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International Journal of Pharmaceutics 193 (1999) 37–47

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Response surface methodology as a predictive tool for determining the effects of preparation conditions on the physicochemical properties of poly(isobutylcyanoacrylate) nanoparticles

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Received 6 May 1999; accepted 2 September 1999

Abstract

Preparation conditions of nanoparticles greatly influence their physicochemical characteristics. A factorial design was used to evaluate the influence of these conditions on the particle diameter, zeta potential, polydispersity, percentage recovery, and molecular weight of poly(isobutylcyanoacrylate) nanoparticles. The relationship between these responses and the effects of simultaneously varying three preparation factors (monomer concentration, surfactant concentration, pH of the polymerization medium) were modelled by response-surface methodology. Three levels were chosen for each factor, giving 27 trials. The responses obtained in the experimental design were found to be modelled by either a reduced quadratic or second-order model. Particle diameter was found to be a function of the pH, whereas zeta potential depended on pH and to a lesser extent of the monomer concentration. Polydispersity depended on the pH and an interaction term between pH and the surfactant concentration. The particle recovery was significantly influenced by all three factors, whereas the pH was the primary influence on the molecular weight. Thus, response surface methodology gave detailed information on the predicted physicochemical characteristics found on poly(isobutylcyanoacrylate) nanoparticles prepared using a wide range of experimental conditions.

Keywords: Nanoparticles; Poly(isobutylcyanoacrylate); Response surface methodology; Synperonic; Zeta potential

1. Introduction

Response surface methodology (RSM) is a rapid technique used to empirically derive a functional relationship between an experimental response and a set of input variables (Khuri and Cornell, 1987). Furthermore, it may determine the optimum level of experimental factors required for a given response. A factor is defined as an input variable whose value can be set during an experiment. The response variable is a measured quantity whose value is affected by levels chosen

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0378-5173/99/$ - see front matter
PII: S0378-5173(99)00311-7

for the factors. RSM reduces the number of experimental runs that are necessary to establish a mathematical trend in the experimental design region. The modelling process begins by identifying factors that are believed to play an important role in the design region. Analysis of variance (ANOVA) is then used to determine the significance of each factor and multiple regression analysis is used to determine the equation of best fit.

RSM is widely used to optimize process parameters, especially in determining optimum conditions for chemical investigations and maximizing yields in large-scale chemical synthesis (Box and Wilson, 1951). It has, however, not been widely applied to pharmaceutical systems, such as the preparation of nanoparticulate carriers. Nanoparticles present obvious advantages for the administration and targeting of drugs. However, it is necessary to have a clear understanding of how preparation conditions determine particle characteristics and, in particular, how particle characteristics are influenced by potential interactions between preparation factors. RSM may provide a useful tool to analyse such interactions.

Poly(alkylcyanoacrylate) nanoparticulate systems have been studied extensively for many years as a potential means for drug delivery and targeting (Douglas et al., 1987; Couvreur et al., 1995). Several well established methods of preparation have been described, including emulsification polymerization (Couvreur et al., 1990), solvent dispersal with interfacial deposition (Fessi et al., 1989) and interfacial polymerization (Krause et al., 1986). Physicochemical characteristics of the resulting particles, such as size, polydispersity, zeta potential and drug entrapment will depend strongly on the chosen method of preparation.

Although not widely used for the preparation of nanoparticles, the direct addition of alkylcyanoacrylate monomer to an aqueous polymerization medium is a simple and efficient technique for the preparation of monolithic polymeric nanospheres, yielding particles with diameters ranging from about 90 nm to just under 1 μm. Particle entrapment or elimination from the body by reticuloendothelial-mediated uptake is influenced by particle diameter and accompanying polydispersity (Illum and Davis, 1982). Other physicochemical characteristics, such as zeta potential, surface hydrophobicity and polymeric molecular weight will influence particle elimination, drug release and organ distribution. Thus, for example, Wilkins and Meyers (1966) showed that negatively charged polystyrene particles were taken up by the rat liver, whereas positively charged colloids were accumulated initially in the lungs.

Factors known to play a significant role in the preparation of poly(alkylcyanoacrylate) nanoparticles include pH of the aqueous polymerization medium, surfactant and monomer concentrations. The effect of varying each of these factors on the physicochemical properties of the resultant nanoparticles is normally difficult to predict because of potential interactions between the factors. Therefore, in the present study, RSM is investigated as a modelling tool to predict the properties of poly(alkylcyanoacrylate) nanoparticles prepared by the emulsification polymerization technique when several preparation factors are simultaneously varied.

