A Design of Experiment Approach to the Synthesis of Alendronate-incorporated Hydroxyapatite

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A design of experiment (DoE) approach is presented for the optimization of Alendronate-hydroxyapatite nanoparticles' synthesis. The synthesis was performed using the chemical precipitation technique from calcium nitrate, diammonium hydrogen phosphate and alendronate. Synthesis temperature, reactant addition rate and ripening time were chosen as the most relevant experimental factors for our synthesis. Design of Experiments was used in order to measure these conclusive process parameters and their effect on controlling some final nanoparticles parameters, such us: alendronate incorporation efficiency (IncorporationEfficiency, %), hydroxyapatite crystallite size (Size_XRD, nm), hydroxyapatite particle size distribution (Size_DLS, Å). Our study found that better HA-AL incorporation efficiency and small nonoparticles can be obtained using the following chemical process parameters: reaction temperature 30°C or smaller, ripening time 108h and addition rate 0.1mol/min. The analysis of more than one nanoparticles characteristics was possible using DoE software, MODDE 9.1. Thus, hydroxyapatite-alendronate incorporation efficiency should be expected to increase with decreasing temperature below 30°C, increasing the maturate time at least 108h, at an addition rate of 0.1mol/min, in an N_2 atmosphere. The same conditions will ensure nanoparticles small size that would be more desirable for the application of implants.

Keywords: Design of Experiment - DoE, MODDE, hydroxyapatite-alendronate nanoparticles

Hydroxyapatite (HA) is a calcium phosphate salt found in human bone and teeth, with the difference that the biological bone apatites contain only a very small percentage of the total number of hydroxyl groups present in highly purified synthetic calcium hydroxyapatites [1]. It has been utilized extensively as implant material for many years due to its superior biocompatibility and bone bonding capacity and also due to its structural and compositional similarity to that of the mineral phase of hard tissue in human bones [2-4]. But osteoporotic patients vary from normal subjects in bone mineral composition, bone mineral content sand crystallinity [5]. A lot of effort must be spent on improving therapies for fractures in the case of osteoporotic patients.

The inclusion of bisphosphonates into the hydroxyapatite layer is an important step for researchers who study bone graft materials with a desirable local anti-osteoporosis property for osteoporotic fracture [6]. A series of studies evidenced advantages for the local drug delivery in osteoporotic bone: local release of osteogenic agents in osteoporotic fracture would increase bone strength and quality and would reduce the period of bone healing, improving implant fixation strength in osteoporotic bone [7].

Nano-scale hydroxyapatite particles were synthesized also for immobilizing heavy metals [8].

We used chemical precipitation method from calcium nitrate, diammonium hydrogen phosphate and alendronate for synthetize hydroxyapatite-alendronate (HA-AL) composites.

The aim of this study is to monitor the production process, to examine the influence of each process variables on HA-AL physicochemical properties and to determine the best set of process variables to produce HA-AL powder of desired characteristics.

The aim of this research is to apply DoE and to create mathematical models that will help researchers, providing the highest reliability, lower process costs and improving powder quality.

A screening DoE was conducted to identify the most important process parameters using a Two-level Full Factorial design, interaction model, with the aid of MODDE 9.1 software.

Design of Experiments is the only technique that enables researchers to observe interaction effects that can improve product quality and obstruct process failure [9]. DoE validates process parameters in a single set of experiments with less time, money and human resources.

Experimental part

Materials and methods

Different methods have been used for HA powders synthesis [10-14]. The nanoparticles were synthesized by the wet precipitation method in N_2 atmosphere as previously reported [15]. All chemicals were analytical reagent (AR) grade: $Ca(NO_3)_2 \cdot 4H_2O$ (Sigma-Aldrich, ≥99%); $(NH_4)2HPO_4$ (Sigma-Aldrich, ≥99%); NH_3 (Sigma-Aldrich, 30 - 33%); alendronate sodium salt (Axxora, ≥ 97%); HPLC Water (LiChrosolv® Merck); hydroxyapatite powder (Sigma-Aldrich).

