Complexation of cationic-neutral block polyelectrolyte with insulin and *in vitro* release studies

Natassa Pippa [a,b], Maria Karayianni[b], Stergios Pispas[b,*], Costas Demetzos[a]

[a]Department of Pharmaceutical Technology, Faculty of Pharmacy, Panepistimioupolis Zografou 15771, National and Kapodistrian University of Athens, Athens, Greece

[b]Theoretical and Physical Chemistry Institute, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, 11635, Athens, Greece

* Corresponding author: Dr. Stergios Pispas, Tel.:+30210-7273824; Fax: +30 210-7273794; E-mail address: pispas@eie.gr

13   **Abstract**
14
15   Insulin (INS) was incorporated into complexes with the block polyelectrolyte

16   quaternized   poly[3,5-bis(dimethylaminomethylene)hydroxystyrene]-b-poly(ethylene

17   oxide) (QNPHOSEO), which is a cationic-neutral block polyelectrolyte. Light

18   scattering techniques are used in order to examine the size, the size distribution and

19   the ζ-potential of the nanocarriers in aqueous and biological media, which are found

20   to depend on the ratio of the components and the physicochemical parameters during

21   and after complex preparation. Circular dichroism and infrared spectroscopy,

22   employed to investigate the structure of the complexed INS, show no alteration of

23   protein structure after complexation. *In vitro* release profiles of the entrapped protein

24   are found to depend on the ratio of the components and the solution conditions used

25   during preparation of the complexes.

26

27   **Keywords:** block polyelectrolyte; insulin; complexation process; stealthiness; *in vitro*

28   release studies

29

1. Introduction

The discovery of insulin revolutionized the use of peptides and proteins as therapeutic agents for several diseases. The evolution of the biotechnological era gave rise to modified insulins to solve some of the bottlenecks in insulin therapy (Pillai, and Panchagnula, 2001; Tibaldi, 2012). Several innovative approaches based on pharmaceutical nanotechnology mimic the endogenous release and kinetics of insulin, and also many improved analogues designed achieve better control and effective treatment of diabetes (Mao et al., 2006; Avadi et al., 2011; Han et al., 2012; Yan et al., 2012).

Asymmetric amphiphilic block copolymers self-assemble in aqueous media, to form core-shell micellar structures and morphologies, with a mesoscopic or nanoscopic narrow size range and are used for biomedical and pharmaceutical applications (in the order of 10-100 nanometers). Polyelectrolyte block copolymers constitute an intriguing class of bio-inspired macromolecules, as they combine the structural properties of amphiphilic block copolymers, polyelectrolytes and surfactants and provide various possibilities for use as delivery nanosystems of genes and proteins through electrostatic complexation (Al-Tahami and Singh, 2007; Hartig et al., 2007; Pispas, 2007; Reis et al., 2008; Karayianni et al., 2011; Karayianni and Pispas, 2012; Becker et al., 2012; Haladjova et al., 2012; Varkouhi et al., 2012). Polymeric delivery systems based on nanoparticles have emerged as a promising approach for insulin delivery. According to the recent literature, the application of polyion complex micelles into therapeutic fields is rapidly increasing due to simple and efficient encapsulation of biopharmaceuticals (peptides and proteins) and outstanding biocompatibility among various polymer-based drug delivery nanocarriers (Lee and Kataoka, 2009).

56 In the present work we employ dynamic (DLS), static (SLS) and electrophoretic

57 (ELS) light scattering in order to examine the complexation process, as well as the

58 structure and solution behavior in aqueous and biological media of the nanosized

59 complexes, formed between quaternized poly[3,5-

60 bis(dimethylaminomethylene)hydroxystyrene]-b-poly(ethylene oxide) (QNPHOSEO),

61 a cationic-neutral block copolymer, and insulin (INS). Furthermore, the secondary

62 structure of the complexed INS was investigated by means of circular dichroism (CD)

63 and infrared (IR) spectroscopy. Finally, the *in vitro* release of INS from the

64 complexes was studied in physiological conditions. The central goal is to create novel

65 and functional hybrid synthetic/biological macromolecular nanostructures and enrich

66 the basic understanding on behavior motifs in aqueous and biological media, as well

67 as widen application potential of nanostructured polymeric delivery systems with

68 controlled release profile of the encapsulated protein.

69 ## 2. Materials and Methods
70
71 ### 2.1 Materials
72
73 The synthesis, the physicochemical characterization, as well as the properties of

74 QNPHOSEO are presented extensively in the recent literature (Mountrichas et al.,

75 2006,2007; Mantzaridis et al., 2009; Stepanek et al., 2011). The QNPHOSEO

76 polyelectrolyte block copolymer was characterized by SEC, ^{1}H-NMR and FTIR and it

77 was found to have the following molecular characteristics: M_w=98,600, M_w/M_n=1.09,

78 15%wt PEO. The molecular structure of QNPHOSEO is presented in Fig. 1(a).

79 QNPHOSEO chains tend to form loose aggregates in aqueous solutions (Fig. S2)

80 (Stepanek et al., 2011). The QNPHOSEO block polyelectrolyte has been used for

81 DNA and RNA complexation and it was found to have cytotoxicity better than other

82 reference cationic polymers utilized in gene delivery (Varkouli et al., 2012). INS with

83 a molecular weight of 5800 g/mol was purchased from Sigma Aldrich and used

84 without any further purification.

2.1.1 Preparation of QNPHOSEO:INS complexes in different aqueous media

86 A pH 7 buffer solution was prepared from NaOH and 5mM sodium phosphate.

87 Moreover, 10mM NaCl were added to solution to maintain a fixed ionic strength,

88 along with NaN_3 in a final concentration of 200ppm, in order to avoid bacterial

89 growth. Stock solutions of INS and QNPHOSEO were prepared by dissolving a

90 weighed amount of the dialyzed sample in the appropriate volume of the buffer and

91 the solutions were left to stand overnight for better equilibration.