2. Materials and methods

2.1. Materials

n-Butylcyanoacrylate monomer was a gift from Loctite® Ireland Ltd (Dublin, Ireland). Phosphoric acid, di-sodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from BDH (Poole, UK). Synperonic (polyoxyethylene-oxypropylene) was obtained from ICI (Cleveland, UK) and tetrahydrofuran was obtained from Labscan (Dublin, Ireland). Other materials were of standard laboratory grade.

2.2. Preparation of poly(butylcyanoacrylate) nanoparticles

n-Butylcyanoacrylate monomer was added dropwise at a rate of 100 μl per minute to a mechanically-stirred phosphate buffer of defined pH and containing a defined concentration of Synperonic surfactant. This polymerization medium was filtered through a 0.45 μm filter (Vericel, Gelman) prior to use in order to remove

contaminating particulate material. The solution was stirred at room temperature for a further 3 h to complete polymerization, filtered through grade one, then grade four, sintered glass filters and freeze-dried for 48 h (Edwards Modulyo, BOC Ltd., Crawley, UK).

2.3. Determination of particle size, polydispersity and zeta potential

The nanoparticle size and its polydispersity was determined by adding approximately 1.0 ml of colloidal suspension to 50 ml of distilled and filtered water. The sample was sized in a Malvern Zetasizer 4 (Malvern Instruments Ltd., Malvern, UK) using light scattering from a laser source (633 nm) determined at a fixed angle (90°).

The zeta-potential was determined on a Malvern Zetasizer 4 using a nanoparticle suspension (0.2 ml) suspended in filtered (0.22 μm) and deionized water (10 ml).

2.4. Determination of molecular weight

Approximately 10 mg of washed and freeze-dried nanoparticles was dissolved in dry, distilled tetrahydrofuran (THF, 0.5 ml) and filtered through a 0.45 μm filter (Whatman). The sample (20 μl) was injected onto a PLGel 5M-Mixed-C-51-16 gel permeation column with THF flowing at 0.5 ml min^{-1} as eluent. Polymer elution was detected using changes in refractive index (Perkin Elmer LC-30 RI) and captured on a chart recorder (Honeywell ElectroniK 194). The column temperature was thermostatically controlled at 23°C (Perkin Elmer LC oven 101).

The column was calibrated using dissolved poly(styrene) standards (30 mg) in dry THF (2 ml). The following molecular weight standards were used; 860, 2550, 7600, 19 600, 47 000, 115 000, and 160 000. The polymeric molecular weight of the unknown sample was determined using a semi-logarithmic calibration plot.

2.5. Determination of particle recovery

Suspensions of nanoparticles were made using the preparation parameters set in the experimental design. Once polymerization was complete, the particulate suspension was filtered through a succession of previously washed and dried sintered glass filters, ranging from grade 1 through to grade 4. Residual polymer in the reaction vessel and material trapped in each filter were dissolved by adding acetone (5 ml), collected and dried in a pre-weighed clean Petri dish. Once dry, the weight of trapped non-particulate matter could be calculated.

The suspension obtained after grade four filtration was washed three times in distilled water and freeze-dried. The recovery was expressed as the mass of dried particles over the total mass of material obtained, as shown in Eq. (1):

$$\%\ \text{recovery} = \left(\frac{\text{mass of particulate matter}}{\text{mass of particulate matter} + \text{mass of non-particulate matter}}\right) \quad (1)$$

2.6. Response surface methodology and statistical anlysis

Data was analysed using a computer software package (Design-Expert, Version 5, Stat-Ease Corporation, USA). Statistical analysis was provided within the package by constructing an ANOVA table, where the variation explained by the fitted model is compared to the variation unaccounted for by the model. From this ratio, an F statistic can be derived allowing rejection of the null hypothesis at the chosen level of significance and inference that the variation accounted for in the model is significantly greater than the unexplained variation.

2.7. Defining the experimental design region and coding of variables

The range of a factor must be chosen in order to adequately measure its effects on the response variable. Furthermore, ranges must be chosen so that they encompass all of the preparation conditions likely to be encountered during nanoparticle formation. The range of surfactant was chosen from 0.2% to 0.6% w/v, pH of the polymerization medium was chosen from 2.5 to 8.0 and monomer

concentration ranged from 0.8% to 2.4% v/v (Table 1).

