Optimisation of the self-assembly process: production of stable, alginate-based polyelectrolyte nanocomplexes with protamine

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Abstract:

The aim of this work was to investigate the possibility of covalent cross-linker free, polyelectrolyte complex formation at the nanoscale between alginic acid (as sodium alginate, ALG) and protamine (PROT). Optimisation of the self-assembly conditions was performed by varying the type of polymer used, pH of component solutions, mass mixing ratio of the components as well as the speed and order of component addition on the properties of complexes. Homogenous particles with nanometric sizes resulted when an aqueous dispersion of ALG was rapidly mixed with a solution of PROT. The polyelectrolyte complex between ALG and PROT was confirmed by infrared spectroscopy. To facilitate incorporation of drugs soluble at low pH, pH of ALG dispersion was decreased to 2, however no nanoparticles (NPs) were formed upon complexation with PROT. Adjusting pH of PROT solution to 3 resulted in the formation of cationic or anionic NPs with a size range 70-300 nm. Colloidal stability of selected AAL/PROT formulations was determined upon storage at room temperature and in liquid media at various pH. Physical stability of NPs correlated with the initial surface charge of particles and was time- and pH-dependent. Generally, better stability was observed for anionic NPs stored as native dispersions and in liquids covering a range of pH.

Keywords: Alginate, protamine, nanoparticles, polyelectrolyte complex, dynamic light scattering, infrared spectroscopy

Introduction

Nanoparticles (NPs) have attracted special interests in the drug delivery area, mainly as carriers for bioactives including peptide drugs (Delie and Blanco-Prieto 2005). NPs formulated from natural polymers have been extensively studied due to their advantages, including the potential to retain protein stability and activity, an increase of the duration of therapeutic effects of proteins and the possibility to be administered by nonparenteral routes (Sarmento et al. 2007). The use of natural polymers as drug carries has also been widely investigated (Liechty et al. 2010; Ngwuluka et al. 2014; Srivastava et al. 2016).

Alginic acid (ALG) is a linear copolymer comprising blocks of (1,4)-linked-D-mannuronate (M) and C-5 epimer α-L-guluronate (G) residues, connected in different sequences or blocks (Fig. 1) (Tønnesen and Karlsen 2002). The wide availability of alginate and its desirable chemical and physical properties makes it an excellent material for formation of micro- and nanoparticles. Using various cross-linking methods, alginate forms an inert, biodegradable hydrogel matrix (Lee and Mooney 2012). Porosity of the gel enables high drug diffusion rates, which additionally can be controlled with polymer coatings (Wee and Gombotz 1998). Alginates are extensively utilised in biomedical applications as mucoadhesive, biodegradable and biocompatible polymers (Sinha and Kurmia 2001). Furthermore, alginate has the ability to ionically cross-link with multivalent cations, such as calcium, forming a network gel, which is stable at low pH and dissolves in neutral or high pH environments (Augst et al. 2006; George et al. 2006). The pH sensitivity of alginate-based hydrogels makes them particularly attractive for oral delivery, as such formulations can contract in the stomach protecting the drug. Passing through the gastrointestinal tract, they subsequently swell and release the drug when pH increases.

The polyelectrolyte complexation (PEC) method of NP preparation has received an increasing attention, as NPs formed by this method have several advantages for cellular uptake and colloidal stability, including suitable diameters and surface charge, spherical morphology and low polydispersity indices (Pdl) (Avadi et al. 2011). Furthermore, the preparation of NPs by PEC methods can be carried out in completely aqueous conditions and at ambient temperature, without the use of organic solvents and/or surfactants, thus the stability and biological activity of loaded peptides is expected not to be affected (Hu et al. 2012). Additionally, NPs formed by the PEC method have been to show to improve encapsulation efficiency of drugs and to control the rate of drug release due to the nature of the bonds formed between the drug and polymer (Cheow and Hadinoto 2012). PECs can be delivered to the intended site, therefore reducing the side effects (Soppimath et al. 2001). The PEC method can also be applied to the preparation of

membranes, coating on films and fibres, isolation and fractionation of proteins as well as isolation of nucleic acids (Lankallapali and Kolapalli 2009).

Silva et al. developed alginate microspheres coated with chitosan (CHIT) prepared by an emulsification/internal gelation method with a mean diameter that ranged from 65 to 106 μm (Silva et al. 2006). An ionic pre-gelation method of alginate with calcium chloride, followed by complexation between alginate and CHIT was used to prepare NPs loaded with insulin. The particle size of the formed NPs increased from 764 to 2209 nm when the CHIT to alginate mass ratio was decreased from 6:1 to 3.1:1 (Sarmento et al. 2006). The PEC formation using alginate and CHIT as oppositely charged polymers has been described by Saether et al. (2008). As reported, the net charge ratio between CHIT and alginate and the molecular weights (MWs) of both the alginate and CHIT components were the most significant parameters that influenced the particle size and zeta potential (ZP) of the formed PECs (Saether et al. 2008). A similar study was conducted by Sarmento's group, where colloidal carriers prepared by complexation of two oppositely charged polymers: dextran sulphate (as an anionic polymer) and CHIT (as a cationic polymer) were used for the delivery of insulin (Sarmento et al. 2007).

The use of cell-penetrating peptides for drug delivery has been extensively studied (Temsamani and Vidal 2004). Protamine (PROT) is an example of a cell-penetrating substance, which was selected herein to form PECs with alginate. PROT has been reported to have *in vitro* membrane-translocating ability, believed to be associated with its positively charged polyarginine chains (Reynolds et al. 2005). The studies performed by Umerska et al. (2014) showed that PROT/hyaluronate can form PECs at the nanoscale, however depending on the ratio of constituents in such PECs their colloidal stability varied considerably. The association efficiency of the drug (salmon calcitonin, sCT) that was loaded into those NPs was up to 100% with notably high drug loading (9.6-39% w/w). The release of the cargo from those NPs was ~70–80% after 24h (Umerska et al. 2014). The release of sCT from chondroitin sulphate/PROT NPs, on the other hand, was susceptible to the pH and ionic strength of release medium (Umerska et al. 2015). Wernig et al. (2008) studied encapsulation of vasoactive intestinal peptide into biodegradable PROT 18mer nonsense oligonucleotides formed by self-assembly of the components. The encapsulation efficiency of the payload was up to 80% with 77-87% of the oligonucleotide released of over 24h. A recent study published by Yu et al. (2016) described chitosan/PROT NPs loaded with fluorouracil with high encapsulation efficiency (82.4%) and sustained release (more than 60% of the drug released into medium over 48h) of the drug.

Our previous study demonstrated that PROT is able to complex to carrageenans (CARs) forming PECs with properties dependent on the type of carrageen used and its concentration (Dul et al. 2015). The complex formation between CAR and PROT was corroborated by infrared analysis and, in the case of iota carrageenan, the polymer chains underwent structural changes when forming NPs.

The aim of current work was to investigate the process of preparation of covalent cross-linker free and stable NPs composed of only alginate and PROT. To achieve this goal, the alginate/PROT PEC manufacturing process was comprehensively studied including examination of the molecular weight of alginate, the component mixing ratios as well as the sequence of addition and speed of mixing of the constituents guided by the outcomes of previous studies (Dul et al. 2015). As one of the most important parameters determining pharmaceutical suitability of a nanoparticle is its colloidal stability, the physical stability of the nanoparticulate dispersions on storage at room temperature and upon the exposure to environments with varying pH was also examined.

2 Materials and methods

2.1 Materials

Alginic acid (as a sodium salt), low (AAL) and medium (AAM) viscosity grades as well as protamine (PROT) (as a sulphate salt, from salmon) were obtained from Sigma-Aldrich (Arklow, Ireland). All other reagents and chemicals used were of analytical grade.

2.2 Preparation and characterisation of polymer and PROT solutions

Aliquots of 100 ml of 0.1% (1 mg/ml) dispersions or solutions of AAM, AAL, PROT, PROT with pH2 (PROT_pH2) and PROT with pH3 (PROT_pH3) were prepared in deionised water. Polymers as powders were dispersed at 25 ºC and stirred at 500 rpm for 30 min on a magnetic stirrer until clear dispersions developed. pH of PROT solutions was adjusted to pH2 or pH3 using 1M HCl.

Viscosity of the liquids containing either alginate or PROT starting/native and NP dispersions was determined using a low frequency vibration viscometer (SV-10 Vibro Viscometer, A&D Company Limited, Japan). Deionised water was employed to calibrate the instrument before use. Triplicate measurements at 25±0.2 ºC were carried out for each sample and temperature of each of the aliquots was equilibrated prior to the measurement in a water bath (Reciprocal Shaking Bath Model 25, Precision Scientific, UK). The results are presented as an average value ± standard deviation (SD).

pH measurements at 25 ºC were carried out with a Thermo Electron Orion 420A+ Basic pH/mV/ORP pH meter connected to an Orion RoseTM 8103SC pH semi-micro electrode. Standard buffer solutions at pH 4, 7 and 10 were employed to calibrate the pH meter on regular basis. The results of measurements are given as an average value of three repeats ± SD.

Gel Permeation Chromatography (GPC) measurements of the molecular weight of alginates were performed using a HPLC system as described previously (Dul et al. 2015). The separative column used was a Plaquagel–OH mixed 8 μm, 300 × 7.5 mm column (Polymer Laboratories Ltd., UK). The mobile phase was composed of 0.2M NaCl and 0.01M NaH2PO4 adjusted to pH 7.4 with NaOH solution and its flow rate employed was 1 ml/min. The calibration curves were made using Pullulan standards (PL Polymer Laboratoires, Germany). Solutions of standards and samples at a concentration of 1 mg/ml were injected onto the column in triplicate (at a volume of 100 μl).

Fourier transform infrared spectroscopy (FTIR) of alginate and PROT was carried out as described previously (Umerska et al. 2012; Dul et al. 2015).

2.3 Synthesis and characterisation of NPs composed of alginate and PROT

Aliquots of 0.1% w/v solutions of AAL, AAM and PROT, PROT_pH2 and PROT_pH3 were prepared according to the method described in Section 2.2. The polymer and PROT dispersions/solutions were combined together in various v/v ratios at room temperature (RT) and mixed under magnetic stirring at 500 rpm for around 10 min to allow stabilisation of the system. Since the concentration of both solid components was the same (0.1% w/v), those v/v ratios were equivalent to alginate/PROT weight mixing ratios (WMRs) and WMRs are used throughout this manuscript.

