International Journal of Pharmaceutics 516 (2017) 178–184

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Microbial biosensors to monitor the encapsulation effectiveness of Doxorubicin in chimeric advanced Drug Delivery Nano Systems: A calorimetric approach

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ARTICLE INFO

*Article history:*
Received 8 September 2016
Received in revised form 9 November 2016
Accepted 10 November 2016
Available online 11 November 2016

*Keywords:*
Doxorubicin
Drug delivery
Liposomes
Dendrimers
Isothermal titration calorimetry (ITC)
*Lactobacillus helveticus*

ABSTRACT

The release of the anticancer drug doxorubicin (DOX) incorporated in a new drug carrier, namely a chimeric nanosystem formed by liposomes and dendrimers, was studied following the influence of the drug on the growth kinetics of the *Lactobacillus helveticus* bacterium, that would mimic the intestinal microflora.

The bacterial growth was followed at 37 °C by means of Isothermal Titration Calorimetry (ITC) and the method was assessed to monitor the overall effect of the delivered drug obtaining simple objective parameters to define the encapsulation effectiveness of the system, discriminating dose effects even in cases of very low release. Traditional microbiological investigations and in vitro release tests were also performed in parallel for validation.

The achieved results suggest that *L. helveticus* is an excellent candidate as biosensor to assess the sealing effectiveness of these DOX drug carriers through ITC investigations. This approach can be extended for quantitative comparison of drug delivery systems with the same drug inserted in other supramolecular bodies for quantitative comparison.

The peculiar results for the DOX drug carrier system investigated, indicate also that, the use of hydrophilic dendrimers in this case, produce a high sealing effect that seems promising in terms of the intestinal flora protection.



## 1. Introduction

The research on drug carriers garnered a substantial improvement thanks to the achievements of nanotechnology. New drug carriers (liposomes, dendrimers etc.) with a size of some tenth of nanometers offer the possibility to increase the therapeutic index (TI) of known or new drugs by improving their effectiveness, diminishing their toxicity against physiological tissues and achieving controlled therapeutic levels for wide time spans (Massing and Fuxius, 2000; Allen et al. 2006; Hatziantoniou and Demetzos, 2006; Wagner, 2007; Park, 2007; Schiffelers and Storm, 2008; Manocha and Margaritis, 2010; Lallana et al. 2012; Liu et al. 2014; Haj Sleiman et al., 2015; Demetzos, 2015a, 2015b; Szulc et al. 2015).

In particular, in the recent years, nanoparticles formed by combining liposomes and dendrimers (Gardikis et al., 2010a, 2010b; Purohit et al., 2001; Khopade et al., 2002; Moraes et al., 2008; Ballut et al., 2009; Tekade et al., 2009; Wrobel et al., 2015; Papagiannaros et al., 2005) were used as carriers of Doxorubicin (DOX), an anthracycline antibiotic for chemotherapy against a wide number of cancers, carcinomas and soft tissue sarcomas (Abraham et al., 2005). These new formulations have recently been categorized as chimeric advanced Drug Delivery nano-Systems (chi-aDDnS) due to the combination of two independent technologies for producing an overall system with unique properties (Gardikis et al., 2010c; Pippa et al., 2014) which could be advantageous compared to conventional liposomal carriers.

Recent studies demonstrate that different type of dendrimers with different molecular size and branching degree, that are able to trigger a phase separation within the lipid bilayer, push the chi-

*Abbreviations:* chi-aDDnS, chimeric advanced Drug Delivery nano-Systems; ITC, Isothermal Titration Calorimetry; DOX, doxorubicin; *Lactobacillus helveticus*, *L. helveticus*.

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http://dx.doi.org/10.1016/j.ijpharm.2016.11.033
0378-5173/

aDDnS-DOX system toward a thermodynamic state that implies the stabilization of the liposomal membrane (Gardikis et al., 2010b). The stability of the liposomal membrane is, in turn, correlated with a limited release of the drug in the blood plasma keeping almost unaltered its TI versus the tumor cells (Gardikis et al., 2010a).

This sealing effect is crucial since one of the main goals of the carrier is to avoid the release of the drug in the plasma before the target cells are reached. Free DOX indeed presents many side effects. A major one is cardiomyopathy: free DOX can lead to congestive heart failure and death (Chatterjee et al., 2010). Another side effect of free DOX is the depletion of the gastrointestinal microflora, namely, the assembly of microorganisms within the intestinal lumen that sustains the host health (Stringer et al., 2009).