92 The complexes were prepared by adding different amounts of the INS solutions to

93 QNPHOSEO solutions of the same volume and concentration, under stirring. Finally,

94 appropriate volumes of buffer solutions were added to achieve a constant final volume

95 and ionic strength (equal to that of the buffer solutions) for all solutions prepared.

96 Thus, the concentration of QNPHOSEO was kept constant throughout the series of

97 solutions, while that of INS varied in order to control the required ratio of the two

98 components (or equivalently the [-]/[+] charge ratio of the mixture). The solutions of

99 the complexes developed a bluish tint or turbidity upon mixing, indicating the

100 formation of supramolecular complexes. Subsequently, the solutions of the complexes

101 were left for equilibration overnight, which in some cases resulted in coacervation, i.e.

102 liquid-liquid phase separation of the solution, depending on the INS concentration and

103 pH. Stable solutions were further characterized as discussed below.

104 For the ionic strength dependent light scattering measurements, the ionic strength of

105 the solution was gradually increased by the addition of appropriate aliquots of 1N

106 NaCl solution at pH=7.00 or 7.40, to 1ml of the previously prepared solutions of the

107 complexes. After each addition the solution was rigorously stirred and left to

108 equilibrate for 15min before measurement. Changes in solute concentrations due to

109 NaCl solution addition were taken into consideration in the analysis of the light

110 scattering data.

111

112 *2.2.2. Insulin association efficiency and in vitro INS release studies*

113 The loading and the release of insulin from complexes was investigated by dialysis

114 method. The percentage of INS incorporated into complexes was estimated by

115 spectrophotometry. INS concentration was estimated with the aid of the following

116 INS calibration curve in PBS (pH=7.4):

117 $$\text{INS concentration} \left(\text{mg}/\text{ml} \right) = \frac{absorbance - 0.0213}{0.2822} \ (R^2 = 0.9966) \ (\mathbf{1})$$

118 The absorbance was measured at 277nm. Encapsulation efficiency (EE) was

119 calculated by using the following equation:

120 $$\%EE = \frac{total\ amount\ of\ INS - free\ INS}{total\ amount\ of\ INS\ (initial)} \times 100 \ (\mathbf{2})$$

121 All samples were measured in triplicate and are reported as the mean value.

122 The release profile of INS from the nanovectors was studied in PBS. Nanovectors

123 incorporating INS (1ml of each sample) were placed in dialysis sacks (molecular

124 weight cut off 12,000; Sigma-Aldrich). Dialysis sacks were inserted in 10 mL PBS in

125 shaking water bath set at 37 °C. Aliquots of samples were taken from the external

126 solution at specific time intervals and that volume was replaced with fresh release

127 medium in order to maintain sink conditions. The amount of INS released at various

128 times, up to 10 h, was determined using spectrophotometry at $\lambda_{max} = 277$ nm with the

129 aid of the calibration curve of the equation (**1**). All the experiments were carried out in

130 triplicate, and the data presented are the average of the three measurements.

2.2. Methods

2.2.1. Dynamic and static light scattering

The hydrodynamic radius (R_h) of nanocarriers and the polydispersity Index (PD.I.) were measured by dynamic light scattering (DLS) and the ratio of radius of gyration to hydrodynamic radius (R_g/R_h) was determined by static light scattering (SLS). Mean values and standard deviations were calculated from three independent samples. For dynamic and static light scattering measurements, an AVL/CGS-3 Compact Goniometer System (ALV GmbH, Germany) was used, equipped with a cylindrical JDS Uniphase 22mV He-Ne laser, operating at 632.8 nm, and an Avalanche photodiode detector. The system was interfaced with an ALV/LSE-5003 electronics unit, for stepper motor drive and limit switch control, and an ALV-5000/EPP multi-tau digital correlator. Autocorrelation functions were analyzed by the cumulants method and the CONTIN software. Apparent hydrodynamic radii, R_h, at finite concentrations, were calculated by aid of the Stokes - Einstein equation:

$$R_h = \frac{k_B T}{6\pi n_0 D} \quad (\textbf{3})$$

where k_B is the Boltzmann constant, η_0 is the viscosity of water at temperature T, and D is the diffusion coefficient at a fixed concentration. The polydispersity of the particle sizes was given as the μ_2/Γ^2 (PD.I.) from the cumulants method, where Γ is the average relaxation rate, and μ_2 is its second moment.

Light scattering has been used widely in the study of the fractal dimensions of aggregates. In static light scattering, a beam of light is directed into a sample and the scattered intensity is measured as a function of the magnitude of the scattering vector q, with:

$$154 \qquad q = \frac{4\pi n_0}{\lambda_0} \sin(\frac{\theta}{2}) \quad (\textbf{4})$$

155 where n_0 is the refractive Index of the dispersion medium, θ is the scattering angle and

156 λ_0 is the wavelength of the incident light. Measurements were made at the angular

157 range of 30º to 150º (i.e. the range of the wave vector was $0.0067 < q < 0.025$ nm^{-1}).

158 The general relation for the angular dependence of the scattered intensity, I(q) is:

$$159 \qquad I(q) \sim q^{-df} \quad (\textbf{5})$$

160 where d_f is the fractal dimension of the aggregates with $1 \leq d_f \leq 3$ (d_f=3 corresponds to

161 the limit of a completely compact Euclidean sphere where less compact structures are

162 characterized by lower d_f values). The above equation is the classical result used to

163 determine the mass fractal dimension from the negative slope of the linear region of a

164 log-log plot of I vs. q.

165 *2.2.2. Electrophoretic mobility – microelectrophoresis*

166 The zeta potential (ζ-potential) plays an important role in colloidal stability of

167 nanoparticles and can be readily measured by the technique of microelectrophoresis.

