} were not statistically significant, a new candidate model
253 was fitted by reducing the Peppas model in equations 4 or 5 to a model without the
254 relaxation related term.
- 255 3. The resulting reduced model was compared with the previous model through a log-
256 likelihood ratio test (Welham & Thompson, 1997).
- 257 4. Steps 2 and 3 were repeated until a model that explained the experimental data with the
258 same ability as the model described in bullet point (1) with a minimum set of parameters.

259 The nonlinear regression methods from the R software were used (R Core Team, 2015).

260 **3. Results**

261 3.1 Molecular modelling of peptides

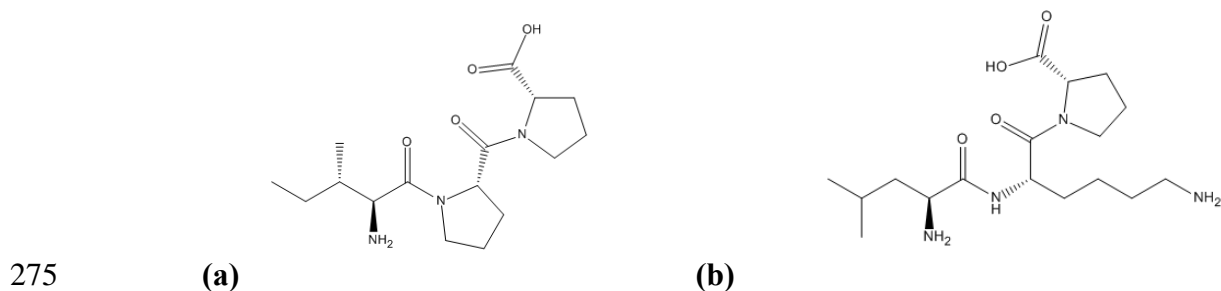
262 Molecular modelling was used to evaluate the conformational structure of the tripeptides.

263 Molecular modelling calculates the energy of a particular molecular structure and then

264 iteratively adjusts the energy through changes in bond lengths and angles to determine the
265 minimum energy structure (Mulloy, Forster, Jones, & Davies, 1993). For MM2, the following
266 are taken into account:

- 267 • A charge-dipole interaction term
- 268 • A charge-dipole interaction term
- 269 • A quartic stretching term
- 270 • Cutoffs for electrostatic and van der Waals terms with 5th order polynomial switching
- 271 function
- 272 • Automatic pi system calculations when necessary
- 273 • Torsional and non-bonded constraints

274 The resultant energy minimised structures are shown in Figure 1.



276 **Figure 1 (a) IPP and (b) LKP chemical structure at its minimised energy state**

277 The minimised total energy calculated for IPP, LKP and chitosan were 35.60 kcal/mol, 8.57
278 kcal/mol and 381.02 kcal/mol, respectively (table 2). LKP was found to have a significantly
279 lower energy compared to IPP and chitosan. Other significant differences were found in the
280 bending, indicating that for chitosan and IPP, more energy is required to deform an angle
281 from its equilibrium state, as presented in table 2. In addition, the torsion for all three
282 components showed significant difference for dihedral angles (torsionals,) which is the
283 energy needed to overcome torsional strain, IPP having more torsional strain than LKP.

284 **Table 2:** MM2 analysis of LKP, IPP and chitosan

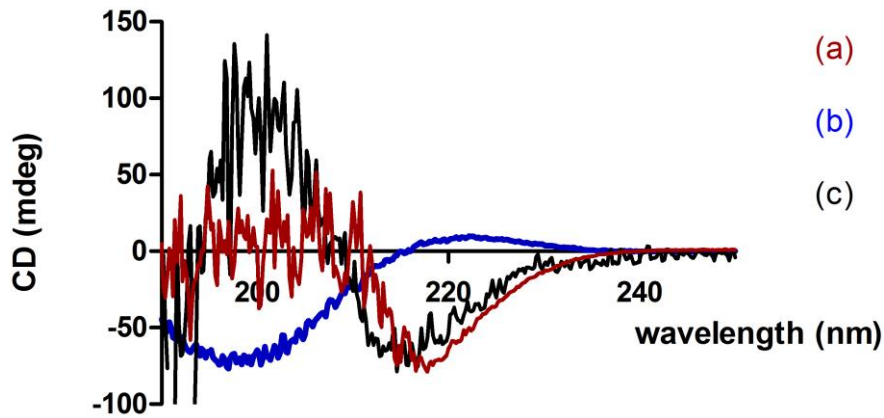
	LKP (kcal/mol)	IPP (kcal/mol)	CL113 (kcal/mol)
Stretch	1.50	1.95	22.64
Bend	12.04	24.26	157.23
Stretch-Bend	0.39	0.43	13.65
Torsion	5.50	15.78	124.29
Non-1,4 Van der Waals	-11.23	-7.77	-151.22
1,4 Van der Waals	12.35	12.95	219.93
Dipole/Dipole	-11.98	-11.99	-5.50
Total Energy	8.57	35.6	381.02

285 3.2 Conformational analysis

286 To further confirm the conformation of the peptides, circular dichroism was used for both
287 peptides and CL113. For our experiment, the spectral range (260–180 nm) was used to
288 measure the light absorption of the peptide bonds to investigate the chiral structure of
289 peptides.