A 3^3 experimental design was used, consisting of three factors set at three different levels. High and low levels of each factor were coded as 1 and -1, respectively, and the mean value was coded as zero. The coded variable, x_i, can be defined using Eq. (2):

$$x_i = \frac{2X_i - (X_{ih} + X_{il})}{X_{ih} - X_{il}} \quad i = 1, 2, 3 \tag{2}$$

where X_{ih} and X_{il} are the high and low value of X_i, respectively. Thus, the means value of each factor, which is coded as zero, is surfactant concentration, 2.75% w/v; pH of the polymerization medium, 5.3; monomer concentration, 1.4% v/v.

Twenty-seven observations are required to complete this 3^3 experimental design. In coded form, {1,1,1} represents the observation where each factor is at its highest level. Similarly, $\{1,0,-1\}$ represents the observation where pH is set high, (monomer) is at its mean value and (surfactant) is set low, and so on. During the study, the order of the observations was randomized.

3. Results and discussion

The application of a factorial design as a means to optimize nanoparticle characteristics has previously been reported (Seijo et al., 1990; Lescure et al., 1992). The classical approach used in these studies, where one factor is varied whilst the others remain constant, is unlikely to reveal the possible presence of factor interaction (Armstrong and James, 1990). Therefore, in this study the interaction of input factors with each other is emphasised, in that the effect of changing one factor will depend on the magnitude of one or more of the other factors.

Several measured responses from the design were chosen for investigation, namely, Z_{ave}, polydispersity, zeta potential, formation efficiency and polymeric molecular weight. These responses represent significant properties of the particle which impact on their physiological fate, such as removal by the reticular endothelial system and entrapment in capillary beds. A factorial design of type 3^n was used, where n is the number of factors, three in this study. Thus, 27 experimental trials were required to complete the design.

Three factors were considered important for this study. Thus, the pH of the polymerization medium was chosen because the polymerization mechanism of poly(isobutylcyanoacrylate) is dependent on hydroxyl and hydrogen ion concentrations. The former acts as an initiator of anionic polymerization, whereas the latter terminates chain growth. The amount of monomer added to the medium was also considered important as it constitutes the primary building block of the formed particle and, ultimately, the nanoparticle. Surfactant concentration was included as the third design factor because it acts as a stabilizer and imparts a protective coating on the surface of the particle, preventing coalescence. An active drug component was not included during the manufacture of these particles. Incorporation of drug into nanoparticles can be achieved in one of two ways. Firstly, the drug may be present in the polymerization medium prior to addition of monomer and this procedure achieves high encapsulation for lipophilic drugs. However, levels of hydrophilic drug incorporation is often poor and drug ad-

Table 1
Factor levels chosen to define the experimental region and their corresponding coded values

Factor		Level		
I	X_i	1	0	−1
1	pH	2.0	4.5	8.0
2	monomer concentration / (mg/ml)	0.5	1.0	2.0
3	surfactant concentration / (mg/ml)	0.02	0.1	2.0

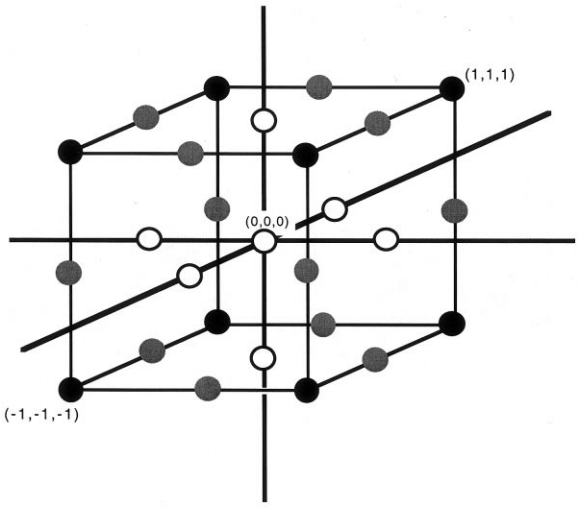

Fig. 1. Three-dimensional region defined by the combination of coded variables. ●, denotes the cube vertices and ○, denotes the centre of the cube faces and the design origin.

sorption on to a preformed particle may be used instead. If the latter procedure is used, drug is not present during particle formation and will not influence the physicochemical outcomes achieved.

