



Oligomerization of a Glucagon-like Peptide 1 Analog: Bridging Experiment and Simulations

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1 **Oligomerization of a Glucagon-like Peptide 1 Analogue:**
2 **Bridging Experiment and Simulations**

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26

27 **ABSTRACT**

28 The glucagon-like peptide 1 (GLP-1) analogue, liraglutide, is a GLP-1 agonist and is used in
29 the treatment of type 2 diabetes mellitus and obesity. From a pharmaceutical perspective, it is
30 important to know the oligomerization state of liraglutide with respect to stability. Compared
31 to GLP-1, liraglutide has an added fatty acid (FA) moiety that causes oligomerization of
32 liraglutide as suggested by small-angle X-ray scattering (SAXS) and multi angle static light
33 scattering (MALS) results. SAXS data suggested a global shape of a hollow elliptical
34 cylinder of size hexa-, hepta or octamer, whereas MALS data indicate a hexamer. To
35 elaborate and further the explanation of the stability of these oligomers and the role of the FA
36 chains, a series of molecular dynamics simulations were carried out on 11 different hexa-,
37 hepta- and octameric systems. Our results indicate that interactions of the fatty acid chains
38 contribute noticeably to the stabilization. The simulation results indicate that the heptamer
39 with paired FA chains is the most stable oligomer when compared to the 10 other
40 investigated structures. In agreement with the SAXS data, the heptamer forms a water-filled
41 oligomer of elliptical cylindrical shape. Theoretical SAXS curves extracted from the
42 simulations qualitatively agree with the experimentally determined SAXS curves supporting
43 the view that liraglutide forms heptamers in solution.

44

45 INTRODUCTION

46 The glucagon-like peptide 1 (GLP-1) receptor is a well-established therapeutic target for the
47 treatment of type 2 diabetes mellitus (T2DM) (1–3), and extensive research has established
48 the physiologic roles of GLP-1 and its endogenous receptor in regulating glucose homeostasis
49 and energy metabolism (4, 5). GLP-1-(7-37) is a 31 amino acid incretin hormone secreted by
50 the endocrine L cells in the gut wall upon glucose intake (6), and is secreted in response to
51 the nutrient content of the gastrointestinal tract and thus potentiate insulin exocytosis from
52 pancreatic β -cells in a glucose-dependent manner (6, 7). Additionally, GLP-1 suppresses
53 appetite, glucagon secretion, and gastric emptying, all of which contribute to inhibition of the
54 postprandial rise in plasma glucose concentrations (8). GLP-1 is responsible for up to 60 % of
55 the postprandial insulin response (9). Recent studies show that GLP-1 is not only a key factor
56 in T2DM treatment, but also has potential in the treatment of obesity (1, 10) and has shown
57 positive effects on neuroprotection in animal models (11), which can potentially be used for
58 the treatment of Parkinson's and Alzheimer's disease (12–15).

59 While GLP-1 is interesting for a pharmaceutical application, it cannot be used for routine
60 treatment, since its biological half-life is only a few minutes (16, 17). The short insulinotropic
61 action of GLP-1 results from the degradation of the peptide by dipeptidyl-peptidase IV (DPP
62 IV) and rapid renal clearance due to its relatively small size (18–20). DPP IV degrades GLP-
63 1 at the N-terminus by cleaving off the first two amino acids, generating the biologically
64 inactive fragment, GLP-1-(9-37) (9, 21). To overcome this shortcoming, different strategies
65 have been used. Those include: *i*) incorporation of the peptides in injectable microspheres; *ii*)
66 fusion with larger carrier molecules like albumin or fragment crystallizable region of
67 immunoglobulin G or polyethylene glycol; *iii*) attachment of a fatty acid (FA) directing
68 oligomerization and promoting reversible binding to endogenous human serum albumin
69 (HSA) (20). All three approaches result in an increased half-life partly due to the increased
70 size of the drug minimizing the renal clearance mechanism. The latter approach, for instance,
71 has been utilized in designing the GLP-1 analogue: liraglutide (Victoza®, Novo Nordisk
72 A/S) (22, 23). An in depth understanding of the oligomerization state of liraglutide is not only
73 instrumental in the understanding of the increased half-life observed for this molecule but
74 also to ensure a stable oligomeric state, since uncontrolled and extensive oligomerization can
75 drive fibrillation (24). As shown in Fig. 1, liraglutide, a C-16 acyl chain (palmitoyl) is linked
76 to Lys26 via a γ -glutamic acid spacer, and the lysine in position 34 of the native GLP-1
77 sequence is exchanged with arginine to ensure homogenous palmitoylation at position 26 (25,
78 26). The general understanding is that the acyl chain allows a non-covalent binding to
79 albumin, which delays both proteolytic inactivation by DPP-IV and renal clearance, resulting
80 in a biological half-life of about 13-14 hours and allowing once-daily administration (2, 23).
81 A further prolongation may also be caused by the fatty acid chain that may sterically hinder
82 DPP IV from degrading liraglutide (5). Furthermore, studies have shown that one way to
83 stabilize GLP-1 is to add a clustering agent that causes the peptide to oligomerize (2), thus,
84 the FA chain in liraglutide could act as a clustering agent.

