Insulin/Poly(ethylene glycol)-*block*-poly(L-lysine) Complexes: Physicochemical Properties and Protein Encapsulation

Natassa Pippa[1,2], Radostina Kalinova[3], Ivaylo Dimitrov[3], Stergios Pispas[2,*], Costas Demetzos[1]

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

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

[3]Institute of Polymers, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

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

ABSTRACT

Insulin (INS) was encapsulated into complexes with poly(ethylene glycol)-*block*-poly(L-lysine) **(**PEG-b-PLys), which is a polypeptide-based block copolymer (a neutral-cationic block polyelectrolyte). These macromolecules can encapsulate INS molecules in aqueous conditions via electrostatic interactions. Light scattering techniques are used in order to examine the complexation process of the hybrid nanoparticles in a gamut of buffers, as a function of protein concnetration. The physicochemical and structural characteristics of the complexes depend on the ionic strength of the aqueous medium, while the concentration of PEG-b-PLys was constant through the series of solutions. As INS concentration increased each polyelectrolyte chain interacts with an increasing number of INS molecules, the degree of charge neutralization becomes higher and the size distribution of the complexes decreased also, especially at the highest ionic strength. The size/structure of complexes diluted in biological medium indicated that the copolymer imparts stealth properties and colloidal and biological stability to the complexes, which could in turn affect the clearance properties *in vivo*. Therefore, these studies could be a rational roadmap for designing the optimum complexes/effective nanocarriers for proteins and peptides.

INTRODUCTION

Insulin encapsulated within nanoparticles is one of many strategies that have been developed in order to enhance the absorption and the bioavailability of the particular therapeutic protein, aiming at achieving successful delivery of insulin.[1-4] Several approaches have been employed in order to realize effective insulin formulations. The structural meta- analysis of insulin in pharmaceutical formulations recently appeared in the literature.[5] An ideal insulin carrier should have reasonably high protein encapsulation efficiency and loading capacity, and sustained/controlled release of the loaded protein while retaining bioactivity.[6,7]

Proteins and polyelectrolytes interact, primarily via electrostatic interactions, to form hybrid complexes, which can have widely varied stoichiometries, morphologies, architectures and shapes. These mixed systems are bio-functional with potential applications in the design and development of delivery systems of biopaharmceuticals, as well as of biosensors. According to the recent literature, polylysine-based block copolymers are effective protein, gene and drug nanocarriers.[8-12] These mixed materials exhibit both pH and temperature-responsive self-assembly and are used as nanocarriers with enhanced properties.[13,14] According to Lee and Kataoka, ionic biopharmaceuticals, such as genes and proteins can interact with ionic block copolymers to form polyion complex micelles with core-shell morphology.[15]

In this investigation, a PEG-polypeptide block copolymer, namely poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-b-PLys) was synthesized and evaluated as INS complexing agent. These macromolecules could encapsulate protein molecules in

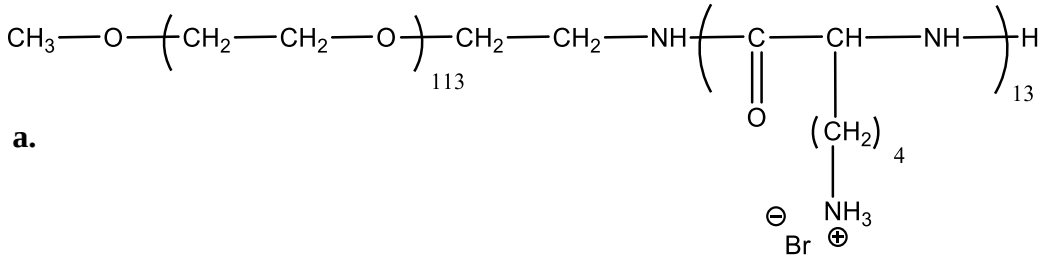
aqueous conditions via electrostatic interactions. In the present work we employ dynamic (DLS), static (SLS) and electrophoretic (ELS) light scattering in order to examine the complexation process, as well the structure and solution behavior of the nanosized complexes in aqueous and biological media, formed between PEG-b-PLys, a cationic-neutral block copolymer, and insulin (INS). It should be pointed out that PEG polymeric chains are employed as the outer shell of the block copolymer micellar type complexes owing to its good water solubility, high degree of biocompatibility, and prolonged circulation time in the blood. The main purpose of this investigation is to develop macromolecular nanostructures as protein carriers in aqueous and biological media and understand aspects of their physicochemical behavior.

EXPERIMENTAL

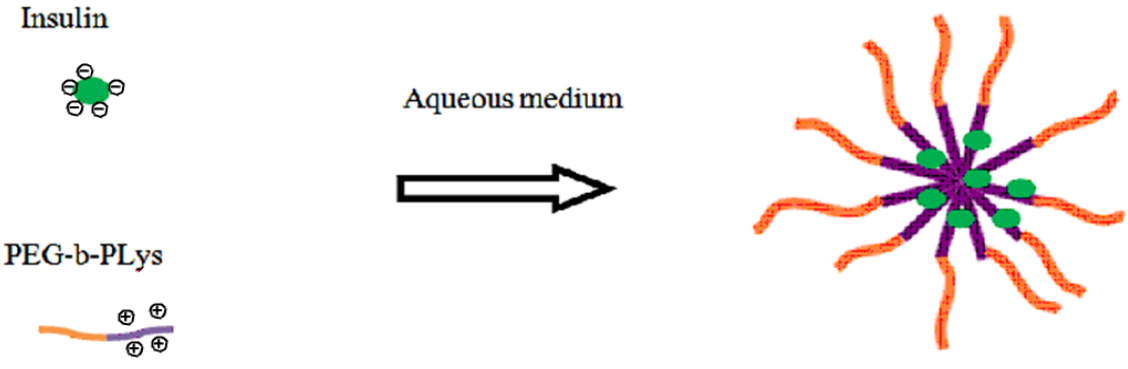
Materials. INS with a molecular weight of 5800 g/mol was purchased from Sigma Aldrich and used without any further purification. N^{ε}-(benzyloxycarbonyl)-L-lysine N-carboxyanhydride (ZLLys-NCA) was prepared from Z-L-lysine and triphosgene in ethyl acetate applying the advanced purification procedure described by Poché and his colleagues.[16] FTIR (cm^{-1}): 3342 (ν, N–H (NCA, Z)); 2936 (ν_{as}, CH_2); 2863 (ν_s, CH_2); 1814, 1774 (ν, C=O (NCA)); 1687 (ν, C = O(Z)); 1533 (δ, N–H(Z); 1258 (δ, C(=O)–O). The polypeptide-based block copolymer PEG-b-PLys was synthesized by controlled ring-opening polymerization of ZLLys-NCA in DMF initiated by primary amino-functionalized methoxy-PEG macroinitiator (M_n = 5000 g/mol, purchased from Sigma-Adrich). The block copolymer was isolated after precipitation in diethyl ether. Due to the copolymer's amphihpilic character it was purified from unreacted macroinitiator by washing of the residue with water/isopropyl alcohol mixture and