The impact of the rate of addition of PROT solution to the dispersion of AAL and the sequence of alginate/PROT mixing have been tested. Variations in the assembly method tested are summarised in Table 1. In all cases after combining both solutions the stirring was continued for 10 minutes.

2.4 Physicochemical characterisation of NPs

The mean particle size (hydrodynamic particle diameter), polydispersity index (PdI) and zeta potential (ZP) values of the polyelectrolyte complexes were determined at 25 ºC by dynamic light scattering (DLS) and laser Doppler velocimetry using a Zetasizer Nano ZS instrumnt (Malvern Instruments Ltd., UK) as described previously (Dul et al., 2015). The results are given as average values of triplicate measurements ± SD. Viscosity and pH measurements were performed as described in Section 2.2. FTIR analysis was performed on AAL/PROT systems produced by combining 0.1% w/v ALG and PROT solutions at a WMR of 1 as described

previously (Umerska et al. 2012). Colloidal stability of NP formulations (in native dispersions) was visually examined instantly after the preparation and prior to conducting any measurements. The presence or absence of instability (for instance aggregation) was noted. Changes in the hydrodynamic particle size, PdI and ZP occurring on storage were ascertained at pre-determined time points. The measurements as well as visual observations were performed up to 3 days (72 h). The following solutions/buffers: 0.01M HCl, 0.1M acetate buffer pH4.5, 0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH6.5 and 0.015M phosphate buffered saline (PBS) pH7.4 were also employed as media to determine the colloidal behaviour and susceptibility of the NPs to changes in pH/ionic strength. The native dispersions containing the nanocomplexes were diluted with each of the above liquid media in the 1:1 v/v ratio. The physical stability of such dispersions was estimated by measuring changes in the mean hydrodynamic particle size over time, for up to 3 days (72 h).

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

As reported by Boddohi et al. (2008) and Umerska et al. (2012) the molecular weight of the polymer and its concentration in solution are the crucial parameters determining the successful polyelectrolyte NP process formation. The study performed by Carneiro-da-Cunha et al. (2011) showed that the polymer concentration affected the mean diameter and ZP of multilayer NPs formed by sodium alginate, carrageenan and CHIT solutions. The mean hydrodynamic diameter of NPs made from a 0.6% w/v solution of alginate was approximately a 2-fold greater than those of NPs formed by 0.2% w/v alginate solution (an increase from 1180 to 2155 nm) (Carneiro-da-Cunha et al. 2011). However, it is important to emphasise that lowering the polymer concentration reduces the production yield of NPs, which can lead to a decrease in the final loading capacity of particles. Therefore, in the present work, to balance the desirable NP characteristics and production capacity of the process, 0.1% w/v solutions of ALG and PROT were used.

3.1 Impact of the rate and sequence of components addition on properties of NPs

As previously reported, the manner in which solutions of polyions in a PEC method are combined influences the properties of formed complexes (Chen et al. 2003; Dragan et al. 2006; Birch & Schiffman 2014; Dul et al. 2015). First, to examine the impact of the rate at which the PROT solution was introduced to an aliquot of ALG dispersion, NPs were prepared by slow (Method_1A) or rapid (Method_1B) addition of components, as described in Table 1. Two

formulations of NPs with an ALG/PROT WMR of 4.5 and 5 were selected for these trials (Table 2).

A significant difference in the particle size was observed. Smaller in size NPs were formed when the PROT solution was rapidly added to the aliquot of ALG dispersion (Method_1B) in comparison to the slow method (Method_1A). A statistically significant difference in the Pdl and ZP values were observed for NPs with an ALG/PROT WMR=5, but not NPs with an ALG/PROT WMR=4.5 (Table 2). Therefore, to obtain NPs with small sizes, a rapid addition of components was used for further studies.

The sequence of mixing of the NP component solutions/dispersions was also investigated. These methods were called Method_2A (PROT solution was added to ALG dispersion) and Method_2B (ALG dispersion was added to PROT solution) as explained in Table 1. Interestingly, Method_2A yielded smaller in size, in comparison to Method_2B, NPs for WMRs of 1, 1.5, 3 and 5 (with negative ZP values), while Method_2B gave smaller particles, in comparison to Method_2A, for WMRs of 0.2, 0.33 and 0.67 (positively charged NPs), as presented in Fig. 2a. However, comparing the Pdl values, it was noticed that NPs with higher Pdl indices (less homogenous) were chiefly generated using Method_2B (Fig. 2b). The order of mixing was seen to impact less the ZP values (Fig. 2c).

The impact of the order of component mixing on the properties of NPs, when preparing PECs, was investigated previously. Birch and Schiffman (2014) showed that that the order of solution addition resulted in differences in particle sizes and ZP values of CHIT/pectin NPs, however the rationale for this behaviour was not provided. Dul et al. (2015) investigated this phenomenon in relation to formation of carrageenan/PROT NPs. Generally, smaller in size, with lower Pdl values and negatively charged NPs were formed when PROT solution was added to the dispersion of carrageenan (Method_2A), similar to what is observed for the ALG/PROT systems here. It was concluded that PROT can either stabilise or destabilise the formed NPs, depending on the mixing method and also on the type of polymer (carrageenan in this case) used (Dul et al. 2015).

3.2 Impact of molecular weight of alginate on the properties of NPs

The molecular weight of AAL was measured to be ~100 kDa, however the molecular range of AAM ranged from 42 to 537 kDa (Table 3). The high polydispersity of AAM may have an impact on the formation and properties of polyelectrolyte complexes. Polydispersity of the polycation has been identified as an issue in studies on DNA complexation and was shown to lead to structural heterogeneity and different biological activity of NPs (Mustafaev 1995; Polexe and Delair 2013), while high viscosity of polymer dispersions may negatively impact

on the mixing process leading to the formation of aggregates (Mackay et al. 2006, Umerska et al. 2012). Viscosity of 0.1% w/v dispersions (used to produce NPs) were in line with the average molecular weights of alginates, with ~2.7 mPa·s for AAL and ~4.2 mPa·s for AAM.

Results presented in Section 3.1 show that both polymers (AAL and AAM) have the ability to form polyelectrolyte complexes with PROT. Furthermore, it was observed that the rate and sequence of components addition have an impact on the properties of NPs. To further explore the difference between NPs formed by alginate with various viscosity/molecular weights (AAL and AAM), a wide range of WMR was tested. The nanocomplexes were prepared using Method_2A (an aliquot of PROT solution was added to an aliquot of ALG dispersion quickly), as this method of preparation was selected to be the most favourable for ALG/PROT NPs self-assembly (Section 3.1).

In was difficult to discern a trend for the particle size when comparing NPs made by AAL and AAM (Fig. 2a and d). The particle size ranged from 51 to 319 nm for the WMR tested (Fig. 2d). An increase in WMR resulted in the formation of NPs, which were smaller in size and for WMRs of 4.5 and above NPs with sizes below 100 nm were formed for both, AAL and AAM. Both combinations tested, AAL/PROT and AAM/PROT, showed the formation of large entities, most likely agglomerates/flocs, occurring for 0.40-0.83 AAL/PROT and 0.77-0.87 AAM/PROT WMRs (Fig. 2d), consistent with charge neutralisation (Boddohi et al. 2008; Umerska et al. 2012, Dul et al. 2015). The intrinsic pKa of ALG is around 3 and at pH of the ALG solution (pH around 6, Table 3) complete ionisation of carboxyl groups is expected, thus notionally all the acidic groups of ALG should electrostatically bind to the basic groups of PROT (Andriamanantoanina and Rinaudob 2010). Pdl values ranged from 0.11 to 0.38 for AAL/PROT NPs and from 0.08 to 0.46 for AAM/PROT NPs and were seen to increase with an increasing ALG/PROT WMR (Fig. 2b). ZP measurements showed that ALG/PROT NPs can be either positively or negatively charged (Fig. 2c). The cationic NPs had similar ZP values of approximately 20 mV for both types of ALG tested, which could be due to the PROT presence on their surface. The anionic NPs composed of AAL/PROT had ZP values of around -110 mV and around -160 mV for AAM/PROT NPs at WMR of 3. The comparison of NP properties containing the different grades of ALG suggested that NPs based on AAL offered more promising characteristics: smaller particles with narrower size distributions. Those NP characteristics have been shown to have an impact on the stability, biodistribution, cellular uptake and bioavailability of those NPs (Elsabahy and Wooley 2012).

Several reports show the use of alginates in drug delivery, however in most of the cases this polymer is combined with CHIT (a different polycation than PROT). Similar to the current

study, NPs of alginate and CHIT were formed by addition of 0.1% w/v alginate solution (pH6.5) under high shear conditions to 0.1% w/v CHIT solution (pH4) (Saether et al. 2008). Those NPs were formed in a one step process, where one polymer solution was added in a dropwise manner into the other solution. Also, homogenisers operating at various speeds were facilitating the mixing process. The size of the obtained particles was 500 nm and greater, reaching values of 2 μm. The component charge ratio and molecular weight were shown to be parameters that affected the particle size, ZP and pH of formed complexes. The mixing order also influenced the size of formed NPs, however without influencing their ZP and pH. At the charge ratios close to 1, the sizes of the largest particles were more variable, however, by using polymers with low molecular weights and employing a process whereby an addition of the polymer solution to an excess volume of the other polymer solution, small in size particles resulted (Saether et al. 2008). Li et al. (2008) also explored alginate/CHIT NPs prepared by the PEC method as a drug delivery system for nifedipine. Described by this group NPs had very small sizes, 20-50 nm, and were prepared by ionic pre-gelation of alginate core (dropwise addition of calcium chloride to aqueous solution of sodium alginate under stirring) followed by CHIT polyelectrolyte complexation (Li et al., 2008).

The importance of the molecular weight ratio of the polycationic and polyanionic components and its charge density was highlighted by Umerska et al. (2014). It was shown that, while it was possible to form NPs between hyaluronic acid (HA, molecular weight ~260 kDa) and PROT (molecular weight ~5 kDa), they were not stable due to the considerable disparity in the molecular weight of HA in comparison to that of PROT. The ratio of molecular weights for AAM/PROT is ~50 (for AAL/PROT ~20), yet this combination yields physically stable complexes in the nanoscale. It could be related to the strength of ionic interactions between then components as HA is regarded as a relatively weak acid with low charge density (Polexe and Delair 2013).

