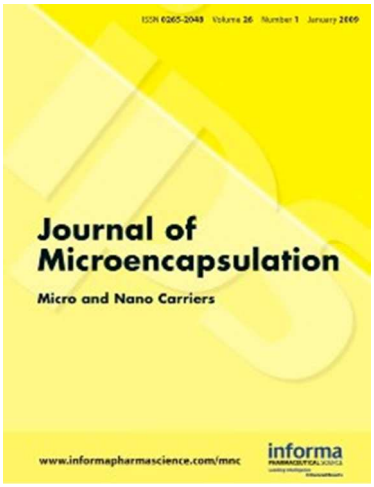

Co-encapsulation of human serum albumin and superparamagnetic iron oxide in PLGA nanoparticles: Part I. Effect of process variables on the mean size

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Co-encapsulation of human serum albumin and superparamagnetic iron oxide in PLGA nanoparticles: Part I. Effect of process variables on the mean size

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ABSTRACT

PLGA (poly D,L-lactic-*co*-glycolic acid) nanoparticles (NPs) encapsulating magnetite nanoparticles (MNPs) along with a model drug human serum albumin (HSA) were prepared by double emulsion solvent evaporation method. This part I will focus on size and size distribution of prepared NPs whereas encapsulation efficiency will be discussed in part II. It was found that mean hydrodynamic particle size was influenced by five important process variables. To explore their effects, a 5-factorial 3-level

experimental design and statistical analysis were carried out using STATISTICA® software. Effect of process variables on the mean size of nanoparticles was investigated and finally conditions to minimize size of NPs were proposed. GAMS™/MINOS software was used for optimization. The mean hydrodynamic size of nanoparticles ranged from 115-329 nm depending on the process conditions. Smallest possible mean particle size can be achieved by using low polymer concentration and high dispersion energy (enough sonication time) along with small aqueous/organic volume ratio.

Keywords: PLGA (poly D,L-lactic-*co*-glycolic acid); albumin; encapsulation.

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INTRODUCTION

Nanotechnologies are wide spread in medical sciences and pharmaceutical industries, namely in controlled drug delivery and disease detection. Nanoparticles designed for drug delivery should be above all biocompatible and biodegradable (Gupta et al. 2005; Xie et al. 2006). The aim of targeted drug delivery and therapy is to transport a drug directly to the disease loci with no or minimal side effects on the human body (Chomoucka et al. 2010). The potential of drug delivery systems based on the use of nanoparticles offer three major significant advantages: (i) the ability to target specific locations in the body, (ii) the reduction of the drug quantity needed to attain a particular

concentration in the vicinity of the target and (iii) the reduction of the drug concentration at non-target sites which minimizes severe side effects (Arruebo et al. 2007).

Poly(*D,L*-lactic-*co*-glycolic acid) (PLGA) is a biocompatible (non-toxic) and biodegradable material often used for preparing nano- and microparticles (Zimmer et al. 1995; Bala et al. 2004). During the last two decades, PLGA has attracted considerable attention due to its excellent drug loading capacity. Drug-loaded PLGA nanoparticles have been extensively studied in the pharmaceutical and medical fields. Many PLGA-based nanoformulations have reached different stages of preclinical development, although they still present distinct challenges for researchers.

Most of the research on magnetic nanoparticles for clinical applications has focused on iron oxide nanoparticles such as magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) due to their biological compatibility and FDA approval for clinical usage (Weissleder et al. 1989; Ibrahim et al. 1983; Muller et al. 1996). Oleic acid coated Fe_3O_4 was selected in this study because it is well dispersible in organic media.

Human serum albumin (HSA) is used as a model drug in this study which is a monomeric multi-domain macromolecule. It is the most abundant plasma protein in the human body with a plasma concentration of 0.6 mM (Yang et al. 2007). HSA consists of 585 amino acids that form into three structurally similar α-helical domains (Yan et al. 2009).

Organic solvent dichloromethane (DCM) has the ability to dissolve a wide range of organic compounds including PLGA. DCM is volatile at room temperature and evaporates very quickly. It can be removed completely by evaporation from a mixture by mechanical stirring. PVA is the most commonly used emulsifier in the formulation of

PLGA nanoparticles (Sahoo et al. 2002). Insufficient amount of the emulsifier fails to stabilize the emulsion, and thus form particles which tend to aggregate.

Some of the common methods to prepare nanoparticles loaded with low-molecular-weight drugs include w/o single emulsification (Niwa et al. 1995), o/w single emulsification (Saxena et al. 2004; Tewes et al. 2007), nanoprecipitation (Barichello et al. 1999; Bilati et al. 2005) and w/o/w double emulsification (Dillen et al. 2006; Ubrich et al. 2004; Song et al. 2008). Among them, double emulsion method was selected in this study. Although single emulsion method is simpler than double emulsion method, it cannot be used for studying the applied three phase system. Because of their compartmentalized internal structure, double emulsions can provide advantages over simple o/w emulsions, especially for encapsulation (Hanson et al. 2008). Double emulsification method has some other advantages e.g. nanoparticle size can be controlled by changing several process parameters and purity of nanoparticles is satisfactory.

Till now, very few works have been published on HSA encapsulation by using PLGA matrix. Moreover, co-encapsulation of HSA or other protein drugs along with magnetic nanoparticles is rarely studied in literature. Size is one of the most important factors for drug-loaded NPs, especially if they are intended for administration by injection. NP size is also crucial to drug release behavior (Wei et al. 2008). Production of spherical particles < 220 nm with a narrow size distribution and without agglomeration has significance in injection drug formulations, allowing sterilization of the product by ultra-filtration via a membrane with 220 nm cut-off value (Feczko et al. 2011).

The observed influences of process variables on the mean size of the resulted PLGA nanoparticles are in good agreement with the earlier works of Feczkó et al.

(Feczko et al. 2011). In that work, the authors encapsulated bovine serum albumin (BSA) into PLGA nanoparticles applying similar conditions and using double emulsion solvent evaporation process. The difference is that study involves BSA encapsulation instead of HSA and the effect of PVA concentration in the external aqueous phase was examined in that study, but no magnetic nanoparticles and their encapsulation were involved in the study of Feczkó et al. (Feczko et al. 2011). Vankova et al. (Vankova et al. 2007) studied five process parameters as our work does to predict the droplet size of liquid-liquid emulsions, although the parameters are different. The main difference between their study and our work is that we used "ultrasonicator" to produce double emulsion and solid nanocapsules were prepared. But Vankova et al. applied a "narrow-gap homogenizer" to produce simple emulsion droplets. Davies et al. (Davies et al. 1985) studied the comparison of droplet sizes with rates of turbulent energy dissipation in various types of practical equipments. The calculations done by the authors support the mechanism of drop break-up. Our findings comply with both Vankova et al. and Davies et al. e.g. because viscosity of the oil phase influenced our particle size, as it was the case with the droplets prepared by them.

