Reducing the Risk in Controlled Drug Release by Using Tannic Acid in Liposomal Formulations

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One of the main challenges in designing a release system is the possibility to control the release rate in order to maintain it at a constant value below a defined limit, to avoid exceeding the toxicity threshold. We propose a method of overcoming this difficulty by introducing the drug into liposomes, prior to its inclusion in the hydrogel. Furthermore, a natural cross linker (as is tannic acid) is used, instead of the toxic cross linkers commonly used, thus reducing the toxicity of the release system as a whole.

Keywords: drug release, pharmaceutical risk, toxicity threshold

Chitosan (CS) is a biosourced polymer with important physico-chemical properties (biocompatibility, biodegradability, non-toxicity [1]) that can interact with proteins, cells and living organisms [2], which recommend it for applications in genes delivery [3], controlled drug release [4], tissue engineering [5].

One of the most common presentation forms of CS for biomedical applications is as hydrogel, that is cross linked CS with the ability to swell in aqueous environments. For biomedical use, most used are physically associated chitosan hydrogels (*physical* hydrogels) and chemically cross linked chitosan hydrogels (*chemical* hydrogels). In *physical* hydrogels, the cross linking is achieved due to physical interactions (electrostatic, hydrophobic interactions or hydrogen bondings) between polymer chains that are reversible interactions, which make the hydrogels unstable, exhibiting reversible gelation. To improve their stability, additive ionic cross linkers were used. However, their mechanical resistance/strength proved to be poor, determining gel dissolution; in consequences, the variation in the size of hydrogel pores were difficult to estimate [2]. Therefore, even if the *physical* hydrogels have the advantage to be obtained easily without the need of toxic additives, essential in biomedical applications, in order to overcome their shortcomings in terms of stability, chemical cross linking, i.e. *chemical hydrogels* have been investigated. Even if they proved to be more stable due to the irreversible nature of chemical bonds, this method has its own disadvantages, of which the most important is the use of toxic residual reactants (glyoxal and glutaraldehyde, epichlorohydrin, tripolyphosphate, ethylene glycol, diglycidyl ether, etc.) that determine a high risk in their administration [2, 6]. This is why there are now interests to remove the toxic covalent cross linker by using some natural products (citric acid [7], caffeic acid, tannic acid [8]), but still keeping hydrogel stability.

Another problem that needs to be solved is the existence of the *burst effect* that occurs due to the initial high release rates driven by the initial high concentration gradient. As a consequence, in the first part of the release process, the drug concentrations can reach or exceed the toxic level, which is pharmacologically dangerous [9,10]. This is why the *burst effect* is regarded in general as an event to be avoided.

We propose two methods to minimize the pharmaceutical risks mentioned above:

-the use of tannic acid (TA) as cross linking agent to reduce hydrogel toxicity; further more, due to its promising results with regard to its interaction with human tissues (biocompatibility, biodegradability, antitumoral activity) [11-14], TA can be considered to have a double role: cross linking agent and therapeutic agent;

-to load the drug into liposomes followed by their inclusion in the hydrogel; thus, the drug molecules will have to pass through two *barriers*: the liposome membrane and afterwards, the hydrogel network. In consequence, a decrease in drug release rate, i.e. a less pronounced *burst effect* is expected, compared to the classical situation of the drug loaded just in hydrogel [15-19].

The release kinetics for both type on release systems, without and with liposomes, will be analyzed and the release parameters will be compared.

Experimental part

Materials and methods

Chitosan (CS), Brookfield viscosity 800.000 cps, poly(vinyl alcohol) (PVA), 80% degree of hydrolysis, molar mass 9000-10000 g/mol^{-1}, calcein, tannic acid (TA) and Triton X-100 extrapure were provided by Sigma-Aldrich. Phospholipon-90G (phosphatidylcholine - PC) was received from Phospholipid GmbH. All other chemicals used were of analitical grade. The degree of deacetylation for chitosan was ~76% as 1HNMR has shown.

Preparation of hydrogels

Hydrogels based on CS and CS/PVA at different molar ratio have been prepared by dissolving 200 mg of polymers in a 2% acetic acid solution (16 mL) at room temperature.

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The impurities were removed by filtration with with filter paper. The cross linking agent, TA, was also dissolved in 2% acetic acid solution (4 mL) in different concentrations and was added dropwise over the polymer solution under stirring to give a final polymer concentration of 1%. The cross linking agent and the polymers were mixed in different molar ratio, as it can be seen in table 1.

