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Response surface methodology and Taguchi approach to assess the combined effect of formulation factors on minocycline delivery from collagen sponges

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An important aspect to be considered in the healing of acute or chronic cutaneous wounds is the associated potential infection management. Collagen, the most abundant protein of the extracellular matrix, with proven properties in wounds healing and tissues regeneration, is one of the most widely used biopolymers as carrier matrix for controlled drug delivery systems. For this reason, the purpose of the current paper is the development of some minocycline-loaded collagen topical sponges uncross-linked and cross-linked with glutaraldehyde, obtained by lyophilization of appropriate hydrogels prepared according to the 3-factor, 3-level face-centered central composite design. The determination of drug delivery from the sponges was performed by assessment of some physicochemical parameters involved in this complex process: sponges surface wettability, swelling ratio and the percentage of minocycline released from the sponges. The application of the response surface methodology allowed the setting of the formulation parameters optimum ranges, which ensure an adequate minocycline release to the application site. The design robustness was checked using the signal-to-noise ratio performance indicator. The optimum collagen-minocycline sponges determined based on the statistical screening technique could be suitable for topical drug delivery in infected wounds healing with moderate to high exudate.

1. Introduction

A major problem encountered in the healing of cutaneous lesions is represented by the bacterial invasion and multiplication control. Infections are among the most serious complications that can occur during wound healing with high costs for treatment. So the first step in the cutaneous wounds healing improvement is the prevention and/or treatment of wound infections (Mi et al. 2002; Elsner et al. 2011).

The main solution for a selective and efficient removal of pathogenic bacteria is the antibiotics use (Ruszczak and Friess 2003; Kim et al. 2005; Prabu et al. 2008; Huang and Fu 2010). Among the commonly used antibiotics, minocycline, a semisynthetic second generation tetracycline with a broad antibacterial activity, was selected as drug model due to its high potential to act against different germs that can infect an existing lesion (Aoyagy et al. 2007; Parolo et al. 2010; Sung et al. 2010).

Antibiotic delivery directly to the wound, and in a controlled manner to maintain a sufficient and effective drug concentration at the infection site, is essential to control the pathogens proliferation rate (Sripriya et al. 2004; Elsner and Zilberman 2009; Elsner et al. 2011). The local drug delivery offers several advantages compared with the systemic administration route: systemic toxicity and associated side effects avoidance, patient increased compliance (Dias et al. 2011), and in addition, in the particular case of the infected cutaneous wound healing, a low rate of bacterial resistance (Ruszczak and Friess 2003; Boateng et al. 2008).

In recent years topical administration of antibiotics in infected lesions is based on the use of natural origin biopolymers as potential vehicles for drug controlled release systems (Malafaya et al. 2007; Huang and Fu 2010).

Particularly, considerable attention was given to collagen, a significant component of the extracellular matrix, easy to purify and isolated from various animal species by chemical and enzymatic treatments. Collagen shows biodegradability, bioresorbability, high biocompatibility, hemostasis ability, no toxicity, low immunogenicity, well-known structure, compliance to mechanical stability, reduced manufacturing cost, possibility to be processed in various forms (Sripriya et al. 2004; Coelho et al. 2010a; Liu et al. 2011; Zhang et al. 2011). These features recommend collagen as a very attractive biomaterial for use as drugs delivery system for different biomedical applications, the porous materials (sponges, collagen matrices) being selected as carrier matrix in the present paper.

The collagen sponges showed significant potential in wound healing and tissue regeneration and, more than that, prevent and/or treat the bacterial invasion infections by the antibiotic presence, protecting also the subsequent tissue damage (Prabu et al. 2008).

When collagen is used as support, its cross-linking treatment is an effective method to model the mechanical and degradation properties of sponges (Ma et al. 2004; Liu et al. 2011). Therefore, glutaraldehyde, one of the most used cross-linking agents, was selected as cross-Linking agent for the collagen sponges with minocycline (Angele et al. 2004; Lungu et al. 2011).

In our previous reported studies (Ghica et al. 2011a), we presented a design and optimization method of some topical collagen-based hydrogels containing minocycline, uncross-linked and cross-linked with glutaraldehyde, with potential applications in infected wounds with low exudate. For the wounds with moderate to high exudate, the lyophilized forms of hydrogel – sponges – are more effective (Elsner and Zileberman 2010), having the ability to absorb large amounts of wound fluid (Sripriya et al. 2004).

Thus, the aim of the current study was the analysis, modeling and optimization of some minocycline-loaded collagen sponges obtained from hydrogels designed according to the 3-factor, 3-level face-centered central composite design (FCCCD), investigating also the influence of the formulation factors selected on some physical-chemical parameters (contact angle, swelling capacity and kinetics characteristics) which determine the complex mechanism of drug release from sponges compared with the hydrogels.

2. Investigations, results and disscusion

2.1. Statistical design of experiments

The collagen hydrogels used for the preparation of the corresponding porous forms through lyophilization were obtained in accordance with the 3^3 FCCCD detailed in our previous studies (Ghica et al. 2011a). The formulation factors selected as well as their variation levels are given in Table 1: collagen (C), minocycline hydrochloride (MH) and glutaraldehyde (GA) concentrations. We investigated the influence of the above mentioned independent variables on some response parameters affecting the drug release properties from sponges, with the specific constraints imposed by the use of these porous forms in the infected cutaneous wounds healing (Table 1). The spongious forms were coded as M1 to M15. The FCCCD applied to conduct the experiments in this paper is illustrated in Table 2.

2.2. Sponges surface wettability

For a better understanding of the drug release profiles from the collagen sponges monitoring of the porous surface properties is needed, as they influence the interactions between the support and the biological environment (Coelho et al. 2010b; Tonda-Turo et al. 2011). In this research the characterization of collagen sponges surface was achieved through the surface wettability and hydrophilicity evaluation (Cheng and Teoh 2004; Liu et al. 2011), quantified in terms of contact angle values (Santiago et al. 2006; Zhao et al. 2007).