Calcium nitrate solution was initially heated to temperatures in the range 30-90° C, and ammonium phosphate was added under continuous stirring (600 rpm, flow rate was varied between 0.1-5 mL/min, added with a peristaltic pump). Aqueous solutions of 5 mM, 10 mM and 20 mM concentration of alendronate were used.

The second synthesis was obtaining hydroxyapatite-alendronate by the addition of sodium alendronate trihydrate into phosphate solution.

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The obtained product was maintained in the reaction medium for 5 hours at a constant temperature under stirring, then centrifuged at 10,000 rpm for 10 min (Eppendorf centrifuge model 5804) and repeatedly washed with distilled water to remove traces of ammonia. The powder was kept overnight at 37°C.

In order to understand this process, we analyzed the mathematical linkage between these complex set of inputs (process conditions or factors) with outputs (powder characteristics or responses). The mathematical model was developed based on the screening analysis of DoE, using Two-level Full Factorial design. These designs support interaction models and require relatively few experiments per explored factor, in which every level of each factor occurs for every combination of the levels of the other factors. Another investigation could be done with Box-Behnken experimental design as Ailiesei et al. [16], but with more experiments (17 experiments) than we done (11 experiments) and the costs would be bigger. Predictable behavior of outcomes were assessed [17] in 36 runs with a model fitted by Partial Least Square (PLS) in the case of 5 factors and 2 responses.

To perform a Two-level Full Factorial design, we have assigned a low level and a high level to each factor. These settings are then used to construct an orthogonal array of experiments. There are some common notations in use to represent such factor settings. Usually, the low level of a factor is denoted by –1 or just -, and the high level by +1 or simply +. As a consequence, the center level, usually chosen for replication, will be denoted by 0.

The experimental trials are represented in a design matrix. Each row of the matrix represents an experiment; each column represents the level of each factor. The Two-level Full Factorial design for the three factors: temperature (X_1), the reactant addition rate (X_2) and the ripping time (X_3), is shown in the design matrix presented in table 1.

The three experimental process parameters were selected, each one at two levels: Temperature (X_1): 30^0C - 90^0C, Addition Rate (X_2): 0.1 mol/min-5 mol/min, Ripening Time (X_3): 0h-72h.

The responses (IncorporationEfficiency, Size_XRD, Size_DLS) were measured in sequential order. The experiments were run in a completely randomized order, as we see in table 2, in order to provide that uncontrolled factors did not influence the final results obtained.

Results and discussions

The experiments were carried out according to the design matrix shown in table 2 in a fully randomized order to avoid any systematic error and the outcomes are shown in table 3.

Statistical analysis of the obtained data was performed using MODDE 9.1 software. Analysis of the regression coefficients of the linear polynomial models describing the relationship between the responses and the tree factors are presented in the following section.

The condition number is a tool that can be used to evaluate the performance of our experiment design prior to its execution. Our Condition number is 1.173 < 3, therefore being considered a very good screening design.

No. experiment	X_1	X_2	X_3	Interaction X_1*X_2	Interaction X_2*X_3	Interaction X_1*X_3	Interaction $X_1*X_2*X_3$
1	-	-	-	+	+	+	-
2	+	-	-	-	+	-	+
3	-	+	-	-	-	+	+
4	+	+	-	+	-	-	-
5	-	-	+	+	-	-	+
6	+	-	+	-	-	+	-
7	-	+	+	-	+	-	-
8	+	+	+	+	+	+	+
9	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0

Table 1
THE TWO-LEVEL FULL FACTORIAL DESIGN, INTERACTION MODEL, FOR THREE FACTORS X_1, X_2, X_3, EACH WITH TWO LEVELS, LOW (-) AND HIGH (+)