final product was collected after centrifugation. GPC in DMF (vs. poly(methyl methacrylate) standards): M_w/M_n = 1.28. ^{1}H NMR (DMSO-d_6): δ): 1.10–2.10 (αCH-(CH_2)$_3$), 2.95 (αCH-(CH$_2$)$_3$-CH_2), 3.23 (CH_3O); 3.51 (O-CH_2-CH_2-O + O-CH_2CH_2-NH), 3.90-4.30 (αC*H*-NH), 4.98 (Z-CH_2), 6.83–7.41 (αCH-(CH$_2$)$_4$-N*H* + C$_6$*H*$_5$), 7.89–8.19 (αCH–N*H*). FTIR (cm^{-1}): 3318 (ν, N–H (NCA, Z); 1697 (ν, C=O(Z); 1651 (ν, C=O (amide I); 1537 (δ, N-H(Z) (amide II), 1103 (ν, C-O-C (ether).

The block copolymer was dissolved in trifluoroacetic acid and was treated with HBr (33 wt.% in acetic acid) to remove the benzyloxycarbonyl protecting groups from the peptide units. ^{1}H NMR (D$_2$O): δ): 1.30-1.95 (αCH-(CH_2)$_3$); 3.02 (αCH-(CH$_2$)$_3$CH_2); 3.40 (CH_3O); 3.72 (O-CH_2-CH_2 + O-CH_2CH_2-NH), 4.33 (αC*H*-NH). The number average degree of Lys polymerization determined by ^{1}H NMR analysis is 13 (approx. 35 wt % Lys units in the block copolymer, Figure S1) The structure of PEG-b-PLys is presented in Fig. 1(a).



a.



b.

Figure 1: (a) Molecular structure of PEG-b-PLys polyelectrolyte block copolymer and **(b)** schematic representation of the formation of PEG-b-PLys:INS complexes.

Preparation of PEG-b-PLys:INS polyelectrolyte aggregates in different aqueous media. A pH 7 buffer solution was prepared from NaOH and 5mM sodium phosphate, Moreover, 10mM NaCl were added to solution to maintain a fixed ionic strength, along with NaN_3 in a final concentration of 200 ppm, in order to avoid bacterial growth. Stock solutions of INS and of PEG-b-PLys were prepared by dissolving a weighed amount of the dialyzed sample in the appropriate volume of the buffer and the solutions were left to stand overnight for better equilibration. Stock solutions of insulin (1mg/ml) and polyelectrolyte in 0.01M pH=7.10 (Tris buffer) were also prepared.[10,17]

The complexes were prepared by adding different amounts of the INS solutions to PEG-b-PLys solutions of the same volume and concentration, under stirring. Finally, appropriate volumes of buffer solutions were added to achieve a constant final volume and ionic strength (equal to that of the buffer solutions) for all solutions prepared. Thus, the concentration of PEG-b-PLys was kept constant throughout the series of solutions, while that of INS varied in order to control the required ratio of the two components (or equivalently the [-]/[+] charge ratio of the mixture). The solutions of the complexes developed a bluish tint or turbidity upon mixing, indicating the formation of supramolecular complexes. Subsequently, the solutions of the complexes were left for equilibration overnight, which in some cases resulted in coacervation, i.e. liquid-liquid phase separation of the solution, depending on the INS concentration and pH. Stable solutions were further characterized as discussed below.

For the ionic strength dependent light scattering measurements, the ionic strength of the solution was gradually increased by the addition of appropriate aliquots of 1N NaCl solution at pH=7.00, pH=7.10 (Tris buffer) or 7.40 (PBS), to 1ml of the previously prepared solutions of the complexes. After each addition the solution was

rigorously stirred and left to equilibrate for 15min before measurement. Changes in solute concentrations due to NaCl solution addition were taken into consideration in the analysis of the light scattering data.

Insulin encapsulation efficiency. The loading of insulin into complexes was investigated by dialysis method. Hybrid nanovectors incorporating INS (3ml of each sample) were placed in dialysis sacks (molecular weight cut off 12,000; Sigma-Aldrich). Dialysis sacks were inserted in 30 mL PBS in shaking water bath, set at 25 ^{o}C (room temperature), for 2 hours. This protocol was found to give consistent results as supported by triplicate dialysis experiments. The percentage of INS incorporated into complexes was estimated by spectrophotometry. INS concentration was estimated with the aid of the following INS calibration curve in PBS (pH=7.4):

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

The absorbance was measured at 277nm. Encapsulation efficiency (EE) was calculated by using the following equation:

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

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

Dynamic and static light scattering. The hydrodynamic radius (R_h) of polyelectrolyte nanocarriers and the size polydispersity index (PD.I.) were determined by dynamic light scattering (DLS). Mean values and standard deviations were calculated on three independently prepared 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:

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

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.

