Published in final edited form as:

Drug Dev Ind Pharm. 2014 November ; 40(11): 1547–1556. doi:10.3109/03639045.2013.838578.

Optimization of the fabrication of novel stealth PLA-based nanoparticles by dispersion polymerization using D-optimal mixture design

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Abstract

Purpose—Nanoparticle size is important in drug delivery. Clearance of nanoparticles by cells of the reticuloendothelial system has been reported to increase with increase in particle size. Further, nanoparticles should be small enough to avoid lung or spleen filtering effects. Endocytosis and accumulation in tumor tissue by the enhanced permeability and retention effect are also processes that are influenced by particle size. We present the results of studies designed to optimize crosslinked biodegradable stealth polymeric nanoparticles fabricated by dispersion polymerization.

Methods—Nanoparticles were fabricated using different amounts of macromonomer, initiators, crosslinking agent and stabilizer in a dioxane/DMSO/water solvent system. Confirmation of nanoparticle formation was by scanning electron microscopy (SEM). Particle size was measured by dynamic light scattering (DLS). D-optimal mixture statistical experimental design was used for the experimental runs, followed by model generation (Scheffe polynomial) and optimization with the aid of a computer software. Model verification was done by comparing particle size data of some suggested solutions to the predicted particle sizes.

Results and Conclusion—Data showed that average particle sizes follow the same trend as predicted by the model. Negative terms in the model corresponding to the crosslinking agent and stabilizer indicate the important factors for minimizing particle size.

Keywords

Free radical dispersion polymerization; macromonomer; optimization; Scheffe polynomial

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Declaration of interest

This work was supported by NCI/NIH Grant #: 1 SC2 CA138179-01, and NIH/NIAID Grant # 5P30A1087714-02 (11-M56R CFDA # 93.855). This work was carried out in facilities supported by NCRR/NIH Grants #1 C06 RR 020608-01 and #1 C06 RR 14469-01.

Introduction

Many approaches have been reported for the fabrication of polymeric nanoparticles. These approaches can be generally divided into two: dispersion of preformed polymers and polymerization^{1,2,3}. A major drawback of nanoparticle preparation by the dispersion of preformed polymers for pharmaceutical purposes is that it requires the use of surfactants to prepare the particles in the aqueous phase, which may generate concerns about toxicity¹.

Dispersion polymerization differs from the classical precipitation polymerization in that it occurs in the presence of a dissolved soluble amphiphilic polymer (or the *in situ* formed graft copolymer product) which is adsorbed on the surface of particles where it lowers the surface free energy and functions as a steric stabilizer^{4,5,6,7,8}. It is a polymerization method in which all reacting monomers are soluble in the solvent medium that is a poor or non-solvent for the polymer being formed^{8,9,10,11}. The starting reaction mixture is a clear, single-phase solution; particles are formed by precipitation of growing polymer chains in the presence of a suitable steric stabilizer. Therefore, the solvent medium becomes a dispersion medium^{8,10,11,12,13}.

Nanoparticles can be administered through various routes such as oral, IV, nasal, ocular and transdermal routes¹⁴. The achievement of a long blood circulation half-life is dependent on two primary factors which are particle size and surface characteristics^{15,16}. The particle should be small enough to avoid the mechanical spleen or lung filtering effects. Also, the cells of the reticuloendothelial system (RES) recognize and rapidly clear nanoparticles from the circulation by phagocytosis. It has been reported that RES uptake increases with particle size^{15,17}. Modifications of surface properties to achieve RES-avoiding characteristics and long-circulation half-lives have also been an intense area of research. It has been reported that adsorption or chemical attachment of hydrophilic, neutral molecules to the surface of nanoparticles enables the particles to evade recognition by the macrophages of the RES (confers "stealth" property) and prolong blood circulation half-lives^{16,17,18}.