As each coded variable, x_i, can take values of -1, 0, 1, it is possible to describe a cuboidal region in three dimensional space representing the design region, R. The geometric centre of the region, with co-ordinates of 0,0,0, represents the experimental observation where each factor is set at its mean value in the defined range. This is illustrated in Fig. 1.

The data obtained for the responses in each trial was fitted to each of three models, as shown in Eq. (3), Eq. (4) and Eq. (5):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \varepsilon \quad \text{first order model} \tag{3}$$

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \varepsilon$$

reduced quadratic model (4)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon$$

second-order model (5)

where X_1, X_2,....X_k are the input factors which influence the response Y; β_0, β_i, β_{ii} ($i = 1,2....k$), β_{ij} ($i = 1,2,...k$; $j = 1,2,....k$) are unknown parameters and ε is a random error term.

Using an F-test, it is possible to test for lack of fit within each model, thereby identifying which of the three models best described the data. Furthermore, the significance of each factor in the chosen model was estimated by testing the hypothesis H_0: $\beta_i = 0$, $\beta_{ii} = 0$, $\beta_{ij} = 0$. If the parameter estimate is zero, then its associated factor plays no significant role in the model.

3.1. Influence of preparation factors on particle diameter

Table 2 shows the data obtained for the size response in terms of a reduced quadratic model. This model was found to generate the highest F value, although each of the three models were found to be highly significant ($P < 0.0001$) with the coefficient of determination (R^2) improving as more higher order terms were added. The pH factor is highly significant in this model, whereas the monomer and surfactant concentrations play only a minor role. Using significant terms ($P < 0.05$) the equation relating particle size to process parameters is:

$$\text{particle size} = 104.02\ (\text{pH}) - 59.84\ (\text{pH})^2 + 271.35$$

Fig. 2 shows the response surface relating both pH and surfactant concentrations to the particle size. It can be seen visually that changes in surfactant concentration do not alter size, whereas decreases in pH reduce the size considerably, a finding confirmed in the equation derived for particle size. Interaction between the factors does not occur.

Colloidal particles with a mean diameter of approximately less than 7 μm can be administered intravenously without significant entrapment by vascular filtration. A Z_{ave} below this level has been shown to significantly influence the location of particle deposition and clearance (Juliano and Stamp, 1975). Thus, control over the mean particle diameter is an important aspect to drug delivery by colloidal systems. Using ANOVA, it was shown that pH has a strong influence on particle size, whereas the amount of surfactant and

Table 2
Values for regression coefficients and their levels of significance for the factors used to model the various responses

Model of best fit (SIZE RESPONSE)	Reduced quadratic model	
R^2 coefficient	0.9045	
F value	39.45	
P value	<0.0001	
Factor	Factor coefficient	P-value
pH	104.02	<0.0001
(monomer)	0.09	0.9899
(surfactant)	1.89	0.7925
pH^2	−59.84	<0.0001
$(monomer)^2$	0.39	0.9727
$(surfactant)^2$	−0.07	0.9951
Intercept	271.35	
Model of best fit (ZETA POTENTIAL RESPONSE)	Full second-order model	
R^2 coefficient	0.6924	
F value	5.50	
P value	0.0005	
Factor	Factor coefficient	P-value
pH	4.61	0.0005
(monomer)	−2.82	0.0200
(surfactant)	1.01	0.3777
pH^2	−2.55	0.1706
$(monomer)^2$	0.89	0.6225
$(surfactant)^2$	−2.01	0.2786
(pH)(monomer)	−2.57	0.0745
(pH)(surfactant)	−5.72	0.0004
(monomer)(surfactant)	−0.01	0.9934
Intercept	−6.54	
Model of best fit (POLYDISPERSITY RESPONSE)	Full second-order model	
R^2 coefficient	0.9174	
F value	27.16	
P value	0.0001	
Factor	Factor coefficient	P-value
pH	0.039	0.0001
(monomer)	−0.011	0.1754
(surfactant)	1.01	0.7650
pH^2	0.002	0.0001
$(monomer)^2$	−0.160	0.4040
$(surfactant)^2$	0.010	0.1564
(pH)(monomer)	0.014	0.0001
(pH)(surfactant)	−0.050	0.8851
(monomer)(surfactant)	-1.390×10^{-3}	0.9934
Intercept	0.3100	
Model of best fit (RECOVERY RESPONSE)	Reduced quadratic model	
R^2 coefficient	0.8523	
F value	24.03	
P value	<0.0001	
Factor	Factor coefficient	P-value
pH	−4.17	<0.0001
(monomer)	−6.65	<0.0001
(surfactant)	4.44	<0.0001