Static light scattering has been used in order to estimate the radius of gyration, R_g, of the chimeric nanoassemblies via the use of Zimm plots, which can be described by the following equation:

$$\left(\frac{KC}{R_{VV}(q)}\right)_{c\to 0} \cong \frac{1}{M_W}\left(1 + \frac{1}{3}R_g^2 q^2\right) \quad (\mathbf{4})$$

where $R_{vv}(q)$ is known as the Rayleigh ratio, $K = 4\pi^2 n^2 (dn/dC)^2 / (N_A \lambda_0^4)$ and $q = (4\pi n/\lambda_0)\sin(\theta/2)$, with N_A, dn/dC, n and λ_0 being the Avogadro number, the specific refractive index increment, the solvent refractive index, and the wavelength of the light in vacuum, respectively. Measurements were made at the angular range of 30º to 150º. Toluene was used as the calibration standard for obtaining absolute values for the scattered intensity. These values were subsequently utilized for the estimation of R_g/R_h ratios which are indicative of the shape of the nanoassemblies.

Electrophoretic Light Scattering-Zeta potential measurements. Electrophoretic Light Scattering is a technique used to measure the electrophoretic mobility of hybrid nanostructures. The fundamental physical principle is that of electrophoresis. A dispersion/solution is introduced into a cell containing two electrodes. An electrical field is applied to the electrodes, and particles that have a net charge, or more strictly a net zeta potential, will migrate towards the oppositely charged electrode with a velocity, known as the electrophoretic mobility, that is related to their zeta potential values. The zeta potential of nanostructures was measured using Zetasizer 3000HAS, Malvern Instruments, Malvern, UK. 50μl of the solutions of the block copolymer/protein complexes was 30-fold diluted in buffer and ζ-potential was measured at room temperature at 633nm. The zeta potentials were calculated from electrophoretic mobilities, μ_E, by using the Henry correction of the Smoluchowski equation:

$$\zeta = \frac{3\mu_E n}{2\varepsilon_0 \varepsilon_r} \frac{1}{f(\kappa a)} \quad (\mathbf{5})$$

where ε_0 is the permittivity of the vacuum, ε_r is the relative permittivity, α is the particle radius, κ is the Debye length, and n is the viscosity of water. The function f(κα) depends on particle shape. While if κα > 1:

$$f(\kappa\alpha) = 1.5 + \frac{9}{2(\kappa\alpha)} + \frac{75}{2(\kappa\alpha)^2} \quad (\mathbf{6})$$

The above function refers to dispersions of the present study.

Results and Discussion

Physicochemical and structural characterization of PEG-b-PLys:INS complexes in aqueous media. The complexation process between the PEG-b-PLys

polyelectrolyte and INS at pH=7, pH=7.1 and at pH=7.4 was first investigated by means of dynamic light scattering. It should be noted that PBS (pH=7.4) was chosen as dispersion medium because the pH and the ionic strength of PBS resembles the physiological conditions of human body. At pH=7 the electrostatic interactions of the system are expected to be strong, since the PEG-b-PLys polyelectrolyte block carries thirteen (+13) positively charged groups per chain (number of lysine amine functionalized monomeric units, Figure 1(a) and INS has a net negative charge of -4. The distributions of hydrodynamic radius (R_h) for INS and PEG-b-PLys exhibit some differences in the different aqueous media, while the R_h were more or less the same. The obtained results from DLS measurements at 90° regarding the values of the light scattering intensity, I_{90}, and hydrodynamic radius, R_h, are shown in Figures 2(a) and (d), as a function of the protein concentration, C_{INS}, in the solutions of the complexes. The structure and the formation process of complexes is schematically represented in Fig.1(b). The concentration of PEG-b-PLys copolymer is kept constant throughout the series of aqueous solutions of different ionic strength investigated. DLS results at low ionic strength (0.01M) at pH=7 show that all solutions exhibit a main peak at high R_h values (ca. 60nm) at low concentration of INS, which apparently corresponds to the formed mixed aggregates (Fig. 2(a)). An increase of R_h values was observed as the concentration of INS was increased. These solutions exhibit a main peak at high R_h values (~120nm at the highest concentration of INS) (Fig. 2(a)). Furthermore, DLS results at low ionic strength (0.01M) at pH=7.10 show that all solutions exhibit a main peak at high R_h values (~40nm) at low concentration of INS, which apparently corresponds to the formed mixed aggregates (Fig. 2(a)). On the other hand, a decrease R_h values was observed at the increased concentration of INS, and these suspensions exhibited a main peak at ~30nm. The Polydispersity Index (PD.I.) values indicate

quite monodisperse colloidal formulations, especially for the dispersions at low ionic strength (0.01M) and at high INS concentrations (Fig. 2(b)). ζ- potential remained unaffected as the concentration of the INS increases (Fig. 2(c)). The values of the scattering intensity, I_{90}, which is proportional to the mass of the species in solution, increased gradually as a function of $C_{INS,}$ providing proof of the occurring complexation (Fig.2(d)), i.e. the mass of the complexes increases as C_{INS} increases. As INS concentration increases each polyelectrolyte chain interacts with an increasing number of INS molecules, the degree of charge neutralization becomes higher and the size distribution of the complexes decreased also, especially at the highest ionic strength. In contrast I_{90} (and mass of the complexes) remains almost constant in the case of PBS solutions (Fig. 2(d)). On the other hand, the ζ-potential values increased in absolute value as the concentration of the INS increases, in PBS (Fig. 2(c)), and become more negative. Additionally, the R_h did not changed significantly as the concentration of INS was increased, while the population of the colloidal nanoparticles became more homogeneous (Fig. 2(b)). Taking into consideration the greater ionic strength of PBS one can assume that the above observations may be due to increased screening of effects on the electrostatic interactions between the components of the complexes. The mass of the complexes is significantly higher at pH=7 and I=0.01M, showing increased interactions, while the size of the complexes is constantly larger in PBS, showing more swelled structures under the latter conditions. Another synergistic phenomenon that could contribute to the above phenomenology is the different solvation of PEG polymeric chains.[18] These differences in the hydration energy of the water molecules make the PEG polymeric chain more hydrophilic. The presence of ions plays a key role for the improved hydration of PEG chains due to the

screening effects in the interactions between the water molecules and the PEG polymeric chains.[19-20]

