Self-assembled carrageenan/protamine polyelectrolyte nanoplexes – investigation of critical parameters governing their formation and characteristics

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Abstract

The aim of this work was to investigate the feasibility of cross-linker free polyelectrolyte complex formation at the nanoscale between carrageenan (CAR) and protamine (PROT). The properties of CAR/PROT nanoparticles (NPs) were dependent on the carrageenan type: kappa (KC), iota (IC) and lambda (LC), concentration of components, addition of divalent cations, weight mixing ratio (WMR) of constituents and mode of component addition. In the case of 0.1% w/v solutions, IC-based NPs had the smallest particle sizes (100-150 nm) and low polydispersity indices (0.1-0.4). A decrease in the solution concentration from 0.1% to 0.05% w/v enabled the formation of KC/PROT NPs. All carrageenans exhibited the ability to form NPs with surface charge ranging from -190 to 40 mV. The inclusion of divalent cations caused an increase in the particle size and zeta potential. Infrared analysis confirmed the presence of a complex between CAR and PROT and showed that IC chains undergo structural changes when forming NPs. Colloidal stability of NPs was related to the initial surface charge of particles and was time- and pH-dependent. IC was found to be the most suitable type of CAR when forming nanoplexes with PROT.

Keywords: Carrageenan; Protamine; Nanoparticles; Polyelectrolyte Complexes; Dynamic Light Scattering; Infrared spectroscopy

1. Introduction

Recent advances in pharmaceutical nanotechnology are focused on using polymeric nanoparticulate systems as carriers for drugs (Delie & Blanco-Prieto, 2005). Nanoparticles (NPs) formulated from natural polymers have attracted considerable interest (Hans & Lowman, 2002). NPs have many advantages, such as the potential to retain protein stability, increase duration of therapeutic effects of proteins and they can be administered by nonparenteral routes (Sarmento, Bibeiro, Veiga, Ferreira and Neufeld, 2007). Natural polymers extensively studied for drug delivery purposes include polysaccharides such as alginate, chitosan, carrageenan (CAR) and proteins, for example casein and gelatin (Sonia & Sharma, 2012).

Polymers, especially those of natural origin, are often composed of subunits capable of bearing charge, thus the polyelectrolyte complexation method of NP preparation has received increasing attention in recent years. NPs formed by this method have several characteristic advantages for cellular uptake and colloidal stability, including suitable diameter, surface charge, spherical morphologies and low polydispersity indices (Bayat et al., 2008). Furthermore, the preparation of NPs by polyelectrolyte complexation methods can be carried out in completely aqueous conditions and at ambient temperature; therefore the stability and biological activity of loaded peptides would not be affected (Hu, Yang and Hu, 2012; Ryan et al., 2013; Umerska et al., 2012, 2014).

Carrageenans (CARs) are an example of such polymers and are capable of forming polyanions. They are a family of hydrophilic sulphated biopolysaccharides extracted from various species of the *Rhodophyta* class of red seaweeds (Campo, Kawano, Braz da Silva and Carvalho, 2009). CARs are composed of a hydrophilic linear sulphated backbone of alternating 1→3 glycosidic-linked β-D-galactopyransose units and 1→4 glycosidic-linked α-D-galactopyranose units or 1→4 glycosidic-linked 3, 6-anhydro-α-D-galactopyranose units (Figure 1) (Berth, Vukovic and Lechner, 2008) and principally differ in the number and position of the sulphate groups, however these reflect only general differences in the composition and degree of sulphation (Necas & Bartosikova, 2013). The actual content of the

sulphate residue (by weight) may vary between 15 and 40% for the various carrageenan types (Nanaki, Karavas, Kalantzi and Bikiaris, 2010). Their applications include experimental medicine, pharmaceutical formulations and are well established as gelling, stabilising and thickening agents for food, cosmetics and industrial uses (Necas & Bartosikova, 2013). CARs are biocompatible and biodegradable polysaccharides with low toxicity and well documented properties of controlling and extending release of various drug substances (Nanaki et al., 2010) as well as improving apparent solubility and dissolution rates of poorly soluble actives (Dai, Dong and Song, 2007). In addition, studies have shown that CARs have a high capacity to interact with proteins due to their strong ionic nature (Malafaya, Silva and Reis, 2007) and form excellent matrices for predictable synthesis of magnetite nanoparticles (Daniel-da-Silva et al., 2007).

To form polyelectrolyte complexes (PECs) with CAR, protamine (PROT) was selected due to its reported *in vitro* membrane-translocating ability, believed to be associated with its positively charged polyarginine chains (Reynolds, Weissleder and Josephson, 2005). The use of cell-penetrating peptides for drug delivery has been extensively studied over the past two decades (Temsamani & Vidal, 2004).

Shumilina and Shchipunov (2002) investigated interactions between CAR and chitosan (CS). They showed that the nature or type of CAR considerably influenced the characteristics of the PECs. The mechanical strength of PEC gels were ranked as follows: lambda carrageenan (LC)/CS > iota carrageenan (IC)/CS > kappa carrageenan (KC)/CS. Moreover, the gels obtained with IC and KC were temperature sensitive due to the helix-coil conformational transitions in their molecules (Shumilina & Shchipunov, 2002). An investigation was performed on the potential of PECs formed between KC, IC or LC and CS to form controlled release systems for glucose oxidase (Briones & Sato, 2010). The complex between CS and KC showed high encapsulation efficiencies for glucose oxidase while having the lowest release rate for this compound. Furthermore, this complex was able to protect the encapsulated glucose oxidase against degradation in pH 1.2 solution, in a chitosanase solution and in a pepsin solution (Briones & Sato, 2010). Another example of

CS/CAR PECs used for delivery of proteins has been reported (Li, Hein and Wang, 2013). As characterised by Li et al. (2013), in acidic solution the negatively charged sulphate groups of KC bound to positively charged amino groups of CS and formed acid-base PECs. When the pH was increased, amino groups protonated and the binding activity between both components became weaker, which resulted in swelling and disintegration of PEC and finally release of the protein. Modulation of drug release to the target site was possible by adjusting the factors that cause swelling properties of PEC (Li et al., 2013).

A number of publications show that carrageenans complex to CS (Briones & Sato, 2010; Li et al. 2013; Shumilina & Shchipunov, 2002), however no work has been published to date which indicates whether complexation between CAR and PROT is possible. Previous work by Umerska et al. (2014) showed that PROT/hyaluronate PECs can form, at the nanoscale, but depending on the ratio of constituents in such PECs their colloidal stability varies considerably. Therefore in this study we have focused on CAR/PROT systems with the aim of undertaking a methodical assessment of the various types of CAR, their concentration, weight mixing ratios, mode of preparation and the additional small cation addition during PECs formation to investigate if CAR/PROT nanoplexes can be obtained and to determine the characteristics of any such nanoplexes formed. CAR/PROT nanoplexes have the potential of being biocompatible, safe as well as capable of peptide binding and protection, as demonstrated for hyaluronate/PROT systems (Umerska et al., 2014), however key parameters determining their pharmaceutical suitability (such as optimum conditions of formation and stability) first need to be assessed.

2. Materials and methods

2.1 Materials

Iota carrageenan (IC, cat. no. C4014), kappa carrageenan (KC, cat. no. 22048), lambda carrageenan (LC, cat. no. 22049) and protamine (PROT) (as a sulphate salt, from salmon, cat. no. P4020) were obtained from Sigma-Aldrich. All other reagents and chemicals used were of analytical grade.

2.2 Preparation and characterisation of polymer and PROT solutions

Loss on drying for polymers was determined by thermogravimetry. A Mettler TG 50 module linked to a Mettler MT5 balance was employed (Paluch et al., 2010). Sample weights between 10–12 mg were used, placed into open aluminium pans and heated isothermally for 3h at 105 ºC under nitrogen purge.

Sulphate content was measured by turbidimetry on 20 mg polymer samples hydrolysed for 5h in 15 ml HCl at 105 ºC in sealed glass tubes, following the procedure of Dogson and Price (1962). Aliquots of 0.2 ml of the hydrolisates were transferred to glass tubes containing 3.8 ml 3% w/v trichloroacetic acid and 1 ml of the barium chloride reagent (0.5 g barium chloride dissolved in 100 ml of 0.5% w/v gelatin solution) was added to each of the tubes. The contents were thoroughly mixed, kept at room temperature for 15-20 minutes and absorbance was measured at 360 nm against the blank. The blank consisted of 0.2 ml 1M HCl mixed with 3.8 ml 3% w/v trichloroacetic acid and 1 ml of gelatin solution. The calibration curve was made using anhydrous potassium sulphate (20-200 µg/ml sulphates).