One mission of a drug product development scientist is to develop drug delivery systems that enhance the optimal performance of the bioactive agent. Many strategies are used to accomplish this purpose, including measuring the effect of several combinations of formulation and process variables on the properties of nanoparticles. By carefully selecting which combinations of these variables to evaluate, it is possible to optimize nanoparticle properties for specific purposes. Common approaches to this endeavor include varying the levels of one factor or variable at a time and examining the effect on desired parameters (particle size, yield, internalization into cells, efficacy, etc) or varying multiple variables simultaneously using a factorial or mixture design of experiment. It would take forever to optimize nanoparticle property using one-factor-at-a-time experiments because every time you change a factor or its level, you would have to re-optimize all of the other factors. Further, one factor at a time experiments could not reveal interaction of factors, and this approach can be resource-intensive in relation to the information generated. However, the design of experiment examines all of the variables simultaneously so it enables the investigator to identify the optimum values for the factors much more quickly. At the same time, information that gives a better understanding of how the factors interact can be easily

captured. Two major approaches to the design of experiments to be able to examine all of the variables simultaneously are factorial and mixture experimental designs. When the responses or nanoparticle properties are known to depend on the proportions of the ingredients, mixture designs are preferred. Thus statistical experimental designs involving mixture methodology is an efficient method for studying products made from the components at various levels.

We have used previously free radical dispersion polymerization technique in the fabrication of polymeric nanoparticles¹⁹. We have extended the technique to the fabrication of novel crosslinked PLA-based stealth polymeric nanoparticles using the macromonomer approach. The nanoparticles are suitable for biomedical applications. We have used the mixture design (D-optimal mixture design) in this work for the response surface method (RSM). The response (particle size) is a function of the proportions of the formulation variables investigated: macromer, initiator, stabilizer, and crosslinker. Aided by a statistical software for the design of the experiments and analysis of the data (Design-Expert®, Stat-Ease Inc.), we examined the effects of the combinations of the variables on particle size. The goal is to generate a model (Scheffe Polynomial) to predict particle size (nm) as a function of the proportions of the formulation variables²⁰. The model (Scheffe Polynomial) was used to optimize nanoparticle fabrication with emphasis on particle minimization. The focus on particle size is based on the fact that particle size plays a key role in determining body distribution of nanoparticles after *in vivo* administration by injection and in facilitating their access to cancer cells (internalization) either by passive or active targeting to tumors^{16, 21–23}.

This article presents the results of studies designed to optimize the size of biodegradable nanoparticles fabricated by dispersion polymerization. The factors that influence nanoparticle size minimization were determined by response surface methodology using the D-optimal mixture design. The significant impact of this work is that the nanoparticles were synthesized by surfactant-free dispersion polymerization technique. Thus the problem associated with the use of surfactant in drug formulation and development will be circumvented. Another significant impact of the work is the use of D-optimal mixture design for experimental design, analysis and optimization. When a formulation is a mixture of various components (proportion of the constituents and not the amounts of constituents is important: macromonomer, crosslinker, PEG and initiator system as studied in this work), mixture design is more useful than a factorial design because it accounts for the dependence of response on proportionality of constituents²⁴.

Materials and methods

Materials

L(–) lactide (Polysciences Inc.) was recrystallized from toluene before use. 2-Hydroxyethyl methacrylate (HEMA) (Aldrich, 97%) was dried over molecular sieves (0.4 nm) for 24 hours and distilled under negative pressure before use. Toluene (Acros, 99 %) and pyridine (Sigma, 99 %) were refluxed over calcium hydride for one hour and distilled prior to use. Methacryloyl chloride (Aldrich, 97 %) was distilled prior to use. Stannous octoate (Sigma, 95 %), phosphorous pentoxide (Aldrich, 97 %), hydroxylamine hydrochloride (Sigma, 99

%), chloroform (Certified A.C.S, Fisher Scientific), hydrochloric acid (Aldrich, 37 %) were used as received. All other solvents were used as received.