The morphology of nanovectors is quite important for their biological behavior and pharmacokinetic profile of the encapsulated bioactive compound.[21-24] The values of R_g/R_h were also calculated and presented in Fig 2.(e)**.** This ratio is sensitive to the shape/morphology of nanoparticles/ nanoparticulate complexes in dispersion and can be used as a rough estimate of the internal morphology of the colloidal particle. This is based on the notion that R_h defines the outer dimensions of the particle while R_g is a measure of the mass density distribution around the center of the nanostructure. According to Burchard, the ratio R_g/R_h takes the values of 0.775 for a hard uniform sphere and 1.0 for vesicles with thin walls, while values of 1.3 to 1.5 indicate a random coil (loose) conformation in the case of macromolecular chains.[25] In the present case, the values R_g/R_h are in the range 0.80-1.00 for PEG-b-PLys:INS at low ionic strength (I=0.01M) and pH=7, indicating a more well-defined spherical, rather dense nanostructure for the complexes (Fig 2.(e)). On the other hand, the values R_g/R_h indicate open (low density) spherical structures for the complexes or a rather low density full spherical structure in PBS (Fig 2.(e)). The differences of the external morphology of complexes indicate that the dispersion medium plays a key role on the electrostatic interactions and the hydration of the hydrophilic PEG chains, due to the different ionic strength of the aqueous dispersion media. In other words, the physicochemical characteristics of the prepared complexes are strongly influenced by the dispersion medium (salinity, pH, ionic strength).

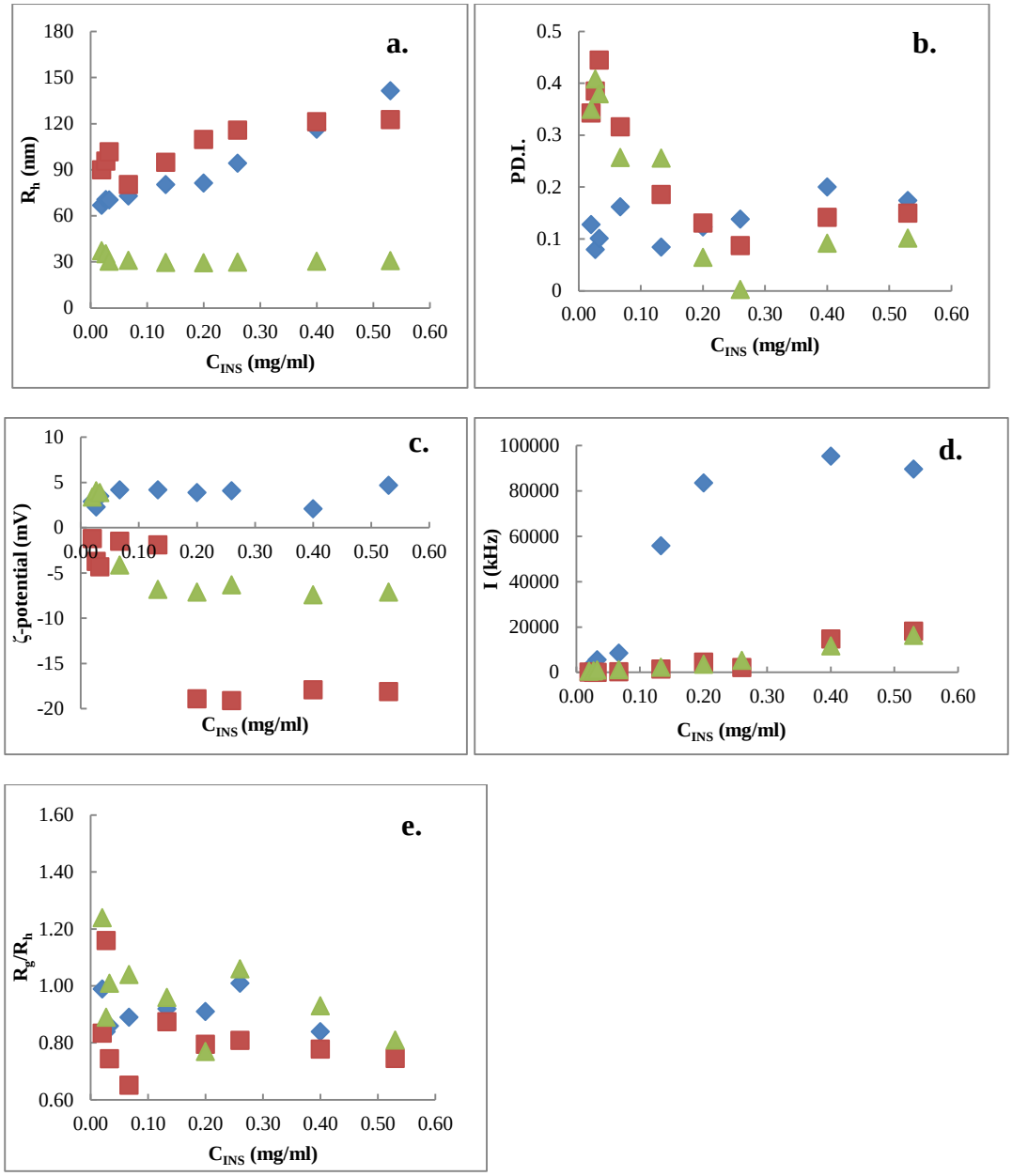


Fig. 2. (a) Hydrodynamic radius, R_h, **(b)** Polydispersity Index, (PD.I.), **(c)** ζ-potential, **(d)** light scattering intensity at 90°, I, and **(e)** R_g/R_h as a function of C_{INS}, for the solutions of PEG-b-PLys:INS complexes formed at pH=7 and 0.01M NaCl (diamonds), pH=7.1 and 0.01M (Tris buffer) (triangles) and at pH=7.4 and 0.154M NaCl (squares).

