Physical and Colloidal Chemistry Department[1], Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy; Collagen Department[2], National Institute for Textile and Leather Research and Development – Division Leather and Footwear Research Institute; Department of Physical Chemistry[3], Faculty of Chemistry, University of Bucharest, Romania

Design and optimization of some collagen-minocycline based hydrogels potentially applicable for the treatment of cutaneous wound infections

M. V. Ghica[1], M. G. Albu[2], M. Leca[3], L. Popa[1], St. Moisescu[1]

Received April 30, 2011, accepted May 30, 2011

Mihaela Violeta Ghica, Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Physical and Colloidal Chemistry Department, 6 Traian Vuia, 020956, Bucharest, Romania
mihaelaghica@yahoo.com, ghica.mihaela@umf.ro

Pharmazie 66: 853–861 (2011) *doi: 10.1691/ph.2011.1061*

Topical delivery systems for the treatment of different infected cutaneous wounds based on natural biopolymers are widely studied. The development of some topical hydrogels with type I collagen and minocycline by the experimental design technique, based on response surface methodology and Taguchi's approach, and the evaluation of their kinetic properties is presented. The concentrations of polymer, drug and cross-linking agent were selected as independent variables for the face centered composite design and low, medium and high concentration as variation levels for each factor. Minocycline kinetic release was investigated using a modified Franz diffusion cell. The drug release followed the Higuchi model. Three formulations having optimal minocycline release profiles were selected. They can be used as potential drug topical release systems for the treatment of infected cutaneous tissue.

1. Introduction

Surgical and nonsurgical wounds are associated with cutaneous infections. Nonsurgical wounds can be superficial or caused by traumatic skin tears, burns or chronic ulcers (Corsetti et al. 2010; Boateng et al. 2008; Zielberman and Elsner 2008). The cutaneous wound healing consists of a succession of events, including inflammation, proliferation, and different cell types migration. Bacterial infections are a serious problem in wound healing (Gopinath et al. 2004).

Administration of antibiotics as a rapid way of treatment and/or prophylaxis of an infected cutaneous wound is to be considered (Ribeiro et al. 2010). Systemically administered antibiotics hardly penetrate into the affected tissue and can induce systemic toxicity. These disadvantages led to attempts to develop new systems able to deliver the therapeutic agent directly at the cutaneous level (Stojadinovic et al. 2008; Zielberman and Elsner 2008). Thus, topical antiinfective delivery systems are currently a continuously developing area of research (Elsner and Zielberman 2010; Lin et al. 2009).

Improvements of topical administration are based on utilization of some biopolymers, which ensure an adequate antibiotic release. Among these biopolymers, an increased interest is given to collagen as a natural matrix for the drug release due to its high biocompatibility, low antigenicity, minimum inflammation, favorisation of cellular growth, differentiation and proliferation (Judith et al. 2010; Goissis and de Sousa 2009; Lin et al. 2009; Gopinath et al. 2004). Furthermore, collagen can be processed in various forms, the hydrogels being the form used in the present study. The high number of studies on hydrogels utilization in topical drug applications can be assigned to their wide utilization and patients compliance (Wang et al. 2010).

The hydrogels are polymeric tridimensional networks having a high internal water content, that can provide a wet environment to the affected surface and can absorb exudates (Sung et al. 2010; Hamidi et al. 2008). They have a limited absorption capacity and are recommended for application on infected wounds with low and moderate exudation (Elsner and Zielberman 2010).

The biological and mechanical properties of collagen hydrogels can be increased by glutaraldehyde crosslinking, which reduces the collagen material imunogenicity and increases the enzymatic degradation resistance (Zhang et al. 2011; Figueiro et al. 2006; Yuadanova and Reshetov 2006; Meade and Silver 1990).

A second generation tetracycline – minocycline – was selected as a model drug for this study. Due to its broad activity spectrum, minocycline is useful in the cutaneous bacterial infections treatment (Sung et al. 2010; Aoyagi et al. 2007). Furthermore it is active on germ strains resistant to tetracycline and doxycycline, imipenem and meticycline, and is also more liposoluble than other tetracyclines (Bishburg and Bishburg 2009). Minocycline also affects a lot of cellular functions with subsequent biological actions different from its antimicrobial activity (Yao et al. 2007).

The starting point in the biopharmaceutical evaluation of a drug incorporated in a topical form is the study of the drug release kinetics *in vitro*, which estimates the easiness of its release into the contact medium. The aim of this study was the development and optimization of topical collagen-based hydrogels containing minocycline, non- and crosslinked with glutaraldehyde, according to the face-centered 3^3 central composite design (Madeira et al. 2011; Fermoso et al. 2010), establishing the influence of formulation factors on some kinetic parameters that quantify the *in vitro* release from the designed systems using the experimental statistic design techniques. Compared with suc-

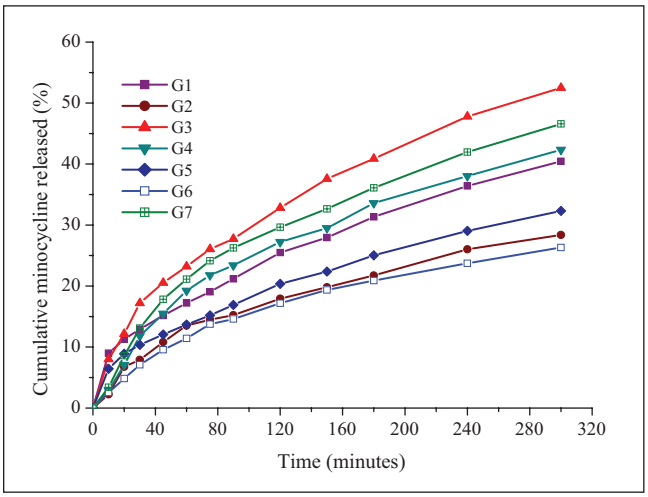

Fig. 1: Cumulative release of MH from collagen hydrogels (Exp. 1–7)

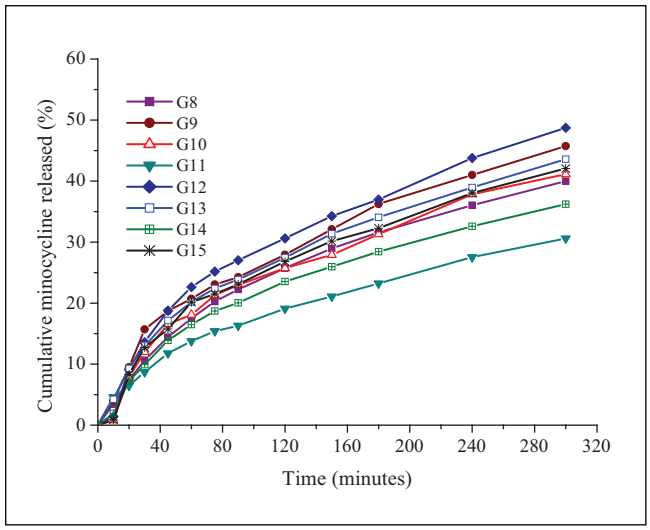

Fig. 2: Cumulative release of MH from collagen hydrogels (Exp. 8–15)

cesive single variable optimization methods, unable to evaluate the interactions between factors (Wu et al. 2009; Rhee et al. 2008; Mannan et al. 2007), the experimental design and response surface methodology in combination with Taguchi's approach offer some advantages: short experimental time, minimum trials for generating useful informations regarding the response of interest (Aggarwal et al. 2008; Bajaj et al. 2009; Aslan 2008), optimization of the products quality and processes robustness.