Table 2 (continued)

pH^2	5.63	0.0002
$(monomer)^2$	1.54	0.2348
$(surfactant)^2$	−2.46	0.0719
Intercept	71.92	
Model of best fit (MOLECULAR WEIGHT RESPONSE)	Reduced quadratic model	
R^2 coefficient	0.8548	
F value	24.54	
P value	<0.0001	
Factor	Factor coefficient	P-value
pH	130300	<0.0001
(monomer)	−18709	0.2647
(surfactant)	20570	0.2213
pH^2	−228500	<0.0001
$(monomer)^2$	−20926	0.3558
$(surfactant)^2$	−12527	0.6393
Intercept	399900	

monomer used play a minor role. Thus, it is possible to use low levels of these two factors, reducing possible toxicity associated with the presence of surfactant and also wastage of monomer.

3.2. Influence of preparation factors on particle zeta potential

Table 2 shows the coefficient estimates for the full second-order model, which generated the highest coefficient of determination and F value. The pH of the polymerization medium is highly significant, whereas the monomer concentration, although significant, played a lesser role. The concentration of surfactant did not contribute to the model. Pure second-order terms contributed little to the zeta potential on the particle, whereas the interaction term containing (pH)(surfactant) did. Using significant terms ($P < 0.05$) the equation relating particle zeta potential to process parameters is:

$$\text{zeta potential} = 4.61(\text{pH}) - 2.82(\text{monomer}) - 5.72(\text{pH})(\text{surfactant}) - 6.54$$

Fig. 3 shows the full second-order response surface for the zeta potential on the particle. High negative values can be achieved by maintaining a low pH of the polymerization medium and increasing the monomer concentration.

The zeta potential on the particle will play an important role in particle recognition, especially by the endoreticular system. The zeta potential can be related to the thickness of absorbed surfactant surrounding the particle (Müller et al., 1992), which in turn can be related to levels of opsonization (Blunk and Müller, 1989). The nature of these adsorbed blood components can have a marked effect on the distribution and clearance of an administered colloid. The reduced quadratic

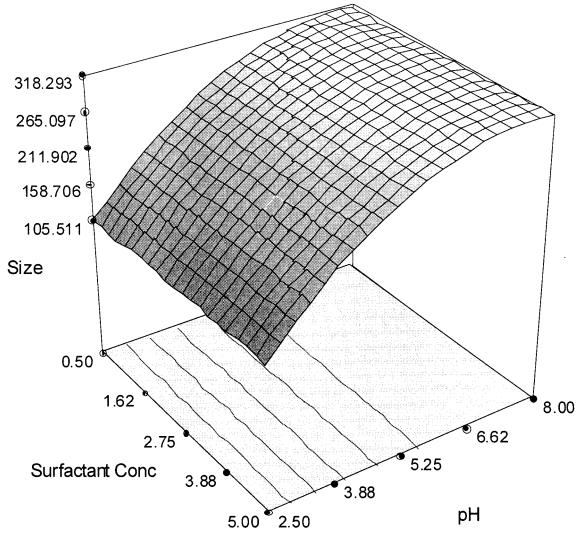

Fig. 2. Reduced quadratic response surface showing particle size as a function of surfactant concentration and pH.

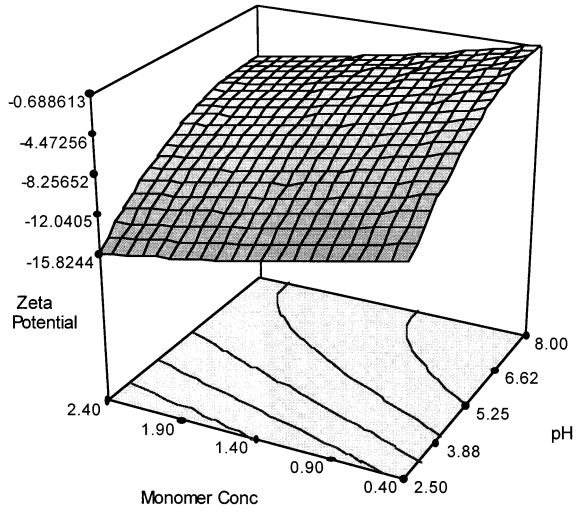

Fig. 3. Full second-order response surface showing the zeta potential on the particle as a function of monomer concentration and pH.