290 As presented in figure 2, CD showed similar spectral profiles for LKP and chitosan over the
291 range 180-260 nm, but markedly different for IPP, indicating different stable side-chain-
292 specific conformations, an unordered peptide structure for IPP and a (disordered) β -strand-
293 like structure for LKP (Gokce, Woody, Anderluh, & Lakey, 2005; Rath, Davidson, & Deber,
294 2005).

295 CL113 is a large polysaccharide which has been reported to adopt a very tight twisted
296 secondary structure, found to be even more complex in aqueous solutions, causing it to
297 unwind the helical structure to a more disordered structure (Y. Wu et al., 2004). Such
298 disorder is evident in figure 2(a). Spectra of LKP and IPP are in accordance with Rath et al.
299 2005, showing a β -strand conformer for LKP and random coil-like structure for IPP.

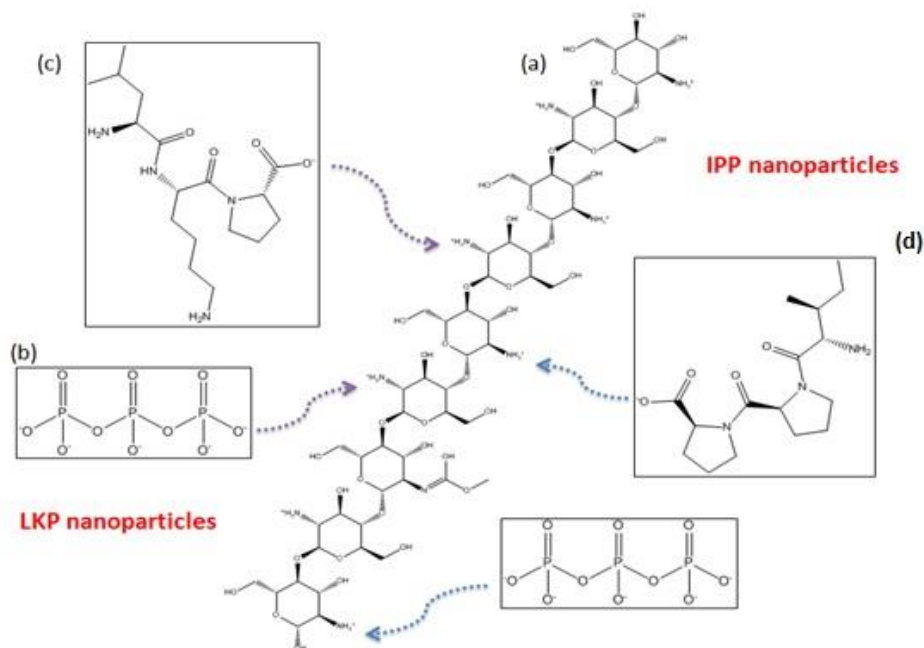


300

301 **Figure 2** CD spectra of (a) chitosan, (b) LKP and (c) IPP

302 3.3 Formulation and physicochemical characteristics of LKP and IPP nanoparticles

303 In the formulation process (section 2.4), the tripeptide was added to the TPP basic solution,
 304 higher than its isoelectric point, to allow ionisation (determined using isoelectric.ovh.org
 305 (2016): LKP = 9.07, IPP = 5.98, TPP = 6.59) predominately protonating the carboxyl (O^-)
 306 and interacting with the deprotonated amine of acidic chitosan, NH_3^+ (Naik 2015), as
 307 illustrated in figure 3.



308

309 **Figure 3** Schematic of LKP and IPP formulation into nanoparticles where (a) chitosan is
 310 protonated in acidic conditions and (b) TPP (c) LKP and (IPP) are oxidised in basic
 311 conditions.

312 The ratio between the polymer and crosslinker is the key to a high EE % (Patil, Marapur,
 313 Gurav, & Banagar, 2015). The same mixture amount design at different ratios of CL113 and
 314 TPP was used for both LKP and IPP in order to help choose the best formulation with
 315 optimal characteristics of delivery (table 2). Different bioactives interact differently to
 316 different ratios of CL113 and TPP for example depending on the size and charge of the
 317 encapsulated bioactive. The PDI, ZP value and EE % were measured for both LKP and IPP
 318 nanoparticles at different ratios, further analysed using experimental design analysis (see
 319 table 4). Formulation B (ratio 6) gave the best physicochemical characteristics with high EE
 320 % (65%). Additionally, formulation D (ratio 4) gave the best physicochemical characteristics
 321 with high EE % (44.8%).