Aliquots of 100 ml of 0.1% w/v (1 mg/ml) and 0.05% w/v (0.5 mg/ml) solutions of IC, KC, LC and PROT were prepared in deionised water. LC and PROT were dispersed at 25 ºC and stirred for 30 min on a magnetic stirrer at 500 rpm until a clear solution resulted. For KC and IC, the polymers were first dissolved with vigorous stirring in deionised water at 80 ºC. When clear solutions were obtained, the polymer solutions were removed from the heat and stirred at 500 rpm for at least 30 min until the temperature reached 25 ºC. The volume of KC and IC solutions was made up to 100 ml to account for any evaporation which occurred during the heating.

A low frequency vibration viscometer (SV-10 Vibro Viscometer, A&D Company Limited, Japan) was employed to measure the viscosity of polymer solutions and NP dispersions. The viscometer was calibrated with deionised water before use. Viscosity measurement of each sample was carried out in triplicate at 25±0.2 ºC. A water bath (Reciprocal Shaking Bath Model 25, Precision Scientific, UK) was used to equilibrate the samples prior to the measurement. The results are given as an average ± standard deviation (SD).

A Thermo Electron Orion 420A$^+$ Basic pH/mV/ORP 25 ºC, Thermo Electron Corporation pH meter equipped with an Orion Rose^{TM} 8103SC glass body pH semi-micro electrode was used to perform pH analyses. pH measurement of each sample was conducted in triplicate at 25 ºC. The pH meter was calibrated with standard buffer solutions at pH 4, 7 and 10 before each batch of sample measurements. The results are given as an average value of three measurements ± SD.

Gel Permeation Chromatography (GPC) measurements of the molecular weight of carrageenans were performed using a system composed of an LC-10 AT VD liquid chromatograph pump system, a SIL-10 AD VP autoinjector, a FCV-10 AL VP low pressure gradient flow-control valve, a DGU-14A degasser, a Waters 410 refractive index detector and an SCL-10A VP system controller (Shimadzu, Japan). A Plaquagel–OH mixed 8 µm, 300 × 7.5 mm column (Polymer Laboratories Ltd., UK) was used. The mobile phase was composed of 0.2M NaCl and 0.01M NaH_2PO_4 adjusted to pH 7.4 with NaOH solution (Umerska et al., 2012). The mobile phase flow rate in each case was 1 ml/min. Pullulan standards (PL Polymer Laboratoires, Germany) were used to construct the calibration curve. Solutions of standards and samples (1 mg/ml) were prepared in the mobile phase and 100 µl of samples or standards were injected in triplicate. Shimadzu CLASS-VP software (version 6.10) with GPC for Class VP (version 1.02) was used for data collection and peak integration.

Fourier transform infrared spectroscopy (FTIR) of various types of carrageenans and PROT was carried out as described previously (Umerska et al., 2012).

2.3 Synthesis and characterisation of NPs composed of CAR and PROT

Aliquots of 0.05% and 0.1% w/v solutions of IC, KC, LC and PROT were prepared according to the method described in Section 2.2. Polymer and PROT solutions were combined together in various v/v ratios at room temperature (RT) and mixed under magnetic stirring for around 10 min to allow stabilisation of the system. Since the concentration of both components was the same, either 0.05% or 0.1% w/v, those v/v ratios were equivalent to CAR/PROT weight mixing ratios (WMRs) and WMRs are used throughout this manuscript.

158 Two methods of NP preparation were considered:

159 Method_A. An aliquot of PROT solution was added to an aliquot of polymer solution under
160 magnetic stirring. Stirring was continued for 10 minutes.

161 Method_B. An aliquot of polymer solution was introduced to an aliquot of PROT solution
162 under magnetic stirring. Stirring was continued for 10 minutes.

163 To examine the impact of divalent cations on IC/PROT NPs formation, $CaCl_2$ (final
164 concentration of 0.05 M and 0.01 M) and $ZnCl_2$ (final concentration of 0.01 M) were
165 dissolved in 0.1% w/v solution of PROT. The polymer and PROT/cation solutions were
166 combined together in various v/v ratios at room temperature and mixed under magnetic
167 stirring for 10 minutes to allow stabilisation of the system.

168 **2.4 Physicochemical characterisation of NPs**

169 The mean particle size (hydrodynamic particle diameter) and polydispersity index (PdI) of
170 prepared polyelectrolyte complexes were determined by dynamic light scattering (DLS) with
171 the use of 173 degrees backscatter detection. The value of material refractive index used
172 was 1.59, while that of absorption 0.01. Actual values of medium viscosity were input and
173 the refractive index of dispersant was kept 1.33, the same as water. The electrophoretic
174 mobility values measured by Laser Doppler Velocimetry (LDV) were converted to zeta
175 potential (ZP) values using the Smoluchowski equation. Both measurements (DLS and LDV)
176 were performed on a Zetasizer Nano ZS series Nano-ZS ZEN3600 fitted with a 633 nm laser
177 (Malvern Instruments Ltd., UK). Samples without dilutions were placed directly into a clear
178 plastic zeta cell (DTS 1061, Malvern, UK) and equilibrated for 2 min at 25 ºC prior to the
179 measurement. The readings for each sample were performed at least three times and at
180 least three batches of each sample were prepared and analysed. The results are presented
181 as an average of size ± SD, polydispersity index (PdI) ± SD and ZP ± SD.

182 Viscosity and pH measurements were performed in the same way as described above in
183 Section 2.2.

184 FTIR analysis was performed on CAR/PROT systems produced by combining 0.1% w/v
185 solutions at a weight mixing ratio of 1 as described previously (Umerska et al., 2012).

The structural and morphological analysis of NPs was performed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). TEM was carried out with a Tecnai G^2 20 TWIN microscope (FEI, USA). Samples were applied to the shiny side of the grid for 30 s and the excess of sample was removed by blotting the grid with a filter paper. Prepared samples were dried for 24h under ambient conditions. SEM was carried out with a Zeiss Supra Variable Pressure Field Emission Scanning Electron Microscope (Germany) equipped with a secondary electron detector. Liquid dispersions of NPs were directly placed onto aluminium stubs and dried for 24h in a desiccator over silica gel. Dried NPs were sputter-coated with gold under vacuum prior to the analysis.

Physical (colloidal) stability of NP formulations was visually inspected immediately after preparation, prior to carrying out any measurements. The presence/absence of aggregation was recorded. The colloidal stability of NPs (in native dispersions) upon storage was determined by measuring changes in the hydrodynamic particle size, PdI and ZP over time. Measurements and visual observations were performed directly after sample preparation (day 0) and were continued every 24h for a further 3 days.

The colloidal, physical stability of NPs in the following liquid media: 0.01M HCl, 0.1M acetate buffer pH 4.5, 0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 6.5 and 0.01M phosphate buffered saline (PBS) pH 7.4 was also determined. The aqueous dispersions of NPs were diluted with a suitable medium in the 1:1 v/v ratio. The colloidal stability of the prepared NPs was determined by measuring changes in the mean hydrodynamic particle size over time. Measurements and visual observations were performed directly after sample preparation (day 0) and were continued every 24h for another 3 days.

2.5 Statistical analysis

The statistical significance of the differences between samples was determined by one-way analysis of variance (ANOVA) using Origin software version 7.5.

3 Results and discussion

3.1 Formulation of NPs composed of various types of carrageenan and PROT

3.1.1 Impact of carrageenan type and solution concentration on NP formation

The molecular weight of the polymers was determined by GPC and it ranged from ~600 to ~700 kDa (Table 1). It was therefore assumed that only differences in the polymer chemical composition and/or structural differences (Figure 1), but not molecular weight, may affect the NP formation. The molecular weight of the polymer is reported as a crucial parameter that determines the success of the polymeric NP formation process, as established by Boddohi, Killingsworth and Kipper (2008) and Umerska et al. (2012).