The poor release rate of chi-aDDnS –DOX therefore is of outmost importance to prevent the action of the drug against non-target cells. This justifies the interest to assess the level of such adverse action through a suitable experimental approach. Indeed, since all the chi-aDDnS-DOX systems are designed in order to provide as low as possible, drug release out of target, the traditional in-vitro release tests may confirm such condition in general, but often may result not appropriate to focus on small differences and peculiarities between such systems. Furthermore, other mechanisms of action of these drug carriers versus the cells (endocytosis etc.) are not contemplated.

This work was undertaken in order to verify the feasibility of a release characterization method that is alternative/complementary to the traditional in-vitro release tests, based on Isothermal Titration Calorimetry (ITC) investigations on a selected microbial culture used as a biosensor of the overall activity of the chi-aDDnS–DOX drug carrier. The related experiments have several advantages: sensitivity, accuracy, continuous real-time data, simplicity and passivity (Braissant et al., 2010). Finally, it must be noted that the heat flow signal (HF) is a nonspecific, net signal related to the sum of all chemical and physical processes taking place in the sample i.e. include all the effects of the drug either in terms of free release or fixation or incorporation of the whole nanoparticle.

In particular, in this work we assembly a new chi-aDDnS–DOX drug carrier following the thermodynamic suggestions previously achieved (Gardikis et al., 2010b) in order to enhance the sealing effect, namely a nanoparticle formed by DSPC/cholesterol liposomes and the PAMAM 3,5 dendrimer.

ITC was used to highlight the difference between free DOX and DOX incorporated in this new chi-aDDnS drug carrier, versus a selected microorganism, namely *Lactobacillus helveticus*. The criteria of this selection was: a) known microorganism with a simple metabolic mechanism, b) suitable to mimic part of the gastrointestinal microbial population (Slattery et al., 2010), c) sensitive to DOX. This homo-fermentative thermophilic lactic acid bacterium (LAB) is extensively used in food fermentation for technological and pro-biotical scopes being a "Generally Recognised as Safe" (GRAS) organism (Giraffa, 2014). Traditional microbiological investigations and in vitro release tests were also performed in parallel for validation.

## 2. Materials and methods

PAMAM G 3,5 dendrimer (MDL number MFCD05865467) and Doxorubicin Hydrochloride (DOX, CID: 443939) were purchased from Sigma-Aldrich. DSPC (CID: 94190) and cholesterol (CID: 5997) were from Avanti Polar Lipids. All solvents were of analytical grade.

### 2.1. Chi-aDDnS-DOX preparation and characterization

The chi-aDDnS-DOX consisted of DSPC/cholesterol/PAMAM 3,5/DOX. The preparation procedure was modified with respect to a previous one where amine terminated dendrimers where used (Papagiannaros et al., 2005): in this case, carboxyl terminated PAMAM 3,5 were used to enhance the surface hydrophilic character of the dendrimer. 0.85 μmol of carboxyl terminated PAMAM 3,5 in methanol were vacuum dried and the dry residue was dissolved in 835.5 μL of HPLC-grade water. The aqueous DOX (2.84 μmol in 164.5 μL of HPLC grade water) was added to this solution. This aqueous system was stirred for 1 h at 70 °C and then mixed with a chloroform solution of cholesterol and DSPC (19.05 μmol DSPC, 9.32 μmol cholesterol; DSPC/cholesterol 6.5:3.5 molar ratio). This mixture was vortexed to form a homogenous emulsion. The organic solvent was removed by heating at 70 °C for 1 h in a rotary evaporator. Traces of chloroform were removed by evaporation under vacuum at 37 °C for 5 min.

The liposomal suspension was subjected to two sonication periods in an ice bath for 5 min interrupted by a 4-min resting period, in order to decrease their mean diameter and size distribution (measured as Polydispersity Index – P.I.). Finally, size exclusion chromatography was used (with HPLC grade water as the mobile phase) to remove extra-liposomal DOX and/or dendrimer molecules.