322 **Table 3:** Physicochemical results of size (nm), ZP (mV), PDI, EE % and LC % of loaded IPP
 323 and LKP nanoparticles

Formulation	Peptide at 100µg/ml	Size (nm)	ZP (mV)	PDI	EE %	LC %
A	IPP	209 ±18	36.6 ±2	0.5 ±0.1	22.6 ±7	1.2 ±0.2
	LKP	207 ±73	38.2 ±5	0.4 ±0.2	40.9 ±11	2.2 ±0.1
B	IPP	173 ±15	32.8 ±2	0.3 ±0.1	30.2 ±12	1.6 ±0.4
	LKP	166 ±14	31.6 ±2	0.3 ±0.1	65.1 ±4	3.3 ±0.0
C	IPP	135 ±23	30.1 ±3	0.3 ±0.1	35.3 ±9	1.8 ±0.0
	LKP	145 ±13	31.6 ±4	0.4 ±0.1	45.3 ±14	2.3 ±0.8
D	IPP	143 ±16	29.6 ±2	0.3 ±0.1	44.8 ±6	3.0 ±0.1
	LKP	145 ±9	32.5 ±4	0.4 ±0.1	50.8 ±7	2.7 ± 0.3
E	IPP	113 ±10	28.6 ±6	0.2 ±0.1	43.9 ±4	2.3 ±0.1
	LKP	125 ±10	29.3 ±3	0.5 ±0.3	46.9 ±5	2.8 ± 0.2

324

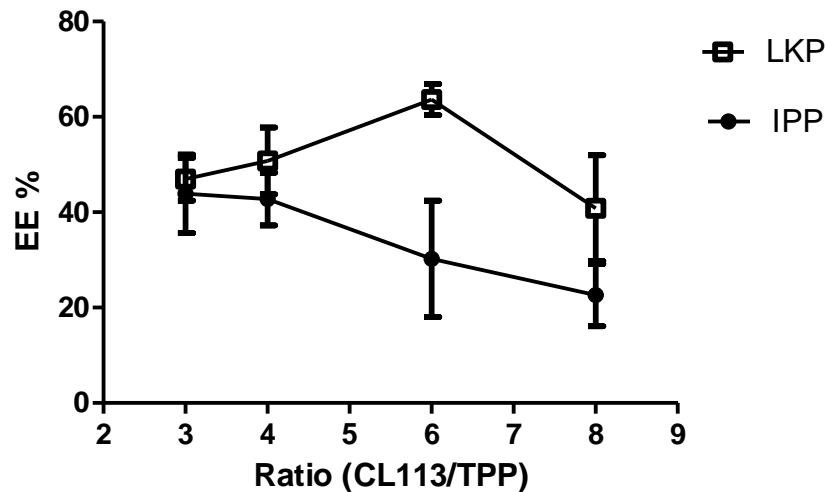
325 The third order polynomial regression coefficients of LKP and IPP parameters, shown in
326 table 4, were analysed and compared from the results in table 3. For size, PDI and zeta
327 potential, a similar trend was observed, showing a positive coefficient for CL113 and TPP
328 and a negative coefficient for the CL113 and TPP interaction (CL113*TPP).

329 A reduction in size, PDI and ZP was observed with the interaction of CL113*TPP, whereby
330 TPP was found to have the strongest effect with the highest coefficient (table 3). In general,
331 all experiments were found to produce nanoparticles within the optimal physicochemical
332 characteristics for oral delivery systems (El-Shabouri, 2002; Sarvaiya & Agrawal, 2014; Yun,
333 Cho, & Park, 2013b). These physicochemical results are in agreement with other studies,
334 previously reporting an optimal ratio of chitosan: TPP between 4-8: 1 (Abdel-Hafez, Hathout,
335 & Sasmour, 2014b; Calvo et al., 1997). For EE %, a different response was found, whereby
336 TPP and CL113 both showed a negative coefficient and CL113*TPP a positive, indicating a
337 reduction of EE % with CL113 and TPP on its own and an increase of EE % with the

338 interaction of CL113 and TPP. LKP was seen to have a more pronounced positive coefficient
 339 for CL113*TPP (1811) compared to IPP (715.7), indicating a stronger interaction for LKP.
 340 **Table 4** Coefficients of polynomial regression equation of LKP vs IPP for normalised (a)
 341 Size, (b) PDI, (c) zeta potential and (d) EE % for LKP and IPP where Coef is coefficient