Preliminary studies were first executed to examine each type of CAR for its ability to form PECs with PROT. CAR and PROT solutions (each at 1 mg/ml) were combined at various CAR/PROT WMRs using manufacturing Method_A, as previously employed by Umerska et al. (2012). The combination of IC and PROT gave the smallest particles with the lowest PdIs when compared with the KC/PROT and LC/PROT systems (Figure 2A and 2B). The particle size and PdI were seen to increase with increasing CAR/PROT WMR. Formation of large particulates, an indication of coagulation or aggregation, was observed for most of the KC/PROT WMRs tested (Figure 2A). High PdI values were measured for the KC/PROT samples with particles in the nano size range (Figure 2B). SEM analysis (Figure 3) showed the presence of NPs and not microparticles, thus it may be concluded that the appearance of large particles for some of the KC/PROT systems was symptomatic of an aggregation process rather than coagulation. NPs with particle sizes of ~100-350 nm were formed when combining LC and PROT solutions (Figure 2A). Generally, LC/PROT NPs were larger in size when the LC/PROT WMRs was lower than 1.

All combinations tested: KC/PROT, IC/PROT and LC/PROT showed the formation of large entities at a WMR of 1, consistent with charge neutralisation occurring at this CAR/PROT WMR (Boddohi et al., 2008; Umerska et al., 2012). This was an interesting finding, as the different types of CAR vary in the number of sulphate residues in their structures (Figure 1) and in theory each of them should have a different, characteristic charge neutralisation point. Nevertheless, the actual sulphate content in the various CAR types varied between 20 and

27% (Table 1), which can be considered similar. Since the pKa value of the anionic sulphate group in CAR is around 2 (Gu, Decker and McClements, 2004), at pH of the CAR solution (Table 1) ionisation of the sulphate groups should be complete and all the groups will be available for potential binding with PROT.

Zeta potential (ZP) values showed that the NPs can bear either negative or positive surface charge (Figure 2C). ZP of positively charged NPs had similar values for each type of NP, possibly due to the PROT presence on the surface. Negatively charged NPs composed of KC/PROT and LC/PROT had similar ZP values (around -100 mV at a CAR/PROT WMR of 3 and approximately -200 mV at a CAR/PROT WMR of 5). A higher charge, around -80 - -100 mV, in comparison to KC/PROT and LC/PROT, was measured for negatively charged IC/PROT NPs. NPs based on IC displayed the most promising results in terms of obtaining NPs with the smallest particle sizes and lowest PdI values (Figure 2).

Thrimawithana, Young, Dunstan and Alany (2010) have characterised properties of carrageenan gels and reported the existence of both KC and IC as random coils in solution and at high temperatures. On reduction of this temperature a double-helix structure is induced to form small independent domains via intermolecular interactions between a limited number of polysaccharide chains. KC forms honeycomb-like, hard gel structures while IC forms soft, elastic gel structures (Thrimawithana et al., 2010). LC does not form gels and it is characterised by a random distribution of polymer chains in solution (Berth et al., 2008). Conformation dependent interactions of carrageenans with other polymers and drugs have been reported in the literature. Thrimawithana, Young, Bunt, Geen, and Alany (2011) highlighted that, in the coiled conformation, the sulphate groups of carrageenans are further apart (at a distance of about 1 nm) than in the helix conformation (about 0.66 nm). As such, long range intermolecular forces between the sulphate groups are weaker in the coiled formation. Therefore, the polyanionic character of carrageenans would appear more important for polyelectrolyte complexation when present in the helix form and inducing this helix conformation of CAR polymers when interacting with the polycation PROT would appear to be most desirable for NP formulation via polyelectrolyte complexation.

KC was seen as the most problematic of the polymers used in initial formulation studies using 1 mg/ml CAR solutions, with large, aggregated particles being produced at almost all KC/PROT weight ratios studied (Figure 2). A lower concentration, 0.5 mg/ml, of KC and PROT solutions was investigated and allowed the formation of particulates in the nano size range for all weight mixing ratios tested (Figure 2A). The NPs had lower PdI values ranging from 0.21 to 0.46 and greater ZP values in comparison to the NP counterparts, if formed, made of 1 mg/ml solutions. While it was possible to obtain NPs based on KC when lowering its concentration, it is important to note that previous reports in the literature have indicated that typically a greater association efficiency of the drug can be achieved when greater concentration of polymers is used in their formulation (Sarmento, Ribeiro, Veiga and Ferreira, 2006; Gupta and Karar, 2011). Thus NPs with a total polymer content of 1 mg/ml would be most desirable in terms of future studies on drug loading.

Overall, the CAR conformation in solution and its solution concentration appear to be the key determinants of success when forming polyelectrolyte nanoplexes with PROT. IC showed the most promising characteristics and small, homogenous and bearing either negative or positive surface charge NPs were successfully formed at 1 mg/ml solution concentrations. Additionally, it should be noted that amines (PROT is rich in arginine moieties) can induce ionic gelation of IC (Yeo, Baek and Park, 2001).

3.1.2 Impact of the preparation method on properties of NPs

Polyion addition mode is known to govern properties of polyelectrolyte nanoplexes (Chen, Heitmann and Hubbe, 2003; Dragan, Mihai and Schwarz, 2006; Birch & Schiffman, 2014). In experiments described in the previous section Method_A (an aliquot of PROT solution was added to a volume of HA solution) was used. A method involving the reverse addition of components (a volume of PROT solution introduced to a volume of PROT solution), i.e. Method_B, was also tested on KC/PROT (with a total polymer content of 0.5 mg/ml) and IC/PROT (with a total polymer content of 1 mg/ml) systems.

Preparation Method_A yielded NPs that were generally smaller in size and with lower PdI values in case of negatively charged NPs (a CAR/PROT WMR of 5, Figure 4A and B) than

298 those generated using Method_B. Also, Method_A allowed the formation of NPs at a
299 CAR/PROT WMR of 2, while Method_B resulted in samples containing large particles at this
300 WMR. Statistically significant differences between the size of NPs were also observed for
301 KC/PROT and IC/PROT at CAR/PROT WMRs of 0.2 and 0.5, but it was Method_B that gave
302 smaller NPs for IC/PROT while Method_A resulted in smaller NPs for KC/PROT (Figure 4A).
303 The variation in the particle size and PdI was not reflected in similar changes in dynamic
304 viscosity and ZP values (Figure 4C and D), and no general trends were discerned.
305 Birch and Schiffman (2014) investigated the order of solution addition, when making
306 chitosan and pectin polyelectrolyte nanoplexes. Substantial differences in particle sizes and
307 ZP values were seen, but the mechanism rationalising the variation was not provided.
308 Dragan et al. found that the charge neutralisation point of the complexes formed between
309 acrylamide or methacrylate derivatives of poly(sulphonate)s and poly(quaternary amine)s
310 was dependent on whether the titrant was the polyanion or polycation (Dragan et al., 2006).
311 In all cases when the polycation was added to the polyanion, the molar charge ratio between
312 the cationic and anionic species was shifted to higher values. This behaviour was explained
313 by the charge localisation on the polycations, making them less efficient in the stabilisation of
314 complex particles with the polyanion; thus larger in size aggregates were formed. The
315 findings of Dragan et al. (2006) can be translated into the observed increase in the particle
316 size of IC/PROT and KC/PROT NPs at a CAR/PROT WMP of 5 as well as KC/PROT at a
317 CAR/PROT WMP of 0.2 and 0.5, when much larger particles, indicative of a looser structure
318 and weaker species interactions, were formed using Method_B (Figure 4A and D). Also,
319 Method_A permitted successful NP production at a CAR/PROT WMP of 2, while extensive
320 aggregation was seen for Method_B.
321 However, Method_B yielded smaller particles, and with lower ZP values, for IC/PROT WMRs
322 of 0.2 and 0.5, in comparison to Method_A, so the supposition of lower polycation efficiency
323 does not hold for these samples.
324 Umerska et al. highlighted the importance of the molecular weight ratio of the polyanionic
325 and polycationic constituents and the difference in charge density (Umerska et al., 2014).