The aim of this study was to prepare PLGA nanoparticles by double emulsion solvent evaporation method with narrow size distribution. Due to experimental design (made by STATISTICA® software), it was possible to explore precisely the influence of different parameters and their combined influences. GAMS™/MINOS software was used for optimization which gave precise result. Earlier no comprehensive studies were carried out to understand the particular effect of the studied five parameters and process conditions on the final sizes of PLGA nanoparticles and no research was carried out on

co-encapsulation of HSA and oleic acid coated magnetite by PLGA (the encapsulation efficiency is discussed in part II) which for sure makes this novel study not only interesting but also creates scope for further research and innovation. The outcome of this study will give us the ideal combination of variables to get desired size of model drug-loaded PLGA nanoparticles which will be exploited with the real drug for further innovation in the field of targeted drug delivery.

MATERIALS AND METHODS

Materials

PLGA (50:50, M_w = 8,000, Resomer® RG 502H) containing free carboxyl end-groups was a gift of Boehringer Ingelheim, Germany. HSA solution was obtained from Trigon Biotechnological Ltd., Hungary. The concentration of bulk HSA solution was 36.87 g/L. Poly(vinyl alcohol) (PVA; M_w = 30,000–70,000) and phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich. Dichloromethane (DCM) was purchased from Spektrum-3D, Hungary. Magnetite was synthesized by co-precipitation method.

Synthesis of oleic acid-coated superparamagnetic iron oxide nanoparticles dispersed in organic media

Neat superparamagnetic nanoparticles were prepared by coprecipitation of Fe(II) and Fe(III) chlorides in aqueous ammonia solution by modification of an earlier published method (Horak et al. 2003). Briefly, $FeCl_3 \cdot 6H_2O$ (24.32 g) and 11.92 g of $FeCl_2 \cdot 4H_2O$ (molar ratio 2:1) were stirred at 400 rpm in Q-water (50 ml) under nitrogen atmosphere. To this solution, 28% NH_4OH solution (50 ml; 50% excess) was added over a period of 20–30 min. To coat the nanoparticles, oleic acid (5 ml) was added to the

reaction mixture at 90°C and the reaction proceeded for 5 h until the NH_3 odor disappeared. After cooling to room temperature, the nanoparticles were washed with Q-water for 4 days (three times 200 ml of water a day). The magnetite particles were separated by a magnet, decanted, washed with water and dried at 80°C and 13 Pa to yield about 16 g of product. Finally, the particles were under sonication resuspended in dichloromethane to a concentration of 1.92 wt% Fe_3O_4. The size of the core of prepared magnetite NPs was 10 ± 5 nm.

Nanoparticle preparation

Nanoparticles were prepared by double emulsion solvent evaporation method (Feczko et al. 2008; Panyam et al. 2003) shown schematically in Fig. 1. At first PLGA (0.05-0.2 g) was dissolved in DCM using magnetic stirrer. Fe_3O_4 (1 to 20% by weight related to the weight of PLGA) was added to the system and sonicated with a Model W-220 probe sonicator (Heat Systems-Ultrasonics) for 30 s. The power of sonicator was 70 W, frequency 20 kHz and probe type was "H-I" type. The total volume of DCM in the system was fixed at 5 ml. Then 0.5 ml model drug solution of preset concentration, diluted with PBS, was added to the system and the two-phase system was emulsified for 60 s using the same sonicator which resulted in w/o emulsion. This emulsion was dispersed in the outer water phase containing 2 wt% PVA (10-30 ml) using the same sonicator for 1-3 min to obtain w/o/w double emulsion. The DCM was evaporated to solidify PLGA NPs under continuous stirring (800 rpm) for 2 h using a magnetic stirrer. After the evaporation of DCM, dispersed solid PLGA NPs with encapsulated model drug and Fe_3O_4 were obtained and stored for further experimental analysis. Sonication process was always carried out in an ice bath. Utilization of an ultrasonic probe leads to an

increase in bulk temperature. If the temperature is not controlled using ice bath, some undesired effects may occur. The most obvious is the degradation of compounds of interest. In addition, as the temperature is increased, the physical characteristics of the liquid media change in such a way that the ultrasonic transmission can be affected and no cavitation is achieved. This phenomenon is known as "decoupling" (Santos et al. 2009).

Fig. 1.

Hydrodynamic size analysis

Hydrodynamic diameter and the size distribution of the resulted particles were analyzed by dynamic light scattering (DLS) method (also called as photon correlation technique) using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25°C. For each sample, five parallel size measurements were carried out.

Experimental design

To elucidate the effect of process conditions on the mean hydrodynamic particle size and to decrease the number of the studied parameter combinations and thus the number of experiments, a $3^{(p-1)}$ type fractional factorial experimental design was carried out using STATISTICA® (version 10.0, StatSoft Inc., USA) software, where "p" is the number of factors (variables). The obtained experimental data were evaluated by statistical analysis, similarly to the method described by Feczkó et al. (Feczko et al. 2011) for bovine serum albumin encapsulated in PLGA nanoparticles and Biró et al. (Biro et al. 2009) for chitosan microparticles.

According to preliminary tests, five process variables summarized in Table 1 (factors F1–F5) were selected, which strongly influenced the hydrodynamic particle sizes and/or the encapsulation process. These variables are the amount of iron oxide in the organic phase (F1) relative to the weight of PLGA used for encapsulation, concentration of PLGA in the organic phase (F2), concentration of HSA in the inner aqueous phase (F3), the outer aqueous/organic phase volume ratio (F4), and time of the ultrasonic treatment in the second emulsification (F5).

Table 1.

As a result of the experimental design (DOE), $3^{(5-1)} = 81$ experiments were needed without repetitions due to variation of five variables. However, to estimate the pure error, 9 repetitions of experiments were also carried out. This resulted in 90 experiments altogether. For each variable 3 different levels (the lowest, mean and highest) were taken into consideration. The main advantage of applying experimental design was the vast reduction of the experimental work without remarkable loss of useful information. Without this, it would be needed to perform $3^5 = 243$ experiments. The experimental program determined by STATISTICA® (including the repetition) is not shown in the manuscript. first six columns of the table in Appendix A. From the table it is seen that repetitions were carried out at the central point of each variable intervals indicated by C with bold numbers. In the second last column of the table, the measured mean particle sizes are listed. In the last column of that table, encapsulation efficiency of model drug is listed which will be discussed in detail in our following paper (Part II).