For the porous structures the contact angle is not unique, but dynamic (Shang et al. 2008). The contact angle decreases in time due to water penetration in the porous structure of the collagen matrix. The KSV CAM 101 device allowed the determination of an average contact angle which represents an useful and quick indicator for the evaluation of the sponges surface wettability, as well as an efficient method for the study of the pharmaceutical forms composition influence on it.

In order to improve the spongious surfaces hydrophilicity and to ensure a good wetting capacity by the biologic fluids at the application site, low values of the contact angle are targeted in the design of these supports. For higher contact angle values the surface becomes more hydrophobic, the fluid being unable to diffuse in the suport structure. The values of the contact angles for all designed systems were inferior to 90°; these values varied between 63.86° and 89.98° (Table 2), indicating that sponges surface had a good wetting capacity by the fluid and allowed in this way its permeation into the sponges.

2.3. Sponges swelling behaviour

By absorbing an adequate amount of biological fluid (exudate) from a cutaneous wound site, the spongious forms get properties similar to living tissues (Tonda-Turo et al. 2011) securing an increased biocompatibility at the application site. Also the penetration, swelling and biologic fluids retention capacity of the porous pharmaceutical forms are some very important aspects in the biomedical applications because they affect the morphology and structure of such forms (Prabaharan et al. 2007; Albu 2011; Thakur et al. 2012), as well as the drugs delivery (Dias et al. 2011). An efficient release profile with an increased drug remanence time at the application site is determined by the fluid uptake ability.

Practically, a sponge for infected cutaneous wound healing has to provide an efficient management of the biologic fluid and to have reservoir properties for a suitable release of drug after the exudate penetration in the spongious support porous structure, simultaneously. As the sponge absorbs an increased amount of fluid and swells more, the drug will diffuse more easily.

In Table 2 the swelling index of the collagen sponges after being kept for 8 h in phosphate buffer 7.4 is presented. The designed supports show an excellent swelling capacity with phosphate

Table 1: Process variables and the experimental conditions in 3^3 face-centered central composite design

Independent variables*	Coded symbol	Coded and uncoded variation levels		
		Low (1)	Middle (2)	High (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	0.0000	0.0015	0.0030

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

Dependent variables	Coded symbol	Constraints
Contact angle, CA (°)	Y_1	Minimize
Swelling ratio, SR (g/g)	Y_2	Maximize
Percent released, PR (%)	Y_3	Maximize

Table 2: Formulation factor values in the different FCCCD experimental assays for the hydrogels used to prepare the corresponding sponges; the observed and predictive responses for the sponges

Trials no.	Independent variables (coded level)			Responses					
	X_1 – C	X_2 – MH	X_3 – GA	Y_1 – CA (°)		Y_2 – SR (g/g)		Y_3 – PR (%)	
				Obs.	Pred.	Obs.	Pred.	Obs.	Pred.
1	1 (0.9)	1 (0.2)	1 (0.0000)	69.12	68.81	42.01	40.38	92.09	94.73
2	3 (1.3)	1 (0.2)	1 (0.0000)	74.11	74.10	30.72	32.04	76.86	77.79
3	1 (0.9)	3 (0.6)	1 (0.0000)	63.86	62.15	41.19	41.94	88.51	88.63
4	3 (1.3)	3 (0.6)	1 (0.0000)	78.87	78.00	31.98	33.60	69.22	71.69
5	1 (0.9)	1 (0.2)	3 (0.0030)	72.03	73.29	38.79	37.93	86.23	84.31
6	3 (1.3)	1 (0.2)	3 (0.0030)	80.97	78.57	29.02	29.59	67.15	67.37
7	1 (0.9)	3 (0.6)	3 (0.0030)	77.52	75.58	33.44	34.59	75.85	78.21
8	3 (1.3)	3 (0.6)	3 (0.0030)	89.98	91.42	27.34	26.25	62.95	61.27
9	1 (0.9)	2 (0.4)	2 (0.0015)	67.08	69.96	43.12	44.23	93.12	89.84
10	3 (1.3)	2 (0.4)	2 (0.0015)	82.30	80.52	37.88	35.89	74.92	72.90
11	2 (1.1)	1 (0.2)	2 (0.0015)	75.18	73.69	34.77	35.36	83.59	83.65
12	2 (1.1)	3 (0.6)	2 (0.0015)	76.66	76.79	36.92	34.47	79.69	77.56
13	2 (1.1)	2 (0.4)	1 (0.0000)	68.16	70.76	44.34	42.89	85.18	83.97
14	2 (1.1)	2 (0.4)	3 (0.0030)	79.82	79.71	35.98	37.99	70.90	73.55
15	2 (1.1)	2 (0.4)	2 (0.0015)	73.01	75.24	40.14	40.44	80.65	81.37

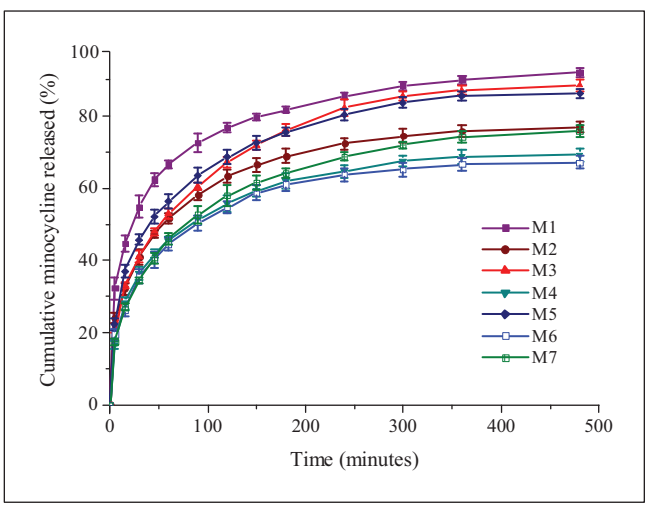

Fig. 1: Cumulative release profiles of MH from collagen sponges as a function of time (Exp. 1–7) n = 3