While it was possible to prepare NPs composed of hyaluronic acid (HA, molecular weight ~260 kDa) and PROT (molecular weight ~5 kDa), they were not physically stable and aggregated within hours due to the large difference in molecular weight of HA and PROT. Interestingly, the molecular weight ratio for KC/PROT is ~130 and that for IC/PROT ~140, yet physically stable NPs were formed (for stability data see Section 3.4). As IC is a much stronger polyanion than HA (Cundall, Lawton, Murray and Phillips, 1979) it is able to form stronger complexes with PROT, but strong, linear polyelectrolytes are also known to form stable, however non-stoichiometric complexes (Chen et al., 2003). The component in excess here, PROT, is expected to charge-stabilise the IC/PROT NPs.

The final conclusion is that PROT can either stabilise or destabilise NPs depending on the mode of mixing and the type of carrageenan. Practically, considering the rather small differences in NP properties depending on the use of Method_A or Method_B for the positively charge particles, Method_A was selected for further experiments.

3.1.3 Impact of Ca^{2+} and Zn^{2+} on NP formation

Calcium ion has been widely used to crosslink negatively charged polysaccharides to aid NP formation and to promote better size distribution and polydispersity of formed (Liu, Jiao, Wang, Zhou and Zhang, 2008). As reported, Ca^{2+} ions favour IC gel formation while K^{+} ions favour KC gel formation (Thrimawithana et al., 2010). Thus the addition of divalent cations (Ca^{2+} and Zn^{2+}) was tested for possible alteration of IC/PROT NPs properties.

At first, Ca^{2+} at a concentration of 0.05M was tested. However, only micron sized particles were formed after incorporation of Ca^{2+} ions to the IC/PROT formulations. To further check the impact of Ca^{2+} concentration, the concentration was decreased to 0.01M. This decrease in the ion concentration brought the particle size of PECs which were formed back into the nano-range (Figure 5A), however PdI values were greater in comparison to those of formulations without Ca^{2+} (Figure 5B). To further optimise the formulation another ion was sought. The presence of Zn^{2+} ions at a concentration of 0.01M resulted in NPs with smaller particle sizes than those containing Ca^{2+} ions (Figure 5A). However, the size of NPs formed with the divalent cation addition was still larger than formulations without ions (Figures 2A

and 5A). As expected, additional cations present in formulations resulted in lowering ZP for all weight ratios tested due to reaction with the anionic sulphate groups of the polymer (Figure 5C).

3.2. Characterisation of interactions between CAR and PROT

FTIR spectra of the different carrageenans, PROT and NPs, along with band assignments for the NP components, are presented in Figure 6. The identity of KC, IC and LC could be easily distinguished by the presence and location of bands of sulphate galactose and sulphate 3,6-anhydrogalactose (Figure 6). The FTIR spectra of NPs were dominated by vibrations characteristic of CAR in the 650-1400 cm^{-1} region with prominent amide I and II peaks of PROT appearing between 1400 and 1700 cm^{-1} confirming the presence of both components in the NP formulations.

No new covalent bonds were created in the process of combining CAR and PROT, but shifts of some absorption bands were evident (Table 2). Interestingly, the peaks associated with sulphate and amine groups of CAR and PROT moved substantially for KC/PROT and LC/PROT systems, while, for the IC/PROT sample, only the amide I band shifted considerably and not the sulphate bands. This implies that the various CAR types may interact with PROT via different mechanisms. Large shifts in the amide I band of chitosan were observed for chitosan/HA (Umerska et al., 2012) and chitosan/KC (Li et al., 2013) systems and attributed to the formation of a polyelectrolyte complex. Therefore it appears that all types of CAR are able to electrostatically complex with PROT, and this mechanism is dominant for KC/PROT and LC/PROT systems, but another process is also likely to occur in the case of IC/PROT. Thrimawithana et al. (2011) noticed that the region between 1000 and 1200 cm^{-1} is the most sensitive to structural changes of CAR as it shows stretching absorption bands of C-O-C and C-O bonds of the polysaccharides. There is a shift by 6 cm^{-1} of the absorption band located at 1155 cm^{-1} towards higher wavenumbers for IC/PROT (shifts by 1-2 cm^{-1} in the opposite direction are seen for KC- and LC-/PROT), consistent with deformation of IC polysaccharide chains. As the driving force for formation of PECs is attraction of oppositely charged moieties (already discussed above) and/or an increase in

382 entropy when the complexation is accompanied by release of counterions (Chen et al.,
383 2003), the shift of the 1155 cm^{-1} band may signify that the latter mechanism is also present
384 and contributes to the formation of small and homogenous NPs.

385 **3.3. Structure and morphology of NPs**

386 Figure 3 shows SEM images of a range of CAR/PROT NPs. It can be observed that NPs
387 with positive surface charge (a CAR/PROT WMR of 0.33) were generally spherical in shape
388 and well defined (KC/PROT and IC/PROT), with the exception of LC/PROT for which a
389 rather fused mass of very small nanoparticulates with sizes of approximately 10-50 nm was
390 observed. It is therefore likely that data presented in Figure 2A pertains to aggregated
391 LC/PROT NPs rather than individual particles. At a CAR/PROT WMR of 3 the particles were
392 still approximately spherical, but a greater degree of fusion was apparent.

393 TE micrographs (Figure 3G and 3H) show spherical and well-defined structures. There is
394 some of the polymeric corona effect (Umerska et al., 2012) visible for the NPs.

395 **3.4 Colloidal stability of IC/PROT NPs**

396 A range of formulations with different IC/PROT WMRs and properties were chosen for
397 colloidal stability testing, which was carried out for up to 72h at RT. A large increase in the
398 particle size was noticed only for the positively charged NPs (IC/PROT WMRs of 0.2 and
399 0.5, Figure 7A) with a very rapid growth in the particle size and flocculation measured for the
400 formulation with an IC/PROT WMR of 0.5. NPs with IC/PROT WMRs of 2 and 4 showed only
401 a small increase in the particle size upon the storage. Interestingly, the increase in the
402 hydrodynamic size of the IC/PROT WMR of 0.5 system after 24h was not accompanied by
403 an increase in PdI (Figure 7B). A gradual decrease in ZP values for NPs with a IC/PROT
404 WMR of 0.5 was observed, indicative of PROT detachment (Figure 7C). The best colloidal
405 stability was determined for NPs with WMRs of 2 and 4.

406 NPs with a positive surface charge (IC/PROT WMRs of 0.2 and 0.5) were physically
407 unstable and aggregated either immediately after mixing with the medium (acetate buffer
408 pH=4.5 and PBS pH=7.4) or extensive aggregation was observed after 24h (0.01M HCl and
409 HEPES buffer pH=6.5) (Figure 8A and B). In contrast, good colloidal stability was seen for all

410 negatively charged formulations (IC/PROT WMPs of 2 and 4) with relatively small variations
411 in the particle size recorded over time (Figure 8C and D).
412 As the component in excess charge-stabilises the PEC complex (PROT for NPs with positive
413 surface charge and CAR for NPs with negative surface charge), its ability to remain on the
414 surface in a range of conditions will define the colloidal stability of particles. As PROT
415 comprises small, approximately 5 kDa fragments, this component can be easily removed
416 from the particle surface, resulting in physical destabilisation of the system. On the other
417 hand, CAR has approximately 100 times greater molecular weight and long, unbranched
418 chains can form entangled structures and thus are removed with more difficultly. Moreover,
419 the impact of the ionic strength of a medium on the binding constant is well known and
420 mathematically presented by the "Record-Lohman" equation, which describes a double
421 logarithmic dependence of the binding constant on the ionic strength (Record, Anderson and
422 Lohman, 1978). Thus in electrolyte media the stability of polyelectrolyte complexes may be
423 even further decreased, as shown in Figure 8.
424

425 **4. Conclusions**

426 In this study, we have demonstrated that carrageenans could be used as suitable polymers
427 for the formation of novel NPs via polyelectrolyte complexation with PROT. The type (KC, IC
428 or LC) and solution concentration of carrageenans used was of particular importance. When
429 0.1% w/v solutions were used, aggregation was seen for KC/PROT, while small nanoplexes
430 with sizes of approximately 100-150 nm were obtained for the IC/PROT combination. The
431 addition of calcium and zinc ions to the environment during the NP formation was seen to
432 increase the particle size and surface potential of particulates and this may be perceived as
433 an undesirable effect. Polyelectrolyte complexation was confirmed as the mechanism of NP
434 formation accompanied by deformation of polysaccharide chains for IC/PROT systems.
435 Good colloidal stability was seen for NPs with negative surface charge stored as native
436 dispersions and with media at various pH.