Optimization of the result

In certain applications, such as production of injectable drug formulations, the smallest possible particle size should be achieved, which obviously depends on the process variables. Due to high number of variables, it was necessary to determine the optimum process conditions mathematically to achieve desirable nanosized PLGA capsules. For this purpose the GAMS™/MINOS Large Scale Nonlinear Solver for Windows Ver. 5.51 (System Optimization Laboratory, Stanford University) software was applied. The software is able to optimize the variables by precise mathematical procedures.

RESULTS AND DISCUSSION

The influencing variables were systematically changed according to the research plan determined by the scheme obtained from the DOE. During this study, the measured size distributions and mean hydrodynamic particle sizes showed characteristic variations depending on the values of independent variables applied in the different experiments. Fig. 2 shows typical particle size distributions selected from the 90 experiments, with various resulted size ranges corresponding to relatively low, medium and high mean particle sizes. It is seen that the studied process variables, such as PLGA concentration in the intermediate organic phase, or the time of the second sonication influenced strongly the obtained size distribution and the mean particle size. For example, low PLGA concentration and long sonication time resulted in smaller mean particle sizes, while high PLGA concentration and short ultrasound treatment during the second emulsification gave larger particle sizes. So, with the increase in PLGA concentration value from low to

high, size distribution changes follow the order a to c in Fig. 2 whereas with the decrease in PLGA concentration, reverse change is observed i.e. from c to a in Fig. 2. Opposite trend has been observed for the variable sonication time and shown in the same Fig. These diagrams show smooth and quite regular curves similar to the usual lognormal distribution. The dependence of the mean hydrodynamic particle size on the process parameters offers good opportunity for optimization. Therefore statistical evaluation on the effect of different variables, and process optimization have been carried out for this purpose.

Fig. 2.a, b, c

As a result of the statistical analysis, the significance and importance of the studied variables influencing the mean hydrodynamic particle size was characterized by ANOVA table (Table 2) and Pareto chart of the standardized effects. From Table 2 it is seen that four factors F1, F2, F4 and F5 (i.e. the relative amount of magnetite, the PLGA concentration, the external aqueous/intermediate organic phase volume ratio, and sonication time, respectively), and the interaction of factors F1 and F4 show statistically significant influences, all of them having much lower p values than the widely accepted significance level ($p = 0.05$). Table 2 also shows that the mean square of residuals (MS) was 762.37 nm^2, i.e. the mean deviation between the measured and estimated mean particle sizes is $\sqrt{762.37} = 27.6$ nm, which was considered acceptable by us. The histogram of residual (not presented here) values showed almost normal distribution. Therefore the estimation made by the multivariable regression was accepted. The pure

error of experimental data determined from the 9 repeated runs was $\sqrt{181.3} = 13.5$ nm, which provides reasonable accuracy.

Table 2.

The Pareto chart (Fig. 3) shows that mean hydrodynamic size was affected most strongly by PLGA concentration (F2) followed by the duration of ultrasonic treatment (F5). Iron oxide/PLGA weight ratio (F1), volume ratio (F4), and the linear-linear interaction of the latter factors (F1L×F4L) also played significant roles. Letter "L" on the scale of the diagram refers to the linear correlation between the given variable and the dependent variable (the mean hydrodynamic particle size). Among the studied five variables, the concentration of HSA in the inner aqueous phase (F3) has no significant influence on particle size although this variable has strong influence on encapsulation efficiency as will be found in our following paper (Part II).

Fig. 3.

As a result of the statistical analysis, a regression equation was obtained by which the dependence of the mean hydrodynamic particle size D_{mean} can be estimated for various combinations of the studied independent variables:

$$D_{mean} = 4.7097 \cdot X_{Fe3O4} + 16.088 \cdot X_{PLGA} + 12.5864 \cdot X_{VOLR} - 18.6089 \cdot X_{time} - 0.7796 \cdot X_{Fe3O4} \cdot X_{VOLR} + 143.667 \quad (1)$$

The effects of the process variables on the predicted mean hydrodynamic particle size are demonstrated in Fig. 4~~–6~~ and discussed below.

Effect of Fe_3O_4/PLGA weight ratio

As can be seen from the 3D diagram of Fig. 4(a), the increase in the relative amount of the Fe_3O_4 nanoparticles (relative to the weight of PLGA) dispersed in the intermediate organic phase (DCM), caused considerable increase in the mean particle size of the final NPs. At medium values of the three other variables (HSA concentration, w/o volume ratio, and sonication time) the predicted (using equation 1, for 1% PLGA) mean particle size of the composite PLGA nanoparticles increases from 174 to 205 nm when Fe_3O_4/PLGA weight ratio is increased between 1 and 20% wt/wt. On the other hand, at higher amounts of iron oxide nanoparticles, the distribution of the obtained composite PLGA nanoparticles was much broader or highly distorted (often having a second peak). The latter corresponded to another solid product differing from the HSA and iron-oxide-containing PLGA nanoparticles. This precipitate was mainly composed of iron-oxide nanoparticles and also contained other unidentified materials, probably a mixture of PLGA, HSA and PVA.

Fig. 4 a,b,c,d.

~~Fig. 5.~~

Both of the precisely non-identified precipitate containing iron oxide and the increase of PLGA nanoparticle size were obtained at high concentrations of iron oxide nanoparticles. This can be explained by the hydrophobic interactions between the oleic acid tails of Fe_3O_4 nanoparticles. These interactions are probably responsible for Fe_3O_4 clustering (Astete et al. 2007) which explains the shifting of the mean particle size and size distribution towards the higher values. The latter explanation may be supported by the study Zhou et al. (Zhou et al. 2008) who studied the size of interferon loaded

magnetic PLA (Polylactic acid) and PLGA microspheres. The authors used Fe_3O_4 and found that size of both types of microspheres increased and the size distribution broadened with the increasing amounts of magnetic nanoparticles. On the other hand it is also expected that higher number of Fe_3O_4 nanoparticles inside the PLGA nanoparticles may adsorb more PLGA, which increases the amount of polymer in a particle, increasing its mass and size.

The effect of Fe_3O_4 is also shown in Fig. 4(b)~~5~~ at different HSA concentrations in which the sonication time was 1 min longer than that in Fig. 4(a) (in Fig. 4(a) the time is 2 minutes whereas in Fig. 4(b)~~5~~, it is 3 minutes). As a consequence of the latter, smallest mean particle size of the product expected at low iron oxide/PLGA weight ratio (1% wt/wt) was smaller, namely 180 nm.

Effect of PLGA concentration

As is seen in Fig. 4(a), particle size significantly increases with the increase in PLGA concentration. At medium HSA concentration (2.2% wt/vol), volume ratio of the intermediate and outer phases (4.0 vol/vol), sonication time (2.0 minutes), low magnetite/PLGA weight ratio (1.0% wt/wt), and the highest PLGA concentration (4.0% wt/vol), large PLGA particles of 223 nm volume mean size results as calculated by Eqn.1. By decreasing the concentration of PLGA in the organic phase to 1.0% wt/vol, the mean particle size decreases considerably to 174 nm while other four parameters were constant.