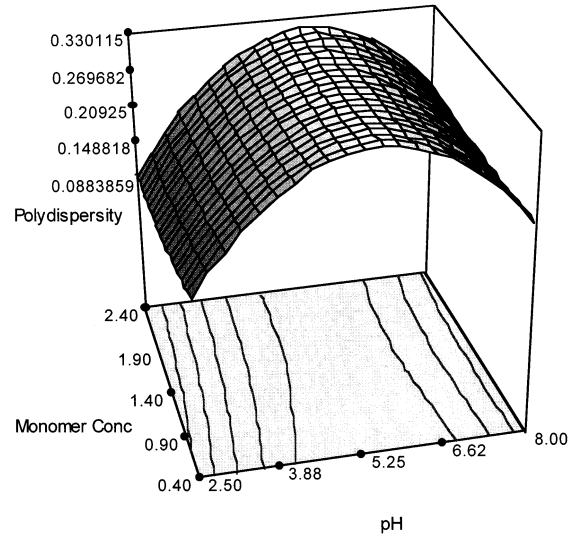

Fig. 4. Full second-order response surface showing the population polydispersity of particle size on the particle as a function of monomer concentration and pH.

model was found to best estimate the zeta potential data. The influence of pH was again highly significant. Monomer concentration was also found to play a role, but was only significant at the $P = 0.0737$ level, with surfactant concentration of no importance.

It is unclear why the monomer concentration has an important role in determining the zeta potential of the particle and, furthermore, why an increase in monomer concentration causes zeta potential to decrease. The response surface shows that an increase in monomer concentration resulted in a small reduction in the zeta potential.

3.3. Influence of preparation factors on particle size polydispersity

The polydispersity index is used to describe the spread in particle diameters produced in a sample of particles. As the index approaches zero, the size range produced becomes narrower. The R^2 value (Table 2) improved as the second-order interaction terms were added. The pH played a highly significant role, whereas monomer concentration and especially the surfactant concentration play very little role. However, when the pH and monomer concentration levels are included as an interaction term, they then became significant. Using significant terms ($P < 0.05$) the equation relating particle polydispersity to process parameters is:

$$\text{polydispersity} = 0.039(\text{pH}) - 0.002(\text{pH})^2 - 0.014(\text{pH})(\text{monomer}) + 0.310$$

Fig. 4 shows that polydispersity achieves a maximum around pH 5. It can be minimized by selecting pH values on either side of this pH, particularly at low pH values around 2.5. Altering the monomer concentration can be seen to produce very little effect.

After particle formation, the size population frequently follows a multimodal distribution. This is often described by the polydispersity index. Values approaching zero are desirable, indicating a narrow size range. A second-order model with interaction terms was required to model the data. The pH was found to play a highly significant and, furthermore, variables including the pH factor, such as $(\text{pH})^2$ or (pH)(monomer), were also significant, as shown in Table 2.

The response surface in Fig. 4 shows that a low pH value will promote a low polydispersity. This may be caused by the plentiful amount of H^+

ensuring that chain termination is an efficient process. As the pH increases, the lower proton concentrations reduces the probability of chain termination, with some polymeric chains having an improved chance to grow longer than others.

3.4. Influence of preparation factors on particle recovery

Particle recovery is an important aspect in the production of poly(isobutylcyanoacrylate) nanoparticles. The preparation procedure must return a useful yield of nanoparticles, particularly if their intended payload is an expensive material to produce, such as a bioactive peptide. If the process is not optimized, large, amorphous, polymeric flakes are produced. This is both wasteful in terms of monomer consumption and loss of incorporated payload. It was found that all three proposed models could represent the data, but the reduced quadratic model was selected, as shown in Table 2. Using significant terms ($P < 0.05$) the equation relating particle recovery to process parameters is:

$$\text{recovery} = -4.11(\text{pH}) - 6.64(\text{monomer}) + 4.44(\text{surfactant}) + 5.63(\text{pH})^2 + 71.92$$

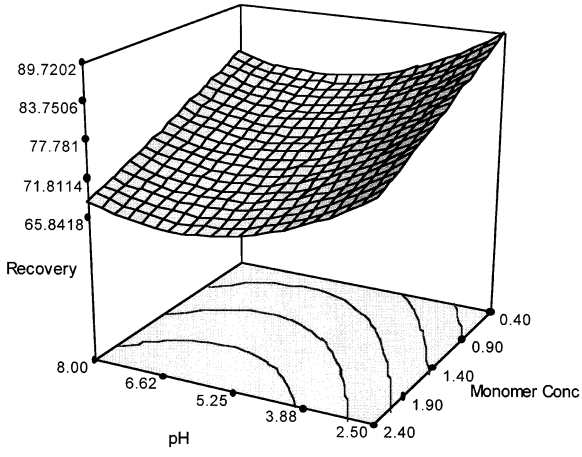

Fig. 5. Reduced quadratic response surface showing the amount of particles recovered as a function of monomer concentration and pH.