3.3 Impact of pH adjustment on the formation and properties of NPs

Comparing the various strategies that have been used to improve drug solubility, changing pH of solution is one of the common approaches for ionisable drug molecules. Therefore, changing pH of one of the polyion solutions prior to mixing with the other polyion solution can facilitate dissolution of an active compound to be loaded into the NPs, however it will also alter binding of components in the nanoparticle via modification of electrostatic interactions. The results presented in Sections 3.1 and 3.2 were generated when pH of the solutions/dispersions was unadjusted (pH values are given in Table 3).

At first, pH of AAL dispersion was adjusted to 2 maintaining pH of PROT solution at the native value (pH5.8). However, only large in size and sedimenting entities (aggregates, floccules and/or microparticles) formed immediately after mixing both solutions, regardless of the WMR. Analysis of physicochemical properties of alginate shows the pH dependent behaviour of this polymer, consistent with this substance being a polyanion. It has been shown that viscosity of sodium alginate dispersion is almost constant in the pH range of 6-8. However, at moderate concentrations and following a pH decrease, viscosity increases and reaches a maximum at pH of 3-3.5 (intrinsic pKa of alginic acid is around 3), as carboxylate groups in the alginate backbone become protonated and form interchain hydrogen bonds (Lee and Mooney, 2012; Guarino et al. 2015). Dispersions of alginic acid at higher concentrations form physical gels at these pH values, where hydrogen bond attractions predominate over electrostatic repulsions.

As no NPs were formed when pH of AAL dispersion was adjusted to 2, further manipulations with solution pH were performed for PROT only. Lowering pH of PROT solution to 2 (PROT_pH2) resulted in the formation of particles larger in size in comparison to AAL/PROT (pH unadjusted) NPs (Fig. 3a) and with only negatively charge, as ZP values ranged from -25 to -113 mV (Fig. 3b).

Aggregates/microparticles were formed at an AAL/PROT_pH2 WMR of 2 and lower. The particle size of anionic NPs ranged from 142 to 262 nm (Fig. 3a), while Pdl values ranged from 0.21 to 0.28. Comparison of AAL/PROT and AAL/PROT_pH2 formulations showed that lowering pH of PROT solution resulted in the formation of NP dispersions with lower dynamic viscosity and higher ZP values for AAL/PROT_pH2 NPs (Fig. 3b and c). As an example, at a WMR of 5 the NPs had ZP of -126 mV for AAL/PROT NPs and -44 mV for AAL/PROT_pH2 NPs. The decrease in viscosity may be due to the different degree of AAL/PROT interactions. Additionally, pH of PROT solution was adjusted using a HCl solution, which resulted in an excess of negatively charged ions (Cl-) in the system, which may have an impact on the surface charge of formed NPs as well as the WMR at which the neutralisation of charge occurs. The addition of PROT solution at such a low pH could have resulted in changes in the nature of interactions between the polymer and PROT driven by partial/local protonation of AAL and/or agglomeration via hydrogen bond interactions (Lee and Mooney 2012).

As decreasing pH of PROT solution to 2 resulted in the formation of anionic NPs only, to enhance the possibility of formation of NPs that would bear a positive charge, pH of PROT solution was adjusted to 3 (PROT_pH3). This time both, cationic and anionic NPs resulted. The particle size of negatively charged AAL/PROT_pH3 NPs ranged from 71 to 137 nm, while

in the case of positively charged NPs they ranged from 172 to 305 nm (Fig. 3a). Aggregation and an increase in the particle size was observed at an AAL/PROT_pH3 WMR of 1 (Fig. 3a). Considering homogeneity (Pdl) of the formed NPs, negatively charged NPs at AAL/PROT_pH3 WMRs of 1.5 to 6 had greater Pdl values, which ranged from 0.17 to 0.37 respectively, compared to cationic NPs (at AAL/PROT_pH3 WMRs of 0.17 to 0.67), ranging from 0.11 to 0.20. ZP values of positively charged AAL/PROT_pH3 NPs were 14-17.9 mV, while in the case of negatively charged AAL/PROT_pH3 NPs the ZP values varied from -49 to -107 mV (Fig. 3b). Generally, lowering pH of PROT solution to 3 changed the WMR at which the agglomeration/flocculation of the formed NPs occurred (Fig. 3a). pH of the NP dispersions ranged from 6.0 for AAL/PROT_pH3 with a WMR of 15 to 3.3 for AAL/PROT_pH3 with a WMR of 0.1. The WMR at which the agglomeration/flocculation occurred was 1 and pH of this formulation was 3.8, which can be regarded as an isoelectric point. For the formulations where pH of the PROT solution was not adjusted, physical instability was observed at AAL/PROT WMRs of 0.50 – 0.83 with pH of those formulations ranging from 5.7 to 6.0.

There were no major differences within the particle size between AAL/PROT_pH3 and AAL/PROT NPs. An increase in the ZP value of anionic AAL/PROT_pH3 NPs was observed, in comparison to AAL/PROT NPs. The differences in the particle size, ZP and dynamic viscosity values for AAL/PROT_pH2, AAL/PROT_pH3 and AAL/PROT NPs are presented in Fig. 3.

3.4 Interactions between alginate and PROT

FTIR spectra of AAL, PROT and NPs are presented in Fig. 4. The spectrum of AAL showed absorption bands associated with hydroxyl, ether and carboxylic functional groups. Bands observed at 1649 and 1460 cm-1 were attributed to asymmetric and symmetric stretching vibrations of the carboxylate ion, respectively. The weak bands at 1301, 1125 and 1094 cm-1 might be assigned to C-C-H and O-C-H deformation, C-O stretching and C-O and C-C stretching vibrations of pyranose rings, respectively. The band at 1035 cm-1 is due to C-O stretching vibrations. The fingerprint region of 950-750 cm-1 is typically the most discussed for carbohydrates. The band observed at 948 cm-1 can be assigned to the C-O stretching vibration of uronic acid residues, while the peak at 888 cm-1 was assigned to the C1-H deformation vibration of β-mannuronic acid residues. The band at 820 cm-1 is characteristic for mannuronic acid residues. In case of PROT the most prominent peaks appeared between 1400 and 1700 cm-1, which are characteristic for amide I and II vibrations. Analysis of NPs spectra showed that no new covalent bonds were formed, however shifts of some absorption

bands were observed (Table 4). It was noticed, that the peaks associated with carboxyl groups of AAL and amine groups of PROT moved slightly (Table 4). Additionally, the band characteristic of mannuronic acid (at 820 cm-1) was less intense following AAL complexation with PROT.

Similar changes were observed for carrageenan complexed to PROT, where the peaks associated with the sulphate and amine groups of carrageenans and PROT moved significantly for the polyelectrolyte complexes containing kappa and lambda carrageenan, while for the iota carrageenan/PROT system only the amide I band, but not sulphate peaks, shifted. It was suggested that, while all the carrageenans studied are able to form polyelectrolyte complexes with PROT, the mechanism of complexation was different for iota carrageenan (Dul et al. 2015). Additionally, another reports show a large shift in the amide I band of chitosan, observed after its complexation with HA (Umerska et al. 2012), chondroitin sulphate (Umerska et al., 2015) and after complexation of chitosan with kappa carrageenan (Li et al. 2013). Therefore, it appears that AAL is another polyanion that has an ability to bind to PROT via electrostatic interactions.

3.5 Colloidal stability of NPs

A challenging, but very important, aspect of NPs characterisation is the stability studies of formulations under conditions that resemble *in vitro* and *in vivo* environment. Stability and the degree of NP agglomeration/aggregation in physiological conditions or different media for biotechnological applications are important parameters to be known. Properties of NPs such as the particle size and/or surface charge determine the quality and applicability of given NPs. A range of formulations with various AAL/PROT WMRs and properties were chosen for colloidal stability testing, which were carried out for up to 72h at RT.

Generally, anionic NPs in their native dispersions were more stable compared to cationic NPs (Fig. 5). A large increase in the particle size was observed only for the cationic NPs (Fig. 5a). A very rapid increase in particle size was observed after 24 h for AAL/PROT NPs with a WMR of 0.5. The AAL/PROT formulation with a WMR of 0.2 showed the best stability comparing the positively charged NPs, as the increase in the particle size was observed after 72 h (Fig. 5a). An increase in the Pdl values was observed only for the positively charged NPs (Fig. 5b). A gradual, but relatively small decrease in the ZP values was observed for the anionic NPs (Fig. 5c), which might be caused by depolymerisation of polymer due to hydrolysis (Holme et al. 2003 and 2008). The best colloidal stability of NPs in the native dispersions was seen for AAL/PROT NPs with WMRs of 2.5 and 5, while the least stable formulation was that with a WMR of 0.5, perhaps as its ZP is closest to the neutralisation point.

Colloidal stability studies of selected AAL/PROT formulations in buffers at various pH were also conducted. Instability and formation of larger particles occurred only in media with very low pH (in HCl) (Fig. 6). Interestingly, better stability in HCl was observed for positively charged NPs (Fig. 6a-c). The cationic NPs (AAL/PROT_pH3 at WMR of 0.2, 0.33 and 0.5) were physically unstable in the other media tested (acetate buffer, HEPES buffer and PBS). Immediate aggregation of cationic NPs occurred in acetate buffer at pH 4.5 and PBS, pH 7.4. Better stability was observed for these NPs in HEPES buffer, pH 6.5. The anionic NPs were stable in most of the media tested (Fig. 6d-f). No considerable difference in the colloidal stability of anionic NPs was observed in the acetate, HEPES and diluted PBS media, even though the concentration of the PBS solution was over a 6-fold lower (0.1M versus 0.015M) than the other two solutions. This might be contributed to the more kosmotropic (order inducing) character of the acetate and 4-(2-hydroxyethyl)-1-piperazineethanesulphonate (HEPES) anions, while the chloride anion is considered more chaotropic (order disrupting) (Umerska et al. 2015). Thus lower concentrations of the chloride anions are needed to induce physical instability of NP dispersions.