Table 1
THE HYDROGELS PREPARATION PARAMETERS

Film code	C/TA molar ratio	CS/PVA molar ratio	Polymers/TA molar ratio
HCT2	20/1	-	-
HCT3	30/1	-	-
HCT4	40/1	-	-
B20	-	3:1	20/1
B30	-	3:1	30/1
B40	-	3:1	40/1

The obtained solutions were kept under vigorous stirring for 30 min, then left at rest to remove the air bubbles. Then, the solutions have been molded into round polyethylene plates, 60 mm diameter, 25 mm height. Subsequently, the gels were placed in an oven at 60°C to remove water and obtain the films. The cross linking time was determined aiming to obtain mechanically stable films that could be removed from the oven and washed. In all cases, 12 h were sufficient to dry the films (other analyzed times were 18h and 20h). Then, the films were washed three times with water (two hours each), 30 min with methanol and keeped for drying at room temperature until the day after.

Preparation of control hydrogels

For control hydrogels preparation 500 μg calcein from stoc solution (32 mg/mL), dilluted in 1 mL acetic acid solution (2%), was added before the cross linker addition.

Preparation of small unilamellar liposomes (SUVs)

SUVs were obtained through the thin film hydration method. The PC was dissolved in chloroform/methanol (2/1 v/v) and the solvent was evaporated at 30°C by rotary evaporation until a thin film was formed on the walls of a round-bottomed flask. The film was hydrated by vortex agitation with 2 mL of calcein solution (32 mg/mL). The resulting large liposomes suspension was sonicated (10 pulses of 60 s duration and 30 s break to allow the sample to cool down). The sample was kept under an ice bath to avoid lipid breakage. To eliminate unentrapped calcein, size exclusion chromatography (25 cm lenght, 1cm diameter) using Sephadex G-25 eluted with PBS buffer (2.2 mL/min) was used**.**

Preparation of complex hydrogels

Liposomes containing calcein were dispersed in polymeric solution prior to cross linker addition.

Calcein release kinetics

Calcein release for control hydrogels

The calcein release was studied by using a technique previously validated: each hydrogel was immersed in covered plastic containers with 50 *m*L PBS *p*H=7.4 solution and placed in a water bath (60 rpm, 37°C). The calcein release was monitored through the spectrophotometrical method. 3 mL of supernatant was removed for determinations and replaced with fresh PBS buffer.

The release efficiency was determined through the relation (1):

$$E(\%) = (m_{ci}/m_{ct}) * 100 \quad (1)$$

where m_{ci} is the amount of calcein released at moment *i* and m_{ct} is the amount of calcein loaded into hydrogels.

Calcein release for complex hydrogels

For studying the calcein release from complex hydrogels, 20μL SUVs suspension was dilluted with 4 mL PBS buffer for measuring the unentrapped calcein. After each measurement, the surfactant Triton X-100 was added in order to disrupt the liposomal membrane and release the entrapped calcein. Then, the amount of released calcein was analized spectrophotometrically and the release efficiency was determined using the above formula. Three measurements were conducted for each determination, and the mean values, (estimated as the most probable values) were considered for the release profiles. Standard deviation was subsequently calculated and plotted on the graph.

Results and discussions

Calcein release kinetics from control hydrogels

The release kinetics, presented in figure 1, reveals that the release is strongly influenced by the characteristics of hydrogel structure determined by preparation parameters.

In all cases, a pronounced burst effect is observed. The burst effect and, generally, the release efficiency reduces with the degree of hydrogel cross linking, logical effect given the slow diffusion of drugs in denser networks.

The CS/PVA behave in a similar manner, but with higher intensity; the explanation may lie in the lower cross linking density of these hydrogels determined by the fact that by reducing the amount of CS (compensated by PVA), the number of the hydrogen bonding interactions of $-NH_2$ groups with calcein reduces, facilitating easy release thereof.

Calcein release kinetics from complex hydrogels

The release kinetics from figure 2, shows that, even in the case of complex hydrogels, when drug is included first in liposomes, subsequently introduced in hydrogels, preparation parameters of hydrogels influences the efficiency of drug release.

A pronounced reduction of the *burst effect*, up its disappearance, can be observed; also, calcein release is significantly delayed, the process efficiency ranging within 5-7% (depending on the CS/TA, polymer/TA ratio) after 21 days. The family of three calcein release curves are placed in a logical order, the release efficiency thereby reducing with the amount of cross linker (cross linking density, respectively).