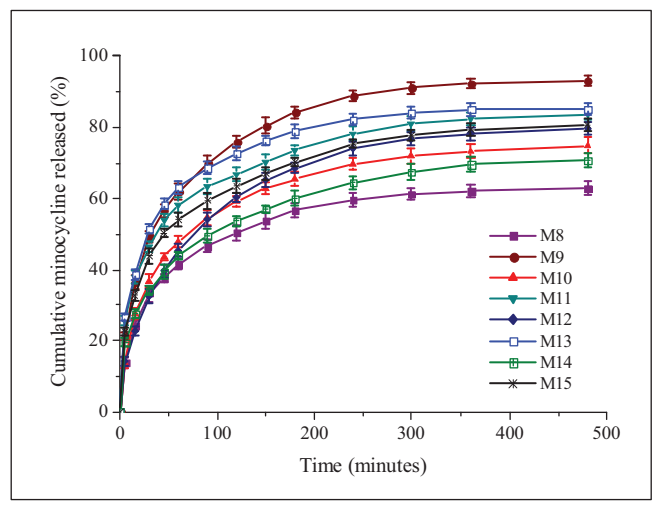

Fig. 2: Cumulative release profiles of MH from collagen sponges as a function of time (Exp. 8–15) n = 3

buffer, all the systems absorbing and maintaining large liquid volumes into the pores. The sponges weight increase was between 27.34 g/g and 44.34 g/g, indicating their high capacity to absorb the wound area exudates.

2.4. In vitro drug release studies and kinetic mechanism

The *in vitro* kinetic patterns of the minocycline-loaded sponges were recorded as drug released percentage vs time and are shown in Fig. 1 and Fig. 2. The minocycline release properties (percent released) from the collagen sponges are shown in Table 2. The cumulative MH released percent varied between 62.95% (M8) and 93.12% (M9).

From Fig. 1 and Fig. 2 we can see that drug release was fast enough in the first about 2 h, the released MH portion being approximately between 50.55% (M8) and 76.75% (M1). After this initial period of burst release we noticed that the antibiotic was gradually and slower released during the next 6 h of experiments, until a plateau was reached.

The kinetic profiles previously described are required for the healing of some infected wounds, when both immediate and long term antibiotic release are targeted for the bacterial proliferation prevention (Mi et al. 2002). The burst effect, which is the consequence of a fast minocycline release from the sponges as soon as the system is in contact with the wound and becomes wet, ensures a rapid reduction of bacteria on an infected wound. On the other side, gradual drug release provides the protective effect of a wound dressing against wound infections over a required period to favour long-term healing.

For the assesment of the MH release mechanism from the designed sponges, the Power law model, Eq. (1), (Phaechamud and Charoenteeraboon 2008; Sung et al. 2010) was applied to the kinetic data, where m_t/m_∞ is the fraction of drug released at time t, k is the kinetic constant and n is the release exponent that can be related to the drug transport mechanism.

$$\frac{m_t}{m_\infty} = k \cdot t^n \qquad (1)$$

The goodness of fit for the sponges varied from 0.9702 (M10) to 0.9903 (M14). The values for the determination coefficients (R^2) are displayed in Table 3, along with the kinetic parameters for the Power law model.

Table 3: Determination coefficients R^2 for minocycline release from collagen sponges obtained using the Higuchi and Power law models; the kinetic parameters for the Power law model

Formulation	Higuchi model	Power law model	Release exponent	Kinetic constant ($1/min^n$)
M1	0.8171	0.9854	0.20	0.276
M2	0.8629	0.9814	0.24	0.188
M3	0.9152	0.9833	0.29	0.158
M4	0.8744	0.9767	0.25	0.153
M5	0.8779	0.9832	0.25	0.196
M6	0.8699	0.9771	0.25	0.154
M7	0.9147	0.9846	0.28	0.136
M8	0.8720	0.9706	0.26	0.134
M9	0.8672	0.9703	0.26	0.205
M10	0.8847	0.9702	0.27	0.145
M11	0.8530	0.9853	0.22	0.217
M12	0.9229	0.9714	0.32	0.116
M13	0.8121	0.9706	0.21	0.246
M14	0.9089	0.9903	0.27	0.142
M15	0.8715	0.9835	0.24	0.189

The release exponent values exhibit an anomalous behavior; values lower than 0.5 are also mentioned in other studies and are correlated with a non-Fickian diffusion transport mechanism (Natu et al. 2007; Phaechamud and Charoenteeraboon 2008; Albu et al. 2010; Sung et al. 2010). The Power law model is used to evaluate the drug release from the formulations for which several processes are associated to the kinetic mechanism (Natu et al. 2007). Thus, in a first stage the wetting of the sponge surfaces by the biologic fluid take place, followed by the desorption for immediate release of the free and closed to the surface drug, not retained in the polymer matrix (phase corresponding to the burst effect). After the progressive penetration of the liquid in the sponge structure simultaneous sponge swelling and polymer relaxation take place, as well as the diffusion of the drug partially imobilized in collagen fibrillar structure (Boateng et al. 2008; Albu et al. 2011; Thakur et al. 2012). All these processes explained the deviation from the squared root of time kinetic model, specific to drug release from hydrogel (Ghica et al. 2011a). The R^2 values for the Higuchi model are listed in Table 3.