85 Although the pharmacological efficiency of liraglutide has been established (23, 27), there is
86 a lack of a molecular understanding of the solution structure of liraglutide. Using analytical
87 ultracentrifugation, Steensgaard and co-workers could show that liraglutide oligomerizes in a
88 concentration independent manner forming predominately heptamers in the concentration
89 range of 0.004–4.501 mg/mL (28). Recently, Wang and co-workers studied the pH
90 dependence of the size and secondary structure of liraglutide oligomers using light-scattering
91 and circular dichroism, respectively (29). The authors report a transformation from an

92 octamer to a dodecamer at pH 6.4 and 6.9 with subsequent partial loss of the α -helical
93 structure of liraglutide. Furthermore, it has been shown that the oligomerization of GLP-1
94 and similar peptide analogues is dependent on the pH and ionic strength (30), and thus
95 different solution structures may exist (3, 31).
96 In order to get further insight in the solution structure of liraglutide, we have performed a
97 series of molecular dynamics (MD) simulations, and simulation results are compared with
98 results from small angle X-ray scattering (SAXS) and multi angle static light scattering
99 (MALS) experiments.

100

101 MATERIAL AND METHODS

102 SAXS: 3 ml commercial Victoza® (contains 18 mg liraglutide (free-base, anhydrous) and the
103 following inactive ingredients: disodium phosphate dihydrate, 1.42 mg; propylene glycol, 14
104 mg; phenol, 5.5 mg in aqueous solution (32)) was dialyzed against 3×1 L buffer containing
105 approximately 0.47 mg/mL (0.376 mM) Na₂HPO₄•2H₂O, pH 8.1, over 3 days. Concentration
106 determinations were performed with the NanoDrop® 1000 Spectrophotometer from Thermo
107 Scientific at 280 nm. The extinction coefficient was calculated to be 6990 cm⁻¹M⁻¹ with the
108 ProtParam (33) tool from ExPASy.org (34) using the primary sequence of the protein.

109 SAXS measurements were performed at the MAX IV laboratories at beamline I911-SAXS,
110 Sweden (35). The sample detector distance and the direct beam position was calibrated using
111 silver behenate (AgBe). Parameters are shown in Table S2 in the Supporting Material.
112 Measurements on pure water were used to get the data on an absolute scale. Buffers were
113 measured both before and after each sample and averaged before subtraction. The sample size
114 was approximately 50 µL injected manually in a flow cell. Measurements were performed on
115 a series of liraglutide samples at approximate concentrations: 1 mg/mL, 2 mg/mL, and
116 4.7 mg/mL.

117 All calibrations and corrections of the SAXS data were done using the in-house software
118 Bli911-4 (35). Buffer averaging and subsequent subtraction prior to data analysis was done in
119 Primus (36). The ATSAS program package version 2.4 (37) was used for further data
120 analysis. Evaluation of the Guinier region was performed within Primus. The pair distribution
121 function, $p(r)$, was evaluated using the interactive program Gnom (38).

122 AF4-UV-MALS: Asymmetric flow field flow fractionation (AF4) separation was performed
123 using a Dionex, Thermo Scientific UltiMate 3000 autosampler and pump (Thermo Fisher
124 Scientific Inc., Waltham, MA, USA) connected to a Wyatt Eclipse AF4 separation system
125 (Wyatt Technology Europe GmbH, Dernbach, Germany) followed by a Dionex, Thermo
126 Scientific UltiMate 3000 RS variable wavelength UV detector set at 280 nm and a Wyatt
127 Dawn Heleos-II 18-angle MALS detector. Separations were performed using a 10 kDa
128 molecular weight cutoff polyethersulfone (PES) membrane in a 17.5 cm separation channel
129 with an S-350 µm spacer. Samples were introduced to the channel at 0.2 mL/min and
130 subsequently focused at the head of the channel at a focus flow rate of 1.5 mL/min. Samples
131 were eluted over 15 min with a channel flow rate of 1 mL/min and a cross flow gradient of
132 4.0 to 2.5 mL/min. Undiluted Victoza® (6 mg/mL liraglutide) and 10x diluted Victoza®
133 (diluted with eluent) was injected and eluted with 20 mM phosphate, 100 mM NaCl, 0.05 %
134 NaN₃, pH 8.1, filtrated through a 0.1 µm filter. Different injection volumes of undiluted and
135 diluted Victoza® were used, and the resulting mass loads were 6, 12 and 18 µg liraglutide.
136 The molecular weight of liraglutide was calculated using the Wyatt Astra software version
137 6.1.2 with $dn/dc = 0.185 \text{ mL/g}$ and UV extinction coefficient (280nm) = 6990 cm⁻¹M⁻¹.