2. Investigations, results and disscusion

2.1. *In vitro release studies*

The face centered central composite design (FCCCD) used to evaluate some of the *in vitro* drug release kinetic parameters from hydrogels was carried out using three independent variables: collagen (C), minocycline hydrochloride (MH) and glutaraldehyde (GA) concentrations. Each input variable was evaluated at three different coded levels: low (1), middle (2) and high (3). The variables considered and their values (uncoded and coded form) are given in Table 1.

The physical level of each independent variable was selected based on our preliminary experiments (not reported here). The FCCCD matrix of independent variables in the form of coded values is summarized in Table 2. The number of systems subjected to experiments is 15, compared to 27 which is the number of experiments for a full factorial design. This experimental plan includes eight factorial points corresponding to 2^3 full factorial design, six axial points corresponding to the face centres of the cube portion of the design and one replicate at the centre of the design.

Minocycline release was determined using a modified Franz diffusion cell, as described in the Experimental section. The method allowed the evaluation of the kinetic profiles of the tested hydrogel compositions.

The cumulative concentrations of MH released at different time intervals were determined from the calibration curve ($A^{1cm}_{1\%} = 274$), which is linear over the concentration range 0–0.00275 g/100 mL (R = 0.9991, with zero intercept).

The *in vitro* kinetic profiles of minocycline from the designed hydrogels, plotted as drug released percentage as a function of time, are illustrated in Fig. 1 and Fig. 2.

In order to establish the drug release mechanism, the experimental kinetic data were fitted to Eq. (1) described by Peppas (1985), where m_t is the amount of drug released at time t, m_∞ is the total drug contents in the designed collagen hydrogels, m_t/m_∞ is the fractional release of the drug at the time t, k is the kinetic constant, reflecting the structural and geometrical properties of the polymeric system and the drug, and n is the release exponent, indicating the mechanism of drug release. Only the kinetic data for which $m_t/m_\infty \leq 60\%$ were used for the evaluation of the release exponent (Loughlin et al. 2008; Natu et al. 2007). The percentage of active compound released ranged between 26.32% (G6) and 52.94 (G3) during 5 h.

Different kinetic models were verified: first order, zero order and Higuchi. The values obtained for the determination coefficients (R^2) of the above mentioned models are summarized in Table 3. Comparing the values of R^2 it can be seen that the highest value is obtained for the Higuchi model for all the tested formulatons (range 0.9946 to 0.9987), which shows that release followed this model. This means that the cumulative amount of MH is proportional to the square root of time, indicating a drug release diffusional mechanism, the rate of drug diffusion through the hydrogel being much smaller that the polymer relaxation rate. The model suggested by Higuchi is applicable both to the dissolved (our case) or suspended substances in the hydrogels network (Ruiz Martinez et al. 2007; Kikwai et al. 2005; Mohamed 2004).

$$\frac{m_t}{m_\infty} = k \cdot t^n \tag{1}$$

$$D = \frac{q^2 \cdot \pi}{4 \cdot C_0^2 \cdot t} \tag{2}$$

$$J = \frac{q}{t} \tag{3}$$

$$Y_1 = 10.869 - 6.965X_1 + 13.295X_2 - 223753.846X_3^2 \tag{4}$$

$$Y_2 = 4.553X_1 - 14.750X_2 + 296.932X_3 + 20.415X_2^2 \tag{5}$$

$$Y_3 = -1.661 + 27.796X_2 - 66441.026X_3^2 - 6.190X_1X_2 \tag{6}$$

$$\frac{S}{Z} = -10 \log \left[\frac{1}{n} \sum_{i=1}^{n} \frac{1}{Y_i^2} \right] \quad \text{[dB]} \tag{7}$$

$$\frac{S}{Z} = -10 \log \left[\frac{1}{n} \sum_{i=1}^{n} Y_i^2 \right] \quad \text{[dB]} \tag{8}$$

Table 1: Physical and coded values of the independent variables used for the experimental design

Independent variables*	Coded symbol	Real values of coded levels		
		1	2	3
Collagen, C (g%)	X_1	0.9	1.1	1.3
Minocycline, MH (g%)	X_2	0.2	0.4	0.6
Glutaraldehyde, GA (g%)	X_3	0.0	0.0015	0.0030

* the amounts of C, MH and GA are reported to 100 g hydrogel

Table 2: Coded values of the variables used in different experimental trials of the FCCCD and the corresponding observed and predictive responses

Runs order	Independent variables (coded level)			Responses					
	X_1 – C	X_2 – MH	X_3 – GA	Y_1 – D·10^6 ($cm^2 \cdot min^{-1}$)		Y_2 – T (min)		Y_3 – J ·10^5 ($g \cdot cm^{-2} \cdot min^{-1}$)	
				Obs.	Pred.	Obs.	Pred.	Obs.	Pred.
G1	1	1	1	7.528	7.259	1.952	1.964	2.834	2.783
G2	3	1	1	3.793	4.474	3.466	3.786	2.183	2.288
G3	1	3	1	12.928	12.578	2.438	2.597	11.731	11.673
G4	3	3	1	8.514	9.792	4.775	4.419	10.075	10.188
G5	1	1	3	4.819	5.246	2.694	2.855	2.396	2.185
G6	3	1	3	3.324	2.460	4.234	4.677	2.067	1.690
G7	1	3	3	10.207	10.564	3.503	3.488	10.908	11.075
G8	3	3	3	7.663	7.778	5.226	5.309	9.525	9.590
G9	1	2	2	9.726	9.415	2.053	1.910	6.915	7.079
G10	3	2	2	7.985	6.629	4.624	3.731	6.021	6.088
G11	2	1	2	4.343	5.363	3.944	3.321	2.291	2.386
G12	2	3	2	11.086	10.681	3.722	3.953	11.506	10.781
G13	2	2	1	8.861	8.526	1.708	2.375	6.632	6.733
G14	2	2	3	6.213	6.512	3.241	3.266	5.711	6.135
G15	2	2	2	8.315	8.022	2.793	2.820	6.471	6.584

2.2. Analysis of response variables

In vitro release kinetic was quantified using two parameters specific to the Higuchi model. The diffusion coefficient (D, $cm^2 \cdot min^{-1}$) was determined according to Eq. (2) (Higuchi 1962), where q refers to the amount of drug released per surface unit of artificial membrane into the donor medium (g/cm^2), C_0 is the initial concentration of the drug in the hydrogel (g/mL), t is the drug release time (min).