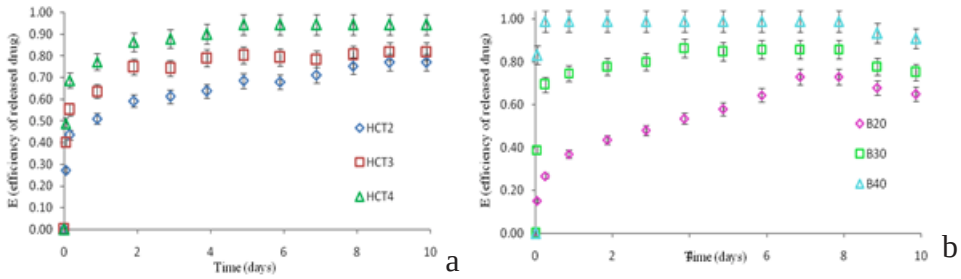

Fig. 1. Calcein release efficiency from control hydrogels based on CS cross linked with TA (a) and CS/PVA cross linked with TA (b)

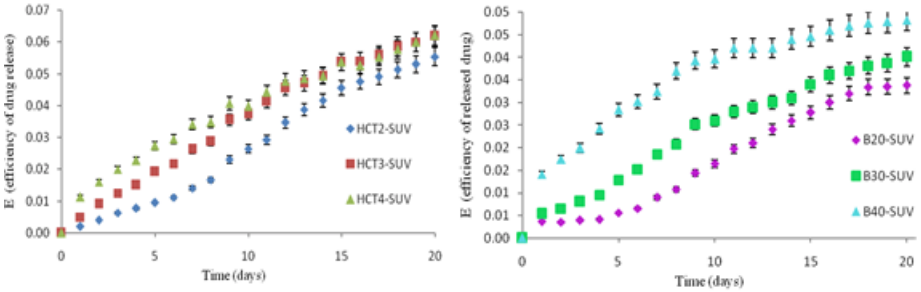

Fig.2. Calcein release efficiency from complex hydrogels based on CS cross linked with TA (a) and CS/PVA cross linked with TA (b)

Two simultaneously release paths can be intuited: 1) while the hydrogel swells, liposomes are driven towards the surface of the hydrogel and, finnaly, in the release environment, where they disintegrate, releasing calcein; 2) the liposomes get broken inside hydrogel (during the cross linking process or during swelling/releasing process due to the network constrains) and calcein is released inside, where can exist both as stand-alone molecules and as well as linked to hydrogel polymer; further it diffuses out of the hydrogel as calcein in free form (*free* calcein). In both cases, the liposome can be seen as a barrier that limits the calcein molecule movement, and, therefore, the calcein release is slower, as confirmed by the experimental data reprezented in figure 2.

It is worth mentioning that even the phenomena that occur simultaneously are numerous, the release kinetics reveals a smooth evolution towars a stable state; we can affirm that the system (drug delivery polymeric matrix in the release environment) is a complex system which behaves like a self-organized structure, in which the release paths find their own equilibrium [20-26].

Theoretical models

We applied the Peppas–Sahlin equation that applies best for systems that evolves until the equilibrium state is reached (the third release phase) [22]. It combines the release determined by the pure diffusion and by the diffusion triggered by relaxation of the polymer network, as a sum of two terms: the first, a *diffusional* term, and the second, a *relaxational* term, both expressed as powers of time:

$$\frac{M_t}{M_\infty} = k_D t^m + k_R t^{2m} \quad (2)$$

where k_D is the diffusional constant (asimilated to release rate), k_R is the relaxation constant, and m is the diffusion exponent [21].

The contribution percentage of relaxation (R) compared to the diffusion one (F_D) will be calculated with equation 3:

$$\frac{R}{F_D} = \frac{k_R}{k_D} t^m \quad (3)$$

Table 2 lists the values of the above parameters determined through mathematical fitting on experimental data, together with the correlation parameter (R^2).

One observation is that both diffusion and relaxation constants - k_D and k_R - have much smaller (even 100 times

Table 2
RELEASE KINETICS PARAMETERS OF PEPPAS–SAHLIN EQUATION

Film code	k_D	k_R	m	R^2
HCT2	0.513	0.009	0.16	0.99
HCT2-SUV	0.004	0.004	0.48	0.99
HCT3	0.956	0.279	0.20	0.99
HCT3-SUV	0.010	0.013	0.31	0.99
HCT4	1.160	0.353	0.19	0.99
HCT4-SUV	0.010	0.0001	0.61	0.99
B20	0.401	0.021	0.30	0.98
B20-SUV	0.002	0.002	0.50	0.99
B30	1.174	0.406	0.26	0.98
B30-SUV	0.002	0.00004	0.99	0.99
B40	1.867	0.863	0.13	0.99
B40-SUV	0.012	0.0007	0.63	0.99

smaller) values in the case of complex hydrogels, indicating that both diffusion and hydrogel relaxation are much slower compared with control hydrogels. Moreover, the decrease in k_D values is smaller that in the case of k_R, indicating that after liposomes inclusion in hydrogel matrix, the relaxation contributes more than diffusion to the release process compared to the control hydrogels (fig. 3). The relaxation mechanism is preponderant in the case of CS/PVA hydrogels (B samples) compared to CS hydrogels (HCT samples) because of their lower cross linking degree.