138 Molecular dynamics (MD) simulations: Several orientations and oligomers of liraglutide have
139 been investigated. All are based on the solution NMR structure of liraglutide (pdb entry:
140 4APD) obtained from the Protein Data Bank (39). The coordinates from the pdb file were
141 copied, translated and rotated in a circle with a radius of 20 Å, corresponding to the results
142 from SAXS experiments. This resulted in several oligomers containing six, seven, or eight
143 monomers, respectively. A set of oligomers was created where the monomers were oriented
144 so that two FA chains were paired in the direction of the elliptical cylinder arrangement,
145 which hereafter will be referred to hexa-, hepta-, and octamer systems (Fig. 2). In the

146 heptamer, one monomer was oriented with the FA chain pointing outward of the elliptical
147 arrangement.

148 Furthermore, another set of oligomers were created where some of the monomers were
149 flipped upside down to see if interactions between the C- and N-terminal charges would
150 stabilize the structures. For this set of oligomers, the hexa- and one octamer were made with
151 every second monomer flipped upside down. In the case of the heptamer, only the monomer
152 with the FA chain pointing outward was flipped upside down. Also, another octameric
153 structure was prepared where every second monomer pair was flipped upside down. These
154 configurations are hereafter referred to as AA6_3ud, AA7_1ud, AA8_4udp, and AA8_4uds
155 systems where ‘ud’, ‘p’, and ‘s’ are short for upside down, pair, and single, respectively (*Fig.*
156 2). To clarify the extent of the stabilizing effect of the FA chain on the structures, another set
157 of oligomers were created. One oligomer is constructed according to the rotation and
158 translation of the first heptamer, but it does not contain any FA chains (hereafter referred to
159 as AA7_glp1 and represented in *Fig. 2*). Three other oligomers were also created; a hexa-,
160 hepta-, and octamer where all the monomers are rotated so that the FA chains are pointing
161 outward of the elliptical arrangement. These will hereafter be referred to as the AA6_FAout,
162 AA7_FAout, and AA8_FAout systems (*Fig. 2*). This gives a total of 11 oligomeric structures.

163 The structures were solvated using the program Solvate from the H. Grubmüller research
164 group (40). Water molecules were described by the TIP3 water model (41). Next, the systems
165 were neutralized by adding 3 Na⁺ ions per monomer. Simulations were performed at an ionic
166 strength of 0.1 M NaCl (see details in Table 1). All simulations were performed using the
167 computer program NAMD (42) with the CHARMM36 force field (43). The same simulation
168 parameters were used as described by Madsen et al. 2011 (44). See Supporting Material for
169 detailed description. Analyses of the trajectories were carried out in VMD (45).

Table 1: System and simulation details of the 11 oligomeric systems.

Structure	No. of atoms	No. of waters	No. of NaCl	Initial box size (Å)	Simulation time (ns)
Hexamer	140203	45615	86	113×118×115	69
Heptamer	53774	16665	31	83×88×89	129
Octamer	136414	44000	83	113×115×115	71
AA6_3ud	57419	18055	34	91×88×85	34
AA7_1ud	57090	17769	33	91×87×87	39
AA8_4udp	54814	16834	32	91×85×85	41
AA8_4uds	54928	16872	32	91×85×85	43
AA6_FAout	52289	16247	31	83×90×85	21
AA7_FAout	53114	16445	31	83×88×89	51
AA8_FAout	54937	16875	32	83×90×90	21
AA7_glp1	51729	16131	30	83×87×87	69

170
171 Theoretical SAXS curves: The program CRY SOL (46), which is part of the ATSAS package
172 version 2.6 (37), was used to compare the SAXS curves of the structures extracted from the
173 MD simulations with the experimental measured SAXS curve of the oligomer. CRY SOL
174 calculates the scattering intensity based on the atomic coordinates of the protein and adds a
175 hydration layer simplified as a continuous outer envelope (47).

177 **RESULTS**

178 The following section is structured as follows: First, experimental results from SAXS and
 179 A4F-UV-MALS are presented followed by the computational results.

180 SAXS: SAXS intensity curves of liraglutide measured at different concentrations are shown in
 181 Fig. 3. Repulsion is observed already at 1 mg/mL while the shape of the curve is consistent
 182 over the concentration range reflecting a similar shape of molecule. This is also reflected in
 183 the Kratky plots shown in Fig. S1 in the Supporting Material. Corresponding pair distribution
 184 functions are provided in Fig. S2.

185 Table 2 summarizes the extracted data analysis parameters. The radius of gyration (R_g),
 186 maximum particle diameter (D_{max}) and $I(0)/c$ show a slight decrease with concentration as
 187 expected from the repulsive behavior.

Table 2: Parameter overview extracted from SAXS measurements.