	LKP	IPP
	Coefficient	Coefficient
(a)		
CL113	207.1	212.0
TPP	1366.7	1238.6
CL113*TPP	-1230.1	-1165.1
(b)		
CL113	0.5	0.5
TPP	9.6	3.2
CL113*TPP	-7.0	-2.9
(c)		
CL113	30.9	30.9
TPP	181.5	177.5
CL113*TPP	-150	-149.9
(d)		
CL113	-3.5	-8.7
TPP	-648.5	-167.9
CL113*TPP	544.8	209.1

342 Taking this into account, the EE % was found to be different for IPP and LKP, within the
 343 experimental conditions. Results showed that, for formulation B (Ratio 6:1 CL113: TPP), a
 344 high EE % was produced for LKP (65 ±4%) and a lower EE % of 30 ±12% for IPP. A
 345 decrease of the CL113: TPP ratio for IPP nanoparticles resulted in an increase of
 346 encapsulation, as shown in Figure 3. Formulation D (Ratio 4:1 CL113: TPP) produced the
 347 best IPP EE % with lowest variability (44 ±6%).



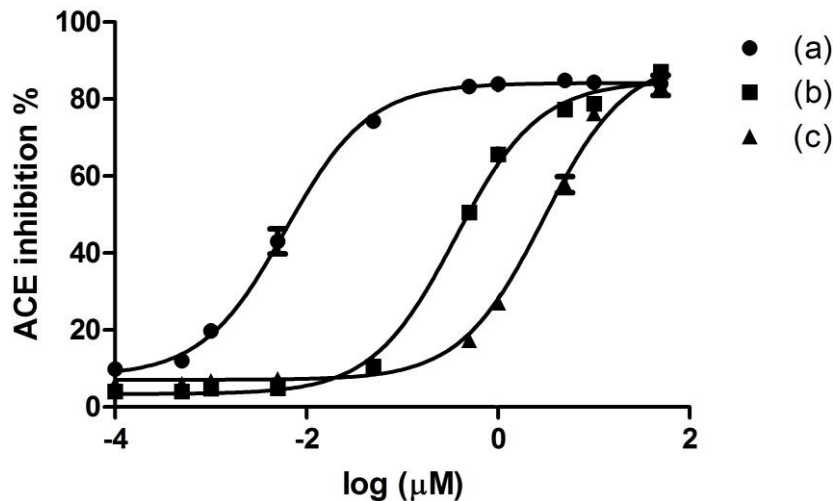
348

349 **Figure 4** Comparison of encapsulation efficiency of LKP-loaded nanoparticles and IPP-
 350 loaded nanoparticles at different ratios (CL113/TPP)

351 3.4 Comparison of ACE inhibitory activity of LKP and IPP and nanoparticles

352 The ACE inhibitory activity of LKP and IPP were compared to captopril, a known ACE
 353 inhibitor, using the substrate, HHL. A concentration-response curve was plotted to determine
 354 the IC_{50} values (figure 5). The IC_{50} for captopril, LKP and IPP were calculated to be $0.006 \pm$
 355 $0.002 \mu\text{M}$, $0.36 \pm 0.01 \mu\text{M}$ and $3.1 \pm 0.6 \mu\text{M}$, respectively. The lowest IC_{50} value was
 356 obtained for captopril, followed by LKP than IPP.

357



358

359 **Figure 5** ACE inhibitory effect on (a) Captopril (b) LKP and (c) IPP. Data are expressed as
 360 the mean \pm SD. Differences among groups were evaluated by one-way ANOVA. For all
 361 measurements, six replicates for three separate experiments were taken for data analysis.

362 When comparing all three ACE inhibitors, a significant difference is observed at 95%
 363 confidence interval (table 5). The ACE inhibitory effects (%) of the peptides released from
 364 the nanoparticles were also determined. The amounts of peptide released from the NPs were
 365 190 μ M LKP and 102 μ M IPP. Peptides were diluted to 10 μ M and were measured for ACE
 366 inhibition using the same assay. A 10 μ M peptide dose of freshly loaded NPs was sonicated
 367 for 1 min without pulse break and ACE inhibition measured for IPP (84 \pm 6%) and LKP (85 \pm
 368 3%). Peptide from controlled release experiments was also collected and assayed for ACE
 369 inhibition, resulting in equivalent LKP (83 \pm 7%) and IPP (82 \pm 5%) bioactivity. Furthermore,
 370 these estimates are comparable to the bioactivity of equivalent doses of fresh IPP and LKP
 371 reported in figure 5 (~80%). These two results confirm that the peptides retained their
 372 bioactivity even after the nanoencapsulation and release process.

