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# Application of Response Surface Methodology in the Preparation of Pectin-Caseinate Nanocomplexes for Potential Use as Nutraceutical Formulation: A Statistical Experimental Design Analysis

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**ABSTRACT**

***Background:*** The formation of electrostatic complexes between two types of biopolymers, sodium Caseinate (a derivative from most abundant milk protein) and Pectin (a natural hetro polysaccharide), was studied as a function of biopolymers concentrations and pH of solutions (3.9- 4.3).
***Method:*** The size and morphology of the resulted complexes were investigated by using of laser light scattering and transmission electron microscopy, respectively. Response surface methodology (A three-factor, three levels Box-Behnken design) was used for the optimization procedure with pH, pectin and sodium Caseinate concentrations as independent variables. Particle size and polydispersity index of nanocomplexes were considered as dependent variables.
***Results:*** Negatively charged nanocomplexes were produced below the isoelectric point of protein (5.4), at pH 4.1 with a suitable colloidal stability and average particle size of about 100 nm. It was found that the particle size of nanocomplexes could be controlled by changing in variables.
***Conclusion:*** In conclusion response surface methodology are simple, rapid and beneficial approach for preparation, optimization and investigation of the effect of independent variables on the properties of products.

## Introduction

Nanotechnology has wide range of applications, which focuses on the characterization, production, and use of biological as well as nonbiological particles smaller than 100 nm.[1] One of the activities of nanotechnology is use of nanocarriers for delivery system.[2] Regardless of successful description of many synthetic polymers as delivery systems, these cannot be used in food usages that need compounds which generally recognized as safe (GRAS). Biopolymer based nanocomplexs could be potentially used as nanocarriers to encapsulate and deliver bioactive or functional components, such as hydrophobic nutraceuticals (e.g, fat-soluble vitamins, antioxidants, carotenoids, phytosterols and polyunsaturated fatty acids) minerals, active peptides, enzymes and antimicrobial compounds.[3-6] Creation of nanocarriers for bioactive components may increase their bioavailability, due to their nanoscopic size and enormous numbers per unit mass, reduce adverse effects such as transparency of clear food systems like beverages, offer protection against degradation of the nutraceuticals by chemical and enzymatic reactions like oxidation during manufacturing and shelf-life, hence inhibiting of increasing of unwanted flavors and odors, as well as loss of metabolic value.[7] Among the several natural or synthetic polymer-based nanoparticle which are potentially available to the food industry, protein-based nanoparticles are generally interesting since they are fairly easy to use and their size distribution can be observed.[8] Throughout the past two decades, interest in developing protein particles as delivery systems has developed and several classes of animal proteins such as casein,[9] albumin,[10] and whey protein[11] and plant proteins like soy glycinin[12] and zein[13] have been investigated. Multiple component matrices such as protein-polysaccharide particles have also been explained.[14]

The interactions between polysaccharides and proteins could be also repulsive and attractive. Attractive interactions encourage inter biopolymer complexes, that almost always arise from electrostatic interactions

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between opposite charges on the biopolymers which there is an adequately robust electrostatic attraction between oppositely charged molecules. Attractive biopolymer interactions mostly take place between positively charged proteins (pH < isoelectric point, pI) and anionic polysaccharides (pH > $pk_a$) or negatively charged proteins (pH > pI) and cationic polysaccharides. At pH values below their pI, proteins transmit positive charges and can interact with polysaccharides bearing sulfate, phosphate, or carboxylic groups. This inter biopolymer complexation of anionic polysaccharides and positively charged proteins can result in the formation of soluble coacervates, complexes or precipitates depending on the charge density of the biopolymers, solution conditions (pH, ionic strength), ratio of protein to polysaccharide, as well as processing conditions (shearing, temperature and time).[15] The pH and the ionic strength are the most important factors affecting the formation, size and stability of these complexes. Both factors influence the number of charges present on the biopolymers, therefore affecting the strength of the electrostatic interactions. Other essential parameters to be considered are the biopolymer charge density and the biopolymer weight ratio, which influence the amount of the electrostatic interaction, the molecular weights of the biopolymers as well as the total biopolymer concentration, where contribute to a low entropy of mixing.[16,17] Soluble complexes may be found when opposite charges passed by the two macro ions within a complex are not equal in number. Nevertheless, when the opposite charges carried by the two biopolymers neutralize each other and the complexes become insoluble which result in coacervation and precipitation.[18] Milk proteins have significant efficient properties such as the capacity to bind hydrophobic molecules, stabilize emulsions, interact with other biopolymers, form gels and slightly delay oxidation. Because of these properties, milk proteins are ideal materials for the entrapment and delivery of bioactive compounds.[19] Casein is an important milk protein and intensely self-associates and precipitates at low pH (<5.4) and he casein micelle is certainly a notable case of natural nanocarriers for nutrient delivery. The stability of casein micelles during processing also makes them a very encouraging nano vehicle. A number of study have mostly focused on interactions between polysaccharides and casein micelles, nevertheless, investigations on complexation between non micellar casein material, such as polysaccharides and Caseinates are hardly found. Pectin is an anionic polysaccharide and contains numerous galacturonic acid groups and arabinose, rhamnose as well as galactose. The ability to control the size of biopolymer nanocomplexe is important not only for defining food product characteristics such as appearance, taste, texture and aroma, but also for control the release rates of the loaded bioactive complexes and finally the total efficiency of the compounds. In this way, the purpose of this study was to study the interaction of LMP with sodium Caseinate and study the effects of different variables on the size and size distribution of prepared complexes. Response surface methodology (RSM) is a group of mathematical and statistical methods that calculates the practical correlation between a number of measured response variables and numerous explanatory factors to find an optimal response by means of a series of tests.[20] To readily reach to this objective, a computer optimization method, based on a RSM using a polynomial equation was used to search for the size range of made complexes and professionally measure the effects of formulation variables on the size and polydispersity.