Concentration mg/mL	R_g Guinier (Å)	R_g $p(r)$ (Å)	D_{max} (Å)	$I(0)$ Guinier	$I(0)$ $p(r)$	$I(0)/c$	Molecular Weight (kDa)
1	23.1	23.8	82.0	0.021	0.021	0.021	26
2	21.7	22.3	75.9	0.040	0.041	0.020	25
4.7	22.2	22.2	74.2	0.096	0.096	0.020	25

188
 189 The partial specific volume, v , used for calculating the molecular weight is chosen to match
 190 pure protein and is set to the average value of $0.73 \text{ cm}^3/\text{g}$. To compare the experimental
 191 results with the model structures extracted from the simulations, the experimental data were
 192 extrapolated to $q = 0$ to avoid inter-particle repulsion.

193 AF4-UV-MALS: Analysis of liraglutide showed a single peak and a uniform molecular weight
 194 across the peak in all analyses (Fig. 4, left) indicating that repulsive behavior is negligible at
 195 the concentration range found in the detector. The average molecular weight across the peak
 196 was 22 kDa, which corresponds to a hexamer assuming a monomer molecular weight of 3.7
 197 kDa. Undiluted Victoza® (6 mg/mL liraglutide) and 10× diluted Victoza® was analyzed and
 198 different injection volumes were used, which resulted in mass loads of 6, 12 and 18 μg
 199 liraglutide. Liraglutide is diluted during analysis by the eluate, and the resulting liraglutide
 200 concentration was quantified in the eluate passing the UV detector (Fig. 4, right). Peak
 201 liraglutide concentrations of 0.012, 0.024 and 0.036 mg/mL were observed.

202 Molecular dynamics simulations: Simulations were performed on several sets consisting of
 203 hexa-, hepta-, and octamer oligomers to study the structural arrangement and stability of the
 204 oligomers including the role of the FA in promoting the stability of the oligomers. The last
 205 structures taken from the simulations are shown in Figs. S3, S4, and S5.

206 Figs. S3, S4, and S5 show that although the internal structures for all 11 oligomers are highly
 207 distorted compared to the start structures (Fig. 2), all of them but AA6_3ud (Fig. S4 a))
 208 maintain a tunnel-like structure which is, however, more or less flattened and resembling an
 209 elliptical shape.

210 The solvent accessible surface area of the oligomers seen in Fig. 5, S6, and S7 relates to the
 211 packing of the monomers.

212 Overall, the packing of the heptamers appears to be more prominent than the octamers
 213 throughout the simulations indicated by the lower SASA. The AA6_3ud structure has a
 214 significant lower packing than the AA7_1ud, AA8_4udp, and AA8_4uds structures, which
 215 most likely is a result of a complete opening of the elliptical structure as seen in Fig S4 a).

216 The relatively high SASA for the AA6_FAout oligomer could be due to the elongation of
217 some of the monomers which appear to unfold from the helix structure (Fig. S5 a)).

218 The time evolution of the root mean square deviation (RMSD) is shown for the 11 structures
219 in Fig. 6, S8, and S9.

220 From the initial steep increase in RMSD, it is evident that the oligomers rearrange to some
221 extend within the first 6 ns. Furthermore, Fig. 6, S8, and S9 show that the RMSD converges
222 for the hexamer, AA6_3ud, heptamer, AA7_1ud, AA8_4udp, AA8_4uds, AA8_FAout, and
223 AA7_glp1 conformations. These structures appear to be stable when it comes to the overall
224 movement of the systems. The octamer, AA6_3ud, AA6_FAout, and AA7_FAout does,
225 however, not converge. Furthermore, the three hexameric systems and AA7_FAout present a
226 significantly higher RMSD value compared to the other systems.

227 To further monitor the movement of the monomers, the 2D positions of the α -carbon in the
228 FA-Lys linker (Fig. S11) for all 11 oligomer conformations, projected onto the yz plane (the
229 monomers are translated and rotated around the x -axis), are shown as a function of simulation
230 time in Fig. 7, Fig. 8 Fig. 9.

231 From Fig. 7, we can conclude that the spread of the hexamer is larger than for the heptamer
232 and the octamer throughout the simulation. Furthermore, the hexamer is squeezed to give a
233 more flattened shape. Whereas, for the octamer it appears that the elliptical structure is
234 unstable since one monomer appears to migrate from the oligomeric structure. In the case of
235 the heptamer, Fig. 7 b), it is evident that the position of the α -carbon is rather dense
236 throughout the simulation and that the structure resembles an ellipse.

237 Fig. 8 a) shows that the AA6_3ud structure flattens drastically which corresponds to the more
238 open structure seen in Fig. S4 a). The AA8_4udp structure maintains a more elliptical
239 arrangement, even though one monomer seems to be leaving the structure (indicated by the
240 circumference). The AA8_4uds structure presents a lot of movement and gains a squared
241 shape. The AA7_1ud structure is, like the hexamer in Fig. 7 a), squeezed so that the elliptical
242 structure is destroyed.