The encapsulation efficiency of the PEG-b-PLys complexes in PBS was also investigated. Values of encapsulation efficiency varied from 35 to 70% for the

different formulation as seen in Figure 3. They were particularly affected by the initial concentration of INS. Encapsulation efficiency increased with the increase of the initial concentration of the protein in PBS (Figure 3). The insulin encapsulation efficiency of these hybrid nanosystems increased at the isoelectric point, where the zeta potential exhibited a shift to negative values (Figure 3 and Fig. 2.(c))[26,27] The higher encapsulation efficiency was observed when the initial concentration of insulin was C_{INS}=0.133mg/ml ([-]/[+] charge ratio equal to 1). At higher concentration of insulin the ζ-potential of complexes became more negative, and this observation indicated that there is repletion in the complexation process/ encapsulation efficiency after the charge equilibration point. This observation may indicate a difference in the internal structure of the complexes at different protein/copolymer ratios. It should be pointed out that the nanocarriers did not release the protein until four hours. In our opinion, this observation is correlated to the strong electrostatic interactions between the protein and the PLys block of the block polyelectrolyte.

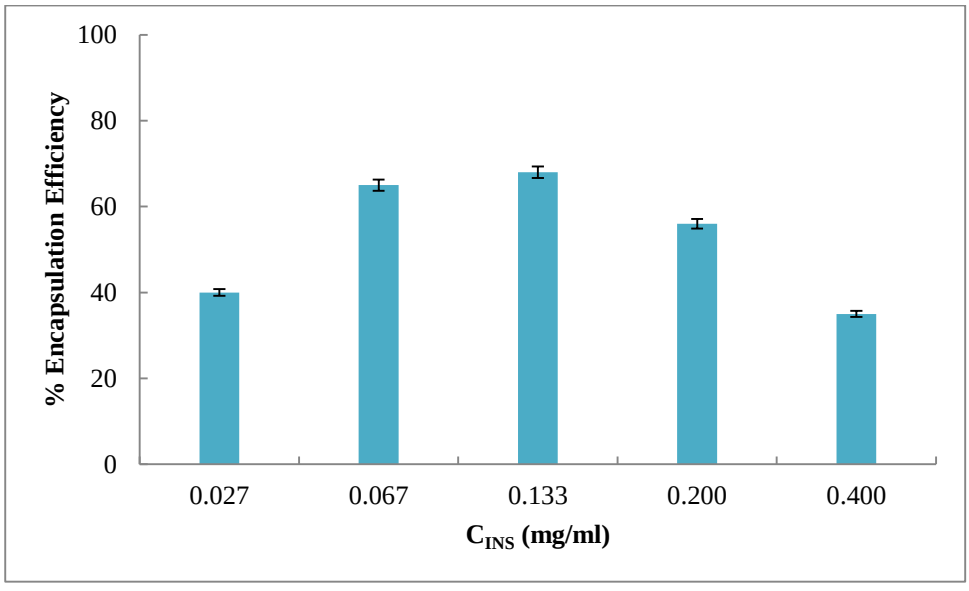
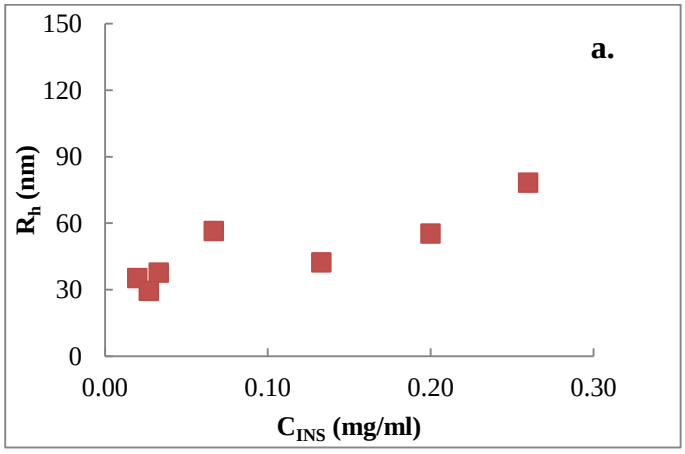


Fig. 3: Encapsulation efficiency (%) of INS in PEG-b-PLys:INS complexes in PBS, as a function of the initial concentration of INS.

Physicochemical characterization of preformed PEG-b-PLys:INS complexes in biological medium (FBS). It is of great importance for the clinical application to characterize the physicochemical properties of the nanostructures in biological media, like FBS, because interaction with plasma proteins of the medium (especially albumin) is expected to alter the physicochemical and morphological properties of the colloidal nanostructures, thereby affecting their stability and clearance/biodistribution properties.[28]

The prepared complexes in aqueous media were diluted in FBS:PBS 10%/90% v/v, in order to investigate the properties of the hybrid complexes in a biological medium. The size of the complexes in biological medium (FBS) are presented in Figs. 4(a) and 5(a). The size of the complexes in FBS decreased by 10 to 25nm, for the complexes prepared in the two different aqueous media, while the reduction of the scattering intensity was larger in comparison to aqueous media. The obtained results indicate the disintegration of the supramolecular aggregates due to the interaction of the components (PEG-b-PLys and insulin) with the serum components, especially with the albumin. After the dilution of the complexes in FBS:PBS 10%/90% v/v, the insulin/PEG-b-PLys hydrid nanostructured exhibited different physicochemical characteristics. In all cases, the supramolecular aggregates of insulin carrier complexes and plasma proteins remained at the nanoscale (smaller than 100nm). In some cases, the observed decrease of the R_h may be due to the interactions of FBS albumins with insulin/PEG-b-PLys complexes, leading to changes in their size, via components redistribution and partial disruption of the initial complexes. This observation indicates that PEG-b-PLys copolymer partially imparts stealth properties and biological stability in the complexes, due to the presence of PEG polymeric chains that shield the nanostructures. A shift of ζ-potential to negative values is

observed presumably due to some binding of FBS proteins, which can alter the nanoparticle's effective size, hydration and surface properties (the main protein component of FBS is albumin which carries a negative charge at physiological conditions).[29,30] We should point out that the ζ-potential of FBS:PBS 10%/90% v/v biological medium is equal to -10mV, while the albumin exhibits -18 negative charges. As a result, in the dispersion, after the dilution in FBS:PBS 10%/90% v/v, serum protein/PEG-b-PLys/insulin complexes, and free serum proteins may be present and influence the values of zeta-potential. However, only one broad peak in electrophoretic mobility was observed, so it is difficult to make any additional conclusions on the present systems. Subsequently, there is a significant decrease in the scattered intensity from the protein–PEG-b-PLys:INS clusters (Figs. 4(b) and 5(b). Alterations in scattering intensity should be attributed to changes in the mass of the species (colloidal polymeric nanoparticles and complexed components of FBS) and/or changes in the relative amounts of the supramolecular aggregates. The results seem to point also toward a change in the internal structure of PEG-b-PLys:INS due to the limited interaction (possible absorption and incorporation) of serum proteins and their aggregation.