The effect of PLGA concentration can also be observed in Fig. 7(d), discussed later in relation of the effect of sonication time. The explanation can be the change of rheological behavior of the mixture during the second emulsification. With the increase

in polymer concentration in the organic phase, its viscosity increases. High viscosity provides higher resistance against the shear forces during the second emulsification and restricts the formation of nanodroplets that are the basis of the formation of final composite PLGA nanoparticles. If cohesive forces in correlation with the viscosity and surface tension are higher in a liquid, it is more difficult to attain better dispersion by cavitation during ultrasonic treatment applied for emulsification. Therefore, high viscosity slows down the rapid dispersion of the polymer containing organic phase, which may considerably influence particle size. It means that insufficient dispersion of phases will result in larger particles with wide size distribution (Duan et al. 2006). If the viscosity of polymeric solution is high, it will slow down the rapid dispersion of organic phase into aqueous phase resulting in the formation of bigger droplets or aggregates (Kollipara et al. 2010). The viscous forces in the aqueous and organic phases oppose the shear stresses in the organic phase. Reducing the organic phase viscosity reduces the viscous forces which result in a net increase in shear stress felt by the organic phase. It decreases the PLGA nanoparticle size (Budhian et al. 2007). With an increase in the applied ultrasonic energy, it may be possible to overcome this viscosity problem. But too high sonication intensities can promote some undesired effects, such as analyte degradation. The increase in the particle size with the polymer concentration was observed by other authors with PLA (Chorny et al. 2002; Quintanar-Guerrero et al. 1996) or poly(lactide-*co*-glycolide) (Kwon et al. 2001). Devi Kusum et al. found that if drug to polymer (Acyclovir:PLGA) ratio increases from 1:1 to 1:2, particle size increases significantly and drug entrapment also increases (Kusum et al. 2009). It was also found by other researchers that for each solvent, above a critical concentration of polymer, large

amorphous polymer aggregates were formed in addition to the desired nanoparticles (Galindo-Rodriguez et al. 2004). Hence, use of polymer above a certain concentration is not beneficial.

Effect of HSA concentration

According to the Equation (1), also seen in Fig. 4(b)~~5~~, the HSA concentration in the inner aqueous phase has no significant effect on particle size. Because this protein was used as a model drug in this study, this is important information. However, apart from size, the concentration of HSA applied in the inner aqueous phase is essential in respect to achieve desired drug concentration within the carrier NPs. Concentration of HSA also influences the efficiency of encapsulation i.e. the proportion of the utilized amount of model drug during the encapsulation process. The latter aspects will be discussed in our following paper (Part II).

Effect of volume ratio of the W_2 and O phases

Volume ratio also has significant effect on the particle size as is seen on Fig. 4(c)~~6~~. Namely, if the ratio between the volumes of external and internal phases of emulsion increases, particle size also increases. This finding is in agreement with the observation of other researchers, (Duan et al. 2006) who pointed out that this ratio play an important role influencing the stability of the emulsion and the size of dispersed globules.

~~Fig. 6.~~

The basic principle governing the size of nanoparticles is that the external energy source (e.g. ultrasound energy) provides shear stresses to the internal organic phase, which results in the formation of nanodroplets, and finally nanoparticles from it. The size of the droplets is inversely correlated to the magnitude of shear stresses (Budhian et al. 2007). Any change(s) in process variables or parameters that reduces these shear stresses will increase the nanoparticle size. The most direct influence on the shear stresses in the system is exercised by the energy density (external energy applied per unit total volume) (Budhian et al. 2007). Increase in energy density directly increases the shear stresses and results in more efficient droplet breakdown which will reduce the nanoparticle size. In our experiments, the introduced ultrasonic energy was constant for different volume ratios. The higher the volume ratio, the higher the liquid volume is which in turn reduces the available energy per unit volume, resulting in weaker emulsification. Weaker emulsification results in larger particles.

From Fig. 4(c)~~6~~ (also confirmed by Equation 1), it can be found that volume ratio and magnetite/PLGA weight ratio have combined effect on mean size. It is seen that decrease in both the iron oxide/PLGA ratio and the volume ratio decrease the particle size very rapidly. This phenomenon can be well utilized for the production of very small nanoparticles, e.g. with mean size below 200 nm.

Effect of sonication time

From the Pareto chart, it can be seen that sonication time has the second strongest influence on particle size (just after PLGA concentration). Fig. 4(d)~~7~~ shows that particle size decreases greatly with the prolongation of sonication time. The reason is that increasing the power and/or the duration of sonication decreases the mean diameter of

nanoparticles, which may also change the population distribution. Higher power and/or longer duration of sonication increases the effect of shear stress and the energy causing more droplet breakdown, resulting in a decrease in particle size (Budhian et al. 2007). The great reduction of particle size is the consequence of stronger disintegration of droplets, due to the longer emulsification process (Feczko et al. 2011). Applying prolonged sonication (e.g. 3 minutes in our case), shear stress is acting for more time in the process leading to better dispersion of polymeric organic phase as nanodroplets of small size. On the contrary, short time of sonication, i.e. insufficient dispersion of phases results in large particles with wide size distribution. Mainardes and Evangelista et al. (Budhian et al. 2007) reported a decrease in particle diameter with increasing sonication time for PLGA nanoparticles system.

~~Fig. 7.~~

Prediction of the expected mean particle size

As was seen in the discussion above on the effects of various process variables, the magnetite weight ratio to that of the polymer (PLGA) matrix, the concentration of PLGA in the intermediate organic phase, the volume ratio of the external aqueous and intermediate organic phases, and sonication time influenced the produced composite (model drug and magnetite loaded) nanoparticles. Knowing the exact values of these variables, the correlation obtained by linear regression (also considering the possibility of quadratic correlation and linear-linear interactions of variables, Eqn. 1), the mean particle size of the product can be predicted in a range of about 100 and 340 nm with a mean error of 27.6 nm, which is acceptable. Fig. 5(a)~~8~~ gives a comparison between the

measured and predicted mean particle sizes. It is seen that the measured and predicted values well correlate along the whole studied size interval. The mean relative deviation between the measured and predicted values is 9.5% and the great majority of the data are within the ±20% range (see dotted lines). However, along the studied size interval, there is slight tendency that in the lowest size region the predicted values are a bit overestimated whereas it is somewhat underestimated at the highest region. Considering that the aim is generally to achieve the smallest possible particle size, this tendency gives more safety than uncertainty.