243 In AA7_glp1, Fig. 9 d), monomers are more mobile than compared with the other structures
244 resulting in a disordered (unstable) structure. In Fig. 9 b), the AA7_FAout structure appears to
245 maintain an elliptical structure, but the movement of the α -carbons is not very spread out. Fig.
246 9 c) shows that the α -carbon movement of the AA8_FAout system is rather centered on the
247 starting position throughout the simulation, which indicates a stable system. However, as
248 seen for the octamer and AA8_4udp systems, one monomer escapes from the elliptical
249 arrangement. The AA6_FAout system (Fig. 9 a)) moves significantly throughout the
250 simulation and this movement results in a flattened structure.

251
252 Mean energy calculations based on the structures taken at every 50 ps throughout the
253 simulations are given in Table 3. The energies calculated for the oligomeric system (peptide-
254 peptide, P-P) and the oligomer-water interactions (P-W) are normalized to the number of
255 monomers in each oligomer to make comparison of the different systems possible. From
256 these, it can be seen that the total P-P van der Waals (vdW) energy for the heptamer is lower
257 than for any of the other oligomers. Considering the P-W interactions, the energy for the
258 heptamer system is less negative than found for the other systems. In general, all the
259 hexameric systems of liraglutide (hexamer, AA6_3ud, AA6_FAout) are higher in P-P vdW
260 energy which might indicate that these structures are less stable than the hepta-, and
261 octameric liraglutide oligomers. The energies show relatively large fluctuations (data not
262 shown) which is also reflected by the relatively large standard deviations. This indicates that

263 internally the 11 oligomeric systems are flexible structures.

Table 3: Mean energies and corresponding standard error of the mean (for P-P and P-W) and standard deviation (for S-S) for the 11 systems are calculated for the simulations. Van der Waals (vdW) energies for the peptide-peptide (P-P), peptide-water (P-W), segment-segment (seg-seg) with FA pairs (S-S FA), seg-seg without FA pairs interacting (S-S), and - for the heptamer – seg-seg interactions for the monomer pairs including the monomer with the FA pointing outward (S-S FA out) are calculated. Energies are also calculated for the systems where all FA chains are pointing outward (S-S all FA out). The energies for the P-P and P-W interactions are normalized according to the number of monomers in the structure. MDEnergy from NAMD was used to calculate the energies in intervals of 50 ps for the simulations.

Structure	P-P vdW (kcal/mol)	P-W vdW (kcal/mol)	S-S vdW (kcal/mol)	S-S FA vdW (kcal/mol)	S-S FA out vdW (kcal/mol)
Hexamer	-188 ± 18.9	-84.7 ± 21.1	-20 ± 4.5	-44 ± 6.1	-
Heptamer	-200 ± 14.8	-68.1 ± 19.3	-37 ± 5.6	-56 ± 4.7	-23 ± 3.8
Octamer	-187 ± 13.7	-84.4 ± 18.0	-21 ± 3.8	-62 ± 4.1	-
AA6_3ud	-185 ± 16.4	-88.1 ± 22.1	-4.6 ± 2.2	-52 ± 7.1	-
AA7_1ud	-191 ± 19.8	-83.0 ± 22.4	-12 ± 4.6	-50 ± 4.7	-38 ± 4.8
AA8_4udp	-189 ± 15.1	-86.8 ± 18.9	-18 ± 3.1	-55 ± 3.5	-
AA8_4uds	-190 ± 16.5	-85.3 ± 21.1	-24 ± 3.6	-54 ± 4.4	-
AA7_glp1	-160 ± 15.1	-84.9 ± 19.0	-23 ± 5.1	-26 ± 5.0	-29 ± 5.2

Structure	P-P vdW (kcal/mol)	P-W vdW (kcal/mol)	S-S all FA out vdW (kcal/mol)
AA6_FAout	-171 ± 13.4	-104 ± 21.5	-23 ± 3.0
AA7_FAout	-187 ± 18.9	-86.5 ± 25.3	-33 ± 3.0
AA8_FAout	-176 ± 13.1	-98.6 ± 22.8	-35 ± 3.0

264
265 The analyses were done for segment-segment interactions where FA chains are facing each
266 other (S-S FA), no FA chains are between them (S-S), one FA chain pointing outward (S-S
267 FA out), and all FA chains pointing outward (S-S all FA out). See Fig. 10 and Fig. 2 for
268 illustration of S-S, S-S FA, S-S FA out, and S-S all FA out (AA6/7/8_FAout structures),
269 respectively. The two last analyses were only done for the heptamer, AA7_1ud, and for the
270 AA6_FAout, AA7_FAout, and AA8_FAout oligomers since these were the only
271 conformations relevant for such investigation. Results for the AA7_glp1 structure are
272 reported as those for the heptamer even though it does not have any FA chains attached, since
273 the monomers in the AA7_glp1 structure are rotated the same way as those in the heptamer
274 with paired FA chains. It can be seen that for the structures with FA pairs, the energies are
275 significantly lower for the monomer pairs that have FA chains facing each other, than those
276 where no FA chains are between them. This supports the view that interactions of the FA
277 chains contribute to the stabilization of the oligomers of liraglutide (2, 5).