Disclaimer

Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Methods

Synthesis of methacrylate-terminated poly(lactide) macromonomer

The macromonomer was synthesized by a modified published method²⁵. L-lactide (6.0234 g, 0.0418 mole; mole fraction of 85 %), HEMA (900 μ L, 0.00738 mole; mole fraction of 15 %) and 3 drops of stannous octoate were placed in a 50 mL round-bottom flask equipped with a magnetic stir bar. The reaction flask was kept under vacuum for 10 min and polymerization was carried out in an inert atmosphere by flushing the flask with nitrogen gas for 36 h on a silicone oil bath kept at 120 °C. The polymer mass was dissolved in chloroform and extracted with 0.1 M HCl to remove the catalyst followed by washing with deionized water. The pure polymer dissolved in the organic layer was then precipitated with excess cold methanol, collected by filtration and dried in a vacuum oven at room temperature under reduced pressure over phosphorous pentoxide for 24 h.

Synthesis of crosslinking agent (MANHOMA)

The crosslinker was synthesized based on literature reports^{26–28}. Hydroxylamine hydrochloride (2.5 g; 0.036 mole) was dissolved in pyridine (26.1 mL; 0.3298 mole). Methacryloyl chloride (8.3 mL; 0.085 mole) and 9.5 mL of chloroform were added to the reaction mixture and stirred at 5 °C for 40 minutes and for 2 hours at room temperature. 28.4 mL of chloroform was added to the reaction mixture and the resulting solution was washed twice with 47.3 mL (1.2 mole) of hydrochloric acid. The organic layer was washed twice with 40 mL of distilled water, dried with anhydrous magnesium sulphate and evaporated under vacuum to give an oily product. Diethyl ether was added and the resulting solution was evaporated under vacuum to obtain a solid crystalline material. Purification of the crystals obtained was carried out by the addition of cold hexane and ethylacetate (5:1) to the solid and placed in the freezer for 1 h. White crystals were then recovered by vacuum filtration and dried *in vacuo*.

Characterization of synthesized monomers

Fourier-transform infrared (FT-IR) spectrophotometric analysis of synthesized compounds was carried out using a Perkin Elmer Spectrum 100 FT-IR spectrometer. Proton nuclear magnetic resonance (¹H NMR) spectrophotometric analysis of the synthesized compounds was done using a Bruker AVANCE 400 MHz NMR spectrophotometer. The samples were dissolved in deuterated chloroform (CDCl₃). Molecular weight and polydispersity index of the synthesized macromonomer were determined by gel permeation chromatography (GPC)

using a Waters 2690 GPC system equipped with a Waters 2410 differential refractive index detector²⁹. Polystyrene standards were used for calibration and tetrahydrofuran was used as mobile phase. Melting point analysis of the crosslinking agent was carried out on a Thomas Hoover Unimelt capillary melting point apparatus (Thomas Scientific, USA).

Statistical experimental design

Based on our preliminary data (data not shown), some constraints were introduced to allow the fabrication of smooth spherical particles. In D-optimal mixture design, there are restrictions on component concentrations (i.e. constraints) such that a lower and an upper limit are specified³⁰ (Table 1).

Using these limits, the component concentrations (i.e crosslinking agent, initiator, stabilizer and PLA macromonomer) of twenty nanoparticle formulations were varied using the Design Expert® statistical software (Stat-Ease Inc., Minneapolis) (Table 2).

Preparation of blank, crosslinked stealth nanospheres by dispersion polymerization using redox initiator system

P(LLA-HEMA) macromonomer, poly(ethylene glycol)*n* monomethyl ether mono methacrylate (PEG-MA), (*n* = 1000), N, O-dimethacryloylhydroxylamine (MANHOMA) as crosslinking agent and benzoyl peroxide/N-phenyldiethanolamine (BPO/NPDEA) redox initiator system were used for *in situ* nanoparticle synthesis by free radical dispersion polymerization. Using this approach, polyethylene glycol macromonomer (PEG macromonomer) was used both as a comonomer and a steric stabilizer.