168 The zeta potential of chimeric nanostructures was measured using Zetasizer

169 3000HAS, Malvern Instruments, Malvern, UK. 50μl of the dispersions was 30-fold

170 diluted in dispersion medium and ζ-potential was measured at room temperature at

171 633nm. The zeta potentials were calculated from electrophoretic mobilities, μ_E, by

172 using the Henry correction of the Smoluchowski equation:

$$173 \qquad \zeta = \frac{3\mu_E n}{2\varepsilon_0 \, \varepsilon_r} \frac{1}{f(\kappa a)} \quad (\textbf{6})$$

174 where ε_0 is the permittivity of the vacuum, ε_r is the relative permittivity, α is the

175 particle radius, κ is the Debye length, and n is the viscosity of water. The function

176 f(κα) depends on particle shape. While if κα > 1:

$$f(\kappa\alpha) = 1.5 + \frac{9}{2(\kappa\alpha)} + \frac{75}{2(\kappa\alpha)^2} \quad (\mathbf{7})$$

178 The above function refers to dispersions of the present study.

179 *2.2.3. Circular dichroism*

180 CD measurements were conducted using a JASCO-715 (Jasco Corp., Tokyo, Japan)

181 spectropolarimeter with a Peltier-type cell holder, which allows for temperature

182 control. Wavelength scans in the far UV region (190 to 260 nm) were performed in

183 Quartz SUPRASIL (HELLMA, GmbH & Co., Müllheim, Germany) precision cells of

184 0.1 cm path length. Each spectrum was obtained by averaging three successive

185 accumulations with a wavelength step of 0.5 nm at a 100 nm/min rate, response time

186 4 s, and bandwidth 5 nm. The absorption spectra were recorded selecting the UV

187 (single) mode of the instrument. An estimation of the secondary structure elements

188 was obtained by analyzing the CD spectra using the CDNN algorithm.

189 *2.2.4. Infrared spectroscopy*

190 Infrared spectra of the protein, polyelectrolyte block copolymer and complexes in thin

191 film form were acquired at room temperature in the range 5000-550cm^{-1}, using a

192 Fourier transform instrument (Bruker Equinox 55), equipped with a single bounce

193 attenuated total reflectance (ATR) diamond accessory from SENS-IR. A small aliquot

194 of each solution was placed on the ATR element and dried under N_2 flow before

195 measurement. For each sample the final spectrum is the average of the three 100-scan

196 measurements at 2cm^{-1} resolution. The measurement of each sample was bracketed by

197 two background spectra in order to allow the elimination of H$_2$O vapor bands by

198 interpolation.

3. Results and Discussion

3.1. Physicochemical characterization of QNPHOSEO:INS complexes in aqueous and

biological media

202 The complexation process between the QNPHOSEO polyelectrolyte and INS at pH=7

203 and I=0.01M, and in PBS (pH=7.40 and I=0.154M) was first investigated by means of

204 dynamic light scattering. It should be noted that PBS (pH=7.40) was chosen as

205 dispersion medium because the pH and the ionic strength of PBS resembles the

206 conditions met within the human body. At pH=7the electrostatic interactions of the

207 system are expected to be strong, since the QNPHOSEO polyelectrolyte block carries

208 two positively charged groups per functionalized monomeric unit and INS has a net

209 negative charge of -4. The obtained results from DLS measurements at 90° regarding

210 the values of the hydrodynamic radius, R_h, and the light scattering intensity, I_{90},

211 (corrected for the concentration increase) are shown in Figs. 2(a) and (b), as function

212 of the protein concentration, C_{INS}, in the solutions of the complexes. The structure and

213 the formation process of QNPHOSEO:INS complexes is represented in Fig. 1(a).

214 The concentration of QNPHOSEO copolymer is kept constant throughout the series

215 of aqueous solutions of different ionic strength investigated. It should be pointed out

216 that at high INS concentration; coacervation of the solutions of the complexes took

217 place, only at pH=7 and I=0.01M NaCl. This phenomenology was not observed for

218 the solutions in PBS. DLS results at low ionic strength (I=0.01M) show that all

219 solutions exhibit a main peak at high R_h values (~80nm), which apparently

220 corresponds to the formed mixed aggregates (QNPHOSEO:INS complexes) and a

221   significantly smaller one at lower R_h values, which most probably denotes the

222   presence of a small number of free unimer diblock copolymer chains in solution. The

223   values of the scattering intensity, I_{90}, which is proportional to the mass of the species

224   in solution, increased gradually as a function of $C_{INS,}$ providing proof of the occurring

225   complexation (Fig.2(b)), i.e. the mass of the complexes increases as C_{INS} increases.

226   As protein concentration increases each polyelectrolyte chain interacts with an

227   increasing number of protein molecules, the degree of charge neutralization becomes

228   higher and the size distribution of the complexes decreases, especially at the highest

229   ionic strength (Becker et al., 2012). In contrast I_{90} (or the mass of the complexes)

230   shows a more steep increase in the case of PBS solutions, although the protein

231   concentration range studied in these systems is wider due to the absence of

232   coacervation. In pH=7, we observed aggregation of the complexes and precipitation

233   of the supramolecular aggregates, when the initial concentration of insulin was higher

234   that 0.07mg/ml. ζ-potential decreases in absolute value as the concentration of the

235   protein increases in PBS, or equivalently the effective positive charge of the

236   complexes reduces as a function of the protein concentration (Fig. 3(a)). On the other

237   hand, ζ-potential values remained unaffected in the lowest ionic strength solutions. It

238   seems that the ionic strength of the solution plays a significant role in the interactions

239   and the structure of the resulting complexes. Due to the primary aggregation of

240   QNPHOSEO chains in aqueous media we expect interactions between QNPHOSEO

241   aggregates with INS molecules and this initial state of the block polyelectrolyte may

242   have a significant effect on the structure of resulting QNPHOAEO:INS complexes

243   under different solution conditions. We also investigated the morphology of the

244   complexes via their fractal dimension (d_f), determined by static light scattering.