278 DISCUSSION

279 The experimental SAXS curves (Fig. 3) show that even at the lowest measured concentration
280 of 1 mg/mL, repulsive interactions between liraglutide oligomers are present which increases
281 with increasing concentration. Repulsive interactions can lead to an underestimation of the
282 molecular weight. The SAXS data suggest that the solution structure of liraglutide is a hexa-,
283 hepta-, or octamer. The uncertainty arises from uncertainties related to the measured
284 concentration and the estimated partial specific volume (v) (and hence the number of
285 monomers in the oligomer). To our knowledge, there is no value of v for liraglutide available
286 in literature. In this study, $v = 0.73 \text{ cm}^3/\text{g}$ (corresponding to an average value for pure
287 proteins) was used. The FA chain could contribute to an increase in the partial specific
288 volume, but to which extent is difficult to estimate. Using $v = 0.74 \text{ cm}^3/\text{g}$ as also reported in
289 literature (48), the molecular weight increases to that resembling an octamer. From the SAXS
290 measurements, it can only be concluded that the solution structure of liraglutide is an
291 oligomer of approximately heptameric molecular size, with a consistent shape of an elliptical
292 cylinder, and that this oligomerization is concentration-independent within the measured
293 range.

294 The MALS results indicate a hexameric solution structure of liraglutide. In contrast to the
295 SAXS data, no repulsion interactions between oligomers were observed in the concentration
296 range of 0.012-0.036 mg/mL. Note that the SAXS data were measured in the concentration
297 range of 1.0-4.7 mg/mL where 1 mg/mL corresponds to the lowest concentration that can be
298 measured.

299
300 Simulation results suggest that the hexamer is an unfavorable arrangement for the monomers
301 as seen from the structural deviation of the oligomers, given by the RMSD (Fig. 6) and the
302 2D plot of the α -carbon in the FA-Lys linker (Fig. 7). However, the packing of the structure
303 is relatively tight with a low SASA (Fig. 5) which indicates that more interactions are
304 obtainable. This is also supported by the relatively low P-P energy of the hexameric system
305 compared to the others (Table 3). The heptamer seems to present a very favorable
306 arrangement with a tight packing indicated by the low SASA (Fig. 5), little displacement
307 given by the low and converging RMSD (Fig. 6), and the overall elliptical shape seen in the
308 2D plot of the α -carbon in the FA-Lys linker (Fig. 7). This is also the structure with the
309 overall lowest P-P energy of $\sim -200 \text{ kcal/mol}$. The octamer, like the hexamer, also appears to
310 present an unfavorable arrangement with a slight opening and disintegration of the elliptical
311 structure as seen in the end structure (Fig. S3), the 2D plot of the α -carbon (Fig. 7), and the
312 high SASA (Fig. 5). The AA6_3ud presents the worst monomer arrangement out of the 11
313 investigated structures based on the end structure (Fig. S4) that opens completely resulting in
314 an unstable conformation as also seen from the relatively large SASA (Fig. S6), increasing
315 RMSD (Fig. S8), and the scattered α -carbon position (Fig. 7). The AA7_1ud, AA8_4udp, and
316 AA8_4uds systems all maintain a relatively well arranged end structure (Fig. S4) even though
317 one monomer appears to be leaving the general elliptical structure of AA8_4up. In contrast to
318 this, the overall packing of the three systems is relatively tight (Fig. S6), but the overall
319 movement is scattered and very spread out as seen in the 2D plot of the α -carbon (Fig. 8).
320 Thus, it seems like interactions between the C- and N-termini are not contributing to the
321 energy, hence, liraglutide is most likely not to arrange like the flipped structures (AA6_3ud,
322 AA7_1ud, AA8_4udp, and AA8_4uds). The AA6/7/8_FAout structures also present
323 unfavorable arrangements of the monomers as can be seen by the large scattering of the
324 individual monomers in the 2D plot of the α -carbon (Fig. 9) and large overall displacement
325 represented by the RMSD (Fig. S9). The most important analysis result for these structures is,

326 however, that the S-S all FAout energies for all three systems are less negative than those of
327 the segment interactions in systems where the FA chains are pointing towards each other
328 (Table 3). This amplifies the hypothesis of liraglutide oligomer structures that give rise to FA
329 interactions. However, when the FA chains are pointing outward they could, in theory, wrap
330 around the elliptical structure in such a manner that they interact, but this appears not to be
331 the case based on the energy calculations (Table 3) and the fact that all the FA chains in all
332 the three structures seem to be randomly laying on the surface of the oligomers (Fig. S5) that
333 could promote clustering of oligomers. However, this is not the case since SAXS and MALS
334 data indicate the presence of one defined oligomeric species. The AA7_glp1 was made as a
335 reference structure when considering the role of the FA chains. The results show that the
336 movement of the individual monomers of this system (Fig. 9) is rather large. This emphasizes
337 the stabilization effect of the FA chains. The system seems to have a tight packing (Fig. S7),
338 however, this fact is more likely to be a result of the missing FA chains in the structure, and
339 hence, less surface area. The S-S energies bear witness of a great lack in possible interactions
340 since these energies are significantly less negative (~ -29 kcal/mol) than for those systems
341 with FA chains present (S-S FA average energy ~ -54 kcal/mol). This corresponds well with
342 the hydrophobic/hydrophilic areas of the monomer shown in Fig. 10 where it is evident that
343 there is a difference in the hydrophobicity around the monomeric structure. All in all, it
344 shows that the FA chains are important in stabilizing the liraglutide oligomer.