Table 3: Determination coefficients R^2 for minocycline release from collagen hydrogels obtained using the specified kinetic models

Formulation	Higuchi	Zero order	First order
G1	0.9976	0.9788	0.9071
G2	0.9967	0.9265	0.8300
G3	0.9987	0.9532	0.8656
G4	0.9970	0.9140	0.8053
G5	0.9980	0.9747	0.8994
G6	0.9949	0.9245	0.8177
G7	0.9977	0.9152	0.8109
G8	0.9976	0.9228	0.8134
G9	0.9966	0.9253	0.8324
G10	0.9946	0.9030	0.7999
G11	0.9986	0.9504	0.8619
G12	0.9971	0.9015	0.7960
G13	0.9983	0.9252	0.8267
G14	0.9976	0.9089	0.8006
G15	0.9971	0.8975	0.7912

From the regression line $q^2 = f(t)$, the intercept (T, min) was determined, associated with the initial period preceding the drug appearance in the receiving medium (corresponding to a diffusion gradient establishment).

Besides the two parameters the drug flux (J – $g \cdot cm^{-2} \cdot min^{-1}$) released during five hours of the experiment was also determined according to Eq. (3) (Güngör and Bergişadi 2003), where, q and t have the above mentioned significance.

The kinetic parameters quantifying drug release from the hydrogels are affected by two categories of experimental factors: depending on the product formulation (donor medium) and depending on the experiment to be performed, and particularly on the membrane and nature of the receiving medium, working temperature, type of apparatus used for the experiment.

The influence of the donor medium and formulation factors respectively on the *in vitro* release kinetic of the MH from topical hydrogels was followed in the present paper.

To do this, a stepwise regression analysis with backward elimination method subroutine (Aggarwal et al. 2008) was conducted to settle the reduced quadratic polynomial models for each response mentioned in Table 2. They were coded as follows: Y_1 – diffusion coefficient (D), Y_2 – intercept (T), Y_3 – flux (J). The regressional models are described by Eqs. (4), (5), (6).

These equations quantitatively reflect the effect of the independent variables and their interactions on the kinetic dependent variables measured and include the terms found to be significant ($p < 0.05$). The terms containing two independent variables represent their interactions and the square of such a variable, the quadratic relationship. The values of the independent variable coefficients, as well as of the terms with two independent variables or squared independent variable are correlated with

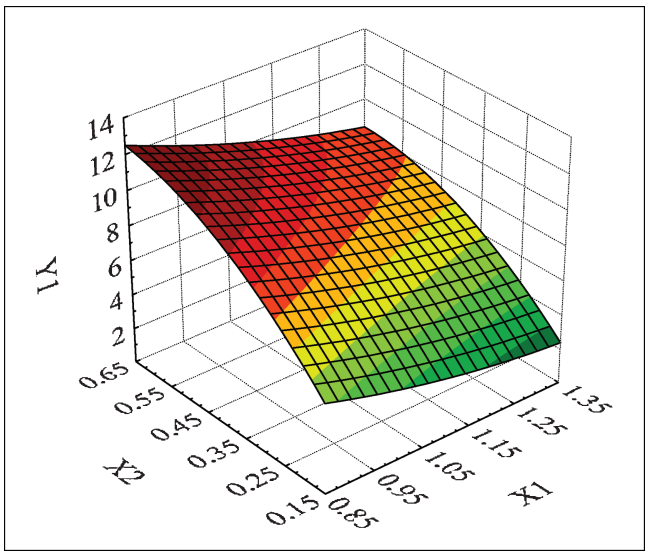

Fig. 3: Plot of response surface for the diffusion coefficient D (Y_1) as a function of collagen (X_1) and minocycline hydrochloride concentrations (X_2)

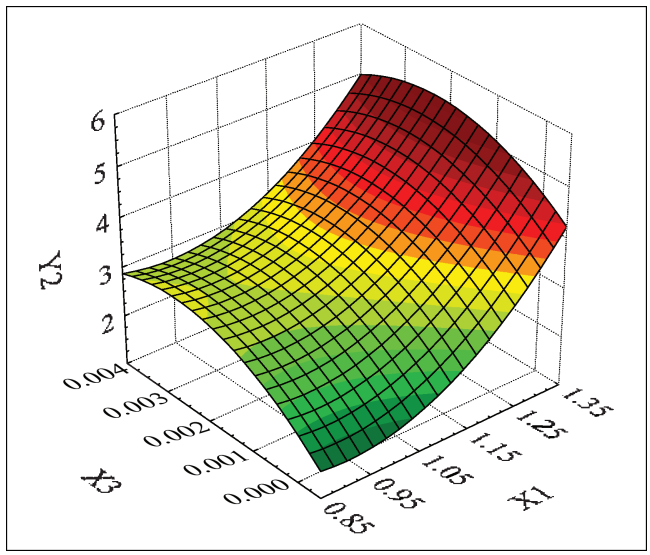

Fig. 4: Plot of response surface for the intercept T (Y_2) as a function of collagen (X_1) and glutaraldehyde concentrations (X_3)

their effects on the Y_i responses (Sun et al. 2010; Motwani et al. 2008). Thus, a positive sign indicates a synergistic effect for the responses to be maximized and an antagonic effect for the responses to be minimized, while a negative term shows a reverse situation (Chang et al. 2007). These coefficients show the conditions for which optimal values are obtained for the system response variables (*in vitro* drug rapid availability characteristics): *maximal values* for the variables Y_1 and Y_3, respectively *minimal value* for variable Y_2.

The Eq. (4) indicates a negative influence of variables X_1 (linear effect) and X_3 (quadratic effect) on MH diffusion coefficient in the hydrogel, phenomenon that can be explained by the increase of viscosity of release medium, which determines a diffusion resistance of MH. In turn, X_2 has an obvious positive effect on variable Y_2.

A positive linear and a negative quadratic effect of variable X_2 on the intercept from Eq. (5) can be seen, while X_1 and X_3 have only a negative linear effect on this response. Eq. (6) shows that X_3 (quadratic effect) and the interaction between X_1 and X_2 have a negative influence on the MH flux, the positive effect being attributed to X_2 (linear effect).

The reduced regressional models determined were validated by testing the model goodness of fit, analysis of variance (ANOVA) and residual analysis respectively.