373

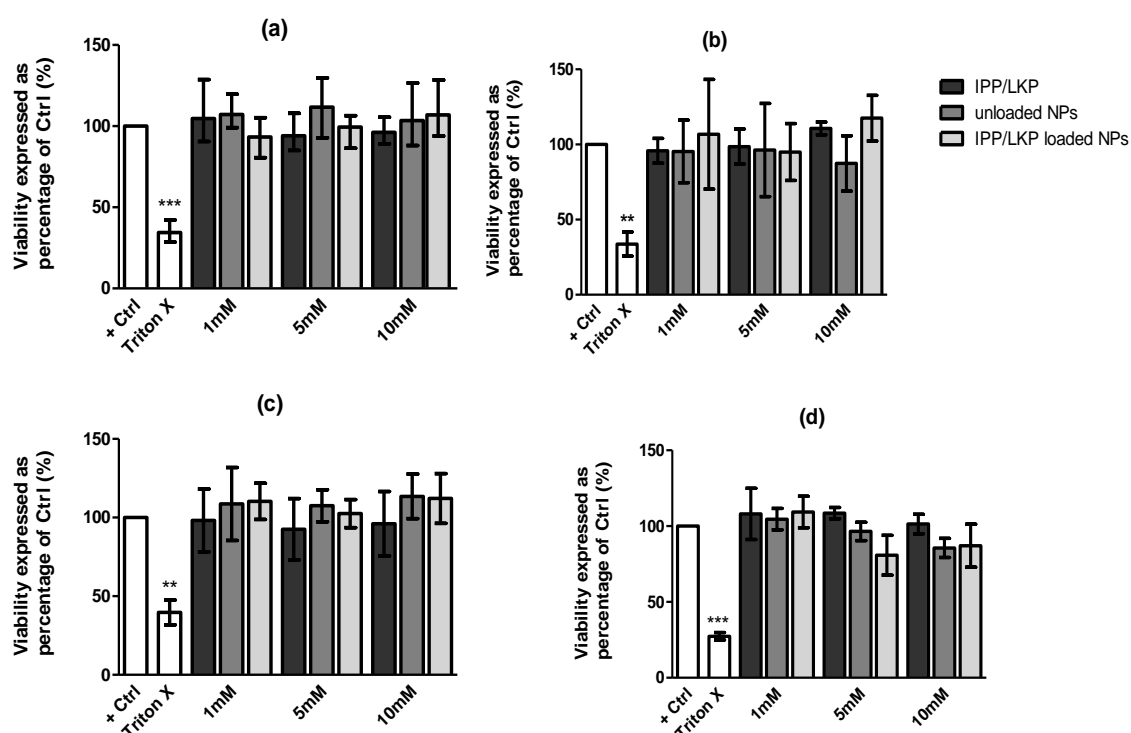
374 **Table 5** Comparison of the IC₅₀ of LKP and IPP at 95% confidence intervals

Lower (μ M)	Est. (μ M)	Upper (μ M)
------------------	-----------------	------------------

Captopril	0.005	0.006	0.007
IPP	2.336	2.819	3.301
LKP	0.309	0.367	0.425

375 3.4 Comparison of Cytotoxicity of LKP and IPP

376 *In vitro* cytotoxicity assessment was performed in Caco-2 and HepG2 cell lines at different
377 exposure times, to mimic the gastro intestinal and liver exposure of the nanoparticles. It has
378 already been reported in the two cell lines that, at therapeutic doses of LKP and IPP (1, 5 and
379 10 mM), no cytotoxicity was observed (Gleeson et al., 2015). Using the same therapeutic
380 dose ranges, IPP and LKP nanoparticles were tested on Caco-2 and HepG2 cell lines and no
381 significant decrease of cell viability was found compared to the negative control using the
382 MTS viability assay (figure 6).



383

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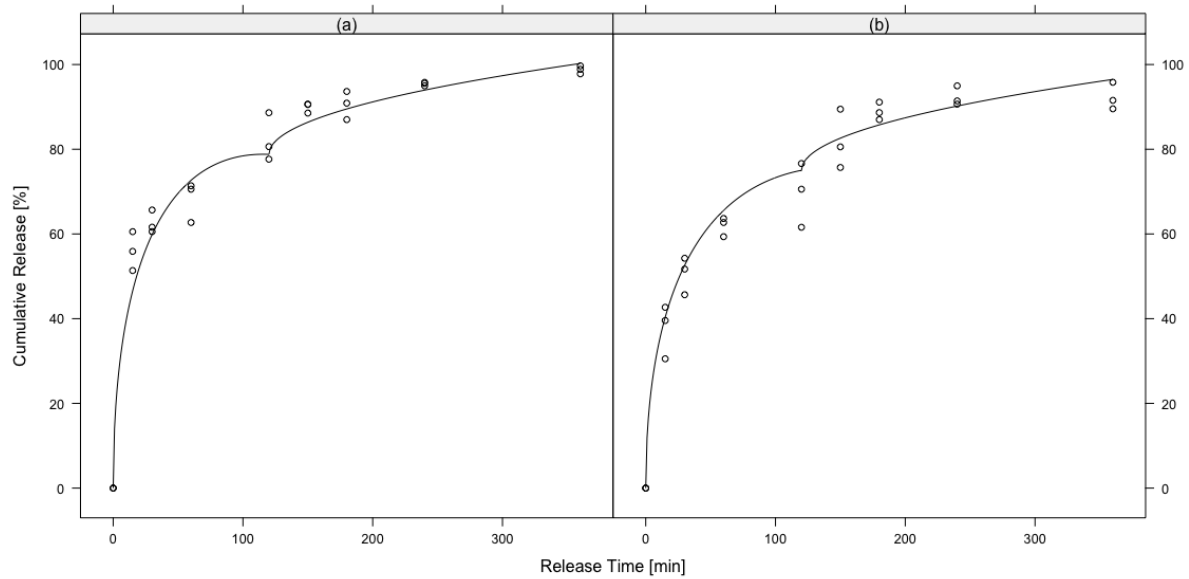
385 **Figure 6** Comparison of cytotoxicity of LKP formulations, as measured using the MTS
386 assay, in (a) Caco2 cell lines 4h exposure and (b) HepG2 cell lines 72h exposure. IPP
387 formulations exposed to (c) Caco2 cell lines exposed to 4h and (d) HepG2 cell lines exposed
388 to 72h. Triton X (0.05%) and 100% cell viability (no treatment) were used as the positive and