345

346 It thus appears that the heptamer is the most favorable arrangement, which is further
347 supported by the comparison between the experimentally determined SAXS (SAXS^{exp}) curve
348 and curves extracted from the simulations. The theoretical SAXS (SAXS^{comp}) curves along
349 with the experimentally determined curve are shown in Fig. S10. SAXS^{comp} curves were
350 calculated from the last structure of the 11 simulations.

351 The discrepancies (χ^2 values) for the 11 curves compared to the experimentally obtained
352 SAXS curve are, together with the radii of gyration, given in Table S1. Besides the radius of
353 gyration for the octamer, the radii of the systems lie very close to those found from the
354 experimental data seen in Table 2. Also, the discrepancies given in Table S1 does not present
355 one specific candidate with the best fit, as several of the systems have low discrepancies
356 between their end structures and the experimentally obtained data.

357

358 To clarify the best fit further, we show the time evolution of the discrepancies between
359 SAXS^{comp} and SAXS^{exp} for the 11 oligomers in Fig. 11.

360 The discrepancy of the heptamer is rather stable and low throughout the simulation. So is χ^2
361 of the hexamer, AA8_4udp, AA8_4uds, and the AA7_glp1 systems. On the contrary, the
362 discrepancy of the octamer and AA6_3ud is very high, and increases with simulation time.
363 That of the AA6_FAout, AA7_FAout, and AA8_FAout systems and AA7_1ud fluctuates
364 significantly throughout the simulations. The results show that the global structure and size
365 seems to be correct, especially for the heptamer and hexamer, but none of our simulated
366 structures capture the precise shape of SAXS^{exp}.

367 Summarizing and combining all the results (see Table 4 for a combined scoring chart), it
368 appears that the most likely solution structure of liraglutide is a heptamer where the
369 monomers are orientated in such a way so that the attached FA chains can interact in three
370 pairs in the direction of the elliptical arrangement and with the remaining monomer oriented
371 so that the FA chain is pointing out.

Table 4: Scoring chart of the 11 oligomeric systems. Each system is evaluated compared to each other in each of the 7 categories; lowest and most stable SASA, lowest and most stable RMSD, most stable 2D projection and elliptical shape, lowest P-P vdW energy, highest P-W vdW energy, lowest discrepancy from measured SAXS curve taken for the end structure, and lowest mean and standard deviation of the discrepancy throughout the simulation (data taken from Fig. 11). The SASA, RMSD, and 2D projection plots are inspected visually. 1 is the best score and 11 is the worst. The total score is normalized.

Structure	SASA	RMSD	2D projection	Energy		SAXS		Total score
				P-P vdW	P-W vdW	χ^2 value	χ^2 value fluctuation	
Hexamer	2	8	3	5	5	7	4	4.9
Heptamer	1	4	1	1	1	3	2	1.9
Octamer	7	10	6	6	3	11	10	7.6
AA6_3ud	10	11	11	8	9	10	11	10
AA7_1ud	4	5	10	2	2	6	7	5.1
AA8_4udp	6	3	5	3	8	5	6	5.1
AA8_4uds	5	2	8	4	7	4	3	4.7
AA6_FAout	11	9	2	10	11	2	8	7.6
AA7_FAout	8	7	9	7	4	9	9	7.6
AA8_FAout	9	1	4	9	10	1	5	5.6
AA7_glp1	3	6	7	11	6	8	1	6.0

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The heptamer, with the above mentioned conformation, presents the best model with a total score of 1.9 being the best scoring structure in 4 out of 7 categories and landing either a second, third or fourth place in the remaining categories. It presents the best energy interactions as well as the least structural deviation of the monomers and highest packing. The hexamer presents the second best solution, but does not score best in any category. The AA6_3ud structure presents the worst arrangement of the monomers with a total score of 10.

379 CONCLUSION

380 From a pharmaceutical perspective, it is important to know the oligomerization state of
381 liraglutide with respect to stability, since uncontrolled and extensive oligomerization can
382 drive fibrillation. Furthermore, oligomerization is important for stabilization of the
383 formulation, which has a 2 year shelf-life in liquid form. Furthermore, MALS provides
384 information about the mass, and SAXS provides information on mass, radius of gyration and
385 shape. In order to get further insight in the solution structure of liraglutide, we have
386 performed molecular dynamics simulations. The SAXS curves indicate that liraglutide
387 undergoes concentration-independent oligomerization. Depending on the partial specific
388 volume used for deducing the molecular weight and in turn the number of monomers in an
389 oligomer, liraglutide may form hexa-, hepta- or octamers in solution. In contrast, the MALS
390 results suggest that liraglutide forms hexamers in solution. The experimental results are not
391 conclusive with respect to the size of the oligomers. Furthermore, no information can be
392 deduced from these measurements regarding the orientation and stabilizing role of the acyl
393 chains in the oligomers.