As the component, which is used in excess is responsible for charge stabilisation of PEC complex (PROT for cationic formulations and ALG for anionic NPs), its ability to remain unchanged on the surface of particle in a varied range of conditions will define the colloidal stability of NPs. In the case of negatively charged NPs, its poor stability at low pH can be explained by the properties of ALG. Similarly to ionically cross-linked hydrogels, PECs exhibit pH-sensitive swelling under acidic conditions (Berger et al. 2004). As the pH value changes, the charge balance between the components inside the formed PECs is altered, resulting in weakening interactions between the two polymers and possibly leading to swelling of particles due to dissociation of the complex. In an acidic medium, the carboxylic groups of the polyacid are protonated, thus forming hydrogen and weaker than ionic bonds with PROT. The study performed by Sankalia et al. (2007) showed that at pH2, the ionic interactions between CHIT and alginate was greatly reduced and folding of alginate was observed with an increased micropore size, which allowed better penetration of particles with the dissolution medium. A similar behaviour of ALG was observed in our study, however an increase in pH of the liquid medium caused instability of cationic formulations. As PROT comprises small, approximately 5 kDa fragments, it can be easily removed from the NP surface. Ionic interactions, which occur between positively charged groups of PROT and ionic groups present in the media often lead to neutralisation of the charge and formation of larger particles/aggregates.

4 Conclusions

We have demonstrated that alginates can be used as suitable polymers for the formation of novel NPs by polyelectrolyte complexation with PROT. The conditions for the formation of nanoparticulate carriers were determined and the self-assembly process was optimised by testing the impact of a range of process and formulation variables, such the grade of polymer (alginate), the mixing ratio of the polymers as well as the speed and order of addition of the components during mixing on the properties of NPs. It was shown that the smallest in size and with lowest Pdl nanocarriers were formed when 0.1% w/v dispersion of AAL and 0.1% w/v solution of PROT were mixed by Method_2A (when an aliquot of PROT solution was introduced into a stirred aliquot of ALG dispersion in a fast way).

To facilitate incorporation of drugs which are soluble at low pH, pH of AAL solution was lowered to 2 (from native 6.4), however no NPs were obtained. Therefore further studies were carried out by adjusting pH of PROT solution. Only negatively charged NPs resulted when pH of PROT solution (from native 5.8) was decreased to 2, while the adjustment of pH of PROT solution to 3 resulted in the formation of both, anionic and cationic NPs, depending on the WMR. Physical stability of selected AAL/PROT NPs formulations was tested upon storage at room temperature and in liquid media at various pH. Good colloidal stability was observed for anionic NPs stored as native dispersions and with media with a range of pH values.

In summary, a simple, organic solvent and surfactant-free production method by mixing aqueous solutions of polyelectrolytes at room temperature was successfully employed to formulate novel, cross-linker free and non-sedimenting NPs comprising alginate and protamine. It was possible to obtain NPs with good physical properties (i.e. small and homogenously dispersed), which could be used for loading a variety of active molecules including proteins and peptides. It is proposed that those NPs (positively or negatively charged) can be used for parenteral and non-parenteral (such as oral, nasal or pulmonary) applications, the route of administration also dependent of the loaded cargo.

Funding

This study was funded by Merrion Pharmaceuticals Ireland. This work was also supported by the Synthesis and Solid State Pharmaceutical Centre funded by Science Foundation Ireland under grant number 12/RC/2275.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

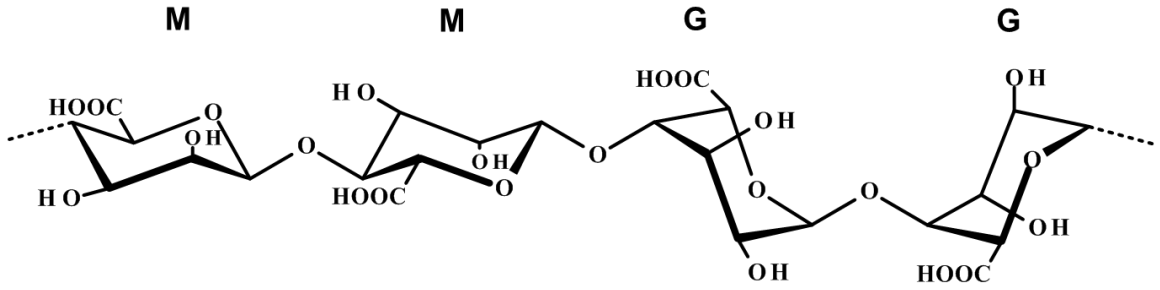
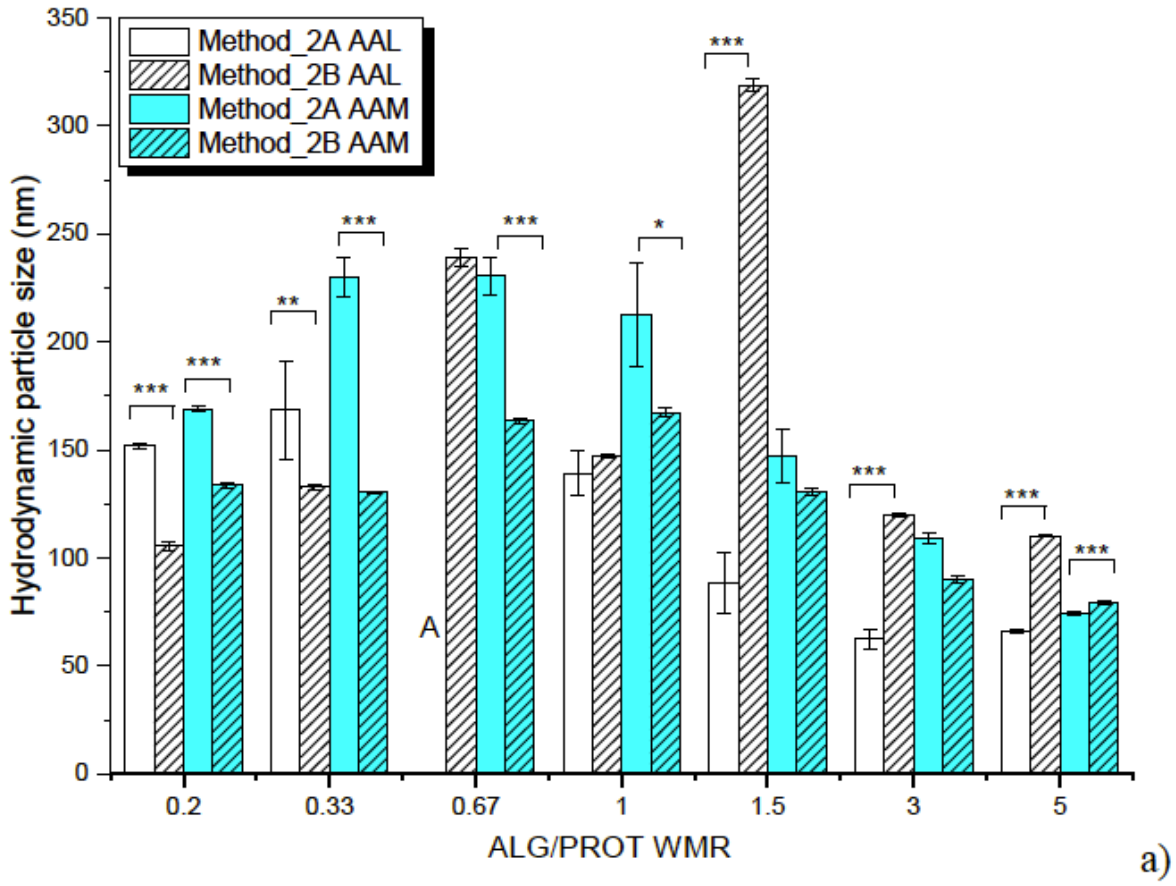
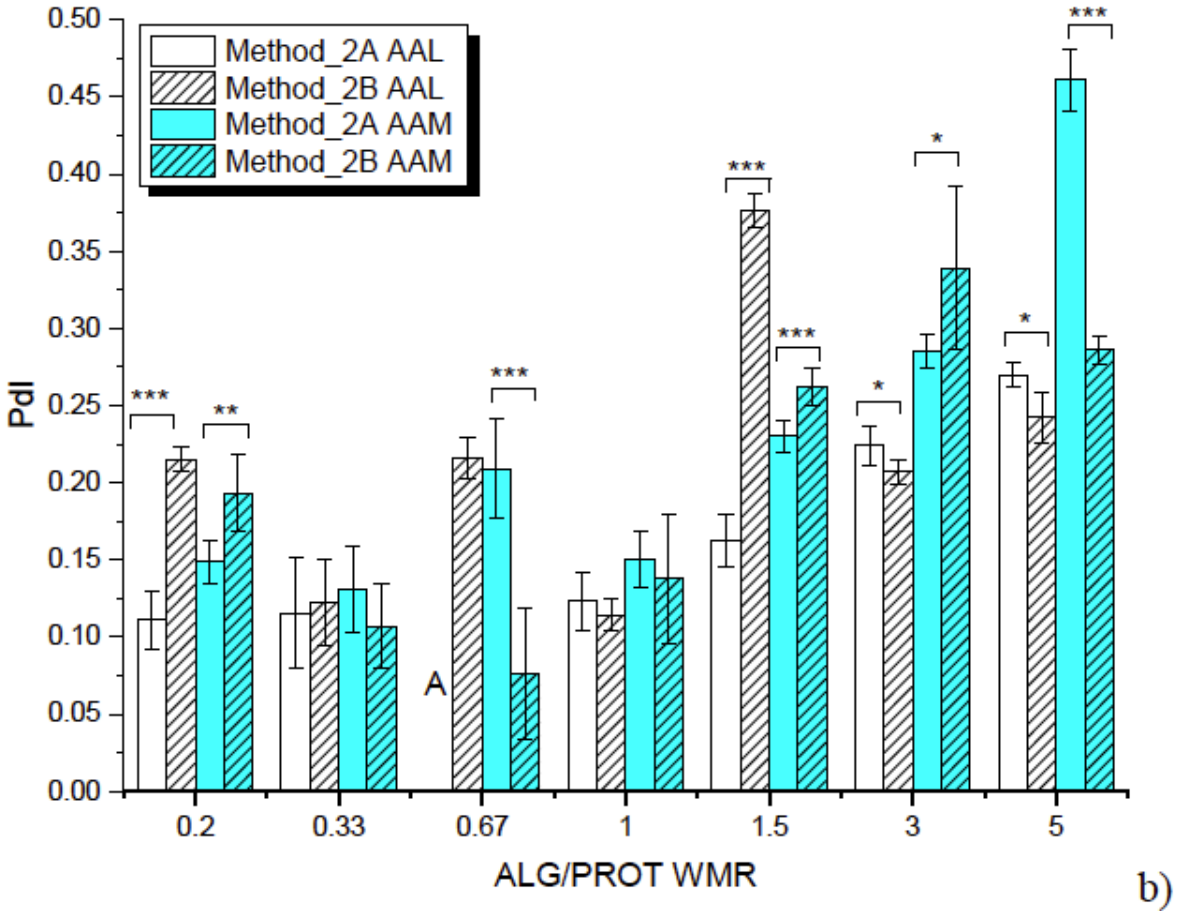