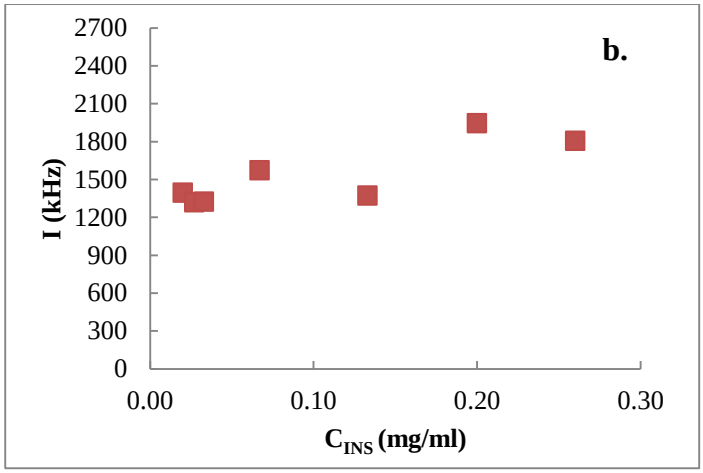
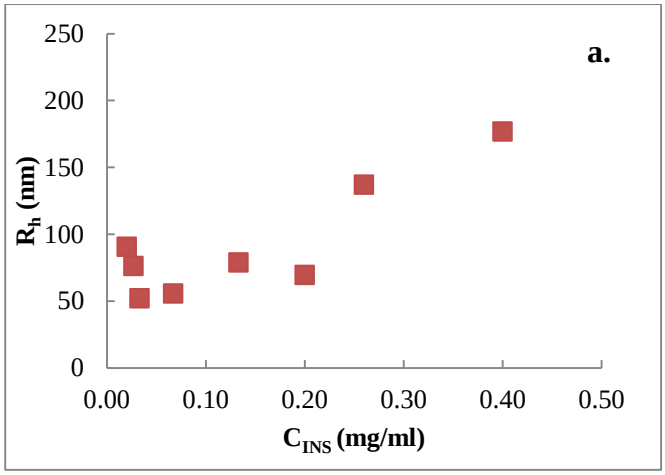


Fig. 4. (a) Hydrodynamic Radius, R_h and **(b)** light scattering intensity at 90°, I, as a function of C_{INS}, for the solutions of PEG-b-PLys:INS complexes prepared at pH=7 and I=0.01M, diluted in FBS:PBS 10%/90% (v/v).



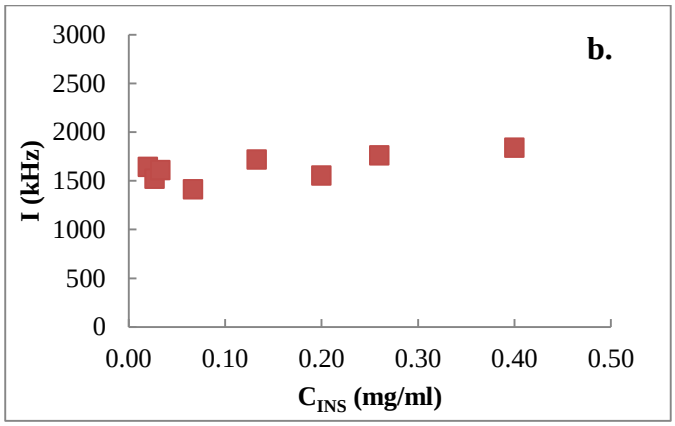


Fig. 5. (a) Hydrodynamic radius, R_h and **(b)** light scattering intensity at 90°, as a function of C_{INS}, for the solutions of PEG-b-PLys:INS complexes prepared at pH=7.4 and I=0.154M, and diluted in FBS:PBS 10%/90% (v/v).

Effect of ionic strength on the physicochemical characteristics of preformed PEG-b-PLys:INS complexes.

According to the literature, it was determined generally that the increase of the ionic strength in the solutions of the polyelectrolyte complexes with peptides/proteins eventually causes the aggregation of the preformed complexes. This effect arises from the induced charge screening that weakens the electrostatic interactions of the systems.

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 performed complexes. In order to investigate this effect, DLS measurements as a function of the added NaCl concentration were conducted, and the resulting I_{90} (corrected for the difference in concentration) and R_h values for representative solutions at low and high C_{INS} values of PEG-b-PLys:INS system at pH 7.00, pH=7.10 and 7.40 are shown in Figs. 6., 7. and 8. At pH 7.00 and pH=7.10, the dispersions of the complexes exhibit more or less the same response (same size, R_h values) to the increase of the ionic strength, apparently due to the particular structure of the formed complexes in each solution (Figs. 6(a). and 7(a).). The scattering intensity decreased significantly (Figs. 6(b) and 7(b)) and this trend was independent of the initial concentration of the insulin. As mentioned previously, at low C_{INS} values the number of interacting INS molecules per polyelectrolyte chain is rather small, while at high C_{INS} values the formed complexes are characterized by rather large number of interacting INS molecules per polyelectrolyte chain. Respectively, an abrupt increase of the mass of the complexes is observed at higher C_{INS} upon addition of the salt at both aqueous media (Figs. 6b and 7b). The R_h of the INS complexes, as a function of INS concentration, increased slightly in the case of PBS buffer (Fig. 8(a)), while the scattering intensity decreased significantly (Fig. 8(b)).[31,32]