Fig. 5 a,b.~~8.~~

Optimization of the process variables

The formal model offered by the statistical evaluation in form of a regression equation (Eqn. 1), gives sufficient opportunity to find out the optimal conditions for producing NPs of required smallest mean particle size in the studied region. As was mentioned, small particle size is advantageous for different reasons e.g. sterilizing them by ultrafiltration is only eligible, if the size distribution does not exceed much above 220 nanometer (the mean size in this case should be much lower, at least 130-160 nm). Small size of NPs is also required to avoid or reduce harmful interactions with the human organisms. To achieve this goal, the independent i.e. decision variables have to be set to optimal values in this respect.

Optimization was relatively easy by the GAMS program package. The program showed that the optimal values of variables to get the smallest mean particle size were at the borders of their studied intervals. The optimal conditions were as follows:

magnetite/PLGA weight ratio, X_{Fe3O4}=1.0% wt/wt (the lowest value), PLGA concentration in the intermediate organic phase, X_{PLGA}=1.0% wt/vol (the lowest value), volume ratio of the external aqueous phase to the intermediate organic phase X_{VOLR}= 2.0 vol/vol (the lowest value), time of second sonication X_{time}=3.0 minutes (the highest value). The concentration of HSA in the inner aqueous phase had no influence in this relation, therefore it does not constrain process optimization. Under these conditions the predictable volume mean particle size is 132 nm, which is more than acceptable in respect of the properties for sterilization and utilization of the product NPs.

Therefore, there is no special reason to use process variables outside the studied parameter intervals, which also may cause technical or economical difficulties (e.g. using too low PLGA concentration decreases the productivity of a given reaction vessel, or applying excessively long time of sonication may lead to degradation of the valuable drug substances).

However, if the magnetite/PLGA ratio X_{Fe3O4}=1.0% wt/wt during encapsulation of the magnetic nanoparticles proves not to be sufficient to achieve suitable level of magnetism in the product NPs, it can be increased with the consequence of obtaining somewhat larger sizes. To clear up this consequence, optimization was carried out with constrain of different volume mean product particle sizes (this time not regarding the efficiencies of HSA and magnetite encapsulation). The results are shown in Fig. 5(b)~~9~~. In the diagram it is seen that the increase of magnetite/PLGA ratio causes a linear increase of the achievable mean particle size, providing optimal conditions regarding the best values of the three other decision variables (X_{PLGA}=1.1% wt/vol, X_{VOLR}= 2.0 vol/vol, X_{time}=3.0 minutes). As a conclusion, if a mean particle size of 160 nm is allowed for the

sterilization by ultrafiltration and in respect of suitable properties as drug carrier, as high as 10% wt/wt magnetite/PLGA ratio can be applied to achieve suitable magnetic behavior of the product nanoparticles.

~~Fig. 9.~~

SUMMARY AND CONCLUSION

Encapsulation of magnetite nanoparticles (MNPs) and human serum albumin (HSA) was carried out into the matrix of biocompatible polymer (PLGA) nanoparticles by double emulsion solvent evaporation method. Size distribution of prepared NPs was determined by using dynamic light scattering method. It was found that mean particle size was influenced by several process variables. To explore these effects a 5-factorial 3-level experimental design and statistical analysis were carried out.

As a summary of the effect of process variables on the mean hydrodynamic particle size of the produced HSA and magnetite-loaded PLGA nanoparticles, it was concluded that the concentration of PLGA in the intermediate organic phase and the duration of the second sonication have the strongest influences. The ratio of introduced magnetite nanoparticles relative to the weight of PLGA, the volume ratio of the external aqueous and intermediate organic phases, and the interaction of the latter with magnetite/PLGA ratio have also significant effect. To achieve the smallest possible mean particle size, relatively low PLGA concentration, high dispersion energy (enough sonication time) and relatively small volume ratio of the intermediate organic/external aqueous liquid should be applied. Low magnetite/PLGA weight ratio is also crucial but, from the utilization aspect, the former should be high enough to provide sufficient level

of magnetism to the produced nanoparticles by its encapsulation. The latter requirement leads to another study dealing with the efficacy of drug and magnetite encapsulation. The morphology of the PLGA NPs were studied by using scanning and transmission electron microscope (SEM, TEM) which showed that the prepared NPs were quite spherical. The SEM and TEM images have been studied (therefore not included in the paper).

Therefore it can be concluded, that the presence of magnetite nanoparticles does not have significant influence on the effect of other variable like polymer concentration, volume ratio of the external and intermediate phases, and the duration of sonication. This knowledge can help the process engineer to make the process well designable.

Optimization of the process variables has been carried out in respect of obtaining the smallest possible mean particle size, allowed by the studied and reasonable intervals of the studied parameters. It was found that by a proper selection of four variables (magnetite/PLGA weight ratio X_{Fe3O4}, PLGA concentration in the intermediate organic phase X_{PLGA}, volume ratio of the external aqueous phase to the intermediate organic phase X_{VOLR}, and duration of the second sonication, X_{time}) the volume mean particle size can be decreased to about 132 nm, which is beneficial for further processing and utilization of the product NPs. If, there is constraint in respect of magnetite/PLGA ratio, i.e. if higher iron oxide/PLGA weight ratio needed to be applied to achieve sufficient level of magnetism, the minimum achievable mean particle size increases but, in the worst case, it remains below 160 nm even using a magnetite/PLGA weight ratio as high as 10.0% wt/wt.

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NOMENCLATURE

Variables

D_{mean}	mean hydrodynamic particle size, nm
X_{Fe3O4}	Weight ratio of the introduced magnetite compared to the mass of PLGA, % wt/wt
X_{PLGA}	Concentration of PLGA in the intermediate oil phase, % wt/vol
X_{HSA}	Concentration of HSA in the inner aqueous phase, % wt/vol
X_{VOLR}	Volume ratio of the outer aqueous phase to the intermediate oily phase, vol/vol
X_{time}	Time of the second sonication, minute

Other notations and indices

C	Central point of the studied interval of all independent variables
F1, F2, F3, F4, F5	Factors (independent variables)
L	Indication of the linear effect of a given variable (factor)
FiLbyFjL	Effect of linear-linear interaction between the factors Li and Lj

O	oily phase (intermediate phase) of the emulsion
W, W2	Aqueous phase, the second (outer) aqueous phase of the emulsion
F	Result of the statistical F test on the studied variable (in ANOVA table)
p	Statistical significance level (in ANOVA table)
df	Degree of freedom (in ANOVA table)
MS	Mean square of the residuals, nm^2 (in ANOVA table)
SS	Sum of deviation squares, nm^2 (in ANOVA table)

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Legends of Figures

Fig. 1. Encapsulation of model drug loaded magnetic PLGA nanoparticles using double emulsion solvent evaporation method.