389 negative control. Data were expressed with 1-Way ANOVA with Dunnetts's post-test *** P<
390 0.001, ** P< 0.01. Each value represents the mean \pm SD. n = 3 independent experiments for
391 each concentration and time point with replicates of three.

392 3.5 *In-vitro* release kinetics of IPP and LKP-loaded nanoparticles

393 The comparative release behaviour of LKP and IPP nanoparticles was studied in SGF and
394 SIF. *In-vitro* controlled release of IPP and LKP nanoparticles showed an initial burst release
395 in the first 2 h at pH 1.2, followed by a 4 h sustained profile at pH 6.8, such that, after 6 h, a
396 cumulative release up 95% was observed for both peptide systems, showing a similar end
397 result.

398 To further understand the release mechanism of IPP and LKP nanoparticles, the dissolution
399 experimental results were fitted using the Peppas kinetic model (Section 2.8.1, (4) and (5)).
400 This model is based on a diffusion-controlled phenomenon, and is commonly used to
401 describe the release profile of polymer based delivery systems (Pandita et al., 2014; Wilson et
402 al., 2010; Jie Wu, Ding, Chen, Zhou, & Ding, 2014). For our experiment, the model for
403 relaxation release (for swellable systems) was observed to fit the experimental observations
404 well. Figure 7 presents the obtained results fitted using a nonlinear regression equation, a
405 modified Higuchi /Peppas equation reported by Siepmann & Peppas (2011, shown in section
406 2.7.1 (equation 4 and 5).



407

408 **Figure 7** Release studies of (a) IPP-loaded and (b) LKP-loaded nanoparticles exposed for a
 409 total time of 720 min (6h) :120 min in SGF followed by 240 min in SIF separated by dashed
 410 lines (n= 3).

411 Table 6 presents the fitted values for the rate constants, k_{s1} , k_{s2} , according to Equations 4 and
 412 5 for LKP and IPP nanoparticles in each simulated GI region. This model predicted the
 413 experimental data well with an estimated R^2 adj of 0.98. For each peptide loaded
 414 nanoparticle, a combination of diffusive and relaxation phenomena was observed in the SGF.
 415 In this medium, k_{s1} and k_{s2} were significantly different for IPP and LKP. IPP was found to
 416 exhibit a higher release rate in the SGF than LKP, a difference of $k_{s1} = 4.27 \pm 0.9$ and k_{s2}
 417 $= 0.34 \pm 0.09$ of IPP vs LKP SGF release.

418 After 2 h incubation in pH 1.2, the nanoparticles were placed in pH 6.8, SIF. No significant
 419 changes were found for the rate constant (k_{i1}) for both IPP and LKP nanoparticles having a
 420 k_{i1} of 1.39 ± 0.15 . No statistically significant ($p < 0.05$) k_{i2} relaxation process parameter was
 421 found, and the same rate for LKP and IPP was observed with no significant difference,
 422 indicating that, in the small intestine (jejunum) at pH 6.8, diffusion is the primary mechanism

423 of release for both peptides. ACE inhibition assay was also carried for the last sample (time =
 424 720 min), no change in bioactivity was observed after NP release.

425 **Table 6** Parameter estimates of Equation 4/5 for LKP and IPP. All parameters were
 426 statistically significant ($p < 0.05$).