## Materials and Methods

### *Materials*

Sodium Caseinate (82 wt% dry protein, 6 wt% moisture, 6 wt% fat and ash, 0.05 wt% calcium) was supplied by DMV international (Veghel, Netherlands), Low-Methoxyl Amidated (LMA) Pectin (31 % *degree of esterification*, 17% degree of amidation) was provided by CP Kelco ApS (Lille Skensved, Denmark). HCl, $K_2HPO_4$, $CaCl_2$, NaOH and potassium citrate were obtained from Merck (Darmstadt, Germany).

### *Solution preparation*

Sodium Caseinate, Pectin and salt solutions were prepared in double-distilled water. These solutions were equilibrated over-night at 4 ˚C for complete hydration and applied freshly for each experiment. To 50 mL solutions of sodium Caseinate , 2 mL 0.4 M potassium citrate, 12 mL 0.08 M $K_2HPO_4$ and 10 mL 0.08 M $CaCl_2$ were gradually added. Finally, pH was adjusted between 6.7 and 7 with dropping of 0.1 N HCl under stirring. The volume was eventually brought up to 100 mL with double-distilled water with the final Caseinate concentration of 1% W/V. Each experiment was performed in duplicate. Pectin was brought into solution by heating to 85 °C for 10 min with vigorous stirring followed by a slow cooling to room temperature with continued stirring for 1 - 1.5 h. This solution was kept in the refrigerator to avoid microbial growth and discarded after a maximum of 4 days. Before addition to Caseinate solution, Pectin solution was vigorously stirred for 1 min.

### *Caseinate-Pectin complexation*

A series of solutions containing various casein and Pectin concentrations were prepared. The Pectin solution was added drop wise into Caseinate solution at the constant temperature of 4 °C. The stirring speed of stirrer was kept on 400 rpm during the experiments. These solutions were titrated slowly with 0.1 N HCl using a 29 G needle to the desired pH and then stirred for 1 h and stored overnight at room temperature prior to determination of the particle size. The studied concentrations of Pectin, Caseinate and desired pH, mentioned at Table 1, were selected according to preliminary experiments (data was not shown).

### *Complex characterization*

#### *Morphology*

Nanocomplexes were observed using transmission electron microscopy (TEM) with the help of negative

staining three days after sample preparation. One drop of the suspension containing complexes was placed on a 200-mesh copper grid (3.05 mm, HF36, Australia) for 5 min and then drained off with filter paper. Consequently, one drop of 2% uranyl acetate was placed on the grid for 20 sec before being drained off. The grid was then placed in a TEM (Philips CM10, the Netherlands) and examined.

### *Particle size measurement*

The particle size of each batch was analyzed with a laser diffraction particle size analyzer (SALD-1100, Shimadzu, Japan). The average particle size was shown as the volume mean diameter (VMD). Each sample was measured three times. The size distribution was assessed with the span value according to the following equation:

$$Span = \frac{D90\,\% - D10\,\%}{D50\,\%} \quad \text{Eq. (1)}$$

Where $D_{N\%}$ (N = 10, 50, 90) is the volume percentage of particles with diameters up to $D_{N\%}$ is equal to N%. The smaller span value indicates the narrower size distribution.[21]

### *Experimental design*

In the current study, to describe the particle size and size distribution of complexes as well as to optimize the preparation process, a three-level four-factorial Box-Behnken experimental design was employed to assess the effects of selected variables on the response. This design is appropriate for exploration of quadratic response surfaces and for production of second order polynomial models, consequently helping to optimize development by means of a small number of experimental runs. Factors considered in the Box-experimental design were: pH, Pectin and sodium Caseinate concentrations. The factor levels were chosen in accordance with the results of our initial studies.[22] Table 1 reviews the factors and their levels. For the three-level four-factorial Box-Behnken experimental design, a total of 30 experimental runs with six replications of the central point, are needed.

The mixture of conditions resultant to the central point of the design replicated six times to approve the validity of the model and reduce the estimation variance of the values expected by the quadratic model. This design also resolves the two-factor interaction effects of individual terms and allows a mid-level setting (0) for the mixture of factors.[23]

## Results and Discussion

It is essential to develop a formulation in straight possible stage, by means of minimum man-hours and raw materials. Usually, formulations are established by changing one variable by trial and error way that is time consuming requiring a lot of inspired efforts. Additionally, it may be difficult to increase an ideal product by means of this classical way, since the combined effects of independent variables are not measured.[24] It is therefore very essential to understand the complication of formulations by using established statistical methods such as factorial design.