Fig. 2. Typical size distributions of the PLGA nanoparticles obtained with different process variables: a – small, b – medium, and c – relatively high size region.

Fig. 3. Pareto chart on the standardized effects of the independent process variables on the mean hydrodynamic particle size.

Fig. 4. The effect of various process variables on the mean particle size; fixed parameters: (a) Fe_3O_4/PLGA weight ratio and PLGA concentration (b) HSA concentration and Fe_3O_4/PLGA weight ratio (c) volume ratio and Fe_3O_4/PLGA weight ratio (d) PLGA concentration and sonication time.~~on the mean particle size.~~

~~Fig. 5. The effect of HSA concentration and Fe_3O_4/PLGA weight ratio on the mean particle size.~~

~~Fig. 6. The effect of volume ratio and Fe_3O_4/PLGA weight ratio on the mean particle size.~~

~~Fig. 7. The effect of PLGA concentration and sonication time on the mean particle size.~~

Fig. 5~~8~~. (a) ~~C~~comparison of the measured and predicted mean particle sizes (b) smallest achievable mean particle size with constrain of various magnetite/ PLGA ratios at optimized other process variables.

~~Fig. 9. The smallest achievable mean particle size with constrain of various magnetite/ PLGA ratios at optimized other process variables.~~

Legends of Tables

Table 1. Process variables (factors) used in experimental design and their studied ranges.

Table 2. Result of statistical analysis on the dependence of the measured mean particle sizes as a function of the influencing factors (ANOVA table).

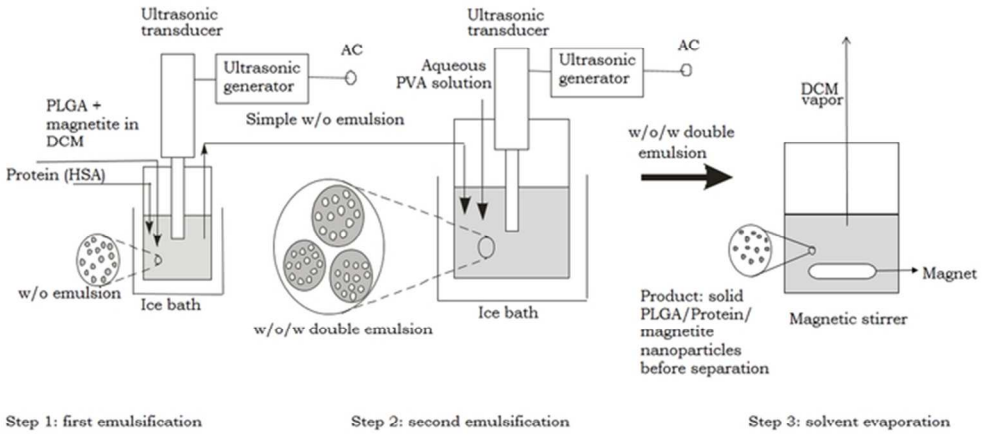

Fig. 1. Encapsulation of model drug loaded magnetic PLGA nanoparticles using double emulsion solvent evaporation method
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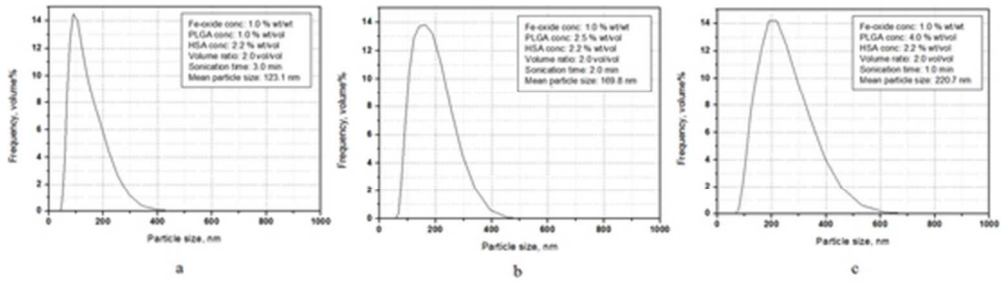

Fig. 2. Typical size distributions of the PLGA nanoparticles obtained with different process variables: a – small, b – medium, and c – relatively high size region
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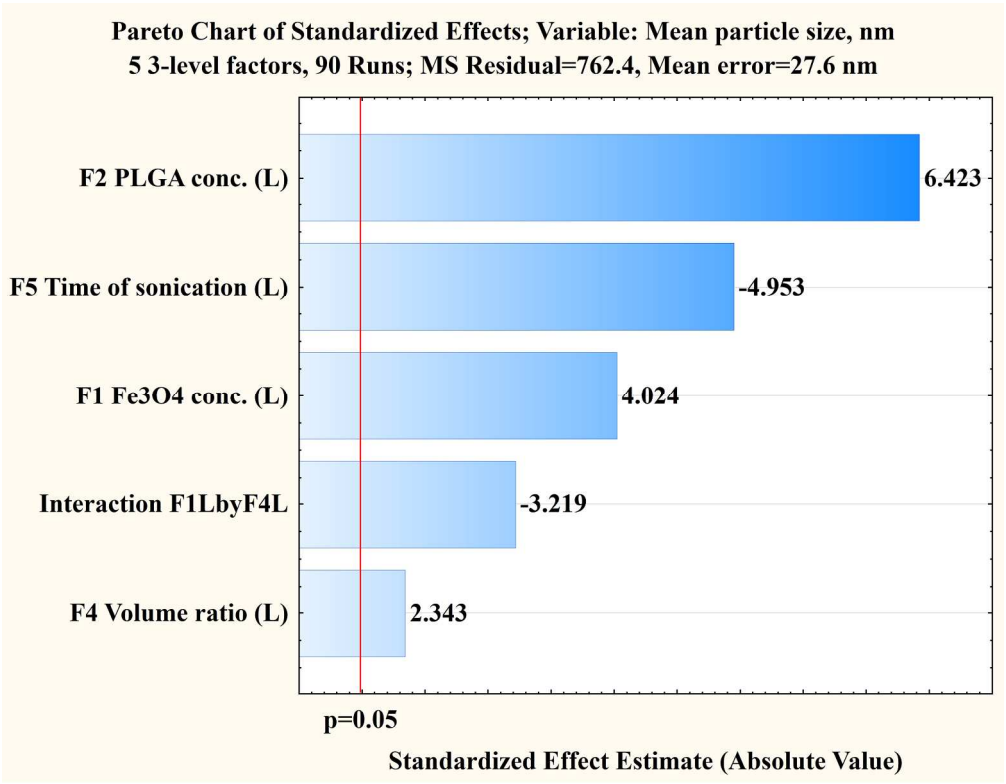