Exp. No.	Run no.	Temperature (^{0}C) X_1	Addition Rate (mol/min) X_2	Ripening Time (h) X_3
N1	1	30	0.1	0
N2	9	90	0.1	0
N3	11	30	5	0
N4	8	90	5	0
N5	3	30	0.1	72
N6	10	90	0.1	72
N7	4	30	5	72
N8	6	90	5	72
N9	7	60	2.55	36
N10	2	60	2.55	36
N11	5	60	2.55	36

Table 2
DESIGN MATRIX FOR SCREENING FULL FACTORIAL DESIGN, EXPRESSED AS REAL VALUES OF FACTORS

Table 3
DESIGN MATRIX FOR SCREENING FULL FACTORIAL DESIGN, EXPRESSED AS REAL VALUES OF RESPONSES

Sample	Incorporation Efficiency (%)	Size_XRD (nm)	Size_DLS (nm)
N1	0.98532	88.9	590
N2	7.626	144.3	356
N3	0.726	125.7	2200
N4	0.9469	117	732
N5	9.84	149.6	439
N6	4.023	311.9	368
N7	3.38525	143.3	259
N8	0.6399	148.5	300
N9	1.61325	123.9	454
N10	1.896	114	754
N11	1.84125	129.2	822

Development of Incorporation Efficiency, Size_ XRD and Size_DLS Models

As a result of analyzing the measured three responses using MODDE software, the insignificant model terms ($p<0.05$) were automatically eliminated. The analysis of experimental data generated through DoE consists of three primary stages.

The first stage, evaluation of raw data – Replicate plot and histograms, concentrates on a general assessment of regularities and specific features in the data. In Replicate plot, the measured responses are plotted against the unique number of each experiment. Since the variation in these three replicates is much smaller than the variation in the entire investigation series, as we can see in figure 1, we can conclude that the replicate error will not confound the data analysis.

It is advantageous in regression analysis if the data of the response variables are normally distributed or nearly so. All histograms have a heavy tail to the right, like in the figure 2, therefore the variables may not be analyzed directly and they need a logarithmic transformation.

The second stage, regression analysis and model interpretation, involves the actual calculation of the model linking the factors and the responses together, and the interpretation of this model. Parameter R^2 is a measure of how well the regression model can be made to fit the raw data, but R^2 alone is not a sufficient indicator for probing the validity of the model. A much better indication of the validity of a regression model is given by the Q^2 parameter, called the goodness of prediction, and estimates the predictive power of the model. It reflects the final goal of modeling – predictions of new experiments. For a model to pass this diagnostic test, both R^2 and Q^2 should have high values and preferably not separated by more than 0.2-0.3. A substantially larger difference constitutes a notice of an unfit model. A $Q^2>0.5$ should be consider as good and $Q^2>0.9$ as excellent, but these limits are application dependent. Model validity might be low in very good models due to high sensitivity in the test or extremely good replicates, like in IncorporationEfficiency model. A good fit of the models was proved by statistical analysis, based on $R^2=0.971$ and $Q^2=0.728$ values for IncorporationEfficiency model and $R^2=0.91$ and $Q^2=0.485$ for Size_XRD model.

Model interpretation with Coefficient plot plays an important role in the data analysis. According to figure 3, there are some small and insignificant two-factor interactions. These terms may be omitted and the model refitted to the data. For IncorporationEfficiency model we can see an important interactions between Temperature and Ripening Time, Temperature and Addition Rate. The important interactions for Size_XRD model are Temp*Rate and Rate*Time, and for Size_DLS model: Rate*Time. We now have a simpler models with better functional predictive ability.

We can clearly see the positive and negative effects over the three responses. The Addition Rate, Ripening Time, Temperature*Addition Rate and Temperature*Ripening Time were the factors which possess the most significant main effect on Incorporation Efficiency. The order of significance for these effects follows the order: Rate>Temp*Time>Time>Temp*Rate.