**Table 1.** Run parameters for three-level four-factorial Box–Behnken and particle size results of experimental design (each number represents mean ± standard deviation, n = 3).

| StdOrder | RunOrder | PtType | Blocks | A[a] | B[b] | C[c] | VMD[d] (nm) | Span |
|---|---|---|---|---|---|---|---|---|
| 6 | 1 | 2 | 1 | 4.3 | 1.0 | 0.20 | 648 ± 58 | 5.82 ± 0.02 |
| 5 | 2 | 2 | 1 | 3.9 | 1.0 | 0.20 | 10529 ± 230 | 3.09 ± 0.15 |
| 23 | 3 | 2 | 1 | 4.3 | 1.0 | 0.70 | 723 ± 25 | 1.33 ± 0.03 |
| 30 | 4 | 0 | 1 | 4.1 | 1.0 | 0.45 | 86 ± 5 | 0.93 ± 0.02 |
| 17 | 5 | 2 | 1 | 4.3 | 0.5 | 0.45 | 1645 ±149 | 4.84 ± 0.01 |
| 11 | 6 | 2 | 1 | 4.1 | 0.5 | 0.70 | 2200 ± 103 | 4.62 ± 0.02 |
| 16 | 7 | 2 | 1 | 3.9 | 0.5 | 0.45 | 1743 ± 141 | 5.95 ± 0.02 |
| 3 | 8 | 2 | 1 | 3.9 | 1.5 | 0.45 | 6157 ± 108 | 2.85 ± 0.06 |
| 13 | 9 | 0 | 1 | 4.1 | 1.0 | 0.45 | 99 ± 6 | 0.89 ± 0.67 |
| 21 | 10 | 2 | 1 | 4.3 | 1.0 | 0.20 | 639 ± 44 | 4.90 ± 0.12 |
| 28 | 11 | 0 | 1 | 4.1 | 1.0 | 0.45 | 93 ± 8 | 0.93 ± 0.53 |
| 14 | 12 | 0 | 1 | 4.1 | 1.0 | 0.45 | 86 ± 5 | 0.87 ± 0.05 |
| 7 | 13 | 2 | 1 | 3.9 | 1.0 | 0.70 | 380 ± 8 | 0.83 ± 0.01 |
| 26 | 14 | 2 | 1 | 4.1 | 0.5 | 0.70 | 2100 ± 86 | 4.42 ± 0.09 |
| 27 | 15 | 2 | 1 | 4.1 | 1.5 | 0.70 | 1094 ± 72 | 0.79 ± 0.15 |
| 2 | 16 | 2 | 1 | 4.3 | 0.5 | 0.45 | 2218 ± 428 | 4.58 ± 0.23 |
| 8 | 17 | 2 | 1 | 4.3 | 1.0 | 0.70 | 793 ± 54 | 0.83 ± 0.15 |
| 24 | 18 | 2 | 1 | 4.1 | 0.5 | 0.20 | 150 ± 305 | 2.67 ± 0.00 |
| 10 | 19 | 2 | 1 | 4.1 | 1.5 | 0.20 | 12025 ± 337 | 2.84 ± 0.01 |
| 15 | 20 | 0 | 1 | 4.1 | 1.0 | 0.45 | 93 ± 6 | 0.96 ± 0.01 |
| 4 | 21 | 2 | 1 | 4.3 | 1.5 | 0.45 | 622 ± 11 | 0.76 ± 0.00 |
| 19 | 22 | 2 | 1 | 4.3 | 1.5 | 0.45 | 626 ± 64 | 0.78 ± 0.03 |
| 20 | 23 | 2 | 1 | 3.9 | 1.0 | 0.20 | 12303 ± 184 | 2.90 ± 0.06 |
| 29 | 24 | 0 | 1 | 4.1 | 1.0 | 0.45 | 94 ± 1 | 0.71 ± 0.09 |
| 12 | 25 | 2 | 1 | 4.1 | 1.5 | 0.70 | 1119 ± 38 | 0.83 ± 0.24 |
| 22 | 26 | 2 | 1 | 3.9 | 1.0 | 0.70 | 322 ± 2 | 0.88 ± 0.01 |
| 25 | 27 | 2 | 1 | 4.1 | 1.5 | 0.20 | 12069 ± 848 | 2.84 ± 0.08 |
| 1 | 28 | 2 | 1 | 3.9 | 0.5 | 0.45 | 1758 ± 140 | 5.95 ± 0.04 |
| 18 | 29 | 2 | 1 | 3.9 | 1.5 | 0.45 | 5921 ± 409 | 2.84 ± 0.29 |
| 9 | 30 | 2 | 1 | 4.1 | 0.5 | 0.20 | 150 ± 218 | 2.67 ± 0.00 |

[a]pH, [b]Sodium Caseinate concentration, [c]Pectin concentration, [d]Volume Mean Diameter

### *Caseinate-Pectin complexation*

Complex creation by interaction of proteins and polysaccharides is fairly simple and includes two main steps, addition of biopolymers and modifying pH. However, the reproducible preparation of nanocomplexes in the preferred properties (desired size and narrow size distribution) can be difficult, because of the large number of factors influencing the properties of complexes, such as biopolymer concentration, type of biopolymer and pH. The influence of each of these factors has to be determined empirically, predictions and scale up remain a problem. Therefore, more information is needed in order to identify the relevant parameters and save development resources. According to above mentioned descriptions, the real variables of Table 1 were considered in the present study. A number of workers have previously described electrostatic complexes designed by milk protein and Pectin. The driving force for complexation was attributed primarily to electrostatic forces between the anionic Pectin and cationic patches on the protein surface, but hydrophobic forces were also thought to play a role for Pectin molecules with higher degrees of methoxylation.[25,26] Complex size reduced when acidification was done after mixing the polymers compared to when acidification was complete before the mixing (data was not shown). Consequently, at the first step of our study, Pectin solution was added into the Caseinate solution at the neutral pH and then the mixture of biopolymers was acidified. This interaction depends on whether the Pectin adsorbs onto the casein particles or not. If Pectin added in sodium Caseinate at a neutral pH, it did not adsorb onto the protein. Non-adsorbing Pectin leads to a phase separation.