Fig. 3. Pareto chart on the standardized effects of the independent process variables on the mean hydrodynamic particle size
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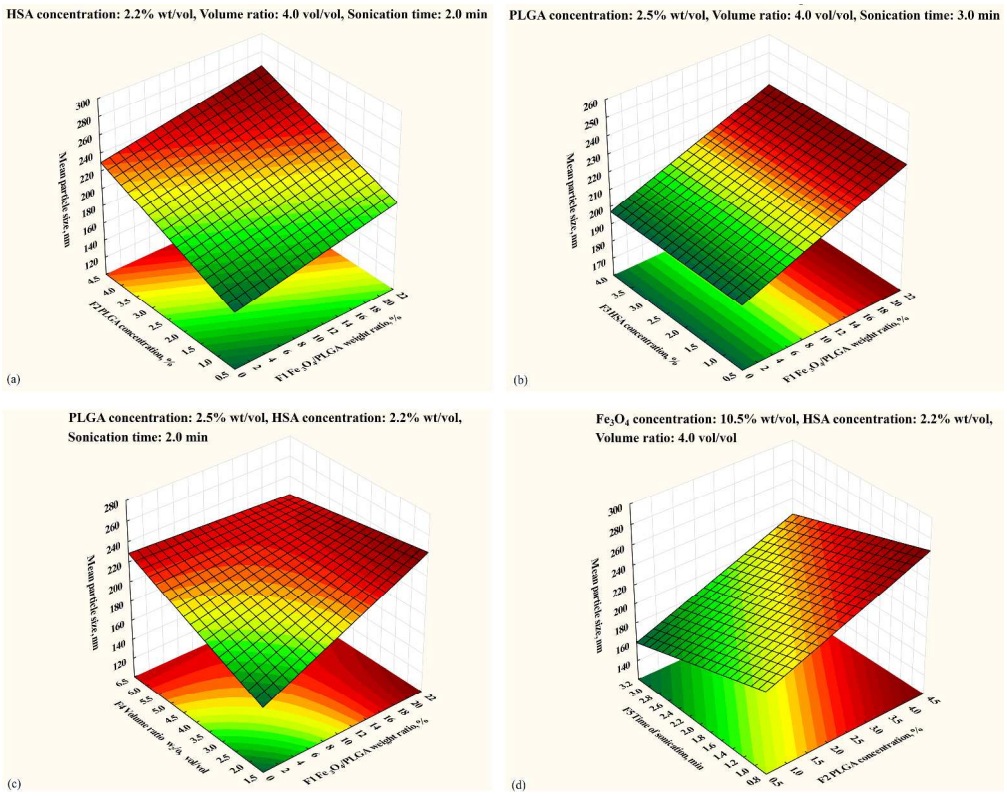

Fig. 4. The effect of various process variables on the mean particle size; fixed parameters: (a) Fe3O4/PLGA weight ratio and PLGA concentration (b) HSA concentration and Fe3O4/PLGA weight ratio (c) volume ratio and Fe3O4/PLGA weight ratio (d) PLGA concentration and sonication time
1048x825mm (96 x 96 DPI)

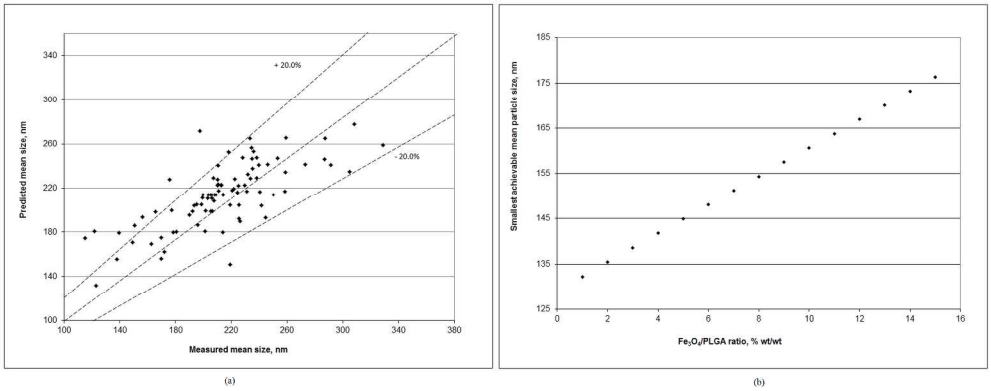

Fig. 5. (a) comparison of the measured and predicted mean particle sizes (b) smallest achievable mean particle size with constrain of various magnetite/ PLGA ratios at optimized other process variables
1016x396mm (96 x 96 DPI)

Table 1
Process variables (factors) used in experimental design and their studied ranges.

Factor	Symbol	Variable	Studied intervals
F1	X_{Fe3O4}	Fe_3O_4/PLGA weight ratio	1-20 wt%
F2	X_{PLGA}	PLGA concentration in the organic phase	1-4 wt%
F3	X_{HSA}	HSA concentration in the inner aqueous phase	0.74-3.69 wt%
F4	X_{VOLR}	Outer aqueous (w_2)/organic phase (o) ratio volume ratio.	2-6 vol/vol
F5	X_{time}	Time of the ultrasonic treatment in the second emulsification	1-3 minutes

Table 2

Result of statistical analysis on the dependence of the measured mean particle sizes as a function of the influencing factors (ANOVA table).

Factor	ANOVA; Var.:Mean particle size, nm 5 3-level factors, 90 Runs; MS Residual=762.4 Mean error=27.6 nm				
	SS	df	MS	F	p
F1 Fe_3O_4 conc. (L)	12343.9	1	12343.89	16.19139	0.000125
F2 PLGA conc. (L)	31447.1	1	31447.08	41.24891	0.000000
F4 Volume ratio W_2/O (L)	4183.7	1	4183.68	5.48771	0.021516
F5 Time of sonication, min (L)	18699.7	1	18699.73	24.52829	0.000004
interaction F1L by F4L	7898.1	1	7898.09	10.35987	0.001831
Error	64039.4	84	762.37		
Total SS	138611.9	89			

Appendix A

Experimental program obtained by 5-factorial 3-level experimental design with the applied process variables (factors), and the measured mean particle sizes and encapsulation efficiencies