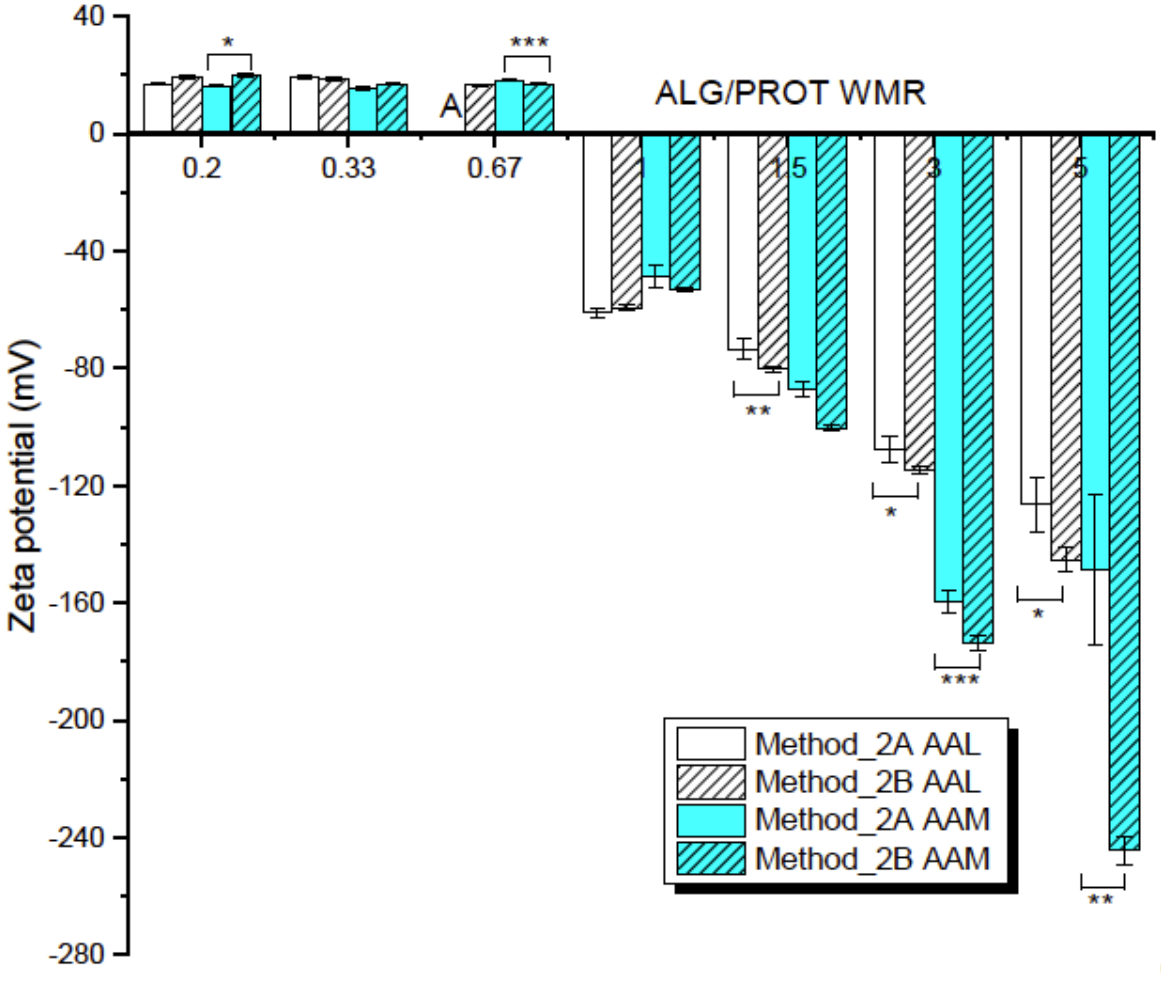
Fig. 1 Structural units of alginate. M = β-D-mannuronic acid, G = α-L-guluronic acid


Method_2A AAL
Method_2B AAL
Method_2A AAM
Method_2B AAM
Hydrodynamic particle size (nm)
350
300
250
200
150
100
50
0
***
***
**
***
***
*
***
***
***
***
A
0.2
0.33
0.67
1
1.5
3
5
ALG/PROT WMR

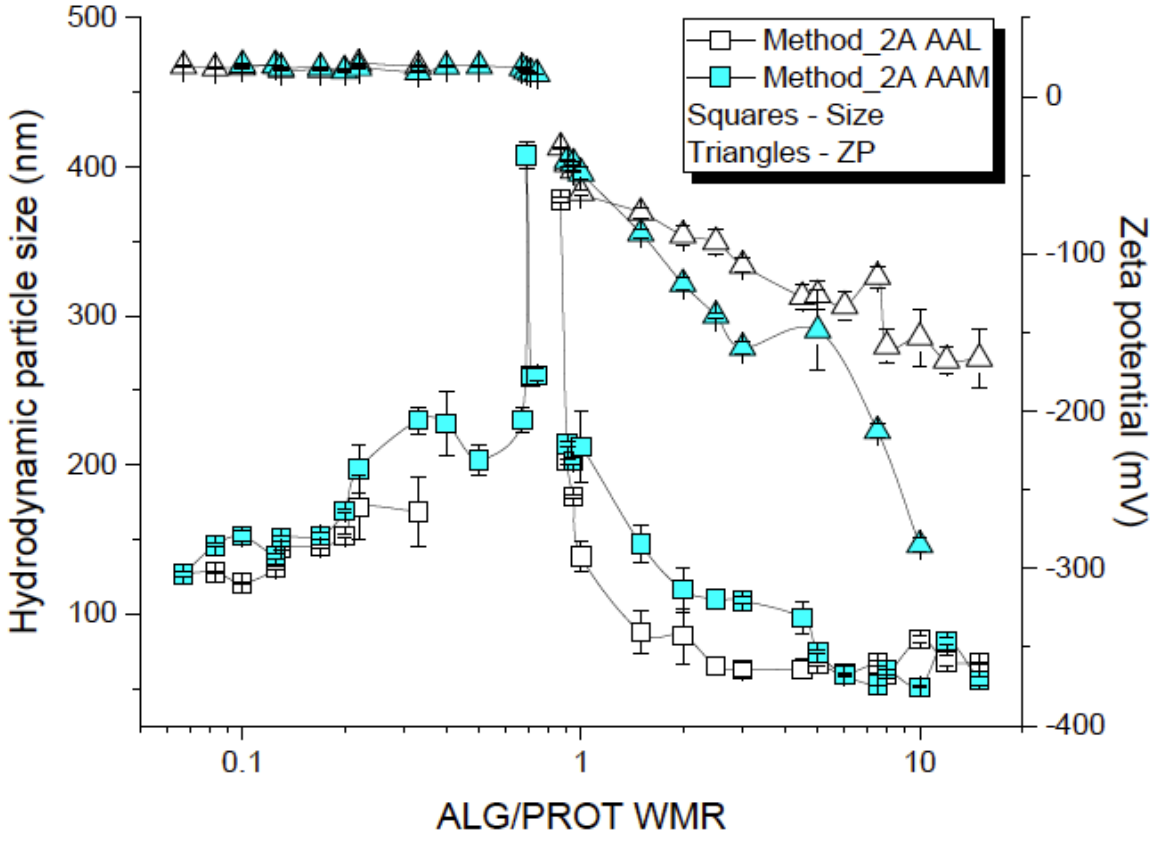
a)


Method_2A AAL
Method_2B AAL
Method_2A AAM
Method_2B AAM
PdI
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
***
**
***
A
***
***
*
*
*
***
0.2
0.33
0.67
1
1.5
3
5
ALG/PROT WMR

b)

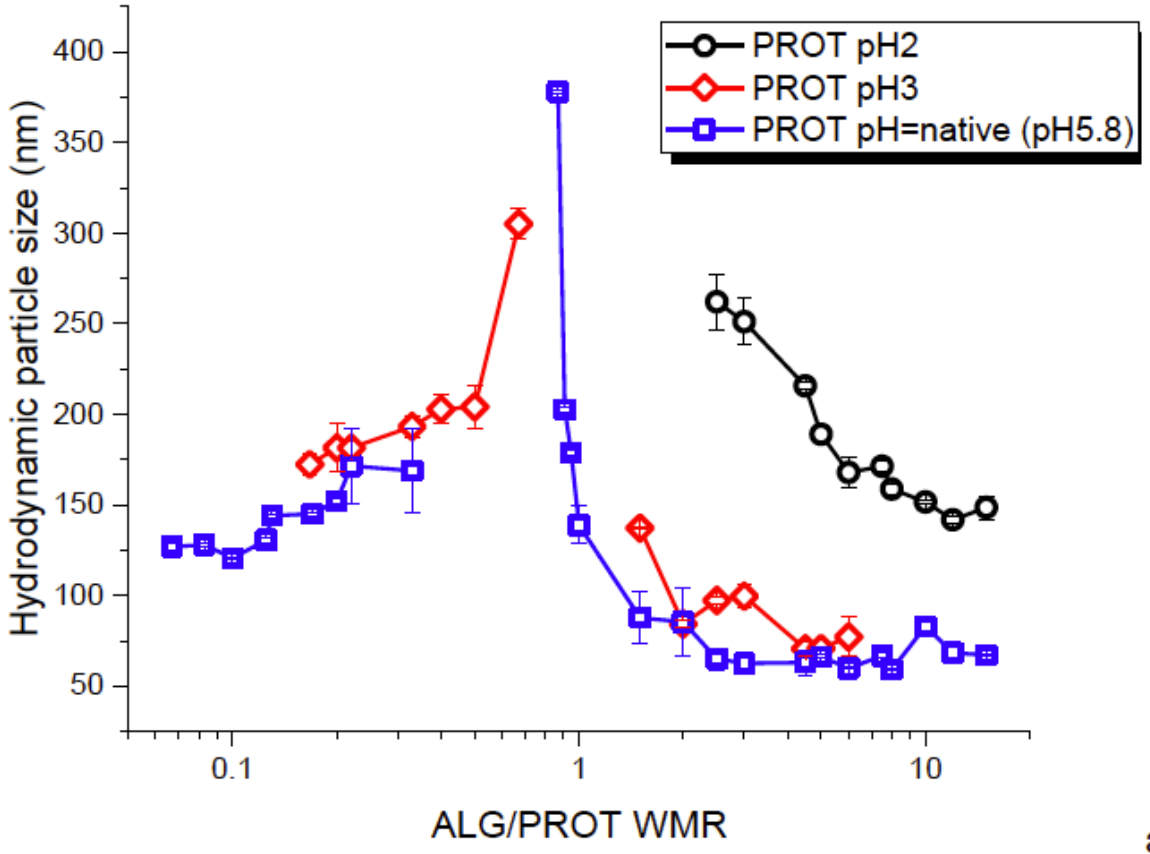

ALG/PROT WMR
Zeta potential (mV)
Method_2A AAL
Method_2B AAL
Method_2A AAM
Method_2B AAM

c)