### *Particle size distribution and morphology*

Negative staining TEM micrograph showed that discrete (Figure 1a) and relatively mono dispersed nanocomplexes (Figure 1b) were prepared.

The mean particle size of complexes was measured and the results presented that the volume mean diameter (VMD) and span were in the range of 86–12303 nm and 0.716–5.952, respectively listed in Table 1. The wide range of observed size and span stated in Table 1 shows that variables are effective in their studied range.

### *Experimental design*

#### *Particle size*

Table 2 shows the effect of independent variables on the size terms of quadratic model. Predicted $R^2$ (ranges between 0 and 100 %) used in regression study to show how well the model predicts responses for new observations, whereas $R^2$ indicates how well the model fits the data.

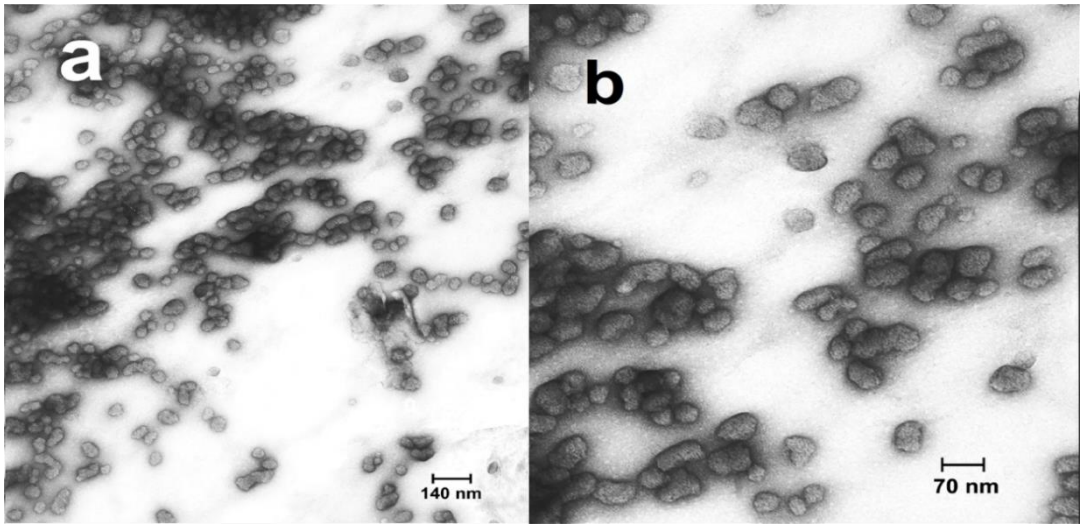


**Figure 1.** Negatively stained transmission electron micrograph of complexes observed in the sodium Caseinate (1%) / Pectin (0.45 %) at pH 4.1.

**Table 2.** Values for regression coefficients and their levels of significance for the factors used to model the best fit size response.

| Term | Coef [a] | SE Coef [b] | T | P |
|---|---|---|---|---|
| **Constant** | 91.83 | 455.2 | 0.202 | 0.842 |
| **pH** | -1949.94 | 278.7 | -6.995 | 0.000 |
| **Sodium Caseinate (%w/v)** | 1729.31 | 278.7 | 6.204 | 0.000 |
| **Pectin (%w/v)** | -2486.38 | 278.7 | -8.920 | 0.000 |
| **[pH]$^2$** | 961.58 | 410.3 | 2.344 | 0.030 |
| **[Sodium Caseinate (%w/v)]$^2$** | 1532.83 | 410.3 | 3.736 | 0.001 |
| **[Pectin (%w/v)]$^2$** | 2238.71 | 410.3 | 5.456 | 0.000 |
| **pH × sodium Caseinate (%w/v)** | -1399.00 | 394.2 | -3.549 | 0.002 |
| **pH × pectin (%w/v)** | 2794.88 | 394.2 | 7.090 | 0.000 |
| **Sodium Caseinate (%w/v) × Pectin (%w/v)** | -3235.13 | 394.2 | -8.207 | 0.000 |

S = 1114.98; R-Sq = 94.46%; R-Sq (pred) = 85.81%; R-Sq (adj) = 91.96%.

[a] Coefficient

[b] Coefficient of Standard Error of Mean.

Predicted $R^2$ can inhibit over fitting the model and can be more beneficial than adjusted $R^2$ for comparing models because it is calculated using observations not included in model estimation. $R^2$ value of this model is admissible and is equal to 85.81. The adjusted $R^2$ is a useful tool for comparing the explanatory power of models with different numbers of predictors. The small difference between the predicted observed R indicates validity of the model.