The evaluation of the goodness of fit is given by the multiple correlation coefficient R and the determination coefficient R^2. Thus, the values of R – 0.9680, 0.9940 and 0.9971 – being closed to 1 indicate a high correlation degree between the observed and predictive values. The determination of R^2 values – 0.9370, 0.9881 and 0.9943 – suggests that about 6.3%, 1.2% and 0.6% respectively from the total variation is not explained by the model for the dependent variables tested. R^2 values being superior to 0.9, the regressional models explained very well the responses (Wu et al. 2009; Wang et al. 2008).

The statistical significance of the regressional model was tested using ANOVA and the results for all the responses are given in Table 4. The ANOVA test for each regressional model demonstrates a high statistic significance of the model, obvious from the values calculated for F-test (greater than F-tabulated in all cases) and the small p probability values (Madeira et al. 2011; Rosa et al. 2007).

In order to ensure the regressional model adequacy the residual analysis was run (Table 2). The residual analysis results show a good correspondence between the experimental values and the theoretical ones determined by the reduced predictor polynomial equations.

The relationship between each dependent variable and the independent variables is further illustrated using the response surface methodology (RSM) that allow the visualization of the formulation factors effects on responses in the tridimensional space. Beside the responses investigation on the full range of the influence factors, the objective of RSM is also the localization of the interest areas where the optimal or near the optimal response values can be found (Aslan 2008; Chang et al. 2007). Some response surfaces (3D) are given for exemplification in Fig. 3 to Fig. 6.

As can be seen in Fig. 3, higher diffusion coefficients are recorded for high MH concentrations and small collagen concentrations, MH having the most pronounced effect. Thus, for the higher concentration of MH, Y_1 increases from $7.663 \cdot 10^{-6}$ to $12.928 \cdot 10^{-6}$ $cm^2 \cdot min^{-1}$ (68.71% increase) when the collagen concentration decreases, while for the smallest concentration of MH it decreases from $4.819 \cdot 10^{-6}$ to $3.324 \cdot 10^{-6}$ $cm^2 \cdot min^{-1}$ (31.02% decrease) with collagen concentration increase.

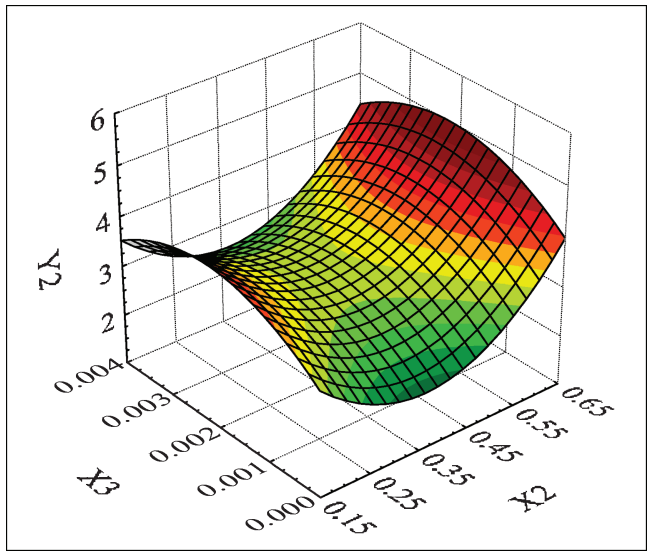

Fig. 5: Plot of response surface for the intercept T (Y_2) as a function of minocycline hydrochloride (X_2) and glutaraldehyde concentration (X_3)

Table 4: Analisis of variance (ANOVA) for the reduced regressional polynomial models

Responses	Sources of variation	Sum of squares	df	Mean squares	F-value	p-value
Y_1	Regresion	101.093	3	33.697	54.616	<0.0001
	Residual	6.786	11	0.617		
	Total	107.879	14			
Y_2	Regresion	183.370	4	45.842	230.229	<0.0001
	Residual	2.190	11	0.199		
	Total	185.560	15			
Y_3	Regresion	180.092	3	60.031	649.245	<0.0001
	Residual	1.017	11	0.092		
	Total	181.109	14			

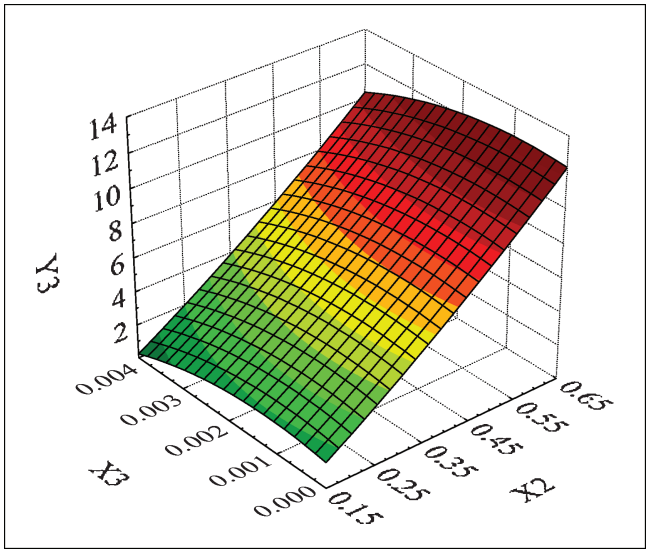

Fig. 6: Plot of response surface for the flux J (Y_3) as a function of minocycline hydrochloride (X_2) and glutaraldehyde concentration (X_3)

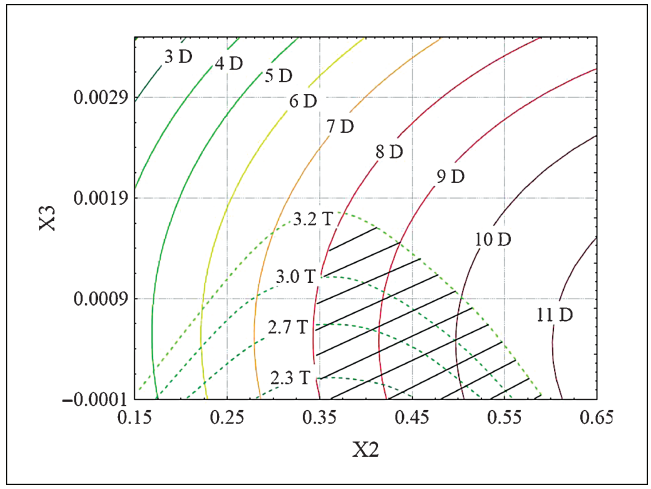

Fig. 7: Superposed contour plots for Y_1 (D ———) and Y_2 (T - - - -) as a function of X_2 (MH) and X_3 (GA)

The response surface plot for the intercept Y_2 (Fig. 4) shows that the smaller values were observed for both low collagen and glutaraldehyde concentrations, the collagen influencing strongly this response. For a high concentration of collagen Y_2 increases from 3.466 to 5.226 minutes (with 50.78%) with the increase of GA, a small concentration of collagen inducing a decrease of Y_2 values from 3.503 to 1.952 min (44.28% decrease).