The Ripening Time, Temperature and Temperature* Addition Rate were the factors which have the most significant main effect on Size_XRD, the two-factor interaction Rate*Time has a minor influence. The order of

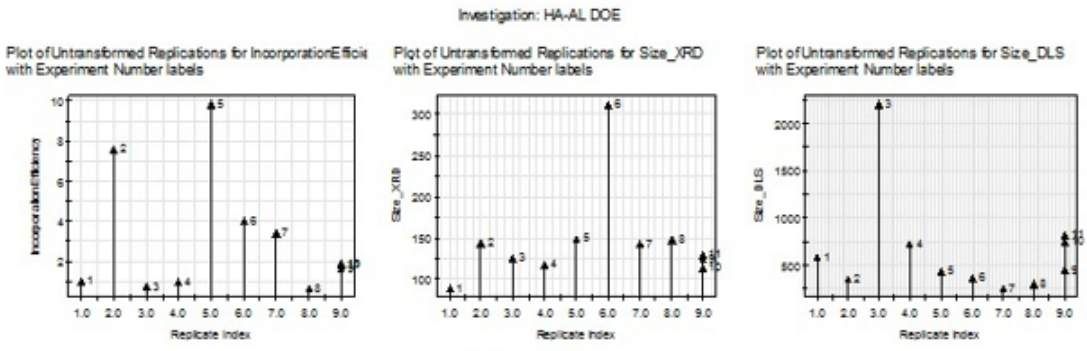

Fig. 1 Replicate plot of IncorporationEfficiency, Size_XRD, Size_DLS

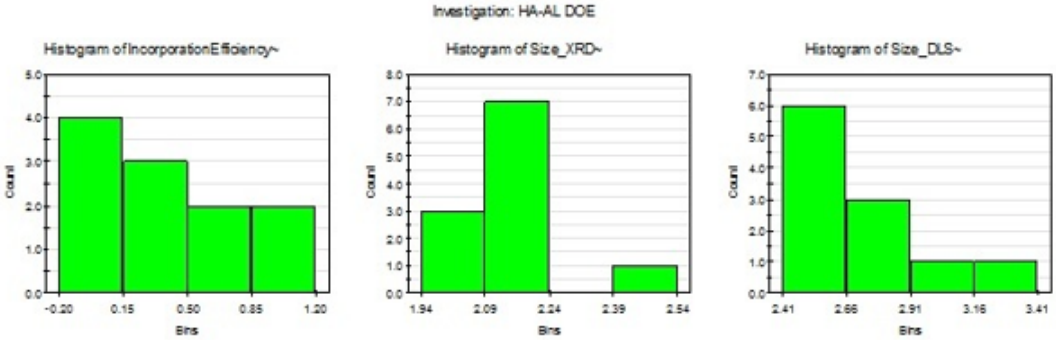

Fig. 2 Histogram of Incorporation Efficiency, Size_XRD and Size_DLS

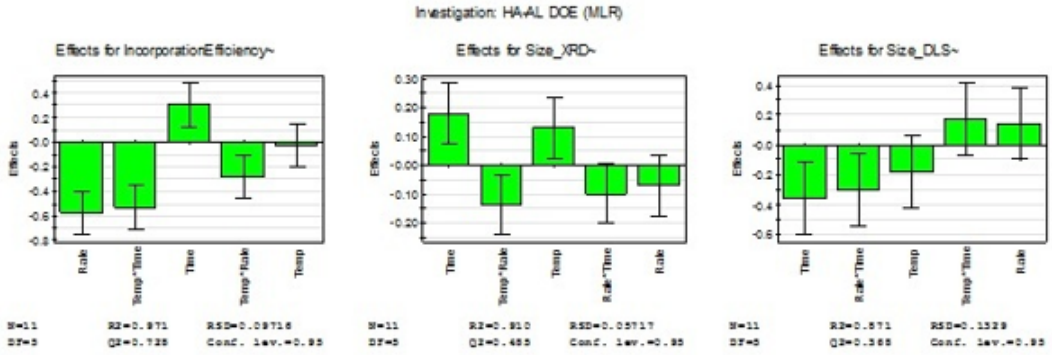

Fig. 3 Regression coefficients of IncorporationEfficiency model, Size_XRD model and Size_DLS model