Release parameters	Estimate
k_{s1} IPP	$15.65 \pm 0.66 \text{ min}^{-1/2}$
k_{s1} LKP	$11.38 \pm 0.66 \text{ min}^{-1/2}$
k_{s2} IPP	$-0.76 \pm 0.07 \text{ min}^{-1}$
k_{s2} LKP	$-0.42 \pm 0.07 \text{ min}^{-1}$
k_{i1}	$1.39 \pm 0.16 \text{ min}^{-1/2}$
R^2 adj	0.98
Residual SE	4.74

427 **4. Discussion**

428 Considering the structural characteristics of IPP and LKP (figure 1), both consist of 3 (LKP)
 429 and 4 (IPP) chiral centres. The formulation of chitosan nanoparticles is obtained by an
 430 electrostatic interaction of the protonated carboxyl (TPP and peptide) interacting with the
 431 deprotonated amine of acidic chitosan (see arrows in figure 4). In addition to this, other non-
 432 covalent inter and intra molecular interaction may also take place between chitosan, TPP and
 433 peptide. This depends on the number of different hydrogen bond donors (HBD) and acceptors
 434 (HBA) available to interact. IPP has 2 HBD and 7 HBA and, LKP has 4 HBD and 8 HBA,
 435 presented in table 7. LKP was found to have a factor of 2 more HBD than IPP, which may
 436 possibly provide better interaction with CL113, as it contains numerous HBA in its polymeric
 437 structure, available to interact with, having a solvent accessible surface area of 2014.39Å.
 438 IPP and LKP have solvent accessible surface areas of 518.48 and 587.97Å (chemicalize.org,

439 2016), respectively, contributing to the differences in encapsulation efficiency, as there is
440 more surface area available for LKP.

441 **Table 7** Number of HBD and HBA for LKP and IPP.

Bond	LKP		IPP	
	HBD	HBA	HBD	HBA
R-OH	1	1	1	1
R=O	0	3	0	3
R-N	0	1	0	2
R-NH	1	1	0	0
R-NH ₂	2	2	1	1

442 Proteins and polypeptides show strong characteristic CD spectra (Greenfield, 2006) but those
443 of tripeptides have also been reported to be characteristic of stable side-chain specific
444 conformations of such peptides (Gokce et al., 2005). Investigations of tri-alanine, tri-valine,
445 tri-lysine and glycerol conformation indicated more ordered, flat β sheet-like conformations
446 rather than what is typically known for short peptides, random coil (Eker et al. 2003). Proline
447 is a cyclic structure which provides rigidity as it locks its ϕ -backbone dihedral angle at
448 $\sim -75^\circ$, giving proline an exceptional conformational rigidity compared to other amino acid
449 groups (Cheng, Cetinkaya, & Gräter, 2010). It has previously been reported that Proline-rich
450 residues are common recognition sites for protein–protein interaction modules (Zarrinpar,
451 Bhattacharyya, & Lim, 2003). Others reported proline based amino acids to have relatively
452 high intrinsic probability of predominately existing as the cis rather than the trans conformer
453 of the preceding peptide bond, compared with other amino acids (Young Ah Shin, Seong Jun
454 Han, 1993). As seen in figure 2, LKP and IPP showed different conformational
455 characteristics. The proline-rich residues in IPP cause more rigidity and steric hindrance to
456 the overall structure, ultimately affecting the intra and intermolecular interactions with
457 chitosan. On the other hand, LKP only has one proline residue, which promotes bending of

458 the structure with less overall rigidity, resulting in significantly lower minimised total energy
459 compared to IPP. CD analysis further confirmed such differences, resulting in an inverse
460 spectrum for both peptides; a (disordered) β -strand was found for LKP and an unordered
461 peptide structure was for IPP. Chitosan is a semi crystalline polymer that exhibits
462 polymorphism, in its liquid phase (Kumirska et al., 2010). CD showed a mixed conformation
463 between a μ -helical and β -sheet.

464 LKP loaded nanoparticles were found to produce better overall characteristics for a
465 nanoparticle delivery system, in comparison to the IPP counterpart. LKP nanoparticles
466 showed higher overall encapsulation efficiency and a slower release profile. LKP showed
467 best EE % at a CL113: TPP ratio of 6:1 (Formulation B) and IPP at 4:1 (Formulation D). This
468 can possibly be due to the number of exposed interactive sites the peptide had with chitosan
469 and the conformation the peptide, IPP needing more TPP (i.e. lower ratio CL113: TPP) to
470 increase the overall negative charge for electrostatic interaction amongst chitosan and IPP,
471 presented in table 4. This result for IPP is in agreement with Gan & Wang (2007), who found
472 that an increase of encapsulation was achieved with a decrease of ratio in bovine serum
473 albumin protein. The authors proposed a possible explanation, that the larger TPP mass may
474 increase the solution pH value, ultimately increasing the overall negative surface charge
475 carried by the protein molecules, which enhanced electrostatic interactions (Gan & Wang,
476 2007). Taking this into account, LKP showed the highest EE % at ratio 6:1 (less TPP) and
477 IPP at ratio 4:1 (more TPP).