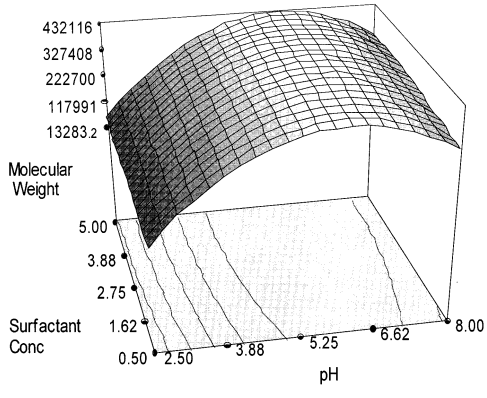

Fig. 6. Reduced quadratic response surface showing the predicted molecular weight of the polymeric chains making up the particles as a function of the pH and the surfactant concentration.

The response surface in Fig. 5 shows that adding more monomer does not increase the yield. Indeed the opposite finding is shown to be the case. There is a small region, as shown on the iso-response projection, where low levels of monomer and pH will maximize the yield. Conversely, yield will be minimized if a pH value around 6.0 is used, particularly with high levels of monomer. Unexpectantly, the addition of more monomer does not lead to greater levels of recovery, as shown in Fig. 5. Indeed, the converse is found to be true. It is conceivable that excessive amounts of monomer polymerize in the form of an interparticulate glue, binding particles into a mass and leading to the production of flaky material.

3.5. Influence of preparation factors on polymeric molecular weight

The data showing the relationship between molecular weight and the three factors is shown in Table 2. The reduced quadratic model was chosen because inclusion of interaction terms marginally increased the R^2 value, but reduced the F value markedly. The pH term was highly significant, both as a linear and quadratic term. The monomer and surfactant concentrations where of almost equally low significance. Using significant terms ($P < 0.05$) the equation relating polymeric molecular weight to process parameters can be estimated as;

$$\text{molecular weight} = 130\,300(\text{pH}) - 228\,500(\text{pH})^2 + 399\,900$$

The response surface in Fig. 6 shows the dependence of molecular weight on the pH of the reaction medium. The surface suggests that a maximum molecular weight can be achieved by using pH values around 6.0. Any further increase in pH causes a reduction. Changes in surfactant concentration do not alter the molecular weight.

The molecular weight could be modelled to a second-order model, but because the R^2 value was not increased noticeably, the interaction terms could be considered to be of negligible contribution. The pH was found to be significant, as noted by several authors (Puglisi et al., 1993). However, monomer and surfactant concentrations were not found to be significant. This is in contrast to Vansnick et al. (1984) who showed that the presence of surfactant in the polymerization medium had a marked effect on the polymer molecular weight. These authors used a similar surfactant to Synperionic, although they compared the molecular weight between particles with and without surfactant. The model described in this study always has surfactant present, albeit down to 0.5% w/v. Altering the design to include particles made without surfactant in this study may have increased the significance of the surfactant term. Puglisi et al. (1993) demonstrated an appreciable increase in molecular weight by increasing the concentration of Triton X-100 in the polymerization medium. They attributed this effect to better wetting of the monomer allowing it to interact better with the aqueous medium. However, this effect was not observed using Synperonic over the range used in this study.

4. Conclusions

This study demonstrates a method to mathematically to evaluate the effect of varying several preparation factors on the physicochemical properties (responses) of the the resulting poly(alkylcyanoacrylate) nanoparticles. Using RSM, it is, therefore, possible to specify regions in the experimental design that will produce nanoparticles with a required property, such as a desired size or zeta potential. This approach may be of value in the design of nanoparticulate carriers for drug delivery and targeting applications.

Acknowledgements

The authors would like to thank Dr J.G. Hamilton, School of Chemistry, The Queen's University of Belfast, for technical assistance in the molecular weight determinations.

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