Preparation of a typical formulation of nanoparticles is as follows. P(LLA-HEMA) macromonomer (0.24 mmol), 0.252 mmol of PEG-MMA macromonomer and 0.016 mmol of crosslinker (MANHOMA) were dissolved in a Dioxane : DMSO : Water (12:5:2.5) solvent system. 0.196 mmol of NPDEA and 0.196 mmol of BPO were injected into the reaction mixture at 10 min and 20 min respectively through a rubber closure under continuous flushing with nitrogen gas and with continuous stirring at 400 rpm. The overall polymerization time was 24 hours. The resulting particles were recovered by dialysis.

Particle size and size distribution analysis

Particle size and size distribution of nanoparticles were determined by dynamic light scattering (DLS) using Zetasizer Nano-ZS (Malvern Instruments, USA). 10 mg of freeze-dried particles was redispersed in 5 mL of filtered distilled water using a probe sonicator (Vibra-Cell; Model VC 750, Sonics and Materials, Inc, Newton, CT) at probe amplitude of 30 % for 60 seconds before measurement. The sonicated suspension was filtered through an Acrodisc syringe filter with a 5 µm Versapor membrane (Pall Corporation). Particle size was determined at 25 °C. The mean of three measurements was recorded. The particle size distribution is given by the polydispersity index ($PDI_{\text{nanoparticle}}$).

Surface morphology

The surface morphology of the nanoparticles was evaluated using scanning electron microscopy (SEM) (FEI Quanta 200F environmental scanning electron microscope). With

this equipment, coating with a metal (e.g. gold) is not necessary and this confers the advantage of imaging accurate morphological features. To evaluate surface morphology, different dilutions of nanoparticle suspension in distilled water were placed on a carbon tape affixed to a specimen stub and dried *in vacuo*. The samples were then viewed using a scanning electron microscope under high vacuum at 10 KV and a working distance of 10 mm. Images were taken at different sample magnifications.

Results and discussion

Macromonomer Characterization

The FT-IR and ^1H NMR spectra of the synthesized P(LLA-HEMA) macromonomer are consistent with the expected structure. Analysis of the IR spectrum (appendix A) reveals the presence of a C = C stretch at 1636 cm^{-1} corresponding to the vinyl functional group of 2-HEMA. The ^1H NMR spectrum (appendix B) confirms the presence of a C=C bond with olefinic protons at $\delta = 5.57\text{ ppm}$ and $\delta = 6.09\text{ ppm}$. These data confirm the incorporation of HEMA into the macromonomer and is consistent with earlier reports²⁵. To determine the composition of the macromonomer, the relative moles of HEMA calculated from the integral of the signal corresponding to the methyl group of the HEMA moiety in the ^1H NMR spectrum of the macromonomer is divided by the total relative moles of L-lactide (calculated from integral of the methine group signal on the repeating units of lactide) and HEMA. Using this method, the percent composition of HEMA in the macromonomer was determined to be a mole fraction of 3.7 %. The number average molecular weight (M_n) was determined by ^1H NMR (2,085) and GPC (3,420) while the weight average molecular weight (M_w) was determined by GPC (4,247). GPC also revealed a single prominent peak (appendix C) showing that the synthesized macromonomer was pure.

Preliminary synthesis of P(LLA-HEMA) macromonomers with mole fractions of 95 % L-lactide and 5 % HEMA and 90 % L-lactide and 10 % HEMA yielded products of large molecular weights (data not shown). To reduce molecular weight and improve reactivity, the P(LLA-HEMA) macromonomer with mole fractions of 85 % L-lactide and 15 % HEMA which gave the lowest molecular weight was selected for nanoparticle fabrication. The polydispersity index ($\text{PDI}_{\text{polymer}}$: which in polymer chemistry is defined as the ratio of weight average molecular weight (M_w) to the number average molecular weight (M_n) of the polymer or macromonomer) is 1.24, which shows that the macromonomer is monodisperse. A perfectly monodisperse polymer would have a $\text{PDI}_{\text{polymer}}$ of unity which in reality is unobtainable; thus the reported PDI of polymers in the literature is always greater than unity and it increases with increasing polydispersity³¹. The discrepancy between the molecular weight (M_n) determined by ^1H NMR and that determined by GPC using polystyrene standards is a common occurrence and has been widely reported in literature. The difference in molecular weight values has been ascribed to the use of polystyrene standards for calibration and is due to differences in the hydrodynamic volume of polystyrene relative to P(LLA-HEMA) macromonomer^{32,33}.