Figure 5 also shows the intercept dependence on MH and GA concentration. A small intercept is favoured by MH concentration at middle level and small concentration of GA. Both formulation factors have a determinant influence on Y_2: the values varies from 4.624 to 1.708 min with the decrease of the GA concentration (63.06% decrease) for collagen concentration at middle level, and from 1.778 to 4.775 min (179.57% increase) with the increase of collagen concentration and the GA at the minimum level.

The MH flux, presented in Fig. 6 as a function of MH and GA concentrations, shows that the highest values of Y_3 are recorded for MH concentrations at maximum level and minimal GA concentrations. Obviously, only the MH concentration has a strong influence on Y_3, with a flux increasing significantly from 2.183 to 11.731 (437.38%) for the GA concentration at minimum level.

The response field of the dependent variables and selection of the pharmaceutical formulations that give the desired kinetic responses, maximal/minimal, was then realized using the level curves superposing method. By superposing the profile charts corresponding to 2 and 3 dependent variables as function of the same 2 independent variables, it is possible to determine the pairs of values that represent a formulation for which the maxim/minim conditions of the paramenters to be optimized are met. The optimum areas for the responses and consequently the acceptable ranges for each formulation factor were found by superposing the individual response surfaces contour plots. As the optimum of individual formulation factors is not located exactly in the same region in the 2D space, it is necessary to set constraints for which all the responses reach their optimum as function of the same variables (Bezerra et al. 2008).

In Fig. 7 and Fig. 8 superposing of the contour plots for Y_1 and Y_2 as function of X_2 and X_3 as well as for Y_1, Y_2 and Y_3 respectively as function of X_1 and X_2 is given as example. The hatched

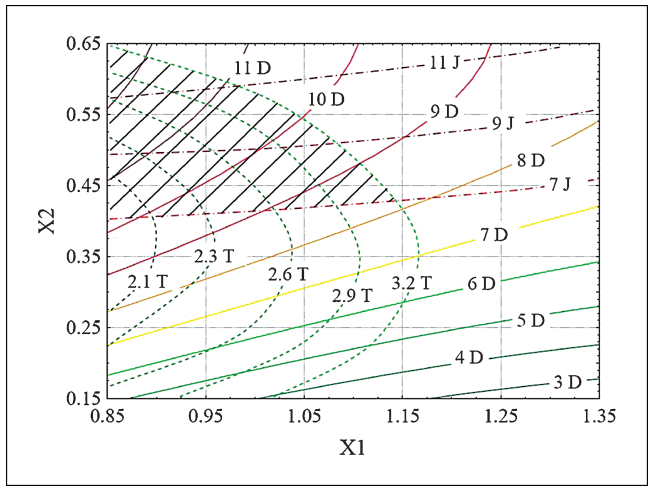

Fig. 8: Superposed contour plots for Y_1 (D ———), Y_2 (T - - - -) and Y_3 (J — · — · —) as a function of X_1 (C) and X_2 (MH)

areas are defining the formulation factor ranges of variation that resulted in optimal responses (maximal for diffusion coefficient and flux and minimal for the intercept).

Figure 8 shows that a GA concentration between the low and middle level and a MH concentration between the middle and high one leads to maximal responses for the diffusion coefficient and minimum responses for the intercept.

In Fig. 8 it can be seen that a MH concentration between the middle and high level and a collagen concentration between the low and middle level is determining maximal responses for the diffusion coefficient and flux and minimal responses for the intercept.

By simultaneous optimization of responses, the optimum variation levels for the formulation factors were set as follows: X_1: [0.90÷1.13]%, X_2: [0.40÷0.60]%, X_3: [0.000÷0.0016]%.

Besides the hydrogels whose formulation factors meet the conditions in the ranges mentioned above, other hydrogels with MH enhanced release characteristics can also be designed by modulation of the composition within the optimum variation range limit.

2.3. Taguchi's technique

The selection of an optimal formulation consists *not only* in obtaining the best values of the responses (maximum, minimum), but also in finding the conditions for which the characteristics vary to the minimum extent (Kuang et al. 2009). Thus, the steps of statistic experimental design previously analyzed were further complemented with Taguchi's approach elements as a method for the determination of the optimization process quality. This technique is a tool for process improvement and not for absolute optimization. The evaluation of performances in the process of modeling the MH release from hydrogels consists in the selection of some systems with proper levels of formulation factors which ensures a kinetic response variability as low as possible and, consequently, a design robustness (Kuang et al. 2009; Aggarwal et al. 2008).

In the frame of Taguchi's approach the independent variables (X_1, X_2, X_3) are considered *control factors*, which need to be optimized for reaching a specified value and to eliminate the variation (Lyu et al. 2010). Besides the control factors, the system responses can be affected by *the noise factors*, influenced by the process deployment conditions, which are defined as the unwanted variability determining the decrease of the optimization process quality. Noise factors always exist, so it is useless to try to eliminate them; this is why their impact on the optimization process (MH release kinetic from topical hydrogels) has to be minimized in order to reduce the variability (Dellino et al. 2010; Pickle et al. 2008).

Taguchi brings in a performance indicator named signal-to-noise ratio (S/N), expressed in decibels [dB]. Its value is a performance measure for the process to be optimized (Aggarwal et al. 2008). Among the indicators suggested by Taguchi, the S/N ratios were used for the criteria that have to be maximized/minimized as follows: (i) S/N ratio for a criterion that has to be maximized– "larger-the-better" – larger values meaning better results, applied to the kinetic parameters Y_1 and Y_3 and calculated according to Eq. (7); (ii) S/N ratio for a criterion that has to be minimized – "smaller-the-better" – smaller values meaning better results, used for the kinetic parameter Y_2 and computed according to Eq. (8), where Y_i is the response measured for the combination of i independent variables and n the number of experiments performed (Pickle et al. 2008).