2.5. Responses analysis and optimization technique

Drug release from spongious systems is a complex process governed by a combined effect of the physical-chemical phenomena previousely described and also by the interactions which can take place between the formulation factors involved in the process: polymer support, drug and cross-linking agent. Therefore, in view of obtaining some spongious systems with desirable release characteristics in relation with the therapeutical action and the application site, the next stage in the sponge design was hydrogel formulation modeling and optimization. For this reason, we used a statistical experimental design technique (Seidi et al. 2011) complemented with response surface methodology (RSM) (Jin et al. 2011; Potur et al. 2011; Acherjee et al. 2012) and Taguchi approach elements (Ghica et al. 2011a; Ghica et al. 2011b, Yusoff et al. 2011; Asilturk and Neseli 2012), a powerful and effective tool which can identify the optimum level of some formulation parameters and evaluate their interaction effects that can affect the drug delivery from collagen sponges. In the current study all references to the optimization of sponge responses were made to the initial concentrations of the formulation parameters of the hydrogels lyophilized to obtain the corresponding sponges.

The experimental data from FCCCD were subjected to a stepwise regression analysis with backward elimination subroutine for setting out the reduced quadratic polynomial equations for each response: Eqs. (2), (3), and (4).

$$Y_1 = 72.149 - 76.082X_2 + 66.031X_1X_2 + 7458.333X_2X_3 \quad (2)$$

$$Y_2 = 30.709 + 114.353X_2 - 9.477X_1^2 - 138.059X_2^2 - 4084.028X_2X_3 \quad (3)$$

$$Y_3 = 133.610 - 42.350X_1 - 19.050X_2^2 - 1157716.224X_3^2 \quad (4)$$

These equations, where only the significant terms ($p < 0.05$) were considered, show mainly the interaction and quadratic effects of the formulation factors, coded as X_i, on the system responses Y_i (Table 1). The regressional coefficient values in the equations set explain their influence on the responses. For the responses that have to be maximized, a positive sign means a synergistic effect and a negative sign means an antagonic effect of the corresponding independent variables, while for the responses to be minimized the meaning of the signs is reversed. Thus, taking into account the constraints determined (Table 1), the coefficients of the reduced model in the eq. (2) indicated that X_2 strongly influences the contact angle: we noticed a positive linear effect, while its interactions with X_1 and X_3 have a negative influence. According to Eq. (3), the negative effect on the swelling ratio is recorded for X_1 and X_2 (quadratic) and for the interaction between X_2 and X_3, while a positive linear influence on the hydrophilicity is given by the MH concentrations. The percent released in Eq. (4) is negatively influenced by all the formulation factors with a linear effect for collagen and a quadratic effect for the MH and GA.

The reduced models generated were evaluated by determination coefficient R, correlation coefficient R^2, analysis of variance (ANOVA) and residual analysis. The values recorded for the multiple correlation coefficient (0.9663, 0.9616, 0.9763) are close to 1 and the values for the determination coefficient (0.9338, 0.9247, 0.9532) are higher than 0.90. The results for the ANOVA are summarized in Table 4. The residual analysis indicated a good correlation between the predicted values and the

Table 4: Analysis of variance for the reduced regressional polynomial models

Responses	Sources of variation	Sum of squares	df	Mean squares	F-value	p-value
Y_1	Regresion	599.128	3	199.709	51.796	< 0.0001
	Residual	42.412	11	3.855		
	Total	641.540	14			
Y_2	Regresion	355.621	4	88.905	30.742	< 0.0001
	Residual	28.919	10	2.891		
	Total	384.540	14			
Y_3	Regresion	1111.571	3	370.523	74.767	< 0.0001
	Residual	54.513	11	4.955		
	Total	1166.084	14			

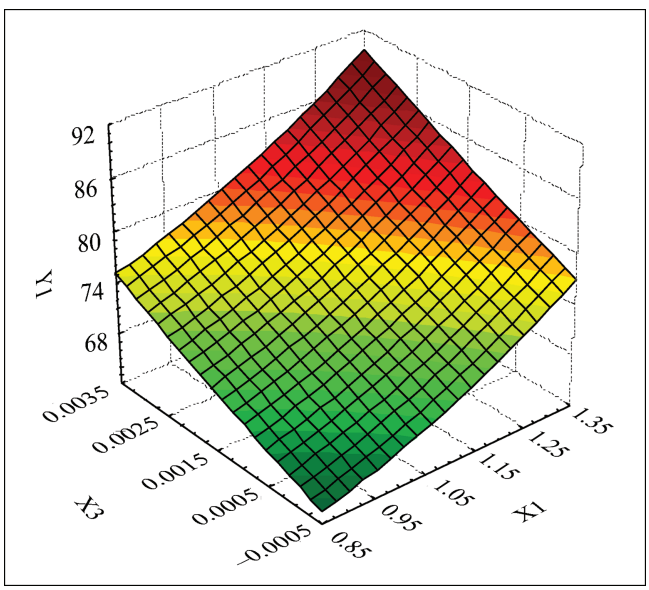

Fig. 3: Response surface plot for the contact angle CA (Y_1) as a function of collagen (X_1) and glutaraldehyde concentrations (X_3)

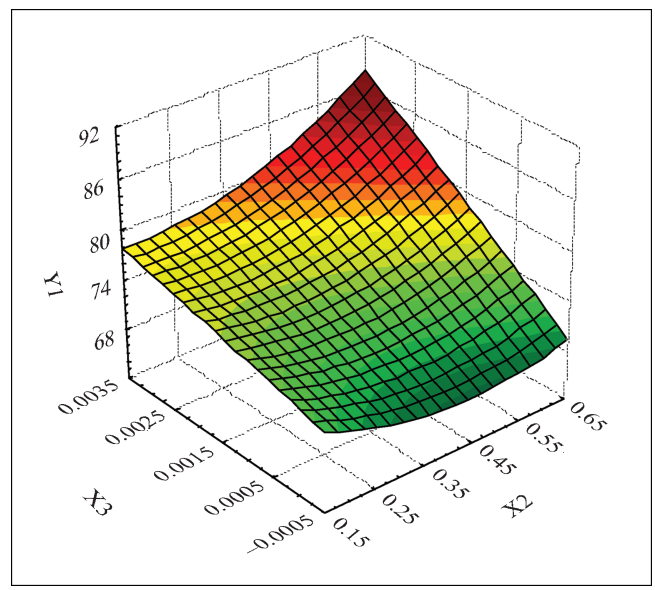

Fig. 4: Response surface plot for the contact angle CA (Y_1) as a function of minocycline hydrochloride (X_2) and glutaraldehyde concentrations (X_3)

observed ones (Table 2). The results of the above mentioned statistical analysis show that the equations set are highly significant and are adequate for correlating and validating the experimental results.