The hydrodynamic diameter of the chi-aDDnS-DOX particles was determined by light scattering. 100 μL of the suspension was 30-fold diluted immediately after preparation and *z*-average diameter and ζ-potential of the empty and DOX loaded formulations were measured at 0, 1, 2, 3, 4 and 7 days at 4 and 37 °C. Light scattering at π/2 (λ = 633 nm) was determined at 25 °C in a photon correlation spectrometer (Zetasizer 3000HS, Malvern Instruments, Malvern, UK) and analysed by the CONTIN method (MALVERN software). Dendrimer and lipid concentration was assessed with HPTLC-FID (Iatroscan) (Hatziantoniou and Demetzos, 2006) using chloroform/methanol/water 60:20:3,2 (v:v:v) as the mobile phase. The incorporation of DOX into all formulations was determined by UV spectrometry (UV – 1700, UV–vis Spectrophotometer, SHIMADZU, Pharmaspec, Kyoto, Japan) at 480 nm wavelength after the addition of methanol to the liposomal suspension at 0, 1, 2, 3, 4 and 7 days at 4 and 37 °C, with the aid of a calibration curve obtained from DOX in methanol. Pure methanol was used as blank.

In vitro release studies were performed as follows: chi-aDDnS –DOX was placed in 12000 molecular weight cut-off (MWCO) 25 mm width dialysis sacks (Sigma-Aldrich). Dialysis sacks were inserted in RPMI 5% medium in a shaking water bath (Selecta) set at 37 °C. Aliquots of samples (1 mL) were taken from the external solution at specific time points and that volume was replaced with RPMI incubated at 37 °C. DOX concentrations were measured with UV spectrometry after the addition of 2 mL HPLC-grade water. As reference sample RPMI at 37 °C, diluted 3-fold with HPLC-grade water was used. The cumulative percentage of drug release was calculated and plotted versus time using the equation: % Released $\text{Dox} = [\text{Dox}]_{\text{released}}/[\text{Dox}]_{\text{initialload}} \cdot 100$.

### 2.2. Microbial cultures

*Lactobacillus helveticus* ATCC (American Type Culture Collection) $15009^{T}$ was maintained as a stock culture stored in 15% (v/v) glycerol, 85% (v/v) MRS (De Man et al., 1960) broth (Difco) at −20 °C. The culture was routinely reactivated overnight at 37 °C in MRS broth (composition in Table 1).

Different concentrations of *L. helveticus* cells, previously grown in MRS broth at 37 °C for 16 h, were used as initial inoculum in MRS broth containing DOX, either free or encapsulated (chi-aDDnS-DOX, 20 and 35 μg/ml). The inoculated test-tubes were regularly examined during the 48 h of incubation at 37 °C to check the microbial growth.

Growth curves were evaluated in triplicate, with an initial population of $10^2$ CFU (colony forming units)/mL The overall

**Table 1**
Composition of the MRS (De Man et al., 1960) broth: pH ≈ 6.2–6.5.

| Component | Conc./g $L^{-1}$ |
|---|---|
| Casein peptone, tryptic digest | 10 |
| Meat extract | 10 |
| Yeast extract | 5 |
| Glucose | 20 |
| Tween 80 | 1 |
| $K_2HPO_4$ | 2 |
| Sodio acetate | 5 |
| Citrate d'ammonio | 2 |
| $MgSO_4{\cdot}7H_2O$ | 0.20 |
| $MnSO_4{\cdot}H_2O$ | 0.05 |

population was determined by plate counts and optical density measurement ($OD_{600nm}$) at different incubation times.

### 2.3. Isothermal titration calorimetry (ITC)

The instrument used was the "Calorimètre E. Calvet pour Microcalormétrie, DAM" (SETARAM, Lyon, France) equipped with 10 $cm^3$ volume stainless steel cells and the injection system Microlab 500 (Hamilton Company, USA). Calibration was performed using the Joule effect calibrator EJ2 (SETARAM). Measurements were performed at 37 °C. A 3 mL suspension containing an initial microbial population of $10^2$ CFU/mL was loaded in the measurement vessel. Free DOX or chi-aDDnS-DOX were added to the microbial culture at the start of the experimental run, or after 16 h incubation in the calorimetry vessel, by injection through an helically wound capillary (to ensure thermal equilibration at 37 °C) that reached the interior of the calorimetric vessel. The added solutions (70 μL) had a concentration adjusted to achieve an overall DOX content (in either form) of 20 or 35 γ (1 γ = 1 μg $mL^{-1}$) within the calorimetric vessel. The term "isothermal calorimetry" will be also used.

The Heat Flow-vs- time raw signal was integrated to obtain the overall thermal effect

$$\Delta H_{overall} = \int_{t=0}^{t=\text{end}} HFdt \text{ and/or the } degree \text{ of reaction } \alpha(t) = \frac{\int_{t=0}^{t} HFdt}{\Delta H_{overall}}.$$

Three replicas were performed for each experiment. The $\Delta H_{overall}$ error was below 6%.