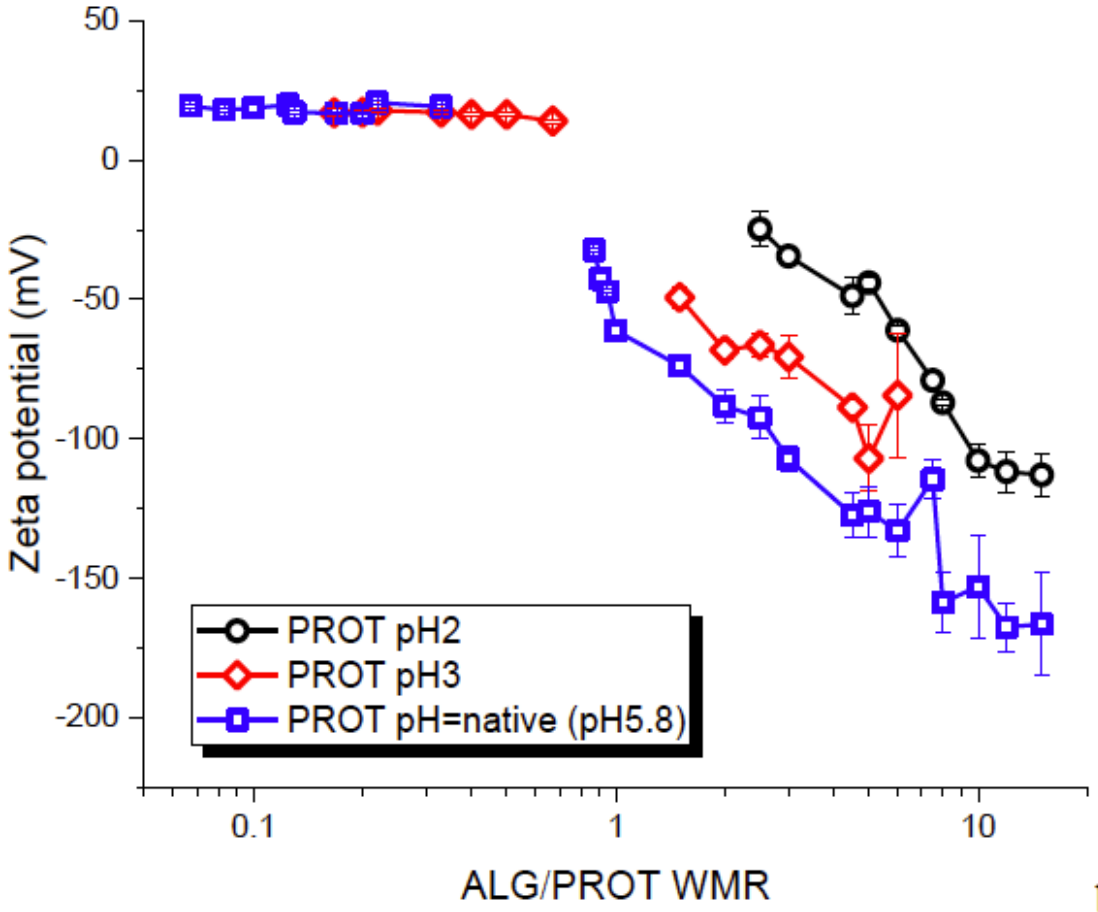

Method_2A AAL
Method_2A AAM
Squares - Size
Triangles - ZP
Hydrodynamic particle size (nm)
Zeta potential (mV)
ALG/PROT WMR

d)

Fig. 2 Comparison of: a) hydrodynamic particle size, b) polydispersity index (Pdl), c) zeta potential and d) hydrodynamic particle size and zeta potential (an extended range of ALG/PROT WMRs) for selected NPs composed of alginate low viscosity/protamine (AAL/PROT) and alginate medium viscosity/protamine (AAM/PROT) formed by the following variation in method preparation: Method_2A: an aliquot of PROT solution was added to an aliquot of ALG dispersion under magnetic stirring and Method_2B: an aliquot of ALG dispersion was introduced to an aliquot of PROT solution under magnetic stirring. A – instantaneous aggregation/flocculation; statistical analysis: *$p<0.05$, **$p<0.01$, ***$p<0.001$


PROT pH2
PROT pH3
PROT pH=native (pH5.8)
Hydrodynamic particle size (nm)
400
350
300
250
200
150
100
50
0.1
1
10
ALG/PROT WMR

a)


50
0
-50
-100
-150
-200
Zeta potential (mV)
0.1
1
10
ALG/PROT WMR
PROT pH2
PROT pH3
PROT pH=native (pH5.8)

b)

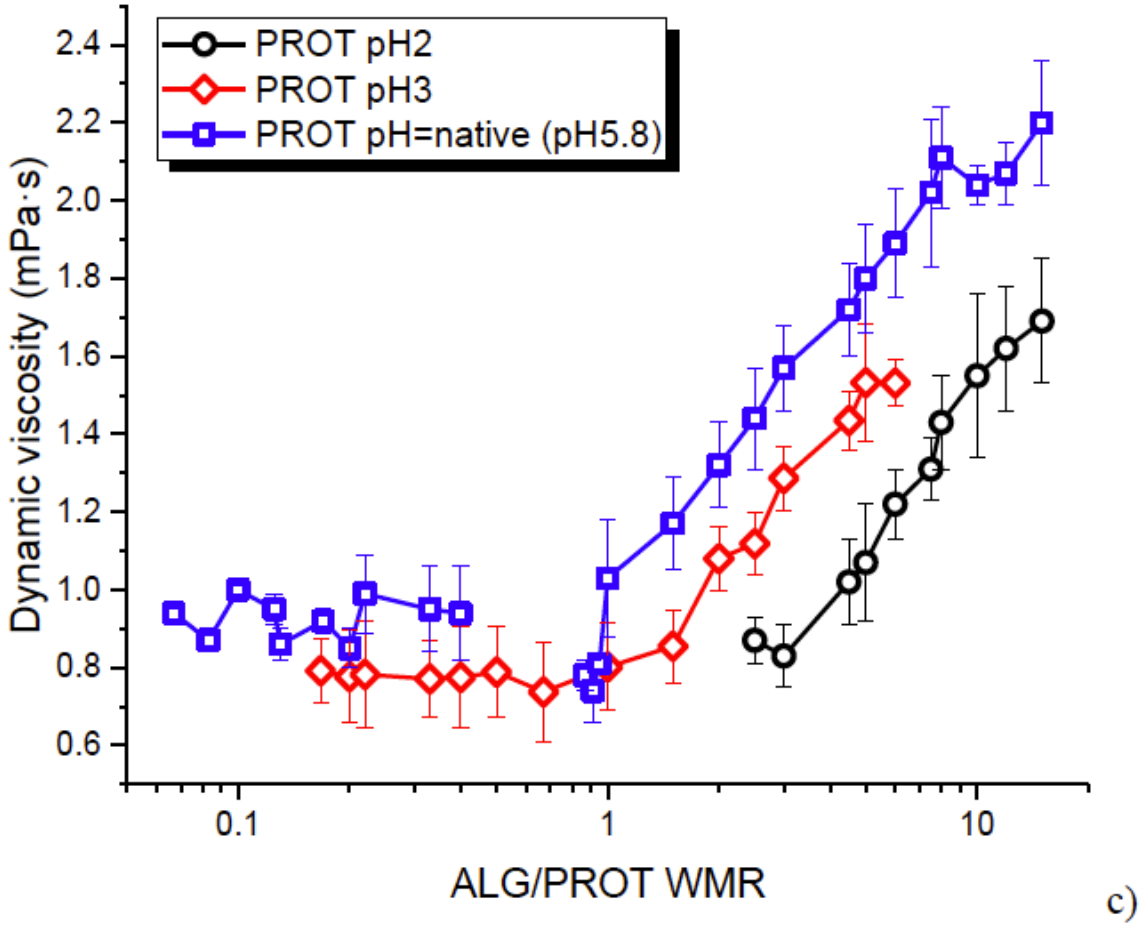


Fig. 3 Comparison of: a) hydrodynamic particle size, b) zeta potential (ZP) and c) dynamic viscosity for NPs made by combining 0.1% w/v dispersion of alginate low viscosity (AAL) and 0.1% w/v solution of PROT with pH2, pH3 and native pH (pH5.8). NPs were prepared by Method_2A: an aliquot of PROT solution was added to an aliquot of AAL dispersion under magnetic stirring