245   Although the nanosystems developed in this study are polydisperse in size we can still

246   make use of this parameter to extract information on morphological changes, since

247   size polydispersity is expected to influence the d_f results of the nanosystems in more

248   or less a similar way (after all the PDI index values determined by cumulant analysis

249   lie in the range 0.2-0.3). d_f was found near to 1.6 for QNPHOSEO:INS aggregates in

250   PBS (Fig. S4). An increase of d_f values was observed for QNPHOSEO:INS

251   complexes in buffer at pH=7.00 as the concentration of the protein increases (Fig.

252   S4), although the concentration range studied in these systems is narrow due to

253   coacervation. This observation may be associated by a small change in the

254   density/morphology of the complexes as INS concentration increases in this case.

255   The values of R_g/R_h were also calculated (Fig 3.(b)) from multiangle light scattering

256   measurements. This ratio is sensitive to the shape of particles in solution and can be

257   used as a rough estimate of the internal morphology of the particles formed after

258   complexation. This is based on the notion that R_g is a measure of the mass density

259   distribution around the center of the structure, while R_h defines the outer dimensions

260   of the particle. According to Burchard (1983), the R_g/R_h ratio takes the values of 0.775

261   for a hard uniform sphere and 1.0 for vesicles with thin walls, while values of 1.3 to

262   1.5 indicate a random coil (loose) conformation in the case of macromolecular chains.

263   In the present case, the R_g/R_h values are near 1.00 for QNPHOSEO:INS in PBS at low

264   INS concentration .These results may indicate a more well-defined hollow sphere

265   (vesicle like) structure for the complexes (Fig 3.(b)) or a rather low density full

266   spherical structure. It should be pointed out that the morphology of mixed aggregates

267   changes significantly as the concentration of the INS increases (as indicated from

268   R_g/R_h values) (Fig. 3(b)). At highest concentration of INS ($C_{INS}>1\times10^{-4}$ g/mL), open

269   (low density) spherical structures are observed for QNPHOSEO:INS complexes (Fig.

270   2(e)). On the other hand, the R_g/R_h values indicate open (low density) spherical

271 structures for aggregates in the pH=7 buffer of low ionic strength (Fig 3.(b)). These

272 differences may be a result of the differences in the salinity of the media, especially at

273 the lower concentration of INS.

274 According to the literature, it is important to characterize the physicochemical

275 properties of the complexes formed under different conditions in biological media,

276 like fetal bovine serum (FBS), because interaction with the proteins of the medium is

277 expected to alter the physicochemical properties of the nanostructures, thereby

278 affecting their stability and clearance properties (Arnida Jánat-Ambury et al., 2011).

279 We investigated the physicochemical characteristics of QNPHOSEO:INS complexes

280 in Fetal Bovine Serum (FBS). The prepared complexes in aqueous media were diluted

281 in FBS:PBS 10% v/v (Arnida Jánat-Ambury et al., 2011; Pippa et al., 2012a,b,

282 2013a,b).. The size of the complexes in biological medium (FBS) was increased from

283 ca. 50 nm to ca. 200 nm, as a function of INS concentration, compared to the initial

284 solutions after complex formation (Figs. 4(a) and 5(a)). In all cases, the

285 supramolecular aggregates of insulin carrier complexes and plasma proteins remained

286 smaller than 300nm (within the nanoparticle scale which is important for

287 nanomedicinal purposes). The values of the scattering intensity, I_{90}, which is

288 proportional to the mass of the species in solution, did increase in FBS, providing

289 proof of some additional, but of relatively low extent, complexation of the block

290 copolymer/INS nanoparticles with components of FBS, especially in PBS. This

291 observation indicates that QNPHOSEO copolymer imparts stealth properties and

292 stability in the complexes, due to the presence of PEO chains that shield the

293 complexes (it should be noted that no precipitation of the complexes was observed

294 when in contact with FBS solutions). A shift of ζ-potential of the complexes to

295 negative values is observed, presumably due to some binding of FBS proteins, which

can alter the nanoparticle's effective size and surface properties (the main protein component of FBS is albumin which carries a negative charge at physiological conditions) (Fig.4(b) and 5(b) (Arnida Jánat-Ambury et al., 2011; Pippa et al., 2012a,b, 2013a,b). The shift of the ζ-potential to negative values explains the absence of precipitation of the complexes, because of additional electrostatic stabilization of the aggregates. In our opinion, these differences are not statically significant and, although there is some protein adsorption on the complexes, their size and surface properties still remain within the limits allowing the use of the present nanosystems as nanocarriers of insulin.

3.2. Effect of ionic strength

The increase of the ionic strength in the solutions of the complexes induces charge screening and weakening of the electrostatic interactions, so it is expected to greatly influence the solution behavior and structure/morphology of the preformed complexes. In order to investigate this effect, DLS/SLS measurements as a function of the added NaCl concentration were conducted, and the resulting R_h and I_{90} (corrected for the difference in concentration) values for representative solutions of QNPHOSEO:INS complexes formed at low and high C_{INS} values at pH 7and 7.40 are shown in Figs. S5 and 6. In the case of complexes prepared at low ionic strength buffer R_h does not change significantly with an increase of the ionic strength (Fig. S5(a)). There is a rather small gradual increase of their size. We recall that at low C_{INS} values the number of interacting protein molecules per polyelectrolyte chain is rather small, while at high C_{INS} values the formed complexes are characterized by rather large number of interacting protein molecules per polyelectrolyte chain (Fig. 5(a)) (Karayianni et al., 2011). Light scattering intensity behavior is different for complexes formed at low and at high INS concentration. At low INS concentration I_{90} (and mass

321 of the complexes) increases, and then drops with ionic strength increase meaning that

322 there is some additional aggregation of the complexes at low ionic strengths (but

323 higher that the initially used ones for complex formation) and by increasing salt

324 concentration the mass decreases again (Fig. 6(b)). In the case of complexes formed at

325 higher INS concentration there are very small intensity changes (and mass changes)

326 with added salt (Fig. S5(b)), which shows rather a small decrease in the mass of the

327 complexes. R_g/R_h values for these systems show a decrease and a plateau at I>0.1 M,

328 reaching at an R_g/R_h value ca.0.9-1.0, which in turn may indicate more compact

329 spherical structures than the initial ones. The observed behavior should be attributed

330 to charge screening effects that weaken electrostatic interactions and promote

331 hydrophobic effects. Due to the hydrophobic nature of the QNPHOSEO chains and

332 their initial aggregation state, addition of salt may result in secondary aggregation or

333 disruption of the complexes, depending on the initial conditions during complex

334 formation.