Concerning the multiple regression analysis, the most significant parameter was the one with the largest t value and the lowest p.[27] The results uncovered that the Pectin concentration and its interaction with Caseinate ($t = 8.920$, $p < 0.0001$ and $t = -8.207$, $p < 0.0001$) had the greatest effect on particle size, followed by the terms pH-Pectin interaction, pH and Caseinate concentration. These results suggest that all studied variables (pH, Pectin and Caseinate concentrations) considerably affect particle size. A positive sign before a factor in polynomial equations represents that the response increases with the factor. On the other hand, a negative sign means the response and factors have a reciprocal relation. The negative coefficient for Pectin concentration could be attributed to the reduction of droplet size with increasing concentration, leading to a decrease in particle size. The positive coefficient of concentration of sodium Caseinate in the equation showed that with enhancing in sodium concentration of Caseinate, the particle size will increase, which is in contrast to Pectin concentration and pH effects on particle size. Figures 2a and 1b illustrate the response surface as a function of sodium Caseinate concentration and pH as well as Pectin concentration and pH, respectively, for the particle size. The response surface in Figure 2a showed that an increase in sodium Caseinate concentration resulted in increase in particle size at the lower pH values which can be indorsed to protein aggregation.[28,29] As it is obvious from this figure, reducing sodium Caseinate concentration at different pH values directed to a reduction of particle size, though, this effect was more noticeable at lower pH value. At lower pH, the effect of sodium Caseinate concentration (at constant concentration of Pectin) becomes critical. Figure 2b shows that at the lower Pectin concentration, pH reduction to the lower amounts leading to an increase in particle size. Whereas, particle size is approximately constant in the higher Pectin concentration during pH variation. The role of pH is absolutely crucial at the lower amounts of Pectin because in such a case little reduction in charge density of Pectin leads to inefficient interaction with sodium Caseinate. But it seems that at the higher amounts of Pectin, even at the lower pH, there is some degree of negative charge on the Pectin which is enough for interaction with sodium Caseinate.[30] Polysaccharides with negative charge such as Pectin prevent Caseinate aggregation in acidic pH due to their interactions and sterically hindered negatively-charged Pectin. Two dimensional contour plots at Figure 2 clearly shows that the suitable pH range for complex formation with sodium Caseinate is above 4.1 possibly due to above mentioned protein aggregation phenomenon at the lower pH range.

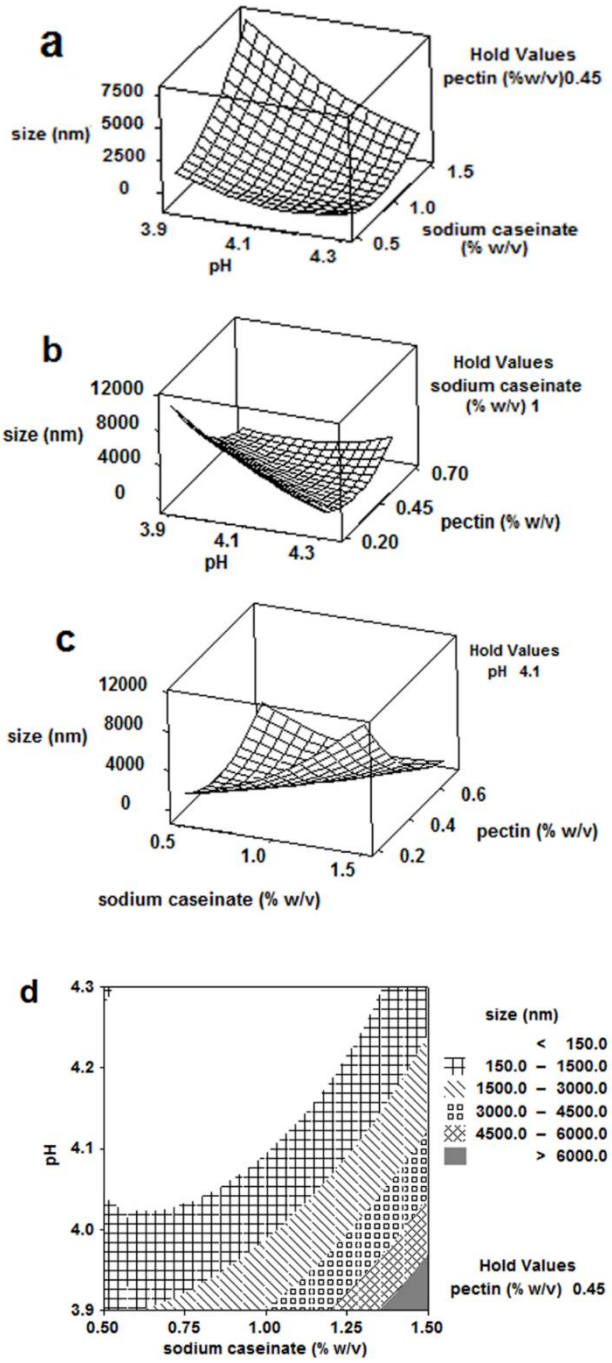


**Figure 2.** Three dimensional surface plots (a-c) and two dimensional contour plot showing the effect of different variables on particle size (d).

Coefficients with more than one factor term denote the interaction terms and coefficients. It can be understood from the equation (Table 2) that there is a significant interaction between all of three factors ($p < 0.003$). The positive coefficient of Pectin-pH term in Table 2 indicates synergist effect of both factors on the response. It means that increasing in pH value leads to growing the negative charges on Pectin and increasing in Pectin concentration causes much more charge density.