The determination of the S/N ratio under optimal conditions leads to the combination of the formulation factor variation levels, respectively their effects size, that determine the kinetic

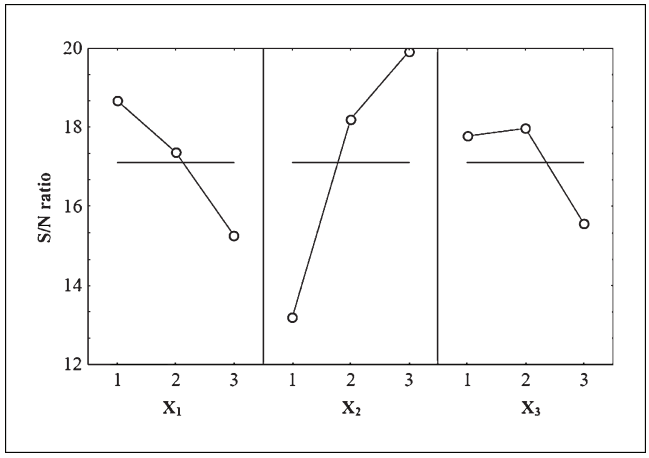

Fig. 9: Control factors effects on S/N ratio for the dependent variable Y_1

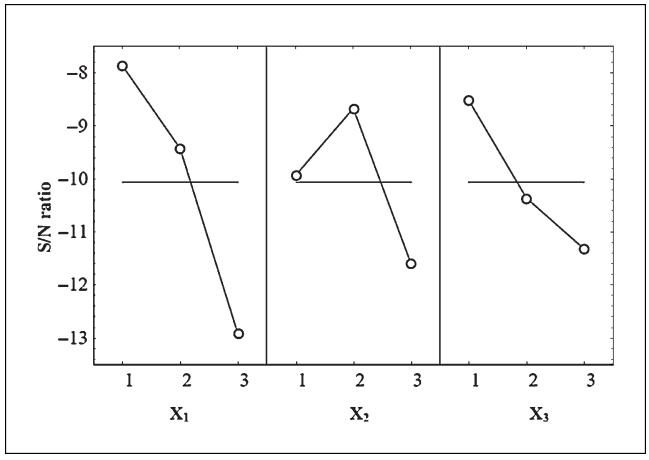

Fig. 10: Control factors effects on S/N ratio for the dependent variable Y_1

responses affected to the minimum extent by the noise factors (Table 5), increasing in this way the performance and quality of the optimization process.

The graphic conversion of the control factors effects on the S/N ratio for each dependent variable emphasizing the optimal combination of the formulation variables is given in Figs. 9–11.

The analysis of the S/N ratio in Figs. 9–11 shows a significant difference between X_2 variation levels for Y_1 and Y_3, which means a strong influence on these responses, while for Y_2 the influence of this formulation factor is less important. The optimal coded levels of this independent variable, which reduce the noise factors effects, are 3 for Y_1 and Y_3 (corresponding to a

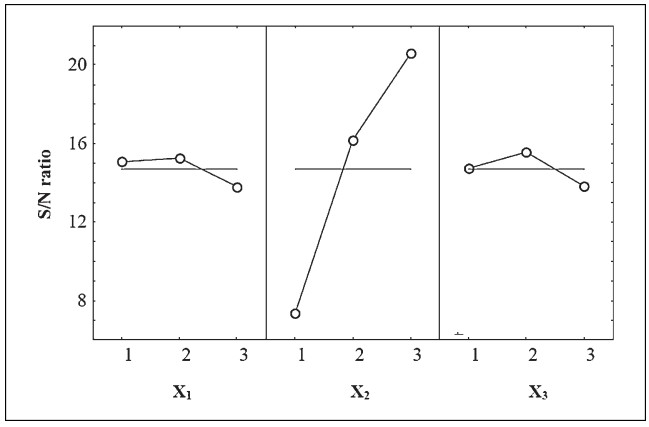

Fig. 11: Control factors effects on S/N ratio for the dependent variable Y_2

Table 5: Optimal combinations of independent variables coded levels, their effect size on S/N ratio for the dependent variables and expected S/N value

Control factors (independent variables)	Y_1		Y_2		Y_3	
	"larger – the– better"	effect size	"smaller – the – better"	effect size	"larger – the– better"	effect size
X_1	1	1.567	1	2.206	2	0.567
X_2	3	2.814	2	1.399	3	5.926
X_3	2	0.865	1	1.551	2	0.732
S/N ratio (dB)	22.351		−4.908		21.899	

MH concentration of 0.6) and 2 for Y_2 (corresponding to a MH concentration of 0.4).

Considering X_1, the biggest difference between the variation levels is observed for Y_2, while for Y_1 it is less important and for Y_3 is low. The X_1 optimal coded levels leading to the noise factors reduction are 1 for Y_1 and Y_2 responses and 2 for Y_3 response.

For X_3, the most important difference between the variation levels is detected for Y_2, those for Y_1 and Y_3 being low. The optimal coded levels for this independent variable resulting in a reduction of the noise factors effects are 2 for Y_1 and Y_3 responses and 1 for Y_2.

Comparing the effect size of the independent variables on the S/N ratio we can see that MH concentration has the main influence on the diffusion coefficient (effect size 1.79 times higher than that of the collagen concentration and 3.25 times higher than the GA concentration) and a strong influence on the flux (effect size 10.45 times higher than the collagen concentration and 8.09 times higher than the GA concentration).

The collagen concentration has an influence on the diffusion coefficient, but the main influence is on the intercept (MH and GA concentration have also an influence on Y_2, the effect size being 1.58 times higher than the MH concentration and 1.42 times higher than the GA concentration). Glutaraldehyde influences more the intercept and less the diffusion coefficient and the flux.

Applying Taguchi techniques we finally selected three optimal formulations (belonging to the ranges previously identified) which individually ensure the responses robustness and the increase of the resistance to the noise factors. The combination of independent variables coded levels under the form 2:3:2 leading to a flux affected to the minimum extent by the noise factors corresponds to the hydrogel G12 included in the 3^3 fractional design, while the combinations of independent variables coded levels under the forms 1:3:2 and 1:2:1 leading to a diffusion coefficient, respectively intercept, affected to the minimum extent by the noise factors do *not* belong to the 3^3 fractional design. These last two formulations, coded G16 and G17, were prepared and tested under the same experimental conditions as the other hydrogels and the kinetic parameters previously described were determined (Table 6).

For the G12, G16 and G17 formulations we also evaluated the theoretical values from the regressional model for the kinetic parameters Y_1, Y_2 and Y_3. A good correspondence between the predictive and observed values can be seen, proving a high predictive power of the reduced polynomial equations (Table 6).

2.4. Conclusion

A statistical approach based on Response Surface Methodology (RSM) and Taguchi technique elements was used for the pharmaceutical design and optimization of some minocycline hydrochloride-collagen based hydrogels with potential application in infected cutaneous wounds. As Taguchi's technique, through the performance indicator S/N, can provide for each response to be optimized only specific given values of the formulation parameters, a RSM is needed in the case of multiple responses to be optimized. Using RSM makes possible the visualization of the entire range of independent variables effect on all the dependent variables and the optimum variation intervals of the formulation factors which lead to the desired responses – *in vitro* drug rapid availability characteristics – were obtained. Combining these two methodologies the optimal responses were obtained, which are also stable, insensitive and robust to the action of different noise factors.

3. Experimental

3.1. Materials

Type I collagen gel having a concentration of 1.99% (w/w) and pH 2.1 was obtained from calf hide by the currently used technology in Collagen Department of Division Leather and Footwear Research Institute (Albu 2011). Minocycline hydrochloride (MH) was purchased from Sigma (Germany). Glutaraldehyde was obtained from Sigma-Aldrich (Germany). Sodium hydroxide, monobasic potassium phosphate and disodium hydrogen phosphate were supplied by Merck (Germany). Water used was distilled and all other chemicals were of analytical grade.