335 The hydrodynamic radius of the complexes, as a function of the added NaCl

336 concentration, remained more or less unaffected in the case of complexes formed in

337 PBS buffer (Fig. 6(a)). The same is true for the mass of the complexes as indicated by

338 the I_{90} values (Fig. 6(b)). The morphology of the complexes showed some changes

339 with more compact spherical structures being observed at higher salt concentrations,

340 as indicated from the decrease in R_g/R_h values (Fig. 6(c)) (Karayianni et al., 2011).

341 For these systems changes in the determined parameters are small and rather smooth.

342 This may imply that the QNPHOSEO:INS complexes formed in PBS are more stable

343 to ionic strength increase and may also have a structure closer to equilibrium.

344 *3.3. Protein structure within the complexes*

The preservation of the enzymatic activity, which is directly correlated with the protein conformation, is an issue of major importance in most applications involving protein-polyelectrolyte complexes. For this reason, the structure of complexed protein was monitored via circular dichroism and infrared spectroscopic techniques, and representative results of QNPHOSEO:INS system at pH=7.40 and 0.154M NaCl are presented below.

Firstly, circular dichroism (CD) was employed for the determination of the complexed protein structure. Fig. 7(a) presents the measured far-UV CD spectra of three representative solutions at C_{INS}=0.07, 0.26 and 0.4 mg/ml of QNPHOSEO:INS system at pH=7.40 and 0.154M NaCl, and of neat INS at 1 mg/ml concentration and same solution conditions. As it can be seen, only small changes in the CD spectra between the neat and the complexed INS are observed. The analysis of the spectra carried out using the CDNN algorithm yielded similar results and the average percentages of protein secondary structure are summarized in Table S1. Obviously, the percentages of protein secondary structure are preserved for all solutions of the complexes, thus proving that the complexation does not cause structural disruption of the protein (Bouchard et al., 2000).

Moreover, infrared (IR) spectroscopy measurements were performed and Fig. 7(b) shows the IR spectra of the same three representative solutions at C_{INS}=0.07, 0.26 and 0.4 mg/ml of QNPHOSEO:INS system at pH=7.40 and 0.154M NaCl in the Amide I and II region, including the corresponding spectrum of neat INS for comparison. The spectra have been normalized to the intensity of the Amide I band, after subtraction of the spectral contribution of the neat block polyelectrolyte copolymer. The constancy of the Amide I and II profiles, peaking at ca. 1658 and 1540 cm^{-1} respectively, indicates the absence of significant protein configuration changes, such as those

370   observed upon denaturation (Bouchard et al., 2000). Therefore, it can be concluded

371   that insulin preserves its structure and activity after complexation.

372   *3.4. Encapsulation efficiency and in vitro release of INS*

373   Values of encapsulation efficiency varied from 10 to 40% for the different

374   formulation as seen in Table S2. They were particularly affected by the dispersion

375   medium and by the initial concentration of INS. Encapsulation efficiency increased

376   with the increase of the initial concentration of the protein in the two aqueous

377   dispersion media. The *in vitro* release of the INS from the nanocomplexes, at different

378   molar ratios of the protein component, is presented in Fig. 8. It is observed that the *in*

379   *vitro* release of the INS from the prepared polyelectrolyte aggregates is quite fast

380   especially for the QNPHOSEO:INS nanoparticles of initial concentration of INS was

381   0.067 mg/ml) (Fig. 8). Full insulin release was only observed with QNPHOSEO:INS

382   nanoparticles (initial concentration of INS was 0.067 mg/ml during complex

383   formation) after 50min (Fig. 8). Up to 80% of the insulin was released almost

384   immediately from QNPHOSEO:INS nanoparticles (initial concentration of INS was

385   0.027 mg/ml). Full insulin release was also observed with QNPHOSEO:INS

386   nanoparticles (initial concentration of INS was 0.260 mg/ml) after 8 hours (Fig. 8).

387   Biphasic drug release patterns were observed for this formulation. The release kinetics

388   of INS from this nanosystem follow zero order kinetics, but separated in two phases

389   with two distinct rates, an initial faster one followed by a slower one (Fig. 8). In our

390   opinion, the determining factor of the release profile of the INS is the initial

391   concentration of the protein, which affects the physicochemical and morphological

392   characteristics of the complexes and consequently the release kinetics. From the

393   application point of view, it can be concluded that encapsulation and release of insulin

394   in the present nanosystems can be controlled via the ratio of the two components and

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395 to a lesser extend by the physicochemical parameters of solution during initial

396 complex formation.

397

398 **4. Conclusions**

399 We have studied the electrostatic complexation process between the block

400 polyelectrolyte quaternized poly[3,5-bis(dimethylaminomethylene)hydroxystyrene]-

401 b-poly(ethylene oxide) (QNPHOSEO), which is a cationic-neutral polyelectrolyte,

402 and INS. It was shown that the structure and solution behavior of the formed

403 complexes depend on the ratio of the two components, as well as on the pH and the

404 ionic strength of the solution during complex preparation. This study provides

405 interesting and useful new insights into the interaction mechanism between oppositely

406 charged block polyelectrolyte loose aggregates with stealth properties and proteins.