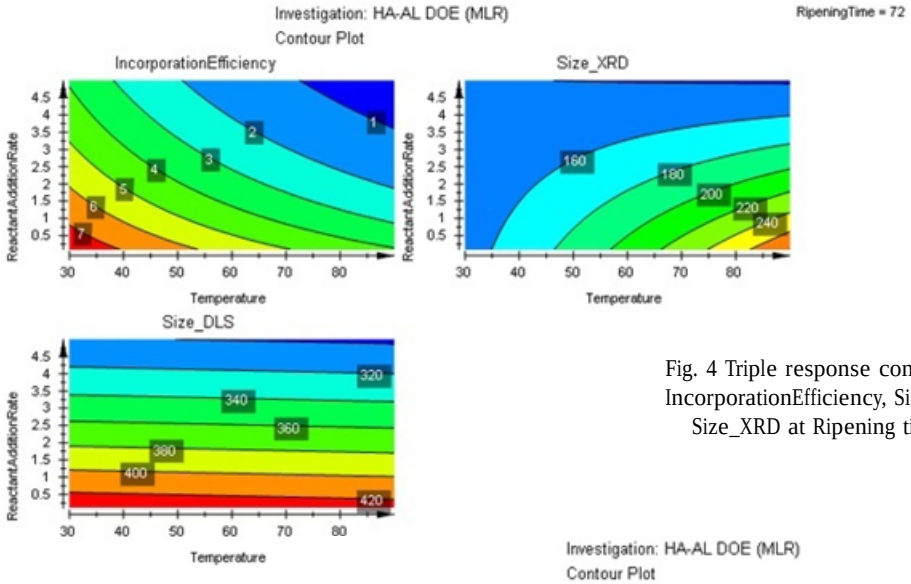

Fig. 4 Triple response contour plot of IncorporationEfficiency, Size_DLS and Size_XRD at Ripening time=72h

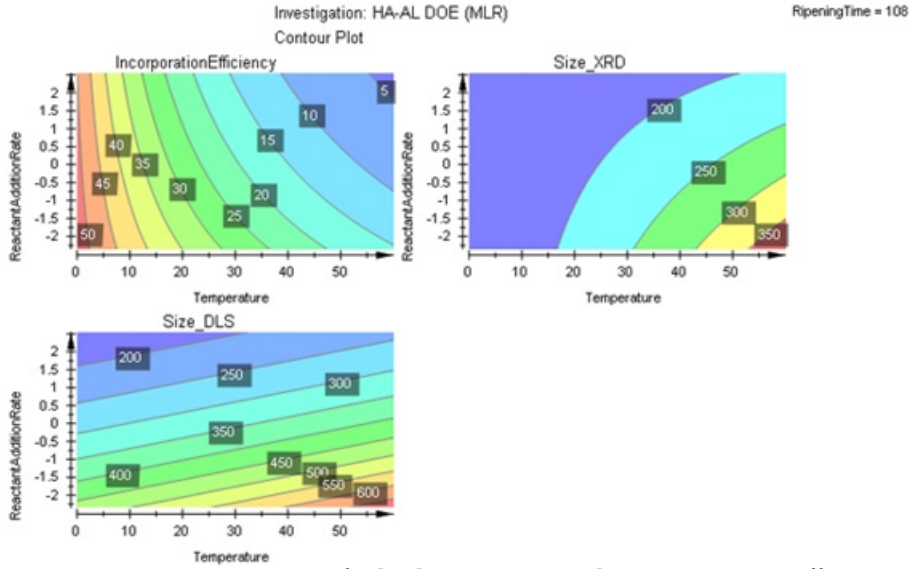

Fig. 5 Triple response contour plot of IncorporationEfficiency, Size_DLS and Size_XRD at Ripening time=108h

significance for these effects follows the order: Time>Temp*Rate>Temp>Rate*Time.

The Ripening Time and Addition Rate*Ripening Time were the factors which have the most significant main effect on Size-DLS. The order of significance for these effects follows the order: Time>Rate*Time.