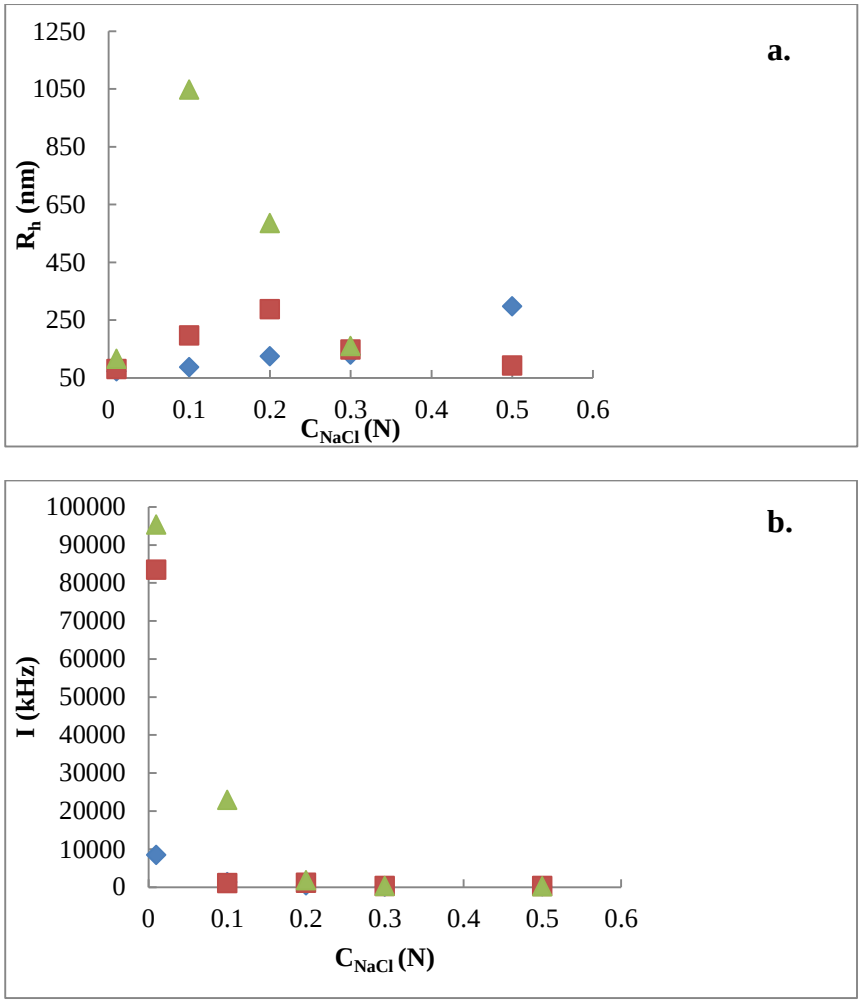


Fig. 6. (a) Hydrodynamic Radius, R_h, and **(b)** light scattering intensity at 90°, I, as a function of the added NaCl concentration of representative solutions (0.067mg/ml – diamonds, 0.200mg/ml – squares and 0.400mg/ml –triangles), corresponding to low and high C_{INS}, of the PEG-b-PLys:INS complexes prepared at pH=7 and I=0.01M.

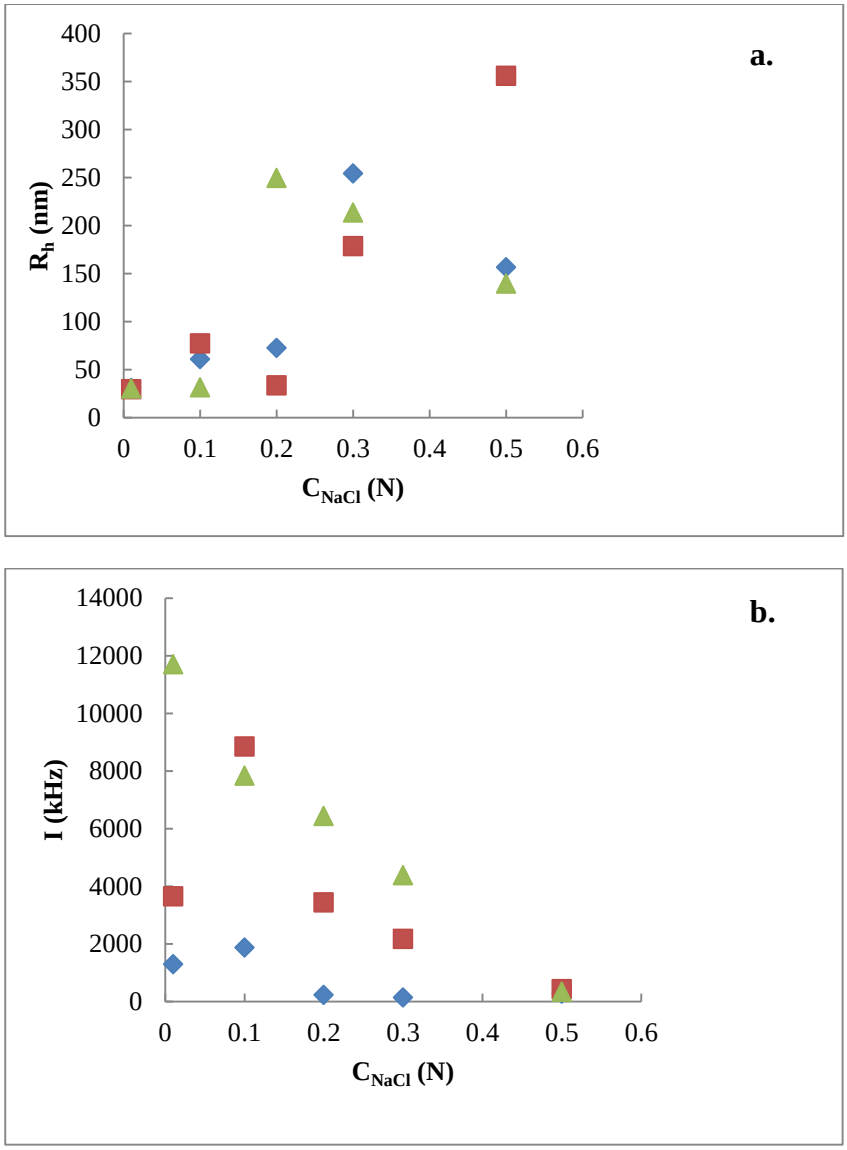


Fig. 7. (a) Hydrodynamic Radius, R_h, and **(b)** light scattering intensity at 90°, I, as a function of the added NaCl concentration of representative solutions, corresponding to low and high C_{INS}, (0.067mg/ml –diamonds, 0.200mg/ml – squares and 0.400mg/ml –triangles),of the PEG-b-PLys:INS complexes prepared at pH=7.1 (Tris buffer).