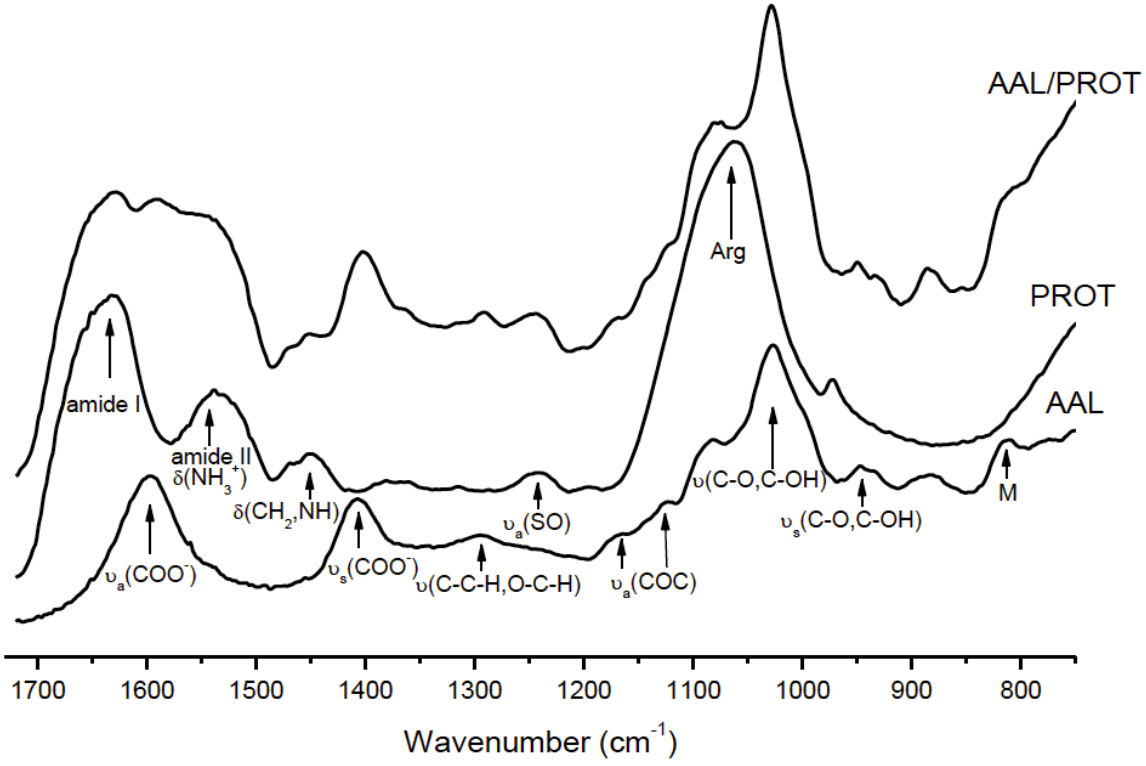
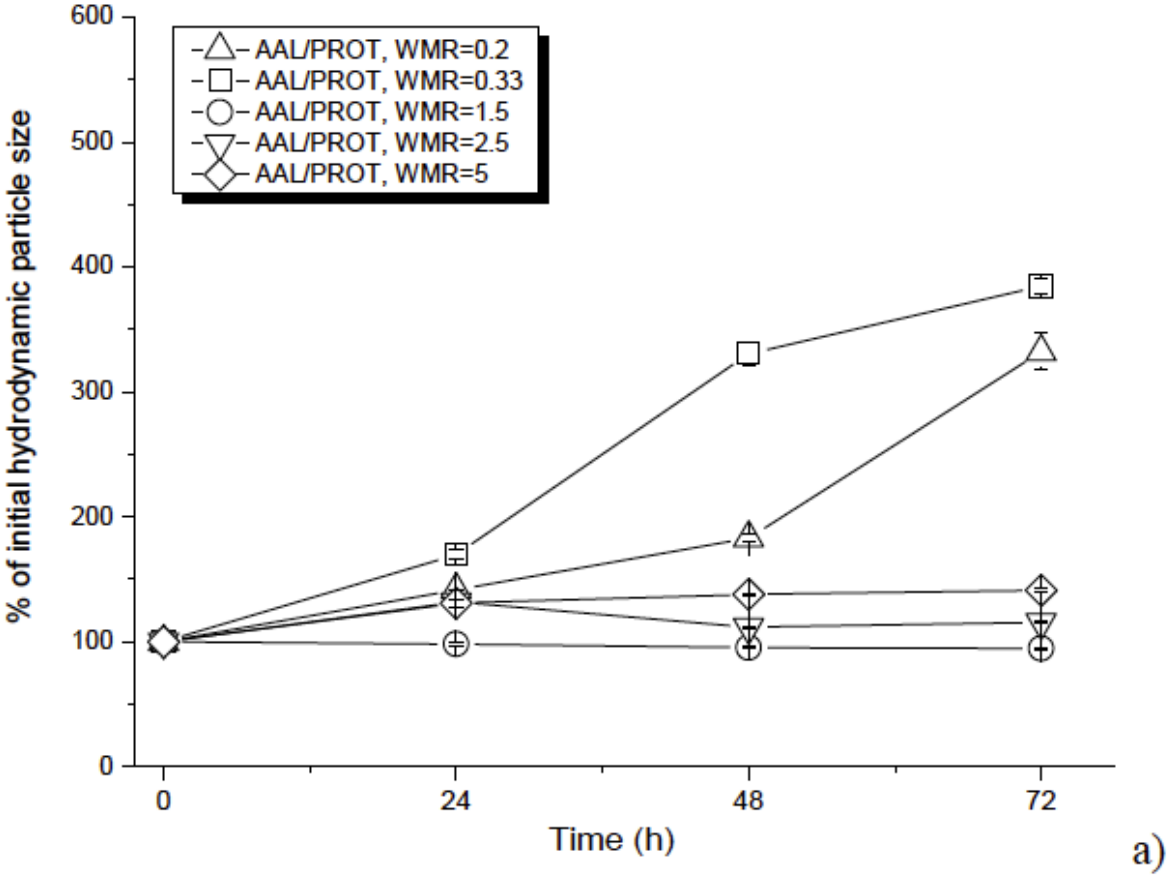
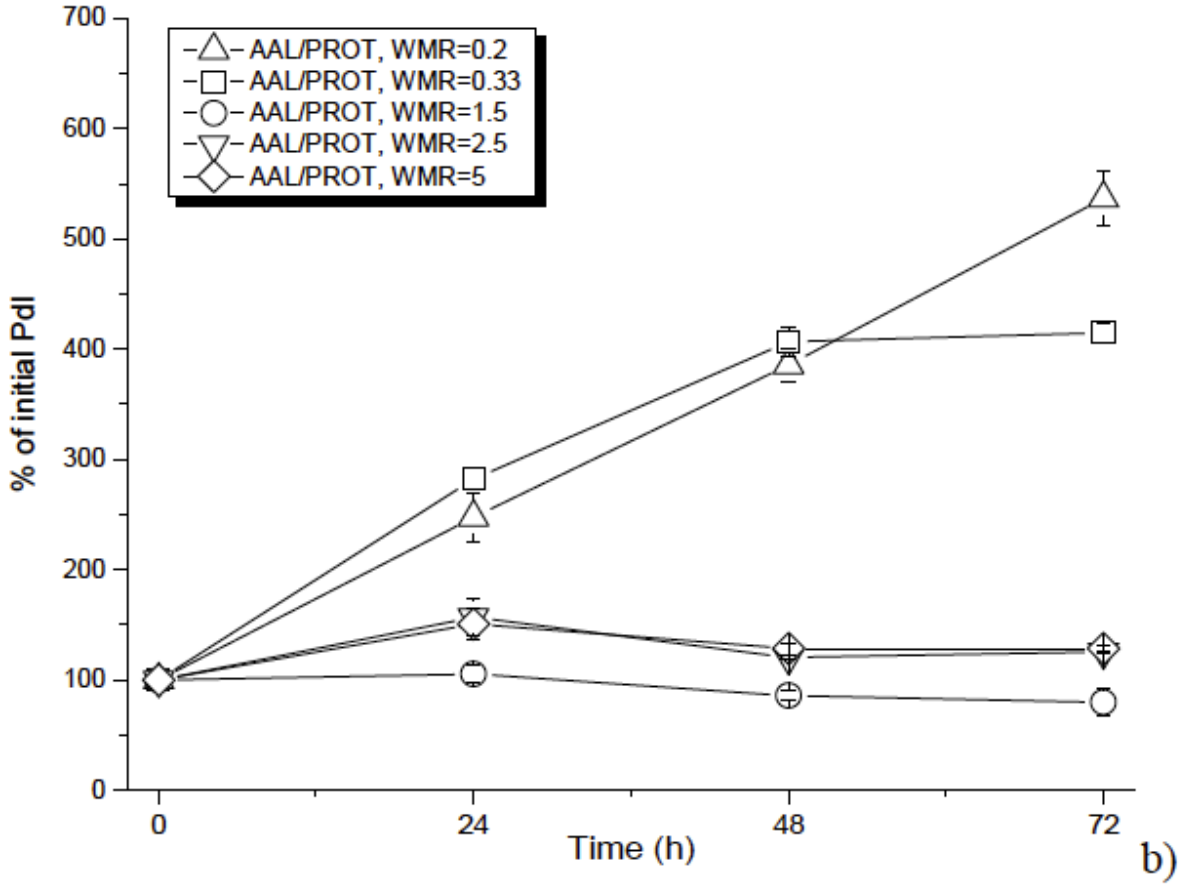


Fig. 4 FTIR analysis of PROT, AAL and AAL/PROT NPs. Band assignments was done based on studies of Bertoluzza et al. (1983), Le-Tien et al. (2004), Leal et al. (2008), Awotwe-Otoo et al. (2012) and Daemi and Barikani (2012). v – stretching, vS – symmetric stretching, vA – asymmetric stretching and δ - bending vibrations. M – mannuronic acid residues, AAL – alginate low viscosity and PROT – protamine


AAL/PROT, WMR=0.2
AAL/PROT, WMR=0.33
AAL/PROT, WMR=1.5
AAL/PROT, WMR=2.5
AAL/PROT, WMR=5
% of initial hydrodynamic particle size
600
500
400
300
200
100
0
0
24
48
72
Time (h)

a)


AAL/PROT, WMR=0.2
AAL/PROT, WMR=0.33
AAL/PROT, WMR=1.5
AAL/PROT, WMR=2.5
AAL/PROT, WMR=5
% of initial PdI
Time (h)
0
24
48
72
100
200
300
400
500
600
700

b)