478 The results of the bioactivity assessment of the peptides are in agreement with the findings of
479 other groups; for example, Henda et al. (2013) reported an IC_{50} value of $0.0151 \pm 0.005 \mu M$
480 for captopril and Stanton (2011) reported the IC_{50} value of LKP as $0.32 \mu M$. Different IC_{50}
481 values have been reported for IPP, varying from 1.89 to $5 \mu M$ (Siltari, Viitanen,
482 Kukkurainen, Vapaatalo, & Valjakka, 2014; van Platerink, Janssen, & Haverkamp, 2007). It

483 has been reported that peptides with specific amino acids at the N- and C-terminus have
484 shown variable inhibition activity with ACE (Iwaniak, Minkiewicz, & Darewicz, 2014). Wu
485 et al. (2006) reported the most favourable tripeptides for ACE inhibition; at the C terminus
486 onwards were aromatic acids, to the second position were positively charged amino acids
487 (middle amino acid) followed by hydrophobic amino acids for the N terminus. Taking this
488 into account, LKP follows a middle positively charge amino acid (Lys) with a hydrophobic N
489 terminus (Pro) and IPP only follows a hydrophobic N-terminus (Pro). Both LKP and IPP
490 have shown ACE inhibition, but with different IC_{50} values, whereby LKP exhibits almost 10
491 fold higher activity *in-vitro* compared to IPP. To confirm that all bioactivity of the peptide
492 was retained after *in-vitro* release studies (spontaneously released NPs); an ACE inhibition
493 assessment of the last sample point was assessed. Results showed no reduction in ACE
494 inhibition compared to the positive control. No degradation or denaturation was observed
495 after in-vitro and control (sonicated release). It has been recently reported that IPP and LKP
496 are stable against low pH, intestinal and liver peptidase, indicating their ability to retain after
497 enzymatic processes (Gleeson et al. 2015). Additionally, MTS cytotoxicity assessment for
498 native and NPs of LKP and IPP found no reduction in cell viability at 1-10 nM concentration.
499 The *in-vitro* release profiles observed in SGF (2 h) at pH 1.2 and SIF (4 h) at pH 6.8 for 6 h,
500 in both optimal peptide formulations were different. The type of bioactive used and ratio of
501 CL113: TPP are the two driving factors that will contribute to the overall release of the
502 particles. Several groups have reported different release profile of different drugs at different
503 ratios, many demonstrating that, at lower ratio (CL113:TPP), a faster release is observed
504 (Gan & Wang, 2007), although others have found that, at lower ratios (CL113:TPP), a slower
505 release is attained (Kouchak & Azarpanah, 2015; Remuñán-López & Bodmeier, 1997). In
506 this case, for both formulations, a burst followed by relaxation (k_{s2}) in SGF and a sustained
507 release in the SIF was observed, but with significant differences in rate constants in the SGF

508 (k_{s1} and k_{s2}). IPP showed a significantly faster release in the acidic pH compared to LKP;
509 this can possibly be due to the rigidity and steric hindrance of IPP restricting the interaction
510 with chitosan. The higher burst of release for IPP may possibly signify more loosely bound
511 IPP in/around the nanoparticle. Both IPP and LKP produced the same intestinal rate constant
512 $k_i = 1.39 \text{ min}^{-1/2}$, consistent with a diffusive release profile. The target site for IPP and LKP
513 absorption is the jejunum in the small intestine. In order for the peptide to provide its
514 inhibitory affect, it needs to bypass the stomach/acid pH and other enzymatic process. For
515 both peptide formulations, over 60% release was observed within the first 2h in the acidic
516 simulated fluid. Similar findings have been reported by Sarmiento et al. (2007), and Ryan et
517 al. (2013) demonstrated up to 50 and 40% release from chitosan based formulations. This
518 suggests that an enteric coating will facilitate to prevent release in the stomach.

519 **5. Conclusion**

520 LKP and IPP were successfully encapsulated into chitosan nanoparticles. The molecular
521 modelling, supported by the CD analysis, indicates that LKP is different to IPP, the former
522 showing better interaction with chitosan. Such differences in the structural characteristics of
523 LKP and IPP resulted in differences in the overall encapsulation efficiency and release
524 properties of the bioactive.. LKP and IPP are quite similar as ACE inhibitors, but yet
525 different, demonstrating that the characteristics of drug interaction with chitosan is important
526 and needs to be investigated for any future peptide formulations. Overall LKP was found to
527 have better compatibility with chitosan because of increased flexibility, more hydrogen bond
528 donors, better encapsulation of the peptide with the particle and release compared to IPP
529 nanoparticles. However the release of the bioactive (IPP and LKP) still needs to be further
530 optimised to overcome the burst release in the stomach pH 1.2

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