In order to visualize the combined effect of two independent variables on the system responses we used the response surface methodology. Some examples of 3D response surfaces are presented in Fig. 3 to Fig. 6.

According to Fig. 3, the best values for the contact angle (lowest) are recorded for low concentrations of collagen and GA. A decrease of 19.03% for the contact angle from 78.87° to 63.86° is observed for collagen concentration decrease when GA is at the minimum level, while for the highest concentration of collagen it increases with 21.41%, from 74.11° to 89.98° for the GA concentration increase. We can also remark that both formulation factors have a similar effect on contact angle (CA).

The influence of MH and GA on the contact angle evolution is given in Fig. 4. In order to get a small contact angle, the low concentrations of GA and the middle concentrations of MH should be targeted. CA decreases from 82.30° to 67.08° (18.49% decrease) with the decrease of GA concentration when MH is at the middle level and increases from 72.03° to 89.98° (24.92% increase) when GA is at maximum level and MH concentration increases.

The swelling ratio profile as a function of collagen and minocycline hydrochloride concentrations (Fig. 5) shows that its highest values are obtained for middle collagen and MH concentrations. The profile indicates that both the collagen and MH influence Y_2 in the same way: the swelling ratio decreases from 44.34 to 35.98 (18.85% decrease) when collagen concentration increases and MH is at the middle level and increases from 33.44 to 43.12 (28.95% increase) for the increase of MH concentration when collagen concentration is at the low level.

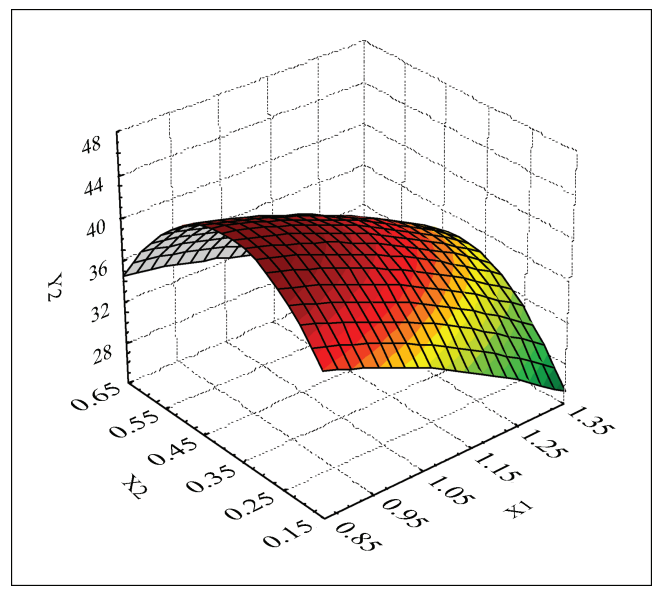

Fig. 5: Response surface plot for the swelling ratio SR (Y_2) as a function of collagen (X_1) and minocycline hydrochloride concentrations (X_2)

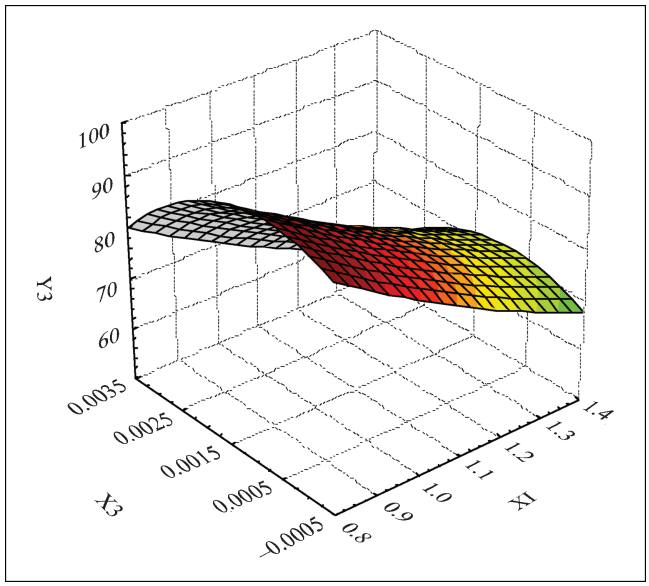

Fig. 6: Response surface plot for the percent released PR (Y_3) as a function of collagen (X_1) and glutaraldehyde concentrations (X_3)

In Fig. 6 we can see that the percentage of drug released is favoured by middle level concentrations of GA and low level concentrations of collagen. Thus the percentage of drug released varies from 74.92% to 93.12% at the decrease of collagen concentration (24.29% increase) when GA concentrations are at middle level and from 93.12% to 75.85% (18.55% decrease) when GA concentrations increase and collagen concentrations are at the minimum level.

The next step of the optimization process was the selection of the variation ranges for the formulation factors that lead to optimum responses and is onwards performed using the contour plots superposition method. Some examples of the contour plots overlapping for Y_1 and Y_3 as function of X_2 and X_3 and for Y_1, Y_2 and Y_3 as function of X_1 and X_2 are given in Fig. 7 and Fig. 8.