The term "isothermal calorimetry" will be also used in some cases for simplicity.

## 3. Results and discussion

This work focuses on the sealing effect and the ITC method proposed for quantification. The other delivery aspects are not contemplated here.

### 3.1. Chi-aDDnS-DOX spectroscopic characterization

Z-average diameter, Polydispersity Index (P.I.), ζ potential and PAMAM/lipid molar ratio and DOX/lipid molar ratio of chi-aDDnS-DOX are summarized in Table 2.

Despite of the relatively prolonged sonication time, Large Unilamellar Vesicles were formed. A possible explanation is that the increased osmotic pressure of the aqueous interior of the vesicles due to the large quantity of the entrapped molecules prevent smaller vesicle dimension (Gardikis et al., 2010a). Indeed, the flexibility of the membrane, enhanced by the presence of cholesterol, leads to a very high loading of the PAMAM and DOX molecules: the DOX encapsulation efficiency was 95%, and respective encapsulation efficiency was found for the dendrimer in accordance with previous studies (Papagiannaros et al., 2005). The chi-aDDnS-DOX did not exhibit significant increase of their mean diameter and P.I. upon storage at 4 °C for 1 week, (significantly lower to the increase observed by Papagiannaros et al. (2005) in the case of the use of amine terminated dendrimers). The increase of the Z-average diameter became, instead, significant after 3 days at 37 °C, possibly because of liposomal fusion (Heurtault et al., 2003) (Fig. 1). Minor deviation was observed for the P.I. (Fig. 2). Taking into account that the parameters emerged from the ITC experiments (see below 3.2 section) was obtained in a time span that was less than two days and only in one case, less than 3 days, this side effect is to be considerate not relevant in our study. The ζ potential did not reveal any change upon storage. The *in vitro* release experiment (Fig. 3) demonstrated that the release of DOX was quite low and was almost assessed in the first 8 h.

The above scenario, i.e. the good stability of this type of systems, is in line with previous studies that indicated that the hydrophilic PAMAM 3,5 in combination with DOX enhanced the stability of the membrane and improved the sealing effect (Gardikis et al., 2010b). Indeed, in the case of such DOX carriers, the hydrophilic nature of the dendrimer play a key role as regard the stability comparing with the use of lipophilic dendriners (Papagiannaros et al., 2005) that are supposed to involve the outer surface of the liposomal membrane (Purohit et al., 2001).

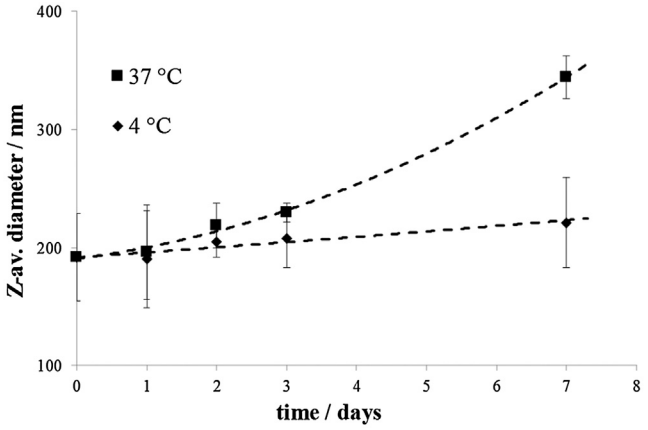


**Fig. 1.** Z-average diameter of chi-aDDnS-DOX stored at 4 °C and 37 °C for 1 week. Dashed lines represent only indicative trends of the experimental data.

**Table 2**
Properties of chi-aDDnS-DOX.

| Z-av. diam./nm | P.I. | ζ-pot./mV | PAMAM/lipid/mol/mol | DOX/lipid/mol/mol |
|---|---|---|---|---|
| 191.7 ± 37 | 0.567 ± 0.05 | −4.8 ± 2.6 | 0.04 ± 0.01 | 0.24 ± 0.03 |