The ANOVA results and its lack of fit test for our responses have been analyzed. In ANOVA, two F-tests are performed: the first one identifies the importance of the regression model and we conclude this test is satisfied when $p<0.5$. Our models are statistically significant as $p=0.001$ for IncorporationEfficiency model, $p=0.012$ for Size_XRD model and $p=0.028$ for Size_DLS model. The second test confronts the model error and the replicate error. The model shows good match to the data when a sufficient low model error is obtained, that is the model has no lack of fit. Hence, this later test is known as the lack of fit test and we say it is satisfied when $p>0.5$. In the IncorporationEfficiency model, $p=0.087$, which is larger than the reference value (0.5) and, therefore, we conclude that the model has no lack of fit. The same with Size_XRD model and Size_DLS model, when $p=0.137$ and $p=0.580$, respectively.

The optimal regression model has been obtained so we can execute the third stage of the data analysis, use of regression model; the achieved model is utilized to predict the best parameters at which to drive next experiments.

Figure 4 show a response contour plot created with the factors Temperature and Addition Rate as axes and Ripening Time fixed at 72 h.

To maximize the Incorporation Efficiency, we should position new (verifying) experiments in the lower left-hand corner, with low Temperature and low Addition Rate. We can see that Incorporation Efficiency has greater value in the lower left-hand corner of Incorporation Efficiency contour plot for a greater value of Ripening Time.

The prevalent objective is that of maximizing the IncorporationEfficiency and minimizing the Size_DLS and we can point out a prediction view in figure 5 in which predictions are made for factor settings expected to be relevant for our goal. All predicted values unanimously indicate that even better IncorporationEfficiency values are achievable outside the explored experimental zone. Hence, we should select one of these suggested values and use them for the verifying experiment.

In the extrapolation phase, we get Incorporation Efficiency=24.4746%, Size_XRD=199,807nm and Size_DLS=323.867nm for Temperature=25, Addition Rate=0.1 and Ripening Time=108.

Conclusions

Wet process has the advantage of low probability of contamination in the time of processing and the process costs are small. Its disadvantage is that the resulting product can be greatly affected by even a slight difference in the reactions conditions. So, design of experiments is vital to the successful control of the chemical precipitation process.

Full factorial 2^3 design is useful for screening few numbers of factors in few experiments and to analyze their influence on HA-AL properties.

Reaction temperature was reported in many studies to affect predominantly the crystalline phase fraction, crystallite size and, as a consequence, specific surface area [18, 19]. Reaction temperature was found to affect incorporation efficiency (greater incorporation for smaller temperature). But we cannot decrease too much the reaction temperature for to obtain a better incorporation efficiency. Neamtu et al. [20] assessed the profile of alendronate from hydroxyapatite-alendronate tablets to improve the efficacy of alendronate.

The rate of reactant addition also affects the characteristics of Al-HA powder. At a constant Ripening Time, a better incorporation efficiency was obtained at smaller addition rate. Thus, we must decrease the addition rate but it is impossible less than 0.1mol/min.

Maturation period has an important role in obtaining better characteristics for alendronate-hydroxyapatite powder. At a 108h of RipeningTime we obtain almost 25 for Incorporation efficiency, but at 36h or 72h of Ripening Time we obtained less than 10 for Incorporation efficiency.

At a constant Ripening Time, as the reaction temperature is decreased, the Size_XRD is also decreased, indicating a decrease in final Al-HA crystallinity. At a temperature of 25^0C, Size_XRD has an average of 100nm for Ripening Time=36h, 150nm for Ripening Time=72h and 200nm for Ripening Time=108h. Thus, we observed that larger agglomerates would be formed for relatively smaller particles for a bigger period of maturation.

Better HA-AL IncorporationEfficiency and small nonoparticles can be obtained using the following chemical process parameters: Reaction temperature 30°C or smaller, Ripening Time 108h and Addition rate 0.1mol/min.