The formulation factors variation ranges for which optimal responses are obtained – minimum contact angle and maximum swelling ratio and percentage of drug released – are defined by the hatched areas.

According to Fig. 7, the minimal responses for the contract angle and the maximal responses for the percentage of drug released

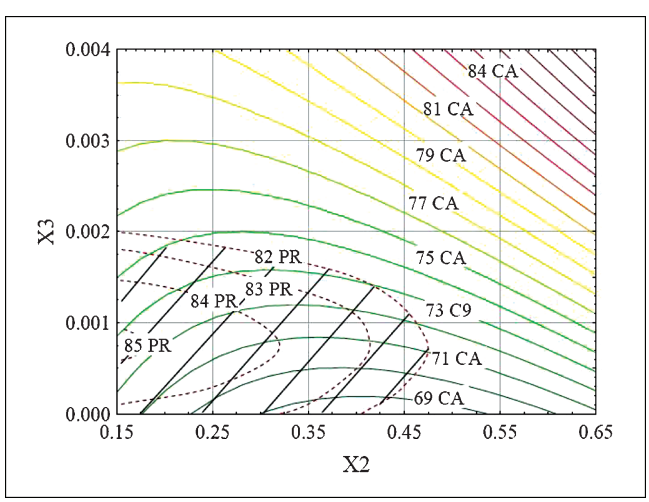

Fig. 7: Overlapped contour plots for Y_1 (CA ———) and Y_3 (PR - - - -) as a function of X_2 (MH) and X_3 (GA)

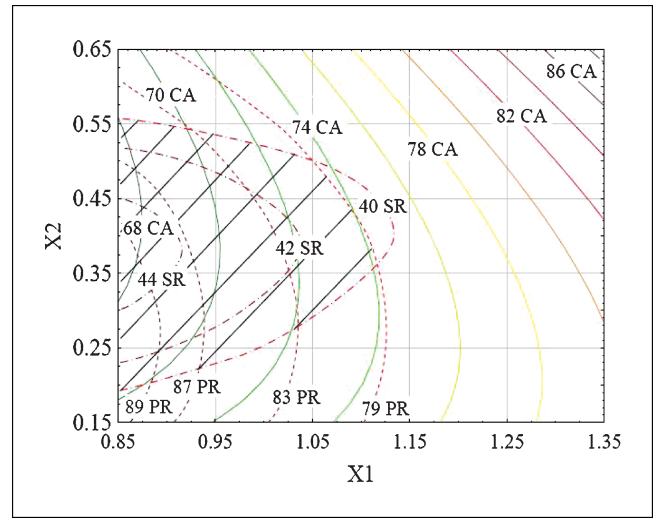

Fig. 8: Overlapped contour plots for Y_1 (CA ———), Y_2 (SR — · — · —) and Y_3 (PR - - - -) as a function of X_1 (C) and X_2 (MH)

are determined by the low to middle levels for both minocycline hydrochloride and glutaraldehyde.

Figure 8 indicates that the low to middle levels for both collagen and minocycline hydrochloride (especially the middle level for the MH) are leading to maximal responses for the swelling ratio and percentage of drug released and to minimal responses for the contact angle.

In order to determine the optimum zone for the sponge preparation, a simultaneous response optimization over the experimental domain was conducted. Consequently, it resulted that the optimum formulation factors ranges for the corresponding hydrogel preparation are as follows: X_1: [0.85÷1.12]%; X_2: [0.20÷0.45]%; X_3: [0÷0.002]%.

An increased amount of collagen and glutaraldehyde determines an increase of the cross-linking density, with reduction of the sponge surface wettability and swelling degree due to the decrease of the collagen protein chain elasticity. An increased MH amount in the hydrogel is not necessarily leading to the increase in drug release from the corresponding sponge probably due to a possible cross-linking in addition to the glutaraldehyde one. This remark is also supported by our previous studies (not reported here) concerning the dynamic and stationary rheometric measurements which indicated an increase in hydrogel viscosity for the increase of drug amount.

Compared to the sponges analyzed in this paper, potentially usable for the treatment of some infections with moderate to high exudate, the optimal preparation zone determined in our previous studies for the hydrogels potentially usable for the treatment of some cutaneous wounds with low to moderate exudate (X_1: [0.90÷1.13]%, X_2: [0.40÷0.60]%, X_3: [0.000÷0.0016]%) (Ghica et al. 2011a) showed optimal variation ranges similar for collagen and glutaraldehyde concentrations, while a higher minocycline percentage in the formulation induced a higher drug release. This could be due to a higher drug concentration gradient in the hydrogel layer in intimate contact with the artificial membrane used for the hydrogel release studies.

The final stage of the optimization technique is to improve the optimization process quality and to make the process performance (drug delivery from sponges) insensitive, robust and stable to the noise factors variation. The noise factors impact on the characteristics targeted was mathematically evaluated through the signal-to-noise ratio (S/N).

The control factors (X_1, X_2, X_3) effects on the S/N for each response (Y_1, Y_2, Y_3), resulting in the optimal combination of

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

Control factors (independent variables)	Y_1		Y_2		Y_3	
	"smaller – the– better"	effect size	"larger – the– better"	effect size	"larger – the– better"	effect size
X_1	1	0.623	1	0.782	1	0.872
X_2	2	0.132	2	0.916	1	0.227
X_3	1	0.516	2	0.539	2	0.383
S/N ratio (dB)	−36.224		33.399		39.394	