IC was found to be the most suitable type of CAR, forming NPs with PROT that were smallest in size and most stable. Furthermore, the ease of formulation and mild preparative conditions required for preparation of these nanoplexes make them promising candidates in terms of use as nanocarriers. Therefore it is proposed to use IC/PROT nanoplexes for further studies involving loading of bioactive compounds for the purpose of drug delivery.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1 Physicochemical properties of carrageenans and their solutions studied in this work. KC – kappa carrageenan, IC – iota carrageenan, LC – lambda carrageenan.

Polymer	Molecular weight (kDa)	Loss on drying (%)	Sulphate content (%)	Viscosity (mPa·s) of solution	pH of solution
KC	672±39	11.3±0.8	20.3±1.4	0.05% - 3.62±0.13 0.1% - 5.49±0.04	0.05% - 6.7±0.1 0.1% - 7.2±0.2
IC	724±175	18.1±0.4	26.6±1.2	0.05% - 2.63±0.05 0.1% - 4.34±0.01	0.05% - 6.3±0.0 0.1% - 6.6±0.1
LC	579±22	14.1±1.2	21.3±0.9	0.05% - 4.58±0.06 0.1% - 6.06±0.19	0.05% - 7.2±0.2 0.1% - 7.9±0.2

Table 2 FTIR band positions (in cm^{-1}) in carrageenans, protamine (PROT) and nanoparticle (NP) formulations. The number in parenthesis indicate the magnitude of the shift in cm^{-1}, * indicates that the group in NP formulation shifted by at least 10 cm^{-1}. KC – kappa carrageenan, IC – iota carrageenan, LC – lambda carrageenan, v – stretching, $v_{s,a}$ – symmetric and asymmetric stretching, v_a – asymmetric stretching.

Band	KC	IC	LC	PROT	KC/PROT NPs	IC/PROT NPs	LC/PROT NPs
v(C-O,C-OH)	1037	1024	1011	-	1033 (4)	1024 (0)	1007 (3)
v_a(COC)	1157	1155	1154	-	1155 (2)	1161 (6)	1153 (1)
$v_{a,s}$(SO)	1228	1215	1219	-	1212 (16)*	1215 (0)	1209 (10)*
$v_a(SO_2)$	1374	1373	1372	-	1363 (11)*	1375 (2)	1363 (9)
amide II and NH_3^+	-	-	-	1538	1535 (3)	1538 (0)	1534 (4)
amide I	-	-	-	1633	1647 (14)*	1645 (12)*	1651 (18)*

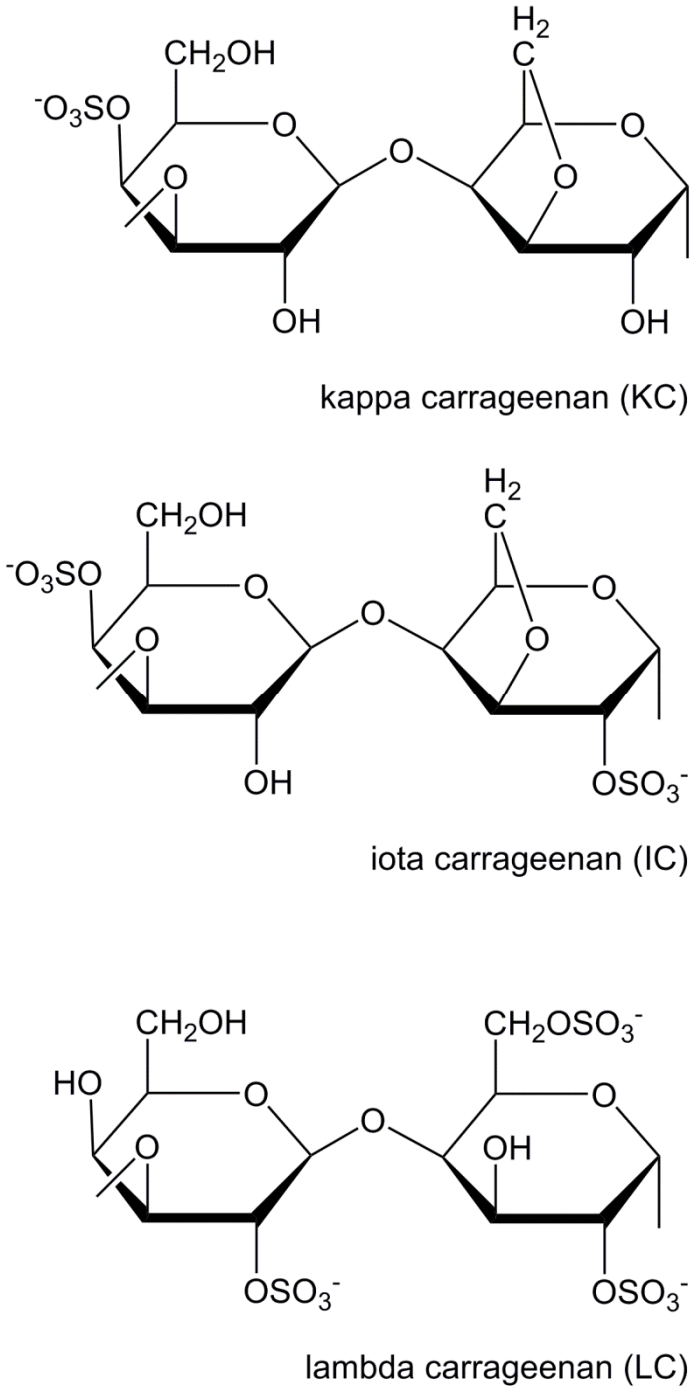

Figure 1. Chemical structure of kappa carrageenan (KC), iota carrageenan (IC) and lambda carrageenan (LC).