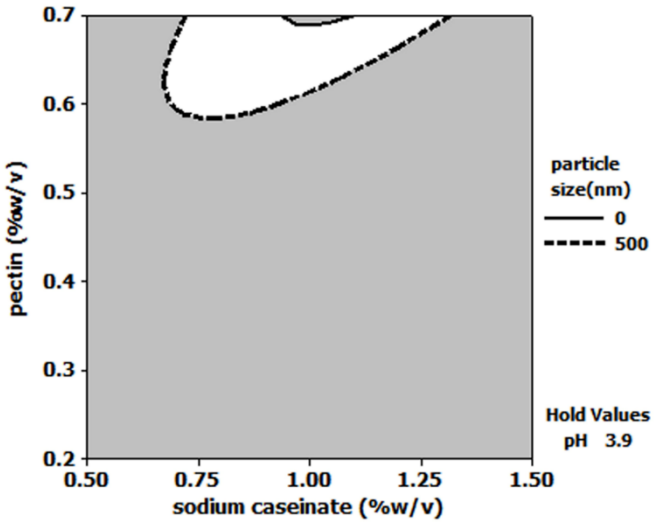


**Figure 3.** Overlaid contour plot showing the effect of different variables on particle size.

But the negative coefficients of pH-Caseinate and Pectin-Caseinate terms state the opposite effect of these factors to each other. A higher pH value causes charge reduction on Caseinate which causes Caseinate aggregation and precipitation.

However, interaction between Caseinate and Pectin concentration is to some extent interesting.

Three-dimensional surface plots are beneficial to represent the interactions. As shown in Figure 2c, increase in sodium Caseinate resulted in increase in particle size at the lower Pectin concentration. An opposite results was shown in higher Pectin concentration. Acidification of medium under isoelectric point (pI =5.4) of sodium Caseinate causes aggregation of uncovered protein. Low amount of Pectin cannot cover sodium Caseinate efficiently leading to instability and precipitation of Caseinate and consequently an increase in particle size after 24 hours.[30] Pectin molecule protected smaller Caseinate aggregates from self-association and consequent precipitation. This effect depends on the concentration of Pectin. At lower Pectin concentrations, the system was probably destabilized by self-association of free Caseinate molecules and charge neutralization of the system. When Pectin concentration is too high, Pectin is down from the surface of the casein micelles. Similarity of such depletion layers makes available an extra volume to the Pectin molecules. If the depletion attraction is strong enough the system phase split up. Overlaid contour plots of particle size (Figure 3) presented the acceptable region met the requirements to nanoparticles production. It is concluded that the desired size particles could be found in narrow regions that are depending on variables. As presented in Figure 3, targeted particles were prepared in Pectin above 0.6 (%w/v) at pH 3.9 and Caseinate concentration in the range of 0.75-1.25 (%w/v).

### *Size distribution*

The degree of dispersity is an essential concern for both quality and efficiency. Table 3 denotes the quantitative effects of the formulation variables on the span as the response in terms of a quadratic model, created by the software. As shown in Table 3, pH has no significant effect ($p \geq 0.05$) on span, unlike to what is detected for particle size. Therefore, pH did not give to the model and control the size distribution of complexes. Concentration of sodium Caseinate is highly significant in this model ($p \leq 0.001$), while, Pectin concentration, also played a significant role ($p = 0.005$). Figure 4 shows the three-dimensional response surface showing the effects of variables on size distribution. Concentration of sodium Caseinate had the most significant influence on span value (Table 3). Commonly, a negative coefficient of sodium Caseinate in the equation of Table 3 showed that increasing concentration of sodium Caseinate caused to a reduction in span value.

**Table 3.** Values for regression coefficients and their levels of significance for the factors used to model the best fit span response.

| Term | Coef [a] | SE Coef [b] | T | P |
|---|---|---|---|---|
| **Constant** | 0.885 | 0.429 | 2.061 | 0.053 |
| **pH** | -0.090 | 0.263 | -0.034 | 0.735 |
| **Sodium Caseinate (%w/v)** | -1.324 | 0.263 | -5.035 | 0.000 |
| **Pectin(%w/v)** | -0.826 | 0.263 | -3.142 | 0.005 |
| **[pH]$^2$** | 1.273 | 0.387 | 3.290 | 0.004 |
| **pH × Sodium Caseinate (%w/v)** | -0.208 | 0.372 | -0.559 | 0.582 |
| **pH × pectin (%w/v)** | -0.538 | 0.372 | -1.443 | 0.164 |
| **Sodium Caseinate (%w/v) × Pectin (%w/v)** | -0.970 | 0.372 | -2.608 | 0.017 |

S =1.05154; R-Sq = 77.0; R-Sq (pred) = 41.40%; R-Sq (adj) = 66.78%.
[a] Coefficient
[b] Coefficient of Standard Error of Mean.

The surface plots in Figure 4 indicate that a minimum span could be achieved at pH=4.1. The response surface as a function of concentration of Pectin and sodium Caseinate is shown in Figure 4a. For instance, if a narrow particle size distribution is appropriate, the combination of higher amount of Pectin and sodium Caseinate should be taken as the preparative condition.