Table 6: Composition of optimal formulations and the observed and predicted values of response variables

Hydrogel	Composition of optimal formulation X_1: X_2: X_3 (g%: g%: g%)	Response variable	Observed value	Predictive value	Predicted error (%)
G12	1.1:0.6:0.0015	$Y_1 \cdot 10^6$ ($cm^2 \cdot min^{-1}$)	11.086	10.681	+3.79
		Y_2 (min)	3.722	3.953	−5.84
		$Y_3 \cdot 10^5$ ($g \cdot cm^{-2} \cdot min^{-1}$)	11.506	10.781	+6.72
G16	0.9:0.6:0.0015	$Y_1 \cdot 10^6$ ($cm^2 \cdot min^{-1}$)	12.269	12.074	+1.61
		Y_2 (min)	2.947	3.042	−3.12
		$Y_3 \cdot 10^5$ ($g \cdot cm^{-2} \cdot min^{-1}$)	11.607	11.524	+0.72
G17	0.9:0.4:0.0000	$Y_1 \cdot 10^6$ ($cm^2 \cdot min^{-1}$)	10.712	9.918	+8.01
		Y_2 (min)	1.545	1.464	+5.53
		$Y_3 \cdot 10^5$ ($g \cdot cm^{-2} \cdot min^{-1}$)	7.142	7.229	+1.20

3.2. Preparation of hydrogels

Collagen hydrogels having the concentrations 0.9%, 1.1% and 1.3% and pH 7.37 (inoLAB pH-meter) were obtained from the initial 1.99% collagen gel with pH 2.1 by diluting under stirring with distilled water, NaOH 1 M solution (680 rpm VELP mechanical stirrer) and proper amounts of minocycline hydrochloride solution (1800 rpm, FALC magnetic stirrer) to obtain the concentrations 0.2%, 0.4% and 0.6% reported to the amount of hydrogel (the density of hydrogel is practically 1 g/cm^3). Some of the hydrogels were crosslinked at 4 °C with 0.0015% and 0.0030% glutaraldehyde for 24 h.

3.3. In vitro release studies

The *in vitro* release of MH was measured by a modified Franz diffusion cell: a peristaltic pump (Masterflex, Cole-Parmer Instrument Company) and a buffer vessel for sample withdrawal were adapted. The peristaltic pump ensures the continuous circulation of the liquid in closed circuit into the receiving compartment allowing a supplementary homogenization, in addition to the one realized by the magnetic stirrer (IKA lab disc white). A phosphate buffer solution of pH 7.4 at 37 ± 0.5 °C (ThermoHaake P5 ultrathermostat) was used as receiving medium to simulate an infected wound environment (Loughlin et al. 2008). Sink conditions were ensured through the continous recirculation of the liquid in the receiving compartment during the whole experiment. After a previous hydration for 24 h in the receiving medium, the cellophane membrane (Autogen Bioclear Ltd) was fitted between the donor and receptor compartments. After 30 min of recirculation of the stirred receiving solution about 1 g from each tested formulation was applied on the artificial membrane surface using a proper syringe, ensuring an intimate contact between hydrogel and membrane. Samples of 5 ml were collected from the buffer vessel at predetermined time intervals, replaced by equal volumes of phosphate buffer solution preheated at 37 ± 0.5 °C. The samples were spectrophotometrically analyzed at 348 nm (Perkin-Elmer spectrophotometer).

3.4. Design of the experiments and statistical data treatment

The hydrogels were obtained according to a 3-factor, 3-level face-centered central composite design summarized in the Table. The kinetic experiments were carried out in triplicate and randomly. The kinetic responses and the formulation variables for all the designed systems were analyzed by Statistica StatSoft Release 8 software. A stepwise regression analysis was conducted to build the second order polynomial equations for all response variables. The significant terms ($p < 0.05$) were chosen for the final equations. The best fitting mathematical model was selected based on the determination coefficient, correlation coefficient, analysis of variance and residual analysis. To estimate the combined effect of independent variables on the responses and to select the optimal formulation, three-dimensional response surface and contour plotting were also drawn. Taguchi Signal/Noise ratio was finally used for the evaluation of the design robustness.

Acknowledgement: This work was financially supported by CNCSIS-UEFISCDI, Postdoctoral Fellowship Programme PN-II-Human Resources, project number 3/28.07.2010, code PD 149/2010, Romania.

References

Aggarwal A, Singh H, Kumar P, Singh M (2008) Optimizing power consumption for CNC turned parts using response surface methodology and Taguchi's technique – A comparative analysis. J Mater Process Tech 200: 373–384.

Albu MG (2011) Collagen gels and matrices for biomedical applications. Lambert Academic Publishing, Saarbrücken, p. 23–24.

Aoyagi S, Onishi H, Machida Y (2007) Novel chitosan wound dressing loaded with minocycline for the treatment of severe burn wounds. Int J Pharm 330: 138–145.

Aslan N (2008) Application of response surface methodology and central composite rotatable design for modeling and optimization of a multi-gravity separator for chromite concentration. Powder Technol 185: 80–86.

Bajaj IB, Lele SS, Singhal RS (2009) A statistical approach to optimization of fermentative production of poly(γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324. Bioresource Technol 100: 826–832.

Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76: 965–977.

Bishburg E, Bishburg K (2009) Minocycline-an old drug for a new century: emphasis on methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii*. Int J Antimicrob Ag 34: 395–401.

Boateng JS, Matthews KH, Stevens HNE, Eccleston GM (2008) Wound healing dressings and drug delivery systems: a review. J Pharm Sci 97: 2892–2923.

Chang JS, Huang YB, Hou SS, Wang RJ, Wu PC, Tsai YH (2007) Formulation optimization of meloxicam sodium gel using response surface methodology. Int J Pharm 338: 48–54.

Corsetti G, D'Antona G, Dioguardi FS, Rezzani R (2010) Topical application of dressing with amino acids improves cutaneous wound healing in aged rats. Acta Histochem 112: 497–507.

Dellino G, Kleijnen JP, Meloni C (2010) Robust optimization in simulation: taguchian and response surface methodology. Int J Prod Econ 125: 52–59.

Elsner JJ, Zilberman M (2010) Novel antibiotic-eluting wound dressings: an *in vitro* study and engineering aspects in the dressing's design. J Tissue Viability 19: 54–66.

Fermoso J, Gil MV, Arias B, Plaza MG, Pevida C, Pis JJ, Rubiera F (2010) Application of response surface methodology to assess the combined effect of operating variables on high-pressure coal gasification for H_2-rich gas production. Int J Hydrogen Energ 35: 1191–1204.