The theoretical model expresed by relation (1), as well as most models for drug release (a short review can be found in [22]), are expressed mathematically by polynomial forms of maximum degree 2 in t^m. However, there are also situations (at extremely small or extremely high temporal scales), for which in addition to the most encountered terms: the diffusional term, $k_D t^m$, and the relaxation one, $k_R t^{2m}$, additional terms must be introduced to describe some *side effects* experimentally observed in the form of fluctuations. These fluctuations decrease significantly the efficiency of drug release and have been

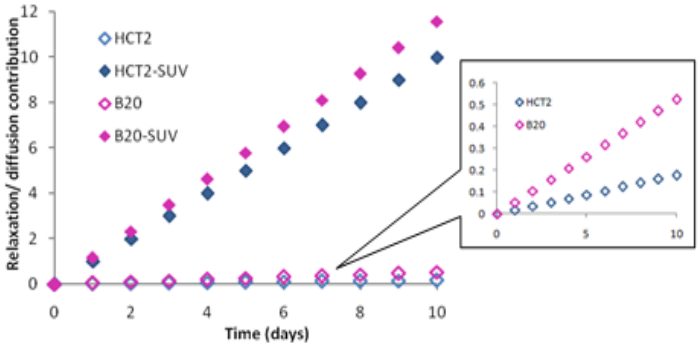

Fig.3. The relaxation vs. the diffusion contribution to the release process

explained by the system self-organization, followed by a delayed release [21].

A theoretical model through which these *side effects* can be taken into considerations will be developed next. The starting point will be to admit that the drug release efficiency can be mathematically expressed through a polynomial of three degree in the form:

$$F(y) = a_0 y^3 + 3a_1 y^2 + 3a_2 y + a_3 \quad (4)$$

$$F(y) = \frac{M(t)}{M_\infty}, \quad y = t^m \quad (5)$$

The cubic F(y) admits the real roots:

$$y_1 = \frac{h + \varepsilon_1 \bar{h} k}{1 + \varepsilon_1 k}, \quad y_2 = \frac{h + \varepsilon_2 \bar{h} k}{1 + \varepsilon_2 k}, \quad y_3 = \frac{h + \varepsilon_3 \bar{h} k}{1 + \varepsilon_3 k} \quad (6)$$

where ε_1, ε_2, ε_3 are the cubic roots of the unit [28-30]:

$$\varepsilon^3 = 1, \quad \varepsilon_1 = 1, \quad \varepsilon_2 = \frac{-1 + i\sqrt{3}}{2}, \quad \varepsilon_{23} = \frac{-1 - i\sqrt{3}}{2} \quad (7)$$

if and only if the discriminator of the cubic F(y) is negative:

$$\Delta = (a_0 a_3 - a_1 a_2)^2 - 4(a_0 a_2 - a_1^2)(a_1 a_3 - a_2^2) < 0 \quad (8)$$

The quantities h and $\bar{h}$ are the roots of the hessian cubic F(y):

$$(a_0 a_2 - a_1^2) y^2 + (a_0 a_3 - a_1 a_2) y + (a_1 a_3 - a_2^2) = 0 \quad (9)$$

and k is a unimodular factor in the form $k = e^{i\phi}$

The simple transitive group with three parameters:

$$y_i \leftrightarrow \frac{a y_i + b}{c y_i + d}, \quad a, b, c, d \in R \quad (10)$$

with y_i - the roots of the cubic F(y), induces a simple transitive group with real parameters for the complex variables in the forms:

$$h \leftrightarrow \frac{ah + b}{ch + d}, \quad \bar{h} \leftrightarrow \frac{a\bar{h} + b}{c\bar{h} + d}, \quad k \leftrightarrow \frac{c\bar{h} + d}{ch + d} k \quad (11)$$

We can affirm that the group (11) contains all the circumstances left unspecified in a drug release experiment (in the sense of Jaynes' probabilistic theory [31]). The elementary measure of the group (11):

$$d\mu = \frac{dh \wedge d\bar{h} \wedge dk}{(h - \bar{h})^2 k} \quad (12)$$

is the probability distribution of the drug release process [32-34].

More specifically, the respective circumstance is not left unspecified for subjective reasons, but because it is not significant in the drug release experiment.

Therefore, this model can explain, for the drug release process, both the *undefined circumstances* as an expression of the group (11) and the probability with which these are *felt* in the form of the elementary measure of the group (11) - see relation (12).

Conclusions

The risks that may occur in the drug release process (variable release rate, exceeding the toxic threshold, unexplained fluctuations) were analyzed from two points of view: experimental and theoretical. In the experimental approach, the drug was encapsulated into liposomes, prior to its inclusion in the hydrogel, procedure that assured a constant drug release over a long period of time. Furthermore, in order to reduce the toxicity, a natural cross linker, tannic acid, is used, instead of the toxic cross linkers commonly used. In the theoretical approach, a model was developed in order to describe both the *undefined circumstances* and their probability with which these are *felt* in a drug release process.

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Manuscript received: 26.06.2017