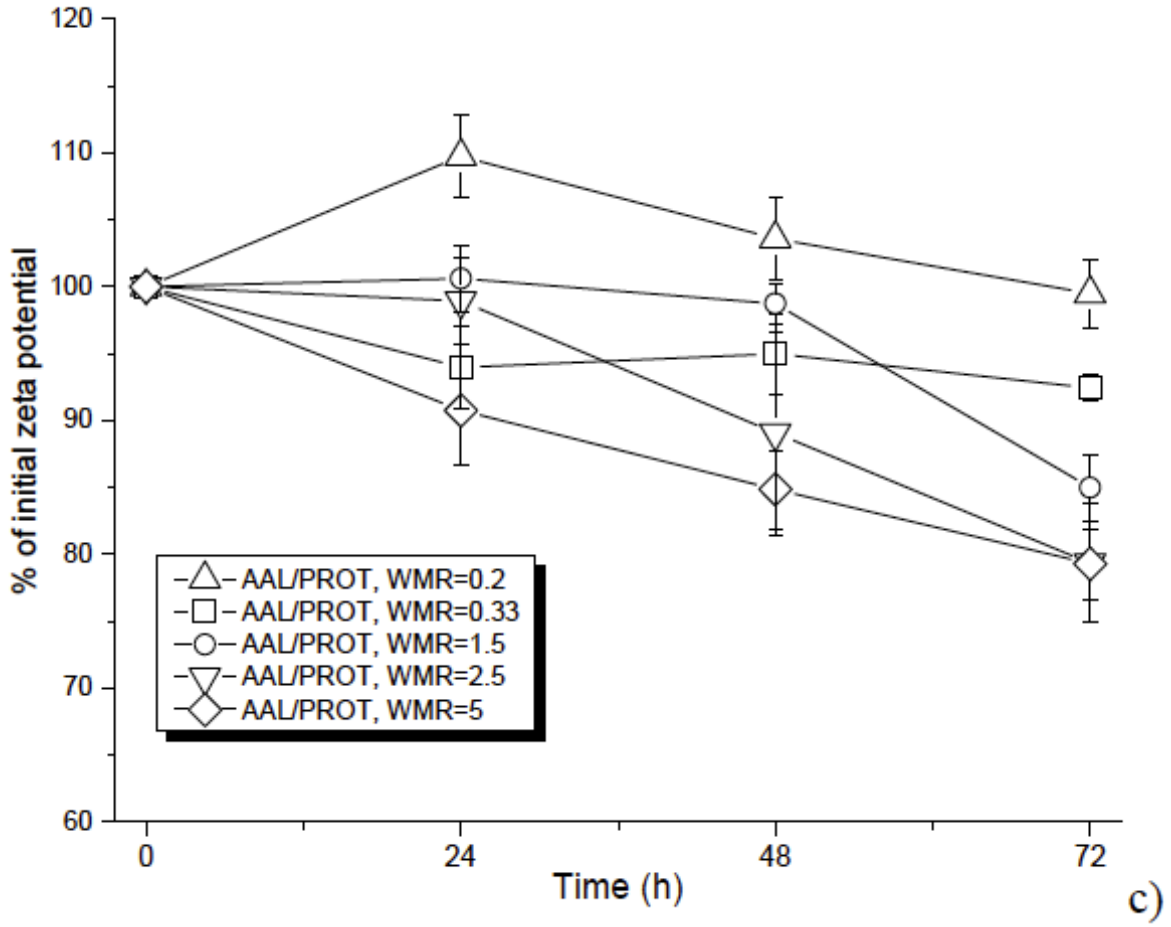


Fig. 5 Colloidal stability of AAL/PROT native dispersions at room temperature: a) hydrodynamic particle size, c) polydispersity index (Pdl) and c) zeta potential. WMR – weight mixing ratio, AAL – alginate low viscosity, PROT – protamine

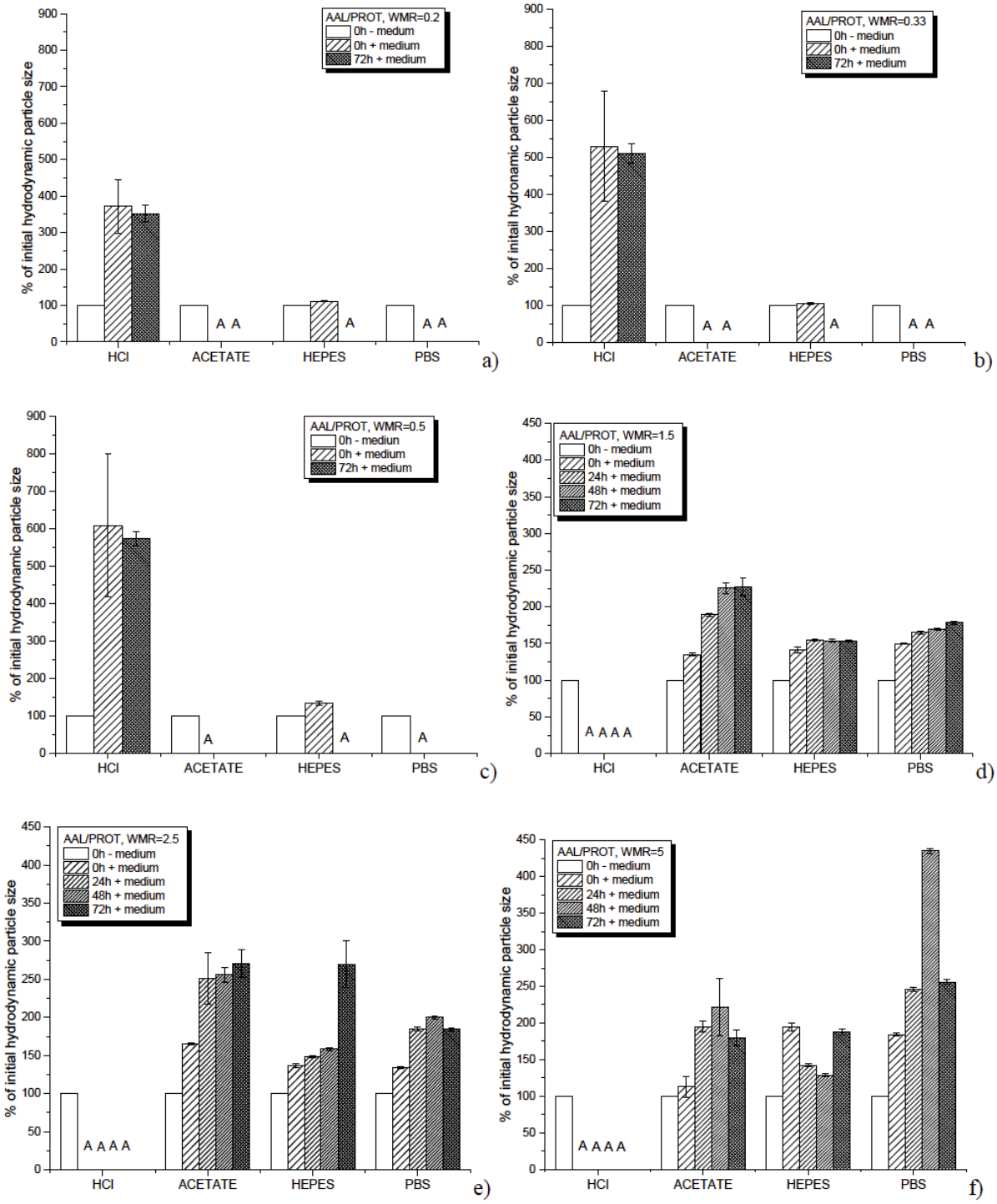


Fig. 6 Colloidal stability studies based on monitoring the change in hydrodynamic particle size of AAL/PROT nanoparticle dispersions in media with various pH. The weight mixing ratios (WMRs) of AAL/PROT were: a) 0.2, b) 0.33, c) 0.5, d) 1.5, e) 2.5 and f) 5. A – aggregation, AAL – alginate low viscosity, PROT– protamine, HCl – 0.01M, ACETATE – 0.1M acetate buffer pH4.5, HEPES – 0.1M HEPES buffer pH6.5 and PBS pH7.4.

Tables

Table 1 Summary of the methods evaluated for NPs preparation. ALG – alginate, PROT – protamine

Factor tested	**Rate** of PROT solution addition to ALG dispersion (PROT solution was added to ALG dispersion)
Method_**1A**	An aliquot of PROT solution was slowly (drop by drop, within 60 seconds) added to an aliquot of ALG dispersion
Method_**1B**	An aliquot of PROT solution was rapidly (within 2 seconds) added to an aliquot of ALG dispersion
Factor tested	**Sequence** of addition of PROT and ALG dispersion (fast addition of components, within 2 seconds)
Method_**2A**	An aliquot of PROT solution was added to an aliquot of ALG dispersion
Method_**2B**	An aliquot of ALG dispersion was added to an aliquot of PROT solution

Table 2 Comparison of properties of AAL/PROT NPs prepared using two various speeds of PROT solution addition to polymer solution. Method_1A: an aliquot of PROT solution was slowly (within 30 seconds) added to an aliquot of polymer dispersion, Method_1B: an aliquot of PROT solution was quickly (within 2 seconds) added to an aliquot of polymer dispersion. Statistical analysis: *p<0.05, **p<0.01, ***p<0.001

AAL/PROT weight mixing ratio	Hydrodynamic particle size (nm)		Polydispersity index		Zeta potential (mV)	
	Method of preparation:					
	1A	1B	1A	1B	1A	1B
4.5	131±1	57±1	0.239±0.010	0.233±0.012	-132.8±2.0	-129.4±6.4
	***		not significant		not significant	
5	133±3	72±0	0.312±0.032	0.380±0.018	-145.6±2.7	-131.6±8.4
	***		**		*	

Table 3 Physicochemical properties of alginates and protamine solutions/dispersions studied in present work. AAL – alginate low viscosity, AAM – alginate medium viscosity and PROT – protamine (* manufacturer data)

Polymer	Molecular weight (kDa)	Viscosity (mPa·s) of 0.1% w/v solution/dispersion	pH of 0.1% w/v, native (pH non-adjusted) aqueous solution/dispersion
AAL	104±10	2.72±0.10	6.4
AAM	42-537 (range)	4.19±0.14	6.4
PROT	5.1*	0.97±0.06	5.8

Table 4 FTIR band positions (in cm-1) in alginate (AAL), protamine (PROT) and AAL/PROT nanoparticle (NP) formulations. The number in parenthesis indicates the magnitude of the shift in cm-1. AAL – alginate low viscosity, PROT – protamine, v – stretching, vS – symmetric stretching, vA – asymmetric stretching.

Band	AAL	PROT	AAL/PROT NPs
ν_S (C-O, C-OH)	947	-	949 (2)
v (C-O, C-OH)	1027	-	1028 (1)
ν_S (COO^-)	1408	-	1403 (5)
ν_A (COO^-)	1597	-	1592 (5)
amide I	-	1632	1628 (4)