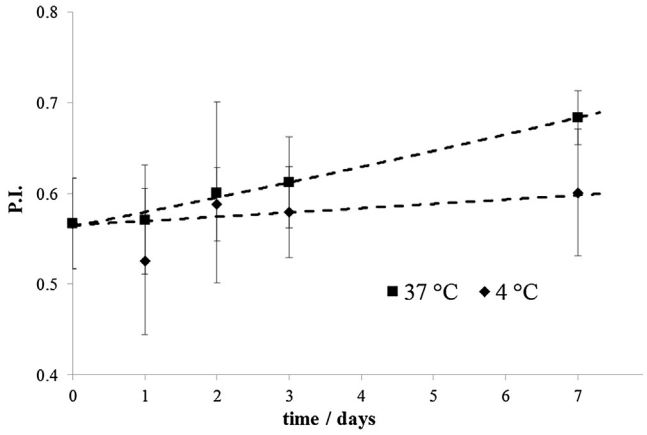


**Fig. 2.** P.I. of chi-aDDnS-DOX stored at 4 °C and 37 °C for 1 week. Dashed lines represent only indicative trends of the experimental data.

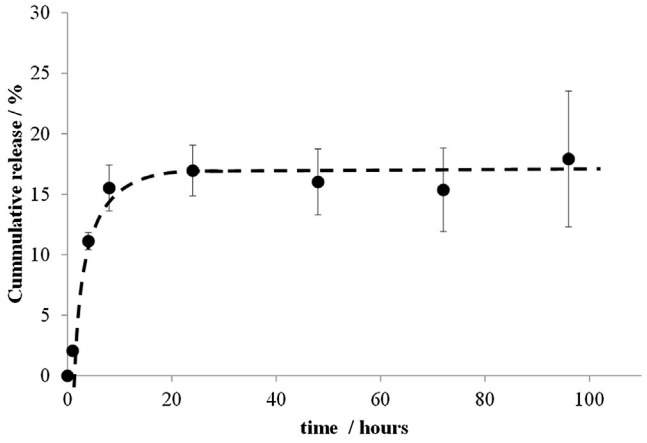


**Fig. 3.** in vitro release of DOX at 37 °C for 96 h. Dashed lines represent only indicative trends of the experimental data.

### 3.2. ITC characterization

Fig. 4 reports the calorimetric trace obtained from a culture of *L. helveticus* in MRS broth at 37 °C with a starting population of $10^2$ CFU $mL^{-1}$, to be used as a reference in order to monitor and compare the effect of the DOX either in the free and *chi-aDDnS –DOX* form. In the secondary axes is also reported the result of the parallel plate counts that very well matched with the degree of advance of the calorimetric trace α(t) once properly scaled (namely, in order to be compared in the same graph, the α(t), that is a dimensionless function in the 0–1 range, was multiplied by the final CFU number obtained from the parallel classical microbiological plate counts). This overlap indicates that the microbial metabolism is strictly related to its growth and does not imply extra contributions to the overall heat released by the microbial culture (Abraham et al., 2005).

In that simple case the heat flow is proportional to the growth rate, $HF = \frac{dN}{dt} \cdot \Delta_{gr}H$, where $N$ stands for the number of microbial cells (i.e. CFU) and $_{\Delta gr}H$ is the growth enthalpy per duplicating cell. The fact that the heat flow vanishes once the nutrient is exhausted and contemporarily the cell count reaches its maximum plateau is a further confirmation of this proportionality. Accordingly the exothermic $_{\Delta gr}H$ was estimated in about 100 nJ $CFU^{-1}$.

It must be noticed that the onset of the calorimetric trace occurred (in the experimental conditions selected) when the microbial population trespassed the threshold of about $10^5$ CFU $mL^{-1}$. With a starting population of $10^2$ CFU $mL^{-1}$, this threshold was reached after about 16 h: the calorimetric signal showed an upward drift becoming reliably distinguishable from the base line (see Fig. 4). This threshold depends on the operating conditions (e.g., volume of the microbial culture, instrumental sensitivity, etc.) and of course on the peculiar metabolism of the microorganism selected. The overall onset time is the result of these factors including also the peculiar lag time of the microorganism.

This calorimetric approach allowed us to verify the effects of DOX on the microbial growth either in the free and the *chi-aDDnS –DOX form* (Fig. 5).

Addition of free DOX (20 γ) to a 16-h incubated culture of *L. helveticus* produced an abrupt downward drift of the growth trend. It must be noticed that the descending trend of the calorimetric signal was not related to the exhaustion of the nutrient (which was in excess because of the smaller uptake), but to the killing effect of the drug that reduced the number of surviving cells which were still able to grow. Indeed, the lower increase of the cell population (which is the balance between growth and death rate) and eventually their depletion (see the microbial count in Fig. 5), is in line with the calorimetric trace trend.