Run # after randomization of their order	F1 (Fe_3O_4/PLGA weight ratio), wt/wt%	F2 (PLGA conc.), wt/vol%	F3 (HSA conc.), wt/vol%	F4 (Volume ratio W_2/O), vol/vol	F5 (Time of sonication), min	Mean particle size, nm	Encapsulation efficiency, %
10	1.0	4.0	0.74	6.0	3.0	175.6	90.40
83 (C)	10.5	2.5	2.21	4.0	2.0	203.3	91.77
18	10.5	4.0	0.74	4.0	3.0	221.7	92.80
1	20.0	4.0	2.21	4.0	1.0	197.6	94.40
26	1.0	1.0	0.74	2.0	1.0	162.8	82.40
16	10.5	4.0	3.69	2.0	2.0	238.2	95.66
19	20.0	2.5	3.69	4.0	1.0	228.0	73.24
9	1.0	4.0	2.21	2.0	1.0	220.7	96.32
11	10.5	2.5	0.74	2.0	2.0	198.5	91.24
25	1.0	2.5	2.21	6.0	3.0	193.3	92.40
17	20.0	1.0	3.69	2.0	3.0	225.5	53.98
13	20.0	4.0	0.74	2.0	3.0	210.6	94.30
2	20.0	2.5	0.74	6.0	2.0	210.3	87.60
3	10.5	2.5	3.69	6.0	1.0	291.2	83.48
24	1.0	2.5	3.69	2.0	1.0	156.2	72.20
15	10.5	1.0	2.21	2.0	2.0	121.8	64.50
5	1.0	1.0	2.21	4.0	2.0	114.9	72.60
12	1.0	1.0	3.69	6.0	3.0	139.6	33.82
84 (C)	10.5	2.5	2.21	4.0	2.0	250.3	90.23
6	10.5	1.0	0.74	6.0	1.0	258.6	81.01
21	1.0	2.5	0.74	4.0	2.0	206.4	88.66
14	20.0	1.0	2.21	6.0	2.0	205.2	74.59
4	10.5	2.5	2.21	4.0	3.0	189.9	85.71
22	10.5	1.0	3.69	4.0	3.0	149.0	60.86
8	20.0	4.0	3.69	6.0	2.0	253.3	79.50
7	10.5	4.0	2.21	6.0	1.0	233.2	90.05
23	20.0	1.0	0.74	4.0	1.0	212.6	72.96
20	20.0	2.5	2.21	2.0	3.0	224.5	87.88
82 (C)	10.5	2.5	2.21	4.0	2.0	206.0	82.00
27	1.0	4.0	3.69	4.0	2.0	212.7	86.68
34	10.5	4.0	3.69	4.0	1.0	234.3	92.70
51	10.5	1.0	2.21	4.0	1.0	207.6	85.30

43	10.5	2.5	0.74	4.0	1.0	232.0	97.20
30	10.5	1.0	0.74	2.0	3.0	171.9	83.50
46	20.0	2.5	3.69	6.0	3.0	225.7	84.10
38	1.0	2.5	0.74	6.0	1.0	239.8	78.90
36	10.5	4.0	2.21	2.0	3.0	203.0	94.40
44	1.0	4.0	0.74	2.0	2.0	201.4	91.00
39	1.0	4.0	2.21	4.0	3.0	219.1	86.40
28	20.0	1.0	3.69	4.0	2.0	195.1	36.32
49	20.0	4.0	3.69	2.0	1.0	308.1	90.20
85 (C)	10.5	2.5	2.21	4.0	2.0	214.2	86.40
29	20.0	1.0	2.21	2.0	1.0	207.1	33.54
41	10.5	2.5	3.69	2.0	3.0	196.1	49.50
40	1.0	2.5	3.69	4.0	3.0	178.4	81.30
54	1.0	2.5	2.21	2.0	2.0	169.8	90.50
50	1.0	1.0	0.74	4.0	3.0	138.1	79.39
48	20.0	2.5	2.21	4.0	2.0	233.8	92.50
52	20.0	2.5	0.74	2.0	1.0	236.0	91.22
86 (C)	10.5	2.5	2.21	4.0	2.0	213.8	89.17
45	20.0	4.0	0.74	4.0	2.0	217.9	88.42
32	20.0	1.0	0.74	6.0	3.0	180.6	67.20
47	10.5	1.0	3.69	6.0	2.0	192.0	50.69
42	10.5	2.5	2.21	6.0	2.0	229.5	85.30
35	1.0	1.0	2.21	6.0	1.0	240.3	55.80
33	1.0	4.0	3.69	6.0	1.0	287.4	88.80
31	1.0	1.0	3.69	2.0	2.0	219.1	18.21
53	20.0	4.0	2.21	6.0	3.0	222.4	65.91
37	10.5	4.0	0.74	6.0	2.0	235.0	87.50
87 (C)	10.5	2.5	2.21	4.0	2.0	209.1	79.40
63	1.0	4.0	2.21	6.0	2.0	286.9	89.78
88 (C)	10.5	2.5	2.21	4.0	2.0	214.2	81.10
55	20.0	1.0	3.69	6.0	1.0	211.0	43.42
81	1.0	1.0	2.21	2.0	3.0	123.1	43.79
70	1.0	2.5	0.74	2.0	3.0	169.9	85.50
66	10.5	2.5	3.69	4.0	2.0	199.7	80.30
71	10.5	2.5	0.74	6.0	3.0	241.7	89.58
72	20.0	4.0	2.21	2.0	2.0	328.9	91.80
79	20.0	2.5	0.74	4.0	3.0	206.1	82.60
67	20.0	4.0	3.69	4.0	3.0	258.7	87.20
64	1.0	1.0	0.74	6.0	2.0	165.5	74.50
61	10.5	4.0	2.21	4.0	2.0	235.1	89.90
78	10.5	1.0	2.21	6.0	3.0	213.8	81.70
90 (C)	10.5	2.5	2.21	4.0	2.0	205.1	87.60
58	1.0	2.5	2.21	4.0	1.0	231.1	84.17
75	20.0	2.5	3.69	2.0	2.0	304.8	34.84

57	10.5	1.0	0.74	4.0	2.0	226.2	77.50
80	1.0	1.0	3.69	4.0	1.0	244.8	33.28
62	1.0	4.0	3.69	2.0	3.0	201.1	87.47
74	1.0	2.5	3.69	6.0	2.0	225.0	83.24
69	1.0	4.0	0.74	4.0	1.0	246.3	91.80
76	20.0	2.5	2.21	6.0	1.0	273.2	88.10
73	20.0	4.0	0.74	6.0	1.0	259.2	81.17
56	20.0	1.0	2.21	4.0	3.0	150.7	74.59
59	20.0	1.0	0.74	2.0	2.0	199.2	83.45
77	10.5	1.0	3.69	2.0	1.0	177.3	39.39
65	10.5	4.0	0.74	2.0	1.0	238.1	94.03
68	10.5	2.5	2.21	2.0	1.0	210.6	92.04
60	10.5	4.0	3.69	6.0	3.0	210.3	87.60
89 (C)	10.5	2.5	2.21	4.0	2.0	208.0	88.90