407 The increase of the ionic strength in the solutions of the complexes induces charge

408 screening and weakening of the electrostatic interactions that lead to different

409 structures of the complexes. The size of complexes in biological medium (FBS) was

410 not increased significantly. This observation indicates that QNPHOSEO copolymer

411 imparts biological stability in the complexes. Moreover, the native protein structure is

412 preserved upon complexation. Encapsulation efficiency increased with the increase of

413 the initial concentration of the protein in PBS. The *in vitro* release of the INS from the

414 prepared polyelectrolyte aggregates is quite slow especially for the QNPHOSEO:INS

415 nanoparticles with the higher initial concentration of INS. The results of our study

416 contribute to the overall scientific efforts to prepare efficient carriers for INS and

417 could be useful in order to develop nanocarriers with increased efficacy, safety and

418 tolerability. The prepared nanocarriers can disclose the pharmacokinetic reality of

419 behavior of INS and improve its therapeutic value and side effects. This is because of

420 the interdependence of particle size distribution and physicochemical characteristics,

421 coupled with the beneficial effects of different biomaterials on biological and

422 pharmacokinetic processes.

423

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532   **Figures**

a.

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537   **Figure 1. a.** Molecular structure of QNPHOSEO block polyelectrolyte (the degrees of

538   polymerization are m=162 and n=334) **b.** Schematic representation of the formation

539   of QNPHOSEO:INS complexes.

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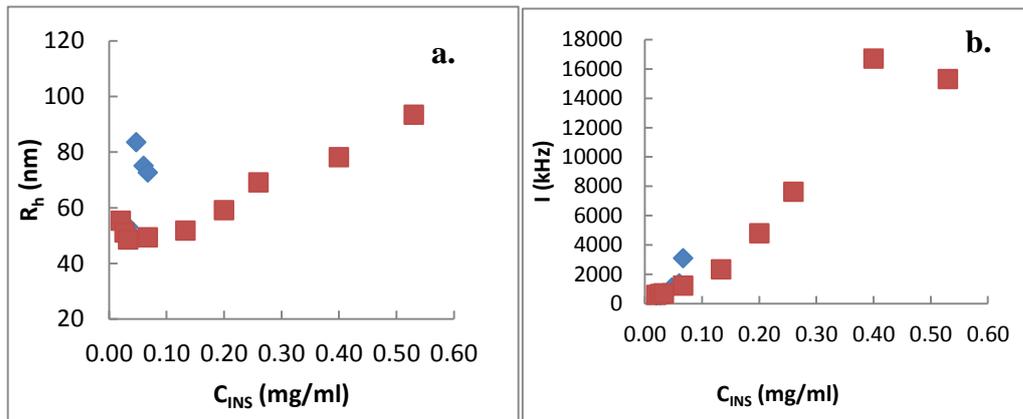
Figure 2. **(a)** Hydrodynamic radius, R_h, and **(b)** light scattering intensity at 90°, I, as a function of C_{INS}, for the solutions of QNPHOSEO:INS system at pH=7.00 and 0.01M NaCl (blue points) and at pH=7.40 and 0.154M NaCl (red points).

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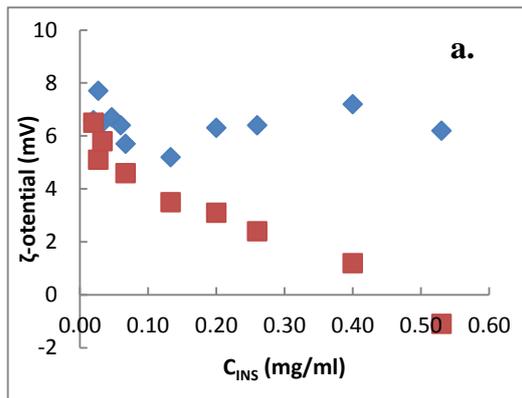
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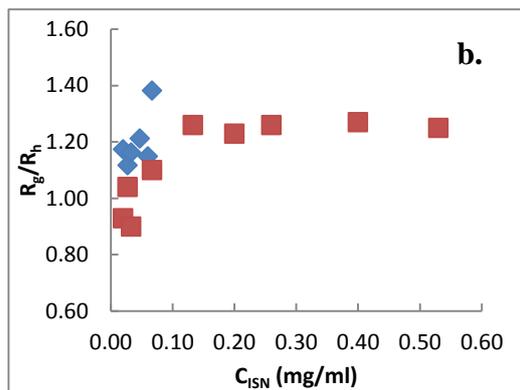
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Figure 3. (**a**) ζ-potential and (**b**) R_g/R_h as a function of C_{INS}, for the solutions of QNPHOSEO:INS system at pH=7.00 and 0.01M NaCl (blue points) and at pH=7.40 and 0.154M NaCl (red points).

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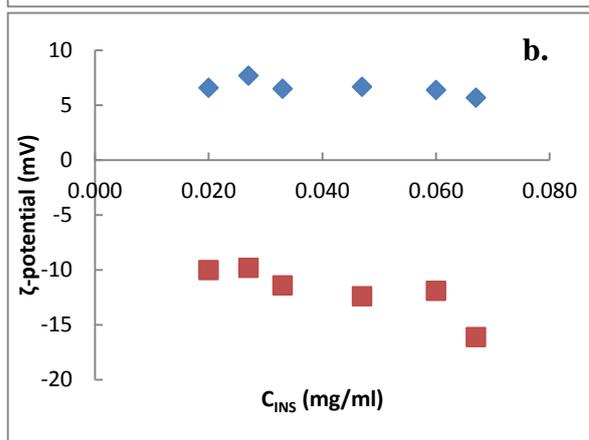
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560 **Figure 4. (a)** Hydrodynamic radius, R_h, and **(b)** ζ-potential as a function of C_{INS}, for
561 the solutions of QNPHOSEO:INS complexes as prepared at pH=7and 0.01M NaCl
562 (blue points) and after dilution in FBS:PBS 10% (v/v) (red points).