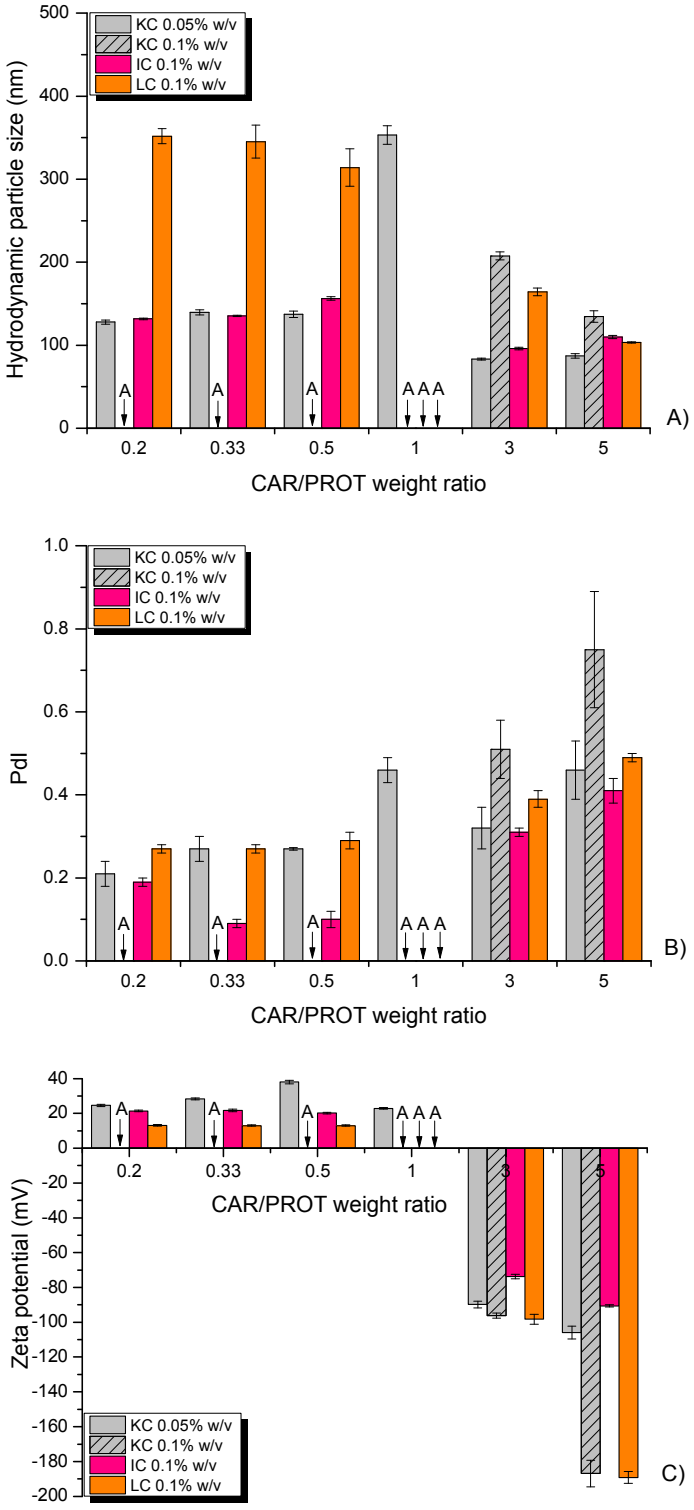

Figure 2. A) Hydrodynamic particle size, B) polydispersity index (PdI) and C) zeta potential for NPs composed of various types of carrageenans and protamine (PROT). A – instantaneous aggregation, KC – kappa carrageenan, IC – iota carrageenan and LC – lambda carrageenan.

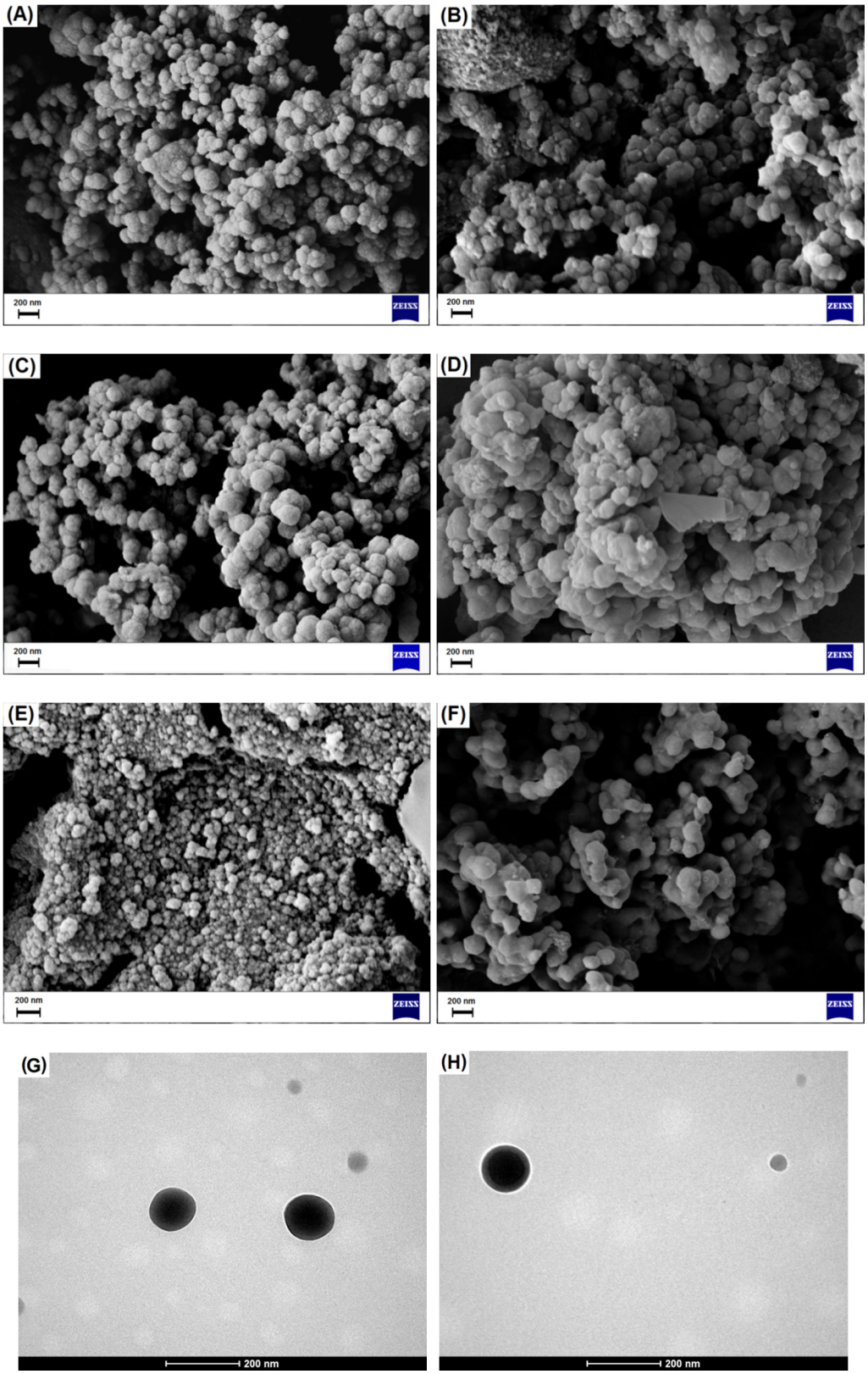

Figure 3. Electron micrographs: A) SEM of KC/PROT NPs with a WMR of 0.33, B) SEM of KC/PROT NPs with a WMR of 3, C) SEM of IC/PROT NPs with a WMR of 0.33, D) SEM of IC/PROT NPs with a WMR of 3, E) SEM of LC/PROT NPs with a WMR of 0.33, F) SEM of

LC/PROT NPs with a WMR of 3, G) TEM of IC/PROT NPs with a WMR of 0.5 and H) TEM of IC/PROT NPs with a WMR of 4. The concentration of constituents was 1 mg/ml. WMR - weight mixing ratio. KC – kappa carrageenan, IC – iota carrageenan, LC – lambda carrageenan and PROT – protamine.