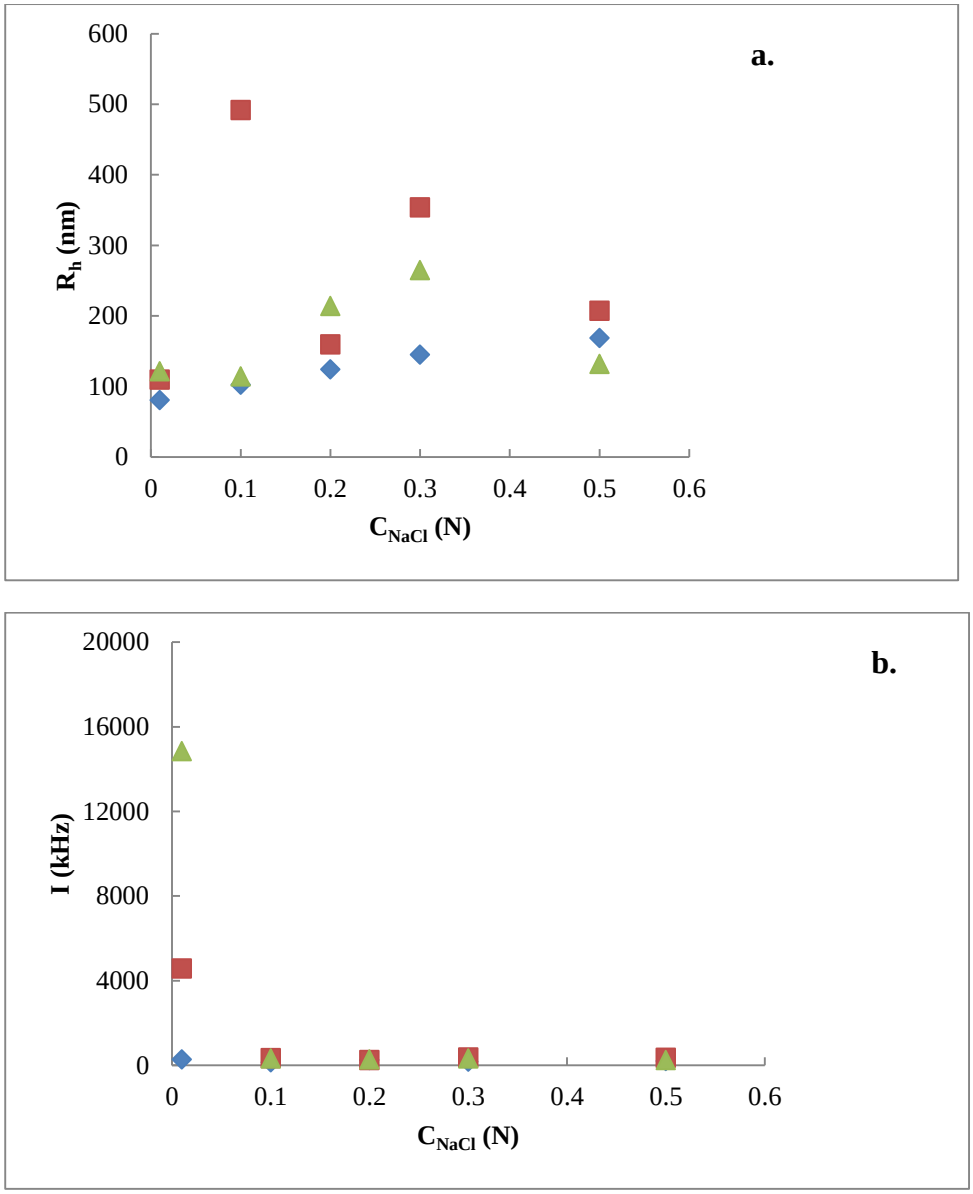


Fig. 8. (a) Hydrodynamic Radius, R_h, and **(b)** light scattering intensity at 90°, I, as a function of the added NaCl concentration of representative solutions, corresponding to low and high C_{INS}, (0.067mg/ml –diamonds, 0.200mg/ml – squares and 0.400mg/ml –triangles), of the PEG-b-PLys:INS complexes prepared at pH=7.4 and I=0.154M (PBS buffer).

CONCLUSIONS

In the present work, we employ a gamut of light scattering techniques in order to examine the complexation process, as well the structure and solution behavior of the nanosized complexes in aqueous and biological media. The concentration of

polyelectrolyte copolymer is kept constant throughout the series of aqueous solutions of different ionic strength investigated. The physicochemical and structural characteristics of the complexes depend on the ionic strength of the aqueous medium. The values of the scattering intensity, I_{90}, which is proportional to the mass of the species in solution, increase gradually as a function of $C_{INS,}$ providing proof of the occurring complexation. The size of complexes in biological medium (FBS) was not increased significantly. This observation indicates that the copolymer imparts stealth properties and biological stability to the complexes, which could affect the clearance properties *in vivo*. The selection of the buffer (aqueous medium) for formulating insulin/PEG-b-PLys complexes is a crucial parameter, because it affects the complexation process, the physicochemical characteristics, the internal/external morphology, the biological behavior and the effects of salinity on the preformed hybrid block copolymer/protein nanostructures. In conclusion, this study provides interesting and useful new insights into the interaction mechanism between oppositely charged block polyelectrolyte and INS resulting in formation of aggregates with stealth properties, and could be a roadmap in order to select the aqueous medium to achieve the desired properties of the nanocarriers.

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Supporting Information Available: NMR spectra of the block polyelectrolyte and size distributions of complexes in different media. This material is available free of charge via the Internet at http://pubs.acs.org.

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Insulin/Poly(ethylene glycol)-*block*-poly(L-lysine) Complexes: Physicochemical Properties and Protein Encapsulation

Natassa Pippa, Radostina Kalinova, Ivaylo Dimitrov, Stergios Pispas*, Costas Demetzos