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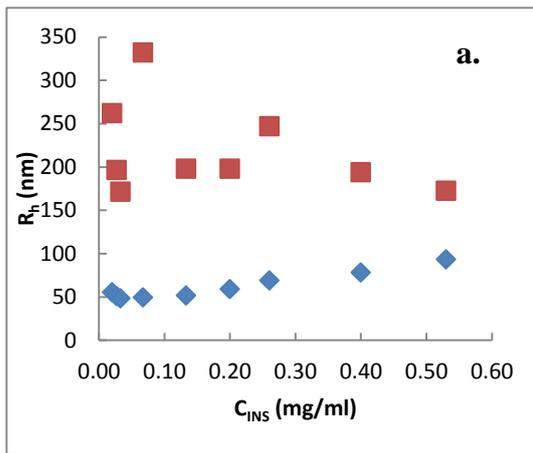
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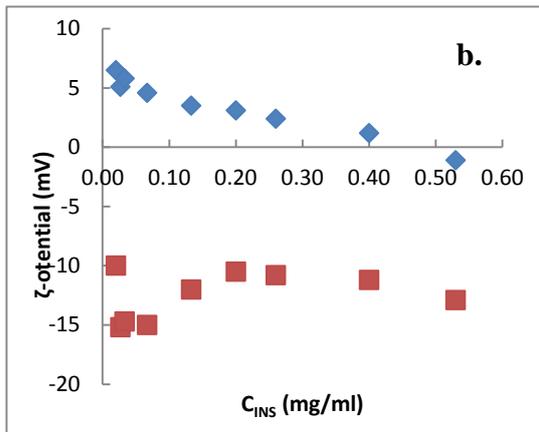
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Figure 5. (a) Hydrodynamic Radius, R_h, and **(b)** ζ-potential as a function of C_{INS}, for the solutions of QNPHOSEO:INS complexes as prepared at pH=7.40 and 0.154M NaCl (blue points) and after dilution in FBS:PBS 10%(v/v) (red points).
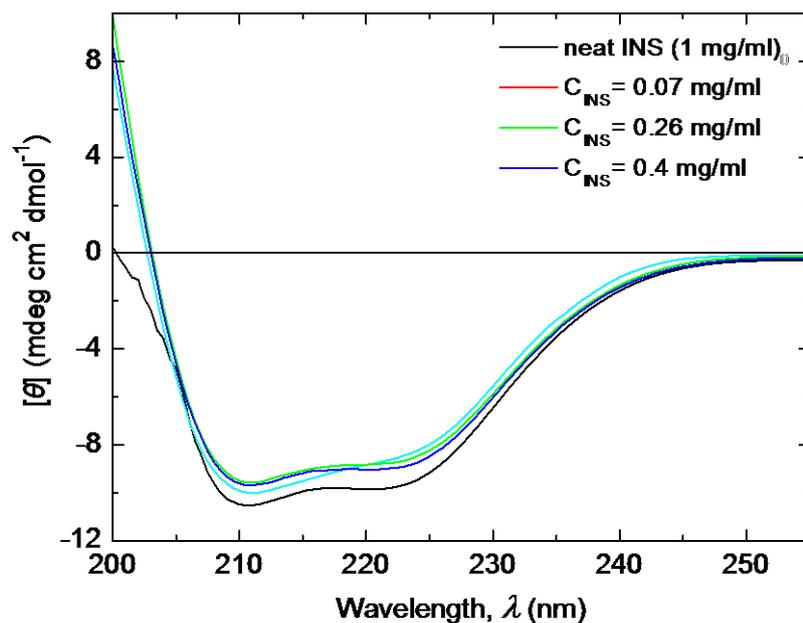
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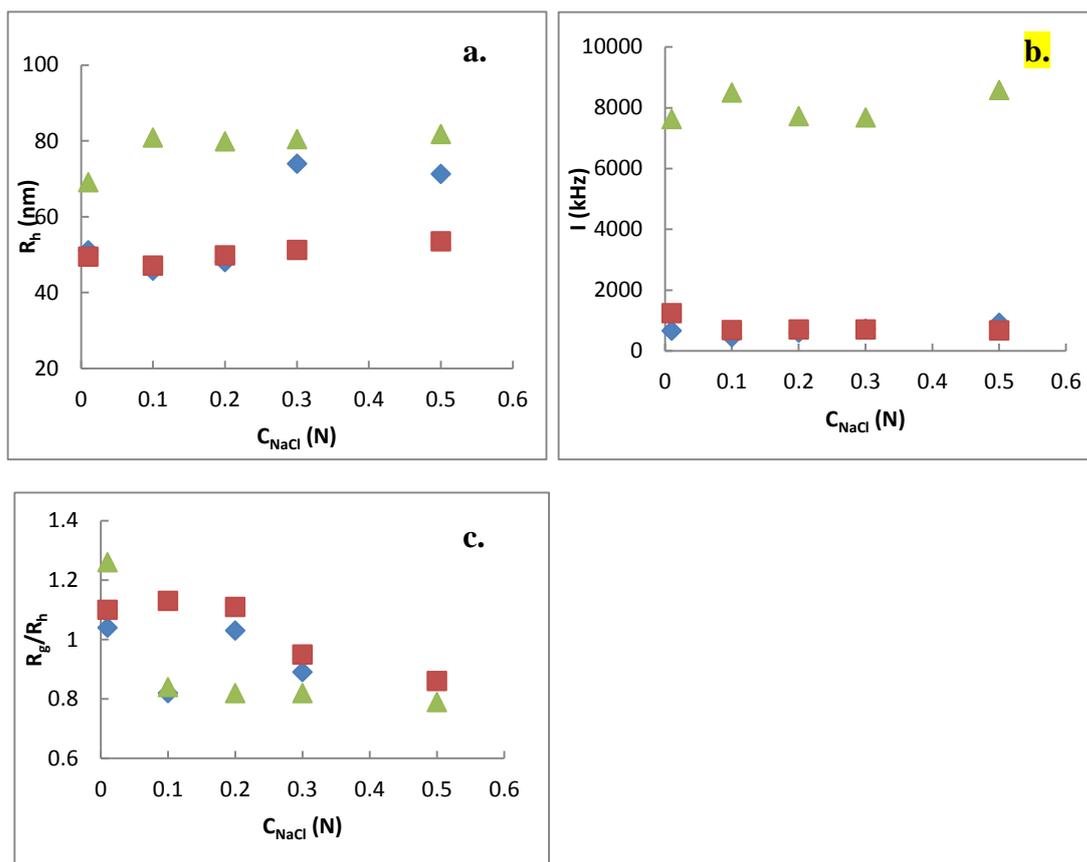
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573 **Figure 6.** (a) Hydrodynamic radius, R_h, (b) light scattering intensity at 90°, I, and (c)
574 R_g/R_h, as a function of the added NaCl concentration of representative solutions,
575 corresponding to low (C_{INS}=0.027mg/ml –blue points and C_{INS}=0.067mg/ml –red
576 points) and high (C_{INS}=0.260mg/ml –green points) C_{INS}, of the QNPHOSEO:INS
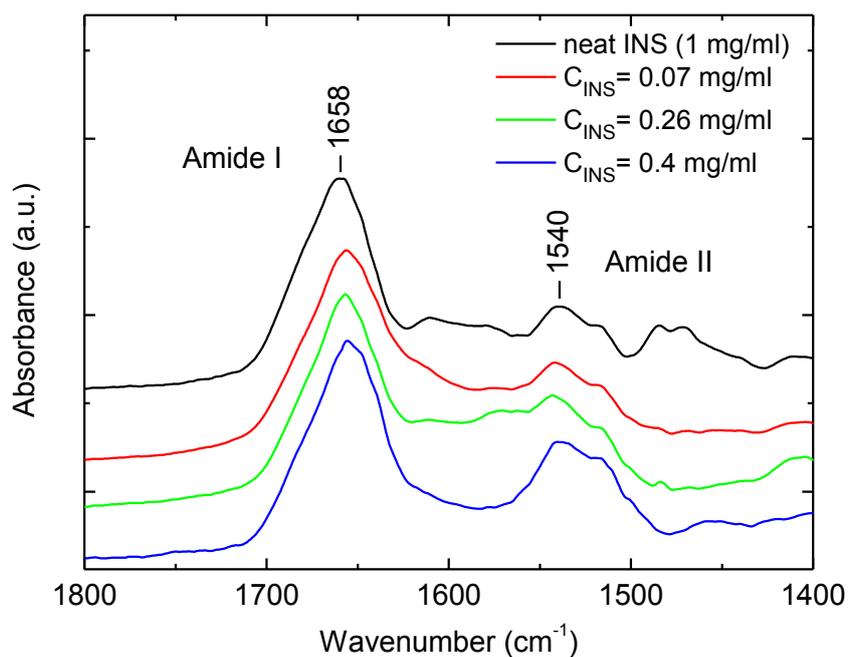577 complexes prepared at pH=7.4 and I=0.154M.