Thus, HA-AL Incorporation Efficiency should be expected to increase with decreasing temperature below 30^0C and increasing the maturate time at least 108h, at an addition rate of 0.1mol/min, in an N_2 atmosphere. These conditions will ensure small size nanoparticles synthesis that would be more desirable for the application of implants.

References

1. REY, C., COMBES, C., ROUET, CD., GLIMCHER, MJ., Osteoporos Int. 2009; 20, p.1013-1021.
2. JAFFE, WL., SCOTT, DF., J Bone Joint Surg Br. 1996; 78A, p.1818-934.
3. LEGEROS, RZ., In: Brown PW, Constantz B (ed) Hydroxyapatite and reated materials. CRC, Boca Raton; 1994. p. 3-28.
4. TYE, C., RATTRAY, K.R., WARNER, K.J., J Biol Chem. 2003; 278, p. 7949–7955.
5. LEGROS, R., BALMAIN, N., BONEL, G., Calcif Tissue Int. 1987; 41, p. 137-144.
6.BIGI, A., BOANINI, E., CAPUCCINI, C., FINI, M., MIHAILESCU, I.N., RISTOSCU, C., SIMA, F., TORRICELLI. P., Biomaterials. 2009; 30, p. 6168-6177.
7.KYLLONEN, L.,D'ESTE, M., ALINI, M., EGLIN, D., Acta Biomater. 2015; 11, p. 412-34.
8.SAPLONTAI, M., BALC, N., SAPLONTAI, V., COJOCARU, I., TOTH, R., MOLDOVAN, M., Rev. Chim. (Bucharest), **63**, no. 12, 2012, p. 1228
9. TORBECK, L.D., Informa Healthcare USA, Inc.; 2007.
10. CONFLANT, P., STP pharma sciences. 1997; 7, p. 282-8.
11.CHAAIR, H., HEUGHEBAERT, J.C., HEUGHEBAERT, M., VAILLANT, M., J Mater Chem. 1994; 4, p. 765-770; DOI: 10.1039/JM9940400765.
12.ARENDS, J., SCHUTHOF, J., VAN der LINDWEN, W.H., BENNEMA, P., VAN der BERG, P.J., Journal of Crystal Growth. 1979; 46(2), p. 213-220.
13.BLUMENTHAL, N., POSNER, A., Calcified Tissue International. 1973; 13(1), p. 235-243.
14.CHAAIR, H., MANSOURI, I., HEUGHEBAERT, M., NADIR, S., Phosphorus Sulfur and Silicon and Related Elements. 2001; 173, p. 163-174. DOI: 10.1080/10426500108045267.
15. BALOSACHE, O.E., BUBULICA, M.V., POPESCU, M., MANDA, C.V., CROITORU, O., BANICERU, M., MELINTE, R., NEAMTU, J., Curr Health Sci J, 2013, 39:39-43.
16. AILIESEI, I., ANUTA, V., MIRCIOIU, C., COJOCARU, V., ORBESTEANU, A.M., CINTEZA, L.O., Rev. Chim. (Bucharest), **67**, no. 8, 2016, p. 1566
17. HUBERT, K.N., BODOKI, E., DOUDOU, B.D., OPREAN, R., SANDULESCU, R., Rev. Chim. (Bucharest), **61**, no. 12, 2010, p. 1186
18. LAZIC, S., ZEC, S., MILJEVIC, N., MILONJIC, S., Thermochimica Acta. 2001; 374:13- 22.
19. KEHOE, S., STOKES, J., J of Materi Eng and Perform. 2011; 20, p. 306. DOI: 10.1007/s11665-010-9671-8.
20. NEAMTU, J., BUBULICA, M.V., ROTARU, A., DUCU, C., BALOSACHE, O.E., MANDA, V.C., TURCU-STIOLICA, A., NICOLICESCU, C., MELINTE, R., POPESCU, M., CROITORU, O., Journal of Thermal Analysis and Calorimetry, 2016, DOI 10.1007/s10973-016-5905-9

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