Figueiró SD, Macêdo AAM, Melo MRS, Freitas ALP, Moreira RA, de Oliveira RS, Goes JC, Sombra ASB (2006) On the dielectric behaviour of collagen–algal sulfated polysaccharide blends: effect of glutaraldehyde crosslinking. Biophys Chem 120: 154–159.

Goissis G, de Sousa MH (2009) Characterization and *in vitro* release studies of tetracycline and rolitetracycline imobilized on anionic collagen membranes. Materials Res 12: 69–74.

Gopinath D, Rafiuddin Ahmed M, Gomathi K, Chitra K, Sehgal PK, Jayakumar R (2004) Dermal wound healing processes with curcumin incorporated collagen films. Biomaterials 25: 1911–1917.

Güngör S, Bergişadi N (2003) *In vitro* release studies on topical gel formulations of nimesulide. Pharmazie 58: 155–156.

Hamidi M, Azadi A, Rafiei P (2008) Hydrogel nanoparticles in drug delivery. Adv Drug Deliv Rev 60: 1638–1649.

Higuchi WI (1962) Analysis of data on the medicaments release from ointments. J Pharm Sci 51: 802–804.

Judith R, Nithya M, Rose C, Mandal AB (2010) Application of a PDGF-containing novel gel for cutaneous wound healing. Life Sci 87: 1–8.

Kikwai L, Babu RJ, Prado R, Kolot A, Armstrong CA, Ansel JC, Singh M (2005) *In vitro* and *in vivo* evaluation of topical formulations of spantide II. AAPS PharmSciTech 6: 565–572.

Kuang SS, Oliveira JC, Crean AM (2009) An analysis of the influence of multiple processing factors on the characteristics of bioactive-loaded beads prepared by extrusion–spheronisation. Food Bioprocess Techn, published on line 30 December.

Lin YC, Tan FJ, Marra KG, Jan SS, Liu DC (2009) Synthesis and characterization of collagen/hyaluronan/chitosan composite sponges for potential biomedical applications. Acta Biomater 5: 2591–2600.

Loughlin RG, Tunney MM, Donnelly RF, Murphy DJ, Jenkins M, McCarron PA (2008) Modulation of gel formation and drug-release characteristics of lidocaine-loaded poly(vinyl alcohol)-tetraborate hydrogel systems using scavenger polyol sugars. Eur J Pharm Biopharm 69: 1135–1146.

Lyu SR, Wu WT, Hou CC, Hsieh WH (2010) Study of cryopreservation of articular chondrocytes using the Taguchi method. Cryobiology 60: 165–176.

Madeira C, Ribeiro SC, Pinheiro ISM, Martins SAM, Andrade PZ, da Silva CL, Cabral JMS (2011) Gene delivery to human bone marrow mesenchymal stem cells by microporation. J Biotechnol 151: 130–136.

Mannan S, Fakhru'l-Razi A, Alam MZ (2007) Optimization of process parameters for the bioconversion of activated sludge by *Penicillium corylophilum*, using response surface methodology. J Environ Sci 19: 23–28.

Meade KR, Silver FH (1990) Immunogenicity of collagenous implants. Biomaterials 11: 176–180.

Mohamed MI (2004) Optimization of chlorphenesin emulgel formulation. AAPS J 6: 81–87.

Motwani SK, Chopra S, Talegaonkar S, Kohli K, Ahmad FJ, Khar RK (2008) Chitosan–sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: formulation, optimization and *in vitro* characterization. Eur J Pharm Biopharm 68: 513–525.

Natu MV, Sardinha JP, Correia IJ, Gil MH (2007) Controlled release gelatin hydrogels and lyophilisates with potential application as ocular inserts. Biomed Mater 2: 241–249.

Peppas NA (1985) Analysis of Fickian and non-Fickian drug release from polymers. Pharm Acta Helv 60: 110–111.

Pickle SM, Robinson TJ, Birch JB, Anderson-Cook CM (2008) A semiparametric approach to robust parameter design. J Stat Plan Infer 138: 114–131.

Rhee YS, Chang SY, Park CW, Chi SC, Park ES (2008) Optimization of ibuprofen gel formulations using experimental design technique for enhanced transdermal penetration. Int J Pharm 364: 14–20.

Ribeiro NFF, Heath CH, Kierath J, Rea S, Duncan-Smith M, Wood F.M (2010) Burn wounds infected by contaminated water: case reports, review of the literature and recommendations for treatment. Burns 36: 9–22.

Rosa PAJ, Azevedo AM, Aires-Barros MR (2007) Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. J Chromatogr A 1141: 50–60.

Ruiz Martinez MA, López-Viota Gallardo J, de Benavides MM, de Dios García López-Duran J, Gallardo Lara V (2007) Rheological behavior of gels and meloxicam release. Int J Pharm 333: 17–23.

Stojadinovic A, Carlson JW, Schultz GS, Davis TA, Elster EA (2008) Topical advances in wound care. Gynecol Oncol 111: S70–S80.

Sun Y, Liu J, Kennedy JF (2010) Application of response surface methodology for optimization of polysaccharides production parameters from the roots of *Codonopsis pilosula* by a central composite design. Carbohyd Polym 80: 949–953.

Sung JH, Hwang MR, Kim JO, Lee JH, Kim YI, Kim JH, Chang SW, Jin SG, Kim JA, Lyoo WS, Han SS, Ku SK, Yong CS, Choi HG (2010) Gel characterisation and *in vivo* evaluation of minocycline-loaded wound dressing with enhanced wound healing using polyvinyl alcohol and chitosan. Int J Pharm 392: 232–240.

Wang C, Varshney RR, Wang DA (2010) Therapeutic cell delivery and fate control in hydrogels and hydrogel hybrids. Adv Drug Deliv Rev 62: 699–710.

Wang L,Yang B, Du X, Yang Y, Liu J (2008) Optimization of conditions for extraction of acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) by response surface methodology. Innov Food Sci Emerg 9: 604–607.

Wu S, Yu X, Hu Z, Zhang L, Chen J (2009) Optimizing aerobic biodegradation of dichloromethane using response surface methodology. J Environ Sci 21: 1276–1283.

Yao JS, Shen F, Young WL, Yang GY (2007) Comparison of doxycycline and minocycline in the inhibition of VEGF-induced smooth muscle cell migration. Neurochem Int 50: 524–530.

Yudanova TN, Reshetov IV (2006) Modern wound dressings: manufacturing and properties. Pharm Chem J 40: 85–92.

Zhang L, Li K, Xiao W, Zheng L, Xiao Y, Fan H, Zhang X (2011) Preparation of collagen–chondroitin sulfate–hyaluronic acid hybrid hydrogel scaffolds and cell compatibility *in vitro*. Carbohyd Polym 84: 118–125.

Zilberman M, Elsner JJ (2008) Antibiotic-eluting medical devices for various applications. J Control Release 130: 202–215.