Characterization of crosslinker

The FT-IR spectrum of MANHOMA (appendix E) shows the presence of C=C stretch at 1627 cm^{-1} . The proton NMR spectrum of MANHOMA (appendix D) confirms the presence of a divinyl structure with a pair of olefinic protons at about $\delta = 5.86\text{ ppm}$ and $\delta = 6.33\text{ ppm}$ and the other at about $\delta = 5.49\text{ ppm}$ and $\delta = 5.76\text{ ppm}$. The spectra are consistent with the expected structure of MANHOMA. Melting point was found to be $(54 \pm 1)^\circ\text{C}$. All the data obtained are consistent with reported literature values^{27,34}.

Morphological evaluation of nanospheres

Nanoparticle fabrication by dispersion polymerization was confirmed by SEM which showed the formation of smooth spherical particles (Fig. 1).

Particle size and statistical experimental design

One of the goals of this project is to generate a model to predict nanoparticle size as a function of the formulation variables and to optimize the particle by numerical optimization (particle size minimization). Particle sizes and PDI were determined by DLS. Typical dynamic light scattering data showing particle size distribution are shown in Fig. 2.

DLS is a widely used technique for the determination of the particle size of colloidal systems^{18,35}. Using this approach the fluctuations in the intensity of scattered light due to the Brownian motion of suspended particles in solution after irradiation with a monochromatic laser is determined. The autocorrelation function is then processed to determine the diffusion coefficient from which the average hydrodynamic radius can be determined^{36,37}. The particle sizes obtained range from $261.5\text{ nm} \pm 3.5\text{ nm}$ to $322.9\text{ nm} \pm 2.5\text{ nm}$. The particle size distribution is given by the $\text{PDI}_{\text{nanoparticle}}$ (A $\text{PDI}_{\text{nanoparticle}}$ value of <0.1 indicates a homogenous monodisperse formulation while a $\text{PDI}_{\text{nanoparticle}}$ of >0.3 indicates polydispersity with large variations in particle size³⁰. The $\text{PDI}_{\text{nanoparticle}}$ values obtained in this work indicate homogeneity of the particle size. It has been reported that particle size plays an important role in determining the drug release behavior of drug-loaded nanoparticles and their fate after *in vivo* administration^{15,16,38,39}. The particles should be small enough to avoid the mechanical spleen or lung filtering effects. Also, the cells of the reticuloendothelial system (RES) recognize and rapidly clear nanoparticles from the circulation by phagocytosis. RES uptake has been shown to increase with particle size³⁹. Furthermore, it has been reported that smaller particles accumulate more in tumors compared to larger-sized ones by the primary mechanism of passive targeting to tumors – the enhanced permeability and retention (EPR) effect³⁸. Thus, efforts were directed at generation of empirical mathematical model and optimization to reduce particle size.

With the mixture design, the response determined by any possible component mixtures can be identified by a point in the experimental domain called the design space. When working with three different variables (components), the experimental domain corresponds to an equilateral triangle with the vertices corresponding to the pure components while different points within the design space correspond to a mixture of components⁴⁰. In this experiment, four components (macromonomer, initiators, crosslinking agent and stabilizer) were combined to prepare nanoparticles; however, the proportion of the macromonomer was kept

constant in all the experiments thereby yielding a triangular experimental domain. Further, as a result of constraints introduced, the region of interest (design space) that allows for the formation of smooth spherical particles is only a fraction of the possible experimental domain (Fig. 3)

The selection of the best model for modeling the particle size data is important since the fitted model is used for optimizing particle size. Model fitting to particle size data was carried out and the quadratic model was found significant and was selected. To improve the model, insignificant terms were removed by backward elimination. Analysis of variance (ANOVA) of the selected model and terms (Table 3) reveal that the selected model is significant ($p = 0.0020$). Further, the model (Scheffe Polynomial) was also selected based on the estimation of several statistical parameters: multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS), provided by the Design-Expert® software. Also, “lack of fit” was not statistically significant ($p = 0.4994$) which is desirable.