394 We, therefore, performed molecular dynamics simulations on several oligomeric sets
395 consisting of hexa-, hepta-, and octamers. Our simulation results indicate that interactions
396 between the FA chains contribute to the stabilization of the structure and that the heptamer
397 presents the best representation of the investigated liraglutide oligomer. Furthermore,
398 comparing the experimentally determined SAXS curve with the SAXS curves determined
399 from the structures extracted from the simulations shows qualitative agreement for the overall
400 size and shape. This indicates that liraglutide in solution is most likely to form heptamers in a
401 hollow, water-filled, elliptical cylindrical shaped structure where the monomers are orientated
402 in such a way so that FA chains can interact pairwise. However, from the simulations, we
403 presently are not able to identify the absolute position of the FA chains in the heptamer, but it
404 is clear that interactions between them are significant.

405

406 **SUPPORTING MATERIAL**

407 2 tables, 11 figures and a detailed description of the molecular dynamics simulation
408 parameters are available at

409 **AUTHOR CONTRIBUTIONS**

410 TMF: Wrote the paper, performed and analyzed simulations.

411 PS: Conducted and analyzed SAXS experiments, contributed in writing the paper.

412 LAR: Performed preliminary simulations, contributed to the paper.

413 PH: Conducted SAXS experiments, contributed in writing the paper.

414 JTB: Conducted SAXS experiments, contributed in writing the paper.

415 AMSP: Conducted and analyzed AF4-MALS experiments, contributed in writing the paper.

416 MNEL: Performed preliminary simulations.

417 GHP: Performed and analyzed simulations, contributed in writing the paper.

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424 VMD (45) was used for all graphical representations of liraglutide.
425

426 **SUPPORTING CITATIONS**

427 References (49–51) appear in the Supporting Material.

428

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569

570

571 **FIGURE TITLES AND LEGENDS**

Fig. 1: Amino acid sequence of liraglutide.

Fig. 2: Representation of the start structures for the 11 oligomeric structures. All structures are shown from a top view. The red line represents the orientation of the FA chain on each monomer, and the ellipses reflect the size and shape of the oligomers.

Fig. 3: Scattering curves normalized for concentration. Plots at concentrations 1, 2, and 4.7 mg/mL. Inset shows scattering over entire measured scattering range. Lir = liraglutide.

Fig. 4: AF4-UV-MALS analysis of undiluted Victoza® (6 mg/mL liraglutide) and 10× diluted Victoza® (F10). Different injection volumes were tested. All analyses showed a single peak in the UV chromatogram and a uniform molar mass of around 22 kDa across the peak (left). No other peaks were observed in the chromatogram. The liraglutide concentration in the eluate passing the UV detector is shown in the right graph.

Fig. 5: Solvent Accessible Surface Area (SASA) as a function of time for the hexamer, heptamer, and octamer. The area has been normalized according to the number of monomers. SASA was calculated every 50 ps along the trajectory using a van der Waals radius of 1.4 Å.

Fig. 6: RMSD of the entire oligomeric structure for the hexa-, hepta-, and octamer. Structures were aligned to the first frame ($t = 0$) and deviations are determined for the backbone chains.

Fig. 7: Position of α -carbon in the FA-Lys linker of the hexamer, a), heptamer, b), and octamer, c), as a function of simulation time. One monomer is highlighted by a circle in the octamer, c), to indicate the possible disintegration of this structure. Coordinates taken from the first 15 ns, 5 ns in the middle of the simulation, and the last ~5 ns are shown. The circles and dashed lines indicate the average structure within the 5 ns intervals shown by the dots. The yz α -carbon atom coordinates for the FA-Lys linker were plotted in intervals of 50 ps.

Fig. 8: Position of α -carbon in the FA-Lys linker of the AA6_3ud, a), AA7_1ud, b), AA8_4udp, c), and AA8_4uds d), as a function of simulation time. One monomer is highlighted in a circumference in AA8_4udp, c), to indicate the possible disintegration of the AA8_4udp system. See figure caption Fig. 7 for details.

Fig. 9: Position of α -carbon in the FA-Lys linker of the AA6_FAout, a), AA7_FAout, b), AA8_FAout, c), and AA7_glp1 d), as a function of simulation time. See figure caption Fig. 7 for details.

Fig. 10: A surface plot of the liraglutide monomer showing the seg-seg orientations. The hydrophobicity is given in green and grey (hydrophobic) and orange (hydrophilic). The FA chain is colored in gray. a) shows the different hydrophobicity on either side of the monomer.

Fig. 11: Discrepancies of the calculated SAXS^{comp} curves and that of liraglutide as a function of simulation time, calculated every 5 ns. The arrows indicate that the χ^2 values continuously increase with simulation time.

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