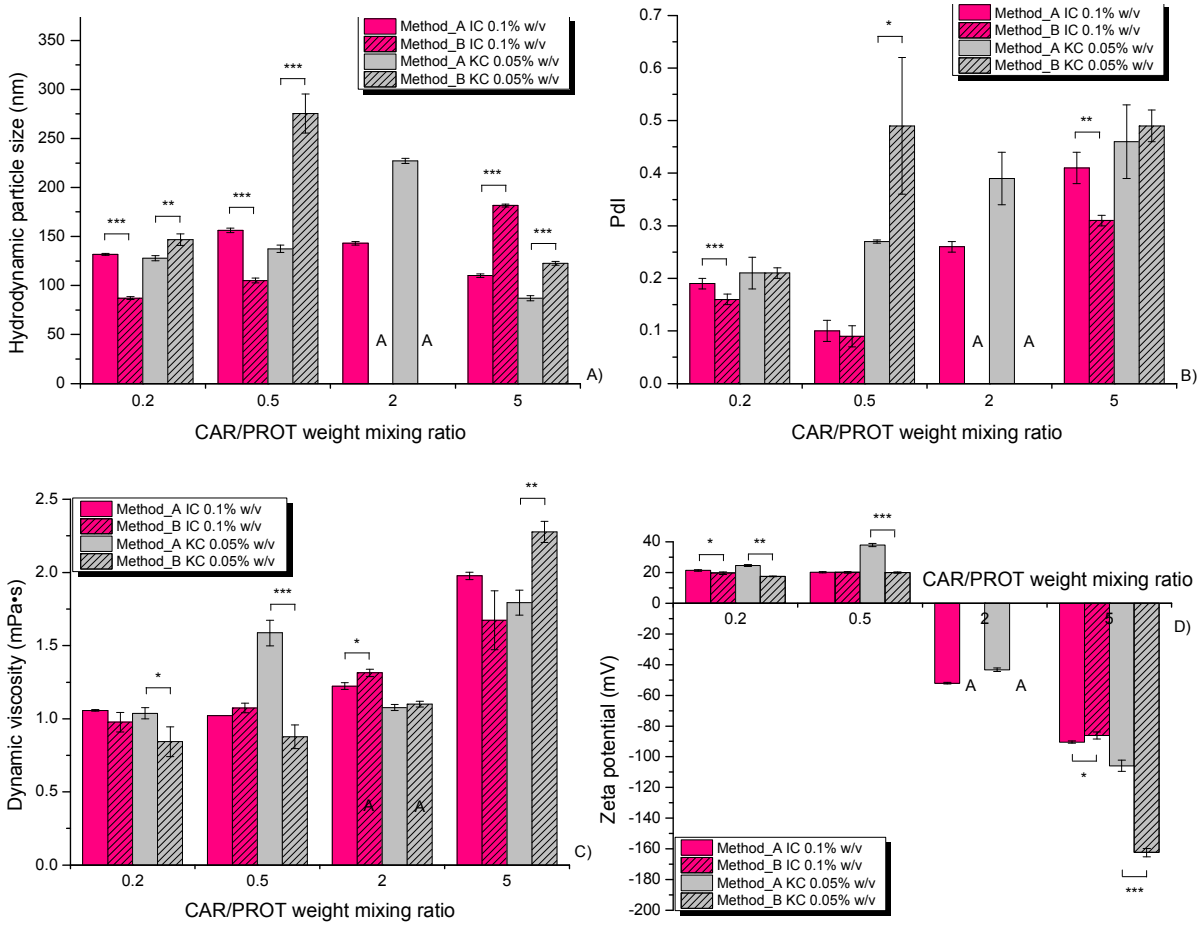

Figure 4. Comparison of: A) hydrodynamic particle size and B) polydispersity index (PdI), C) dynamic viscosity and D) zeta potential for selected NPs composed of kappa carrageenan/protamine (KC/PROT, 0.05% w/v) and iota carrageenan/PROT (0.1% w/v) formed by the following variation in the method of preparation: Method_A: an aliquot of PROT solution was added to an aliquot of carrageenan solution under magnetic stirring and Method_B: an aliquot of carrageenan solution was introduced to an aliquot of PROT solution under magnetic stirring. A - instantaneous aggregation, statistical analysis: *p<0.05, **p<0.01, ***p<0.001.

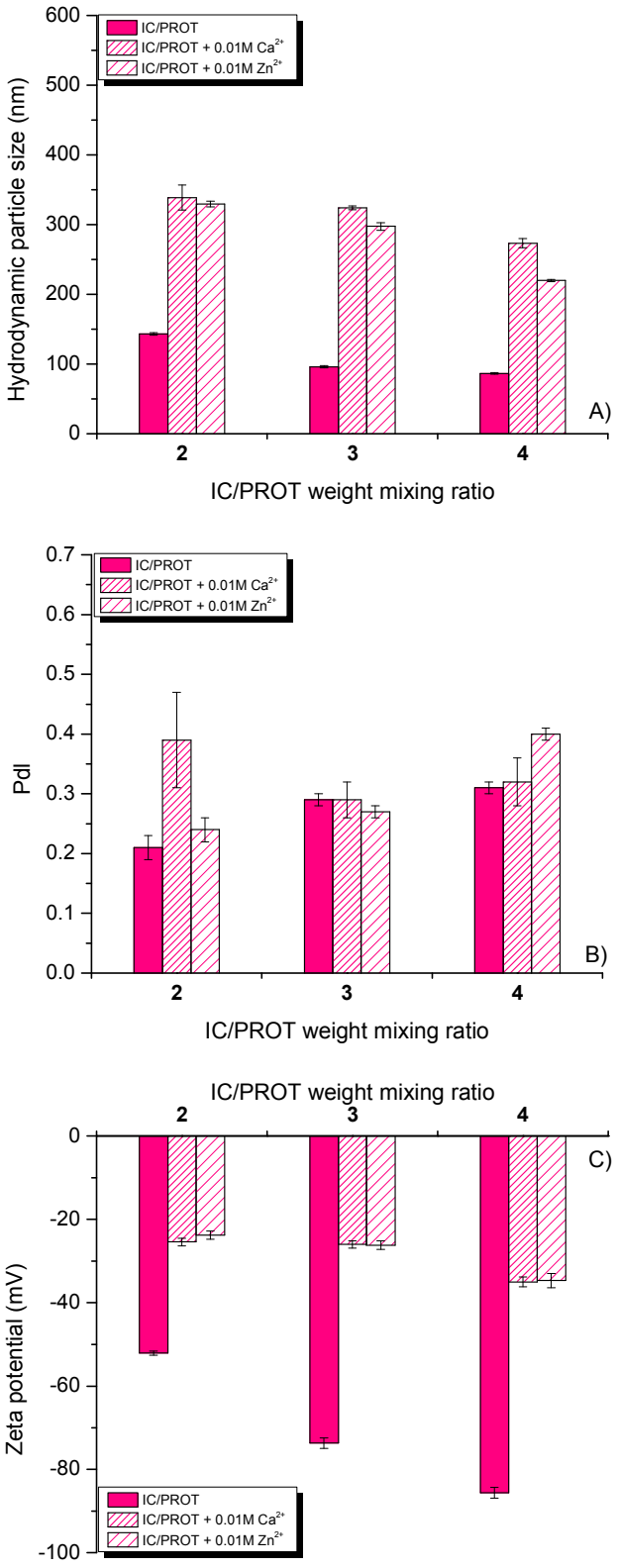

Figure 5. Comparison of: A) hydrodynamic particle size, B) polydispersity index (PdI) and C) zeta potential values for NPs composed of iota carrageenan (IC) and protamine (PROT) without/with addition of calcium and zinc cations.

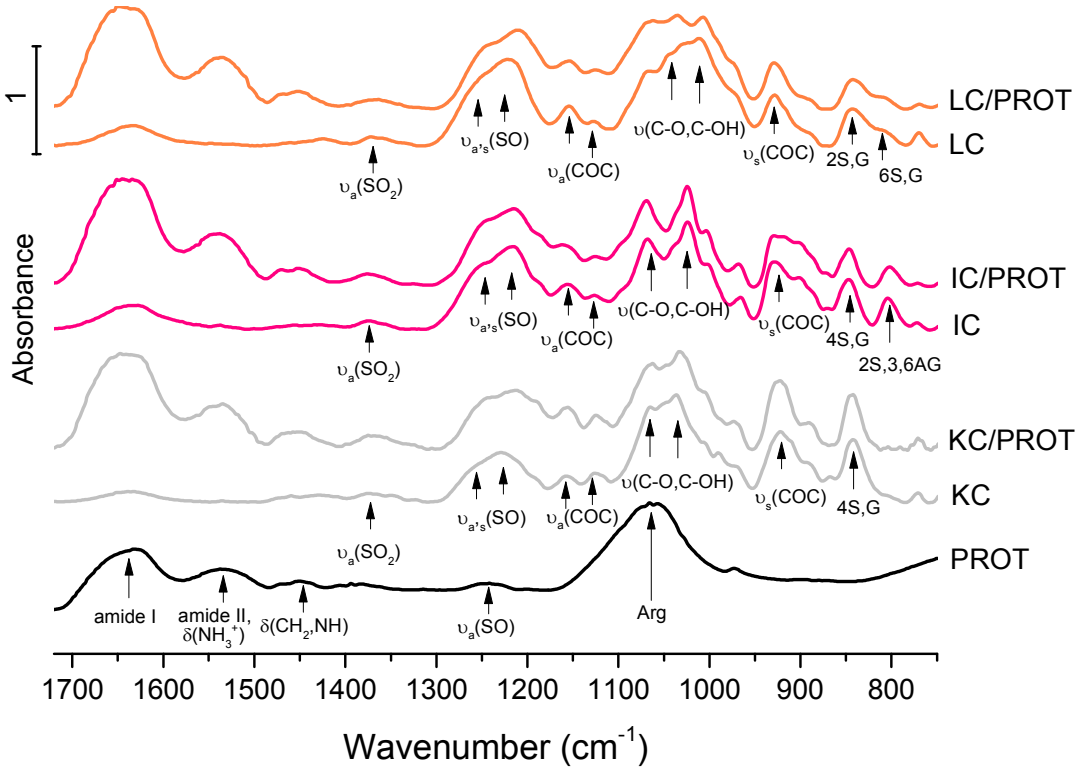

Figure 6. FTIR analysis of PROT, KC, IC, LC, KC/PROT NPs, IC/PROT NPs and LC/PROT NPs. Band assignment was done based on studies of Bertoluzza et al. (1983); Melo, Feitosa, Freitas and de Paula (2002); Nanaki, Karavas, Kalantzi and Bikiaris, (2010); Awotwe-Otoo et al. (2012); Arman & Quader (2012). v – stretching, $v_{s,a}$ – symmetric and asymmetric stretching, v_s – symmetric stretching, v_a – asymmetric stretching and δ – bending vibrations. Arg – arginine, 2S,G - 2-sulphate galactose (LC only), 4S,G - 4-sulphate galactose (KC and IC only), 6S,G - 6-sulphate galactose (LC only) and 2S,3,6AG - 2-sulphate 3,6-anhydrogalactose (IC only). KC – kappa carrageenan, IC – iota carrageenan, LC – lambda carrageenan and PROT – protamine.