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Figure 7. a. Circular dichroism spectra in the far-UV and **b.** infrared spectra in the Amide I and II region of three representative solutions at C_{INS}= 0.07, 0.26 and 0.4 mg/ml of QNPHOSEO:INS system at pH=7.4 and 0.154M NaCl. The spectrum of neat INS at 1 mg/ml concentration and same solution conditions is included for comparison.

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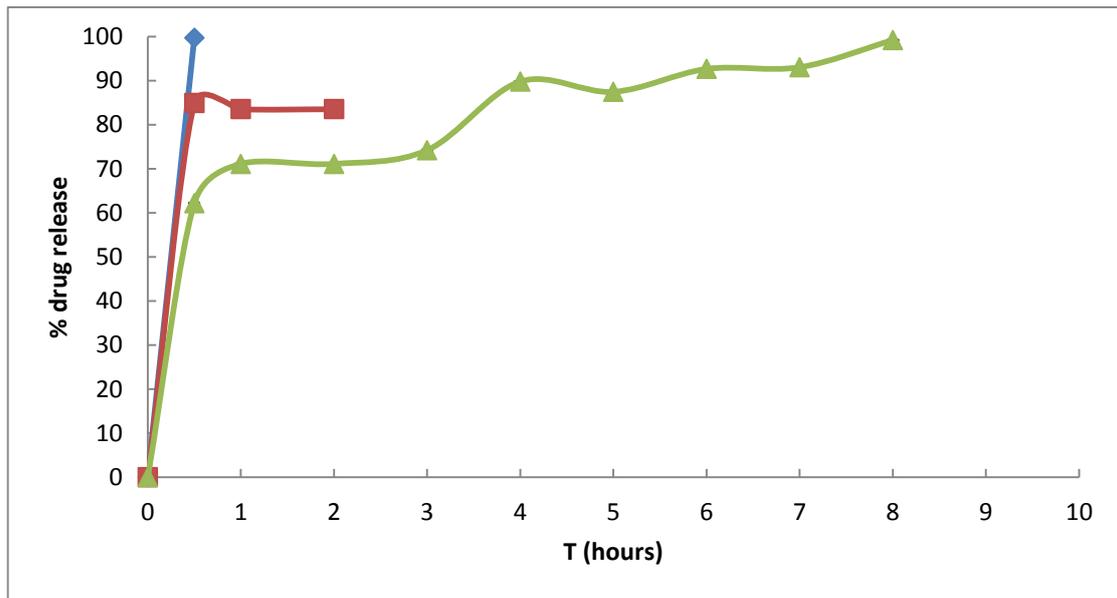
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Figure 8. Cumulative INS release from three different QNPHOSEO based nanocarriers (whereas the initial concentration of protein was $C_{INS}=0.027$ mg/ml –red line, $C_{INS}=0.067$ mg/ml –blue line and $C_{INS}=0.260$ mg/ml –green line) 37°C in PBS. Mean of three independent experiments run in triplicate, SD<10%.

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Caption List

Figure 1. a. Molecular structure of QNPHOSEO block polyelectrolyte (the degrees of polymerization are m=162 and n=334) **b.** Schematic representation of the formation of QNPHOSEO:INS complexes.

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