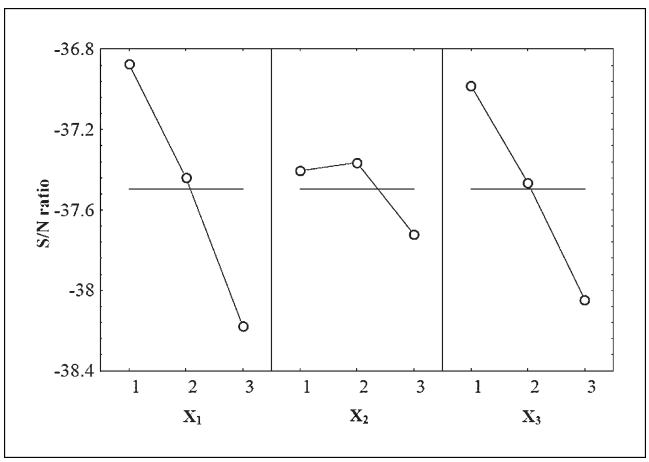

Fig. 9: Control factors effects on S/N ratio for the contact angle (Y_1)

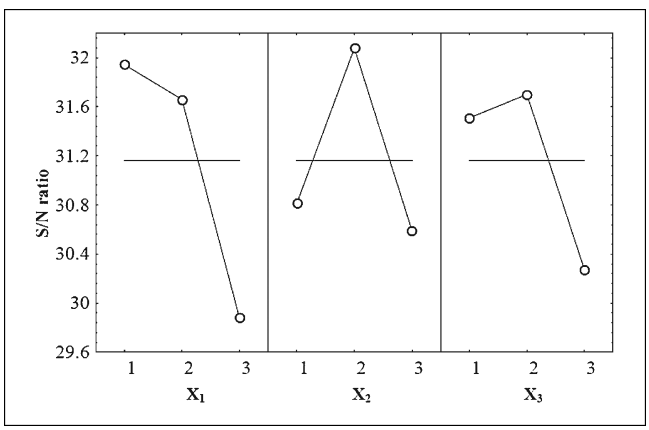

Fig. 10: Control factors effects on S/N ratio for the swelling index (Y_2)

formulation factors, are illustrated in Table 5 and Fig. 9 to Fig. 11.

In these Figures we see that X_1 has a significant influence on Y_1, Y_2 and Y_3. The optimal coded level of this formulation factor is 1 for all the responses, meaning that this level involves a reduction of the noise factors effect.

For X_2, we remark a small influence on Y_1 and Y_3, while for Y_2 the influence is more important. The noise factors effect reduction is consequently obtained for the following independent variables coded levels: 2 for Y_1 and Y_2 and 1 for Y_3.

Concerning Y_3, it shows a moderate influence on all the responses: higher on Y_1 and Y_2 and lower on Y_3. From the Figures it results that the noise factors effect is reduced in the case of the coded level 2 for Y_2 and Y_3 and for level 1 for Y_1.

The effect size (Table 5) of the formulation factors on the S/N ratio gives us the informations concerning their influence degree on the system responses. Thus, collagen concentration is the main influencing factor for the contact angle (effect size 4.72 times higher than the MH and 1.2 times higher than the GA) and the percentage of drug released (effect size 3.84 times higher than the MH and 2.77 times higher than the GA).

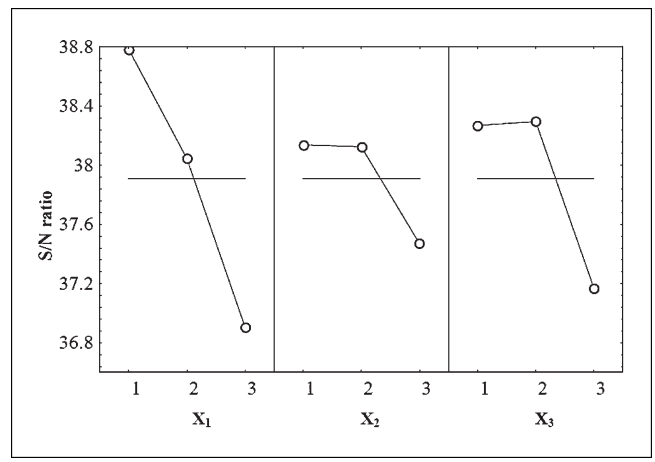

Fig. 11: Control factors effects on S/N ratio for the percent released (Y_3)

The minocycline concentration has the main influence on swelling ratio (effect size 1.17 times higher than the collagen and 1.70 times higher than the GA), its influence on the contact angle and the percent of drug released being comparatively low.

Concerning the glutaraldehyde, its main influence is recorded for the contact angle, being quite similar to the collagen, the other two response variables being less influenced by this factor.

The use of Taguchi approach resulted in the selection of three hydrogel formulations with the composition included in the optimal ranges previously set. The responses of the sponges obtained from these hydrogels were robust, stable and insensitive to the uncontrollable factors.

In Table 6 we present the composition of hydrogels for the optimal sponge preparation leading either to contact angle (M16), swelling ratio (M9) or percentage of drug released (M17) affected to the minimum extent by the noise factors. M9 was prepared from a hydrogel included in the initial experimental matrix, while M16 and M17 were obtained from hydrogels not initially designed in FCCCD. M16 and M17 were prepared and tested under the same experimental conditions.

The experimental values recorded from the M9, M16 and M17 sponges responses were compared to the theoretical ones found based on the reduced polynomial equations. A high predictive power of the regressional models was remarked (Table 6).

2.6. Conclusions

Drug delivery from sponges can be controlled by varying the collagen content, the cross-linking degree and the drug concentration. The swelling ratio, the contact angle and the kinetic

Table 6: Composition of hydrogels used to prepare the corresponding optimal sponges; the observed and predicted values of response for the optimal sponges

Sponges	Composition of hydrogel for optimal sponge preparation X_1: X_2: X_3 (g%: g%: g%)	Response variable	Observed value	Predictive value	Predicted error (%)
M9	0.9:0.4:0.0015	Y_1 (°)	67.08	69.96	−4.11
		Y_2 (g/g)	43.12	44.23	−2.51
		Y_3 (%)	93.12	89.84	+3.65
M16	0.9:0.4:0.0000	Y_1 (°)	65.88	65.48	+0.61
		Y_2 (g/g)	45.85	46.68	−1.77
		Y_3 (%)	95.34	92.45	+3.12
M17	0.9:0.2:0.0015	Y_1 (°)	70.85	71.05	−0.28
		Y_2 (g/g)	39.48	39.15	+0.84
		Y_3 (%)	89.52	92.12	−2.82

characteristics reffering to the complex process of drug delivery from sponges are parameters that have to be evaluated and optimized in view of sponges development for an adequate release of the drugs used in infected wound healing.