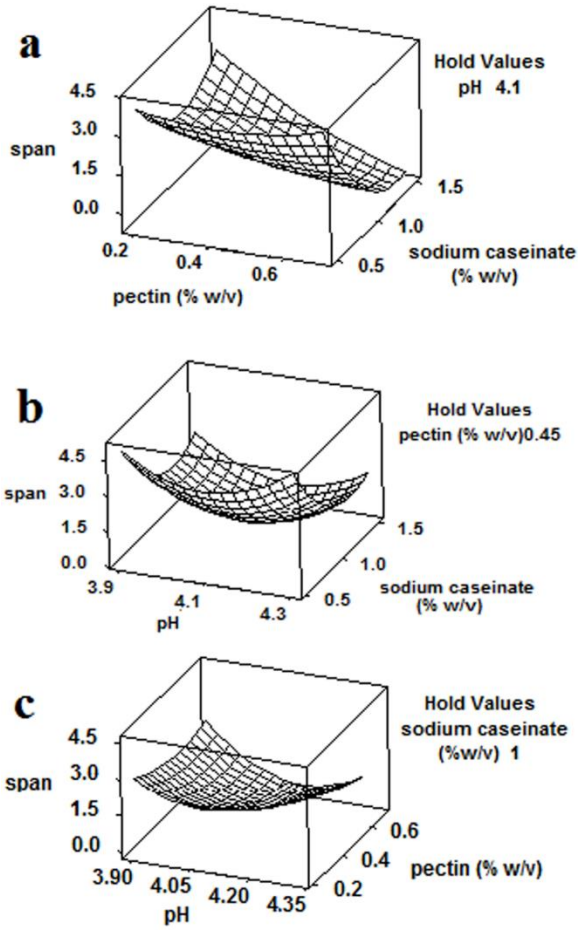


**Figure 4.** Three dimensional surface plots showing the effect of different variables on span.

Coefficients with higher order terms or more than one factor term in the regression equation denote quadratic relationships or interaction terms, respectively. This means the relation between responses and factors are not always linear. A factor can created different degree of effects on a response when used at different levels. A similar situation may arise when more than one factor are changed at the same time. Figures 4b and 4c clearly indicate the fact of interaction between Pectin and Caseinate concentrations and pH. The terms of interactions is also appeared in Table 3. Sodium Caseinate concentration represented different effects on span at the selected pH (Figure 4b). An equivalent result was revealed in Figure 4c, that showed the influence of Pectin concentration and pH on the response (span).

## Conclusion

Formation of sodium Caseinate-Pectin nanocomplexes occurred in a pH just below the isoelectric point of Caseinate (pI=5.4). It is expected that the Pectin-Caseinate complexes have a net negative charge at this pH. Therefore, the stabilization of the Pectin-Caseinate system is assumed to be caused by the repulsion between the negatively charged Pectin-Caseinate complexes. The presented investigation was revealed that a suitable statistical design can be effectively employed in the preparation of sodium Caseinate-Pectin nanocomplexes with probable size properties. By using response surface methodology it is possible to specify areas in the experimental design that will produce nanoparticles with required properties. This study is concluded that, when a small mean particle size and a narrow distribution is desired, it is essential to prepare the particles by means of a suitable pH (4.1) and concentrations of sodium Caseinate and Pectin.

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## Conflict of interests

The authors claim that there is no conflict of interest.

## References

1. Iavicoli I, Leso V, Beezhold DH, Shvedova AA. Nanotechnology in agriculture: Opportunities, toxicological implications, and occupational risks. Toxicol Appl Pharmacol. 2017;329:96-111. doi:10.1016/j.taap.2017.05.025
2. Xie J, Yang Z, Zhou C, Zhu J, Lee RJ, Teng L. Nanotechnology for the delivery of phytochemicals in cancer therapy. Biotechnol Adv. 2016;34(4):343-53. doi:10.1016/j.biotechadv.2016.04.002
3. Barat A, Crane M, Ruskin HJ. Quantitative multi-agent models for simulating protein release from plga bioerodible nano- and microspheres. J Pharm Biomed Anal. 2008;48(2):361-8. doi:10.1016/j.jpba.2008.02.031
4. Oh JK, Drumright R, Siegwart DJ, Matyjaszewski K. The development of microgels/nanogels for drug delivery applications. Prog Polym Sci. 2008;33(4):448-77. doi:10.1016/j.progpolymsci.2008.01.002
5. Rahimnejad M, Najafpour G, Jahanshahi M. Evaluation of effective parameters on fabrication of bsa nanoparticles. Afr J Biotechnol. 2008;7(4):362-75.
6. Muxika A, Etxabide A, Uranga J, Guerrero P, de la Caba K. Chitosan as a bioactive polymer: Processing, properties and applications. Int J Biol Macromol. 2017;105(2):1358-68. doi:10.1016/j.ijbiomac.2017.07.087
7. Zimet P, Livney YD. Beta-lactoglobulin and its nanocomplexes with pectin as vehicles for ω-3 polyunsaturated fatty acids. Food Hydrocoll. 2009;23(4):1120-6. doi:10.1016/j.foodhyd.2008.10.008
8. Barouti G, Jaffredo CG, Guillaume SM. Advances in drug delivery systems based on synthetic