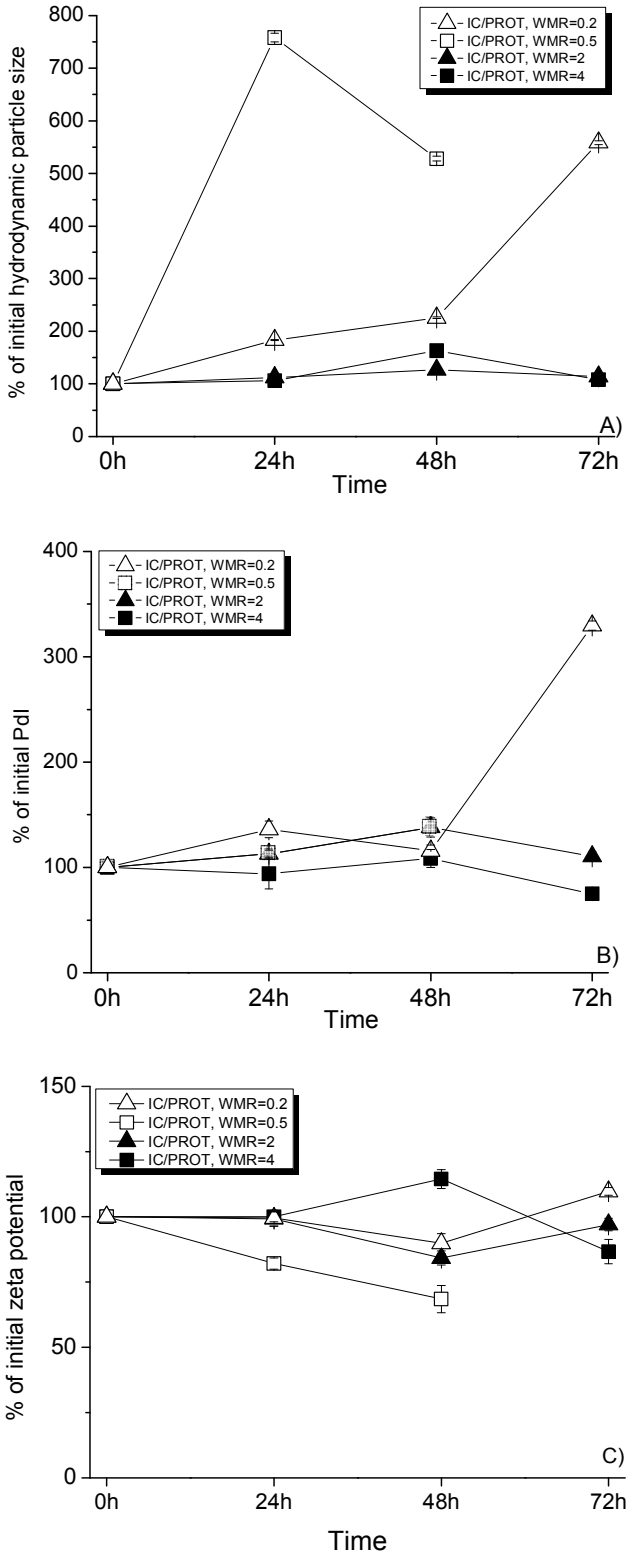

Figure 7. Colloidal stability studies of IC/PROT nanoparticle native dispersions at room temperature: A) hydrodynamic particle size, B) polydispersity index (PdI) and C) zeta potential. WMR – weight mixing ratio, IC – iota carrageenan and PROT – protamine.

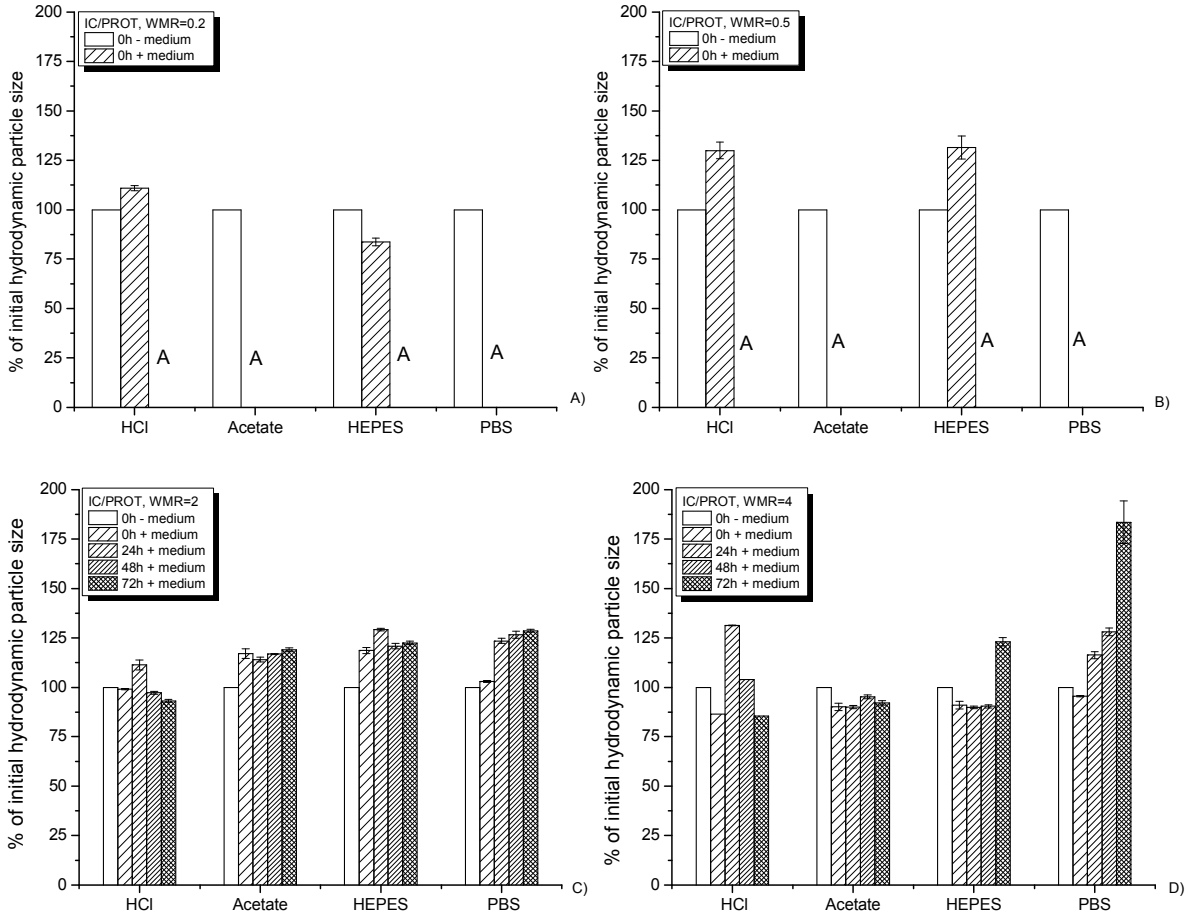

Figure 8. Colloidal stability studies based on monitoring hydrodynamic particle size changes of IC/PROT nanoparticle dispersions in media with various pH. The weight mixing ratios (WMRs) of IC/PROT were: A) 0.2, C) 0.5, D) 2 and F) 4. A – aggregation, IC – iota carrageenan and PROT – protamine, HCl – 0.01M HCl, Acetate - 0.1M acetate buffer pH 4.5, HEPES - 0.1M HEPES buffer pH 6.5 and PBS - 0.01M PBS pH=7.4.