The empirical models of the mixture design are different from the models of other regression analyses in that the models of the mixture design have no constant term⁴⁰. Thus, the resulting model (Scheffe polynomial) is shown in eq. 1 below:

$$\begin{aligned} \text{Mean particle size (nm)} = & -1772.43 (A) \\ & +275.78 (B) \\ & -1089.88 (C) \\ & +270.51 (D) \\ & +2481.55 (AB) \\ & +7504.21 (AC) \\ & +1453.29 (BC) + 2509.21 (CD) \end{aligned} \quad (\text{eq. 1})$$

Where:

A = Crosslinker (mmol)

B = Initiators (mmol)

C = Stabilizer (mmol)

D = Macromonomer (mmol)

Diagnostic plots (Fig. 4) show the validity of the model. The normal probability plot of the residuals (Fig. 4A) is the most important diagnostic and it checks for non-normality in the error term. A linear normal probability plot of the residuals which indicates normality in the error term was obtained. Fig. 4B shows a diagnostic plot that tests the assumption of constant variance. Both plots show no problem with our data.

The model was subsequently used for numerical optimization: minimization of nanoparticle size as a function of the composition of the formulation (Table 4). Solutions 3, 4, 5 and 9 out of ten solutions were used to experimentally check the model and compare predicted particle

sizes with experimental data. The average particle size ($n = 3$) of selected solutions (depicted as actual size) are also shown in Table 4.

The negative terms in the empirical model (equation 1) corresponding to the amounts of crosslinking agent and stabilizer included in the reaction mixture are the terms to be controlled for particle size minimization in agreement with published literature^{12,28,41–43}. The predicted sizes for the four solutions or formulae are 276.7 nm, 283.6 nm, 291.6 nm and 302.7 nm while the experimentally obtained sizes are $244.2 \text{ nm} \pm 4.20 \text{ nm}$, $246.6 \text{ nm} \pm 0.87 \text{ nm}$, $250.4 \text{ nm} \pm 4.04 \text{ nm}$ and $271 \text{ nm} \pm 4.62 \text{ nm}$ respectively (Table 4). The corresponding PDI values are 0.23, 0.28, 0.27 and 0.29 respectively. Comparative analyses show that although the particle sizes obtained were lower than the predicted sizes, the same trend of increasing particle size was observed.

Conclusion

Crosslinked, biodegradable, stealth polymeric nanoparticles were prepared by free radical dispersion polymerization. D-optimal mixture statistical experimental design was used to generate an empirical model to predict particle size as a function of reaction mixture composition. The crosslinking agent and stabilizer have been shown to control particle size. An optimized formulation yielding an acceptable particle size was selected for drug loading and *in vivo* applications.

Acknowledgments

The authors wish to thank Dr. John Small, Division Chief, Surface and Microanalysis Science Division, National Institute of Standards and Technology for access to the environmental scanning electron microscope.

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Appendices

Appendix A. Infrared Spectrum of P(LLA-HEMA) Macromonomer

Appendix B: Proton NMR Spectrum of P(LLA-HEMA) Macromonomer

Appendix C: GPC Chromatogram of the Synthesized P(LLA-HEMA) Macromonomer

Appendix D: ^1H NMR spectrum of N,O-dimethacryloylhydroxylamine **Appendix E: IR spectrum of N,O-dimethacryloylhydroxylamine**

Fig. 1.
Typical SEM image of blank nanoparticles prepared by *in situ* dispersion polymerization

Fig. 2.
Typical particle size distribution data of nanoparticles prepared by dispersion polymerization

Design-Expert® Software
Component Coding: Actual
Highs/Lows inverted by U_Pseudo coding
Size
326.4
261.5
X1 = A: A
X2 = B: B
X3 = C: C
Actual Component
D: D = 0.278

Fig. 3. Model graph showing the design space and variation in particle size as a function of the mixture composition. A = Crosslinking agent; B = Initiators; C = Stabilizer and D = Macromonomer.