poly(hydroxybutyrate) (co)polymers. Prog Polym Sci. 2017;73:1-31. doi:10.1016/j.progpolymsci.2017.05.002
9. Latha MS, Lal AV, Kumary TV, Sreekumar R, Jayakrishnan A. Progesterone release from glutaraldehyde cross-linked casein microspheres: In vitro studies and in vivo response in rabbits. Contraception. 2000;61(5):329-34. doi:10.1016/S0010-7824(00)00113-X
10. Yeo JH, Lee KG, Lee YW, Kim SY. Simple preparation and characteristics of silk fibroin microsphere. Eur Polym J. 2003;39(6):1195-9. doi:10.1016/S0014-3057(02)00359-2
11. Picot A, Lacroix C. Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. Int Dairy J. 2004;14(6):505-15. doi:10.1016/j.idairyj.2003.10.008
12. Lazko J, Popineau Y, Legrand J. Soy glycinin microcapsules by simple coacervation method. Colloids Surf B Biointerfaces. 2004;37(1-2):1-8. doi:10.1016/j.colsurfb.2004.06.004
13. Liu X, Sun Q, Wang H, Zhang L, Wang JY. Microspheres of corn protein, zein, for an ivermectin drug delivery system. Biomaterials. 2005;26(1):109-15. doi:10.1016/j.biomaterials.2004.02.013
14. Semenova M. Protein–polysaccharide associative interactions in the design of tailor-made colloidal particles. Curr Opin Colloid Interface Sci. 2017;28:15-21. doi:10.1016/j.cocis.2016.12.003
15. Il'ina AV, Kurek DV, Zubareva AA, Il'in MM, Mestechkina NM, Varlamov VP. Preparation and characterization of biopolymer nanoparticles based on lactoferrin-polysaccharide complexes. React Funct Polym. 2016;102:33-8. doi:10.1016/j.reactfunctpolym.2016.03.003
16. Azarikia F, Wu Bc, Abbasi S, McClements DJ. Stabilization of biopolymer microgels formed by electrostatic complexation: Influence of enzyme (laccase) cross-linking on ph, thermal, and mechanical stability. Food Res Int. 2015;78:18-26. doi:10.1016/j.foodres.2015.11.013
17. Park SB, Lih E, Park KS, Joung YK, Han DK. Biopolymer-based functional composites for medical applications. Prog Polym Sci. 2017;68:77-105. doi:10.1016/j.progpolymsci.2016.12.003
18. Sing CE. Development of the modern theory of polymeric complex coacervation. Adv Colloid Interface Sci. 2017;239:2-16. doi:10.1016/j.cis.2016.04.004
19. Arranz E, Corredig M. Invited review: Milk phospholipid vesicles, their colloidal properties, and potential as delivery vehicles for bioactive molecules. J Dairy Sci. 2017;100(6):4213-22. doi:10.3168/jds.2016-12236
20. Ghanbarzadeh S, Khorrami A, Arami S. Preparation of optimized naproxen nano liposomes using response surface methodology. J Pharm Investig. 2014;44(1):33-9. doi:10.1007/s40005-013-0098-8
21. Ghaderi S, Ghanbarzadeh S, Hamishehkar H. Evaluation of different methods for preparing nanoparticle containing gammaoryzanol for potential use in food fortification. Pharm Sci. 2015;20(4):130-4.
22. Ghanbarzadeh S, Valizadeh H, Zakeri Milani P. Sirolimus nano liposomes. Pharm Ind. 2013;75(8):1-13.
23. Singh SK, Dodge J, Durrani MJ, Khan MA. Optimization and characterization of controlled release pellets coated with an experimental latex: I. Anionic drug. Int J Pharm. 1995;125(2):243-55. doi:10.1016/0378-5173(95)00135-6
24. Ghanbarzadeh S, Valizadeh H, Zakeri Milani P. Application of response surface methodology in development of sirolimus liposomes prepared by thin film hydration technique. BioImpacts. 2013;3(2):75-81. doi:10.5681/bi.2013.016
25. Sagalowicz L, Leser ME. Delivery systems for liquid food products. Curr Opin Colloid Interface Sci. 2010;15(1-2):61-72. doi:10.1016/j.cocis.2009.12.003
26. Chanasattru W, Jones OG, Decker EA, McClements DJ. Impact of cosolvents on formation and properties of biopolymer nanoparticles formed by heat treatment of β-lactoglobulin–pectin complexes. Food Hydrocoll. 2009;23(8):2450-7. doi:10.1016/j.foodhyd.2009.07.003
27. Roldan Carrillo T, Martinez Garcia X, Zapata Penasco I, Castorena Cortes G, Reyes Avila J, Mayol Castillo M, et al. Evaluation of the effect of nutrient ratios on biosurfactant production by serratia marcescens using a box-behnken design. Colloids Surf B Biointerfaces. 2011;86(2):384-9. doi:10.1016/j.colsurfb.2011.04.026
28. HadjSadok A, Pitkowski A, Nicolai T, Benyahia L, Moulai Mostefa N. Characterisation of sodium caseinate as a function of ionic strength, ph and temperature using static and dynamic light scattering. Food Hydrocoll. 2008;22(8):1460-6. doi:10.1016/j.foodhyd.2007.09.002
29. Kobori T, Matsumoto A, Sugiyama S. Ph-dependent interaction between sodium caseinate and xanthan gum. Carbohydr Polym. 2009;75(4):719-23. doi:10.1016/j.carbpol.2008.10.008
30. Maroziene A, de Kruif CG. Interaction of pectin and casein micelles. Food Hydrocoll. 2000;14(4):391-94. doi:10.1016/S0268-005X(00)00019-9