The statistical approach used in the design and development of minocycline-loaded collagen sponges is time and cost effective and allows the selection of the optimum, stable and robust topical formulations. Based on the results obtained, we can estimate that the collagen sponges with optimized composition could be successfully used with prophylactic and therapeutical potential in infected cutaneous wounds secreting moderate and high amounts of exudate.

3. Experimental

3.1. Materials

Type I collagen gel having a concentration of 1.99% (w/w) and pH 2.1 was extracted from calf hide by the currently used technology in Collagen Department of Division Leather and Footwear Research Institute (Albu 2011). The chemicals were purchased from commercial suppliers as follows: minocycline hydrochloride from Sigma (Germany), glutaraldehyde from Sigma-Aldrich (Germany), sodium hydroxide, monobasic potassium phosphate and disodium hydrogen phosphate from Merck (Germany). The water used was distilled and all other reagents used for analysis were of analytical grade.

3.2. Preparation of sponges

Collagen gels having the concentrations 0.9%, 1.1% and 1.3% were obtained from the initial 1.99% collagen gel by dilution under stirring with distilled water and the initial 2.1 pH was adjusted to 7.37 pH with NaOH 1 M solution under mechanical stirring (680 rpm VELP mechanical stirrer). 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) were added maintaining at the same time the hydrogels concentrations in collagen (0.9%, 1.1% and 1.3%). Some of the hydrogels were cross-linked at 4 °C with 0.0015% and 0.0030% glutaraldehyde for 24 h as shown in Table 1 and designed according to the face-centered 3^3 central composite design.

The hydrogels prepared were lyophilized (freeze-dried) using the Delta LSC 2-24 Martin Christ lyophilizer (Germany) using the method previously described (Lungu et al. 2011) and the M1-M17 collagen sponges with minocycline were obtained.

3.3. Contact angle measurements

The sponges surface wettability, expressed through contact angle values, was measured using a KSV Scientific Instrument (Finland) equipped with a video camera for image capturing and a CAM-101 software for data acquisition. The pendant drop dynamic method was used, specific to the porous structure systems. After dispensing the distilled water drop with a Hamilton syringe on the sponge dry surface, the drop images were automatically analyzed by the camera connected to the device and the contact angles from the images were recorded, calculating the average contact angle. To evaluate the contact angle (expressed in degrees), we applied the Young-Laplace equation which mathematically described the formed drop shape, the contact angle being computed as the slope of the contour line at the intersection point between the liquid, gas and solid phases. The contact angles were measured on sponge both sides and in different places, performing three determinations for each side. The results were averaged.

3.4. Swelling studies

The sponges swelling capacity was determined by a gravimetric method, at 37 °C. Collagen sponges accurately weighed in dry condition (W_0) were immersed in the absorption medium represented by phosphate buffer pH 7.4 which simulates the wound pH (Ramli and Wong 2011). At preset time intervals of more than 8 h, the swollen sponges were removed from the absorption medium and hanged for one minute until no drops were anymore formed, slowely blotted with filter paper to remove the liquid excess from the surface and then weighed (W_t) with a four decimal point electronic microbalance. The swelling ratio (g/g) was evaluated from the support weight modification before and after swelling with buffer solution, according to the following formula: $(W_t - W_0)/W_0$. Each experiment was performed in triplicate and the results were averaged.

3.5. In vitro antibiotic release kinetics

The *in vitro* minocycline release studies were conducted using a dissolution equipment in conjunction with paddle stirrers (Essa Dissolver, Italy). The collagen sponge having round shape (3 cm diameter) and known weight, was fixed in a transdermal sandwich device which was then immersed in the apparatus release vessel. The release medium (200 mL), a phosphate buffer solution of pH 7.4 as wound simulated environment, was continuousely and constantly stirred at a rotational speed of 50 rpm and maintained at the temperature of 37 ± 0.5 °C. Aliquots (5 mL) were collected at fixed periods of time and an equivalent volume of fresh phosphate buffer solution preheated at 37 ± 0.5 °C was immediately replaced into the release vessel after each sampling in order to maintain a constant volume. The concentrations of the MH solutions released in the receiving medium were spectrophotometrically assayed at 348 nm (Perkin-Elmer UV-Vis spectrophotometer) and computed from the calibration curve ($A^{1cm}_{1\%} = 274$), previously determined. The kinetic studies were performed for 8 h, in triplicate.

3.6. Data analysis and statistical methods

A face-centered 3^3 central composite design was used to evaluate the combined effect of the formulation factors (Table 1) on the physical-chemical parameters that determine drug delivery from collagen sponges. The experiments were performed in triplicate and randomly to minimize the errors due to systematic trends in the factors (Baby et al. 2009; Pongstabodee et al. 2012). The statistical analysis was done with the software package Statistica StatSoft Release 8. The stepwise regression method was used to obtain quadratic polynomial equations, which automatically eliminates the insignificant terms. The adequacy of the reduced models was verified through the determination coefficient, correlation coefficient, analysis of variance and residual analysis. The response surfaces in the three- and bidimensional space were further built to determine the formulation optimum conditions for the sponges preparation. The statistical analysis was complemented with Taguchi's approach elements, namely the signal-to-noise ratio performance indicator, for the determination of the optimization process quality and robustness.

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