Design-Expert® Software
Size
Color points by value of
Size
326.4
261.5

A

Design-Expert® Software
Size
Color points by value of
Size
326.4
261.5

B

Fig. 4. Diagnostic plots. (A) Normal Plot of Residuals, (B) Residuals vs Predicted

Table 1

Constraints on component concentrations

	Lower limit (mmol)	Upper Limit (mmol)
Crosslinking agent	0.018	0.087
Initiator system ^a	0.183	0.625
Stabilizer	0.091	0.304
Macromonomer	0.204	0.445

^a Benzoyl peroxide/N-phenyldiethanolamine (1:1)

Table 2
Composition and response of D-optimal mixture experimental design for the fabrication of stealth nanoparticles

Standard order	Run order	A: Crosslinking Agent (mmol)	B: Initiator System (mmol)	C: Stabilizer (mmol)	D: Macromonomer (mmol)	Response (Particle size (nm))
9	1	0.048	0.359	0.304	0.289	297.6
8	2	0.056	0.285	0.215	0.445	306.6
1	3	0.087	0.377	0.091	0.445	261.5
5	4	0.018	0.565	0.214	0.204	270.6
19	5	0.087	0.377	0.091	0.445	268.1
12	6	0.042	0.452	0.259	0.247	286.4
20	7	0.018	0.565	0.214	0.204	295.8
17	8	0.087	0.183	0.304	0.426	326.4
14	9	0.018	0.312	0.304	0.367	290.3
15	10	0.053	0.415	0.148	0.384	322.3
3	11	0.055	0.625	0.115	0.204	293.1
4	12	0.087	0.183	0.304	0.426	322.9
13	13	0.036	0.522	0.163	0.279	320.7
2	14	0.018	0.446	0.091	0.445	313.1
11	15	0.087	0.500	0.091	0.321	273.3
16	16	0.018	0.446	0.091	0.445	293.8
18	17	0.055	0.625	0.115	0.204	305.9
6	18	0.018	0.234	0.304	0.445	320.9
7	19	0.018	0.625	0.091	0.265	295.6
10	20	0.087	0.394	0.198	0.321	314.9

Table 3

Analysis of variance table [Partial sum of squares – Type III]

Source	Sum of Squares	df	Mean Square	F Value	P-value	
Model	6011.26	7	858.75	6.83	0.0020	Significant
Linear mixture	1431.12	3	477.04	3.79	0.0401	
AB	693.79	1	693.79	5.52	0.0368	
AC	2723.37	1	2723.37	21.65	0.0006	
BC	694.88	1	694.88	5.52	0.0367	
CD	1247.85	1	1247.85	9.92	0.0084	
Residual	1509.25	12	125.77			
Lack of Fit	895.66	7	127.95	1.04	0.4994	Not significant
Pure Error	613.59	5	122.72			
Cor Total	7520.52	19				

Table 4

Optimised formulations showing predicted and actual particle sizes

Solutions	Linker (mmol)	Initiator (mmol)	Stabilizer (mmol)	Macromer (mmol)	Predicted size (nm)	Actual Size (nm)
1	0.087	0.377	0.091	0.445	263.305	---
2	0.018	0.389	0.304	0.289	273.053	---
3	0.018	0.435	0.280	0.267	276.747	244.2
4	0.018	0.547	0.223	0.212	283.668	246.6
5	0.033	0.511	0.233	0.222	291.678	250.4
6	0.033	0.535	0.221	0.211	292.65	---
7	0.061	0.625	0.091	0.223	295.408	---
8	0.068	0.535	0.127	0.270	301.539	---
9	0.049	0.512	0.216	0.223	302.676	271.0
10	0.061	0.501	0.207	0.232	308.162	---