On the other hand, the addition of the same dose of doxoribucin in the form of chi-aDDnS –DOX (in the same conditions, i.e., a 16-h incubated culture) produced an almost negligible effect on the microbial growth (the corresponding calorimetric trace was practically overlapped to the reference trace, see Fig. 5). Similar result was obtained by addition of chi-aDDnS-DOX 35 γ dose (not shown). This evidence confirms the good sealing effect (under our conditions) of the chi-aDDnS encapsulation of DOX versus this microorganism, that is related with the gut bacteria (Braissant et al., 2010), with respect to the free DOX.

Namely, when the microbial population was at or above the $10^5$ CFU level, the growth rate (which is proportional to the size of the microbial population) was large enough to overbalance the rate of internalization of chi-aDDnS –DOX injected and related killing effect. In other words, since we suppose that the overall effect is the result of a "competition" between the growth and death rates we expect that it depends on the (cell population)/(chi-aDDnS-DOX dose) ratio. For this reason, in order to maximize this ratio and highlight the dose differences, even if the effect is very small, we performed experiments with addition of the DOX (either free or chi-aDDnS-DOX) directly to the starting microbial culture ($10^2$ CFU).

As expected, addition of free DOX (20 and 35 γ) to the starting microbial culture produced a quick decline of the cell population and the corresponding calorimetric trace therefore was flat and close to zero (not shown).

When the same DOX doses were supplied as chi-aDDnS-DOX, the cell population was still able to grow although with increased delay: the higher the dose, the longer the delay. This delay could be partially attributed to the slight release of DOX from the chi-aDDnS-DOX during the first 8 h, as observed by *in vitro* release experiments but it includes, in any case, all the action mechanisms of the chi-aDDnS-DOX – cell system. The corresponding calorimetric traces (Fig. 6) evidence such differences compared with the reference.

The areas beneath either calorimetric peak were practically the same ($11.4 \pm 0.5, 11.3 \pm 0.5\ 10.7 \pm 0.5$ J $mL^{-1}$, for reference, *20 and 35 γ of DOX in the* chi-aDDnS-*DOX form,* respectively), which means that the microbial population was able to attain the same size (this fact was also confirmed by the final plate count after the experiment, i.e. about $10^8$ CFU/mL, as in the reference culture), no matter the dose of chi-aDDnS –DOX used. However, the two traces show clearly a different delay, namely, the time span before the detection of a reliable signal and, also, a different profile.

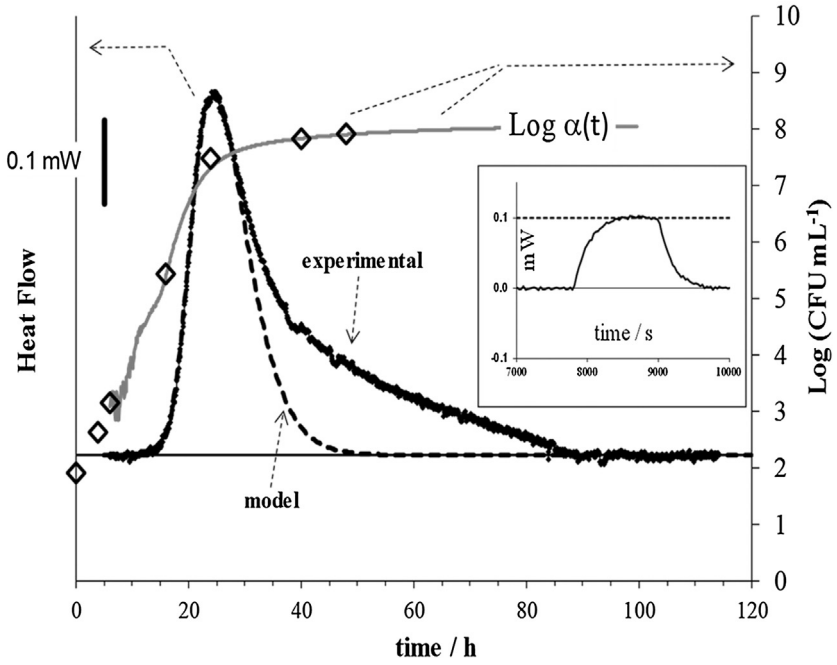


**Fig. 4.** Isothermal calorimetry trace (black bold line) of *L. helveticus* in MRS broth at 35 °C. The light gray line is the logarithm of the degree of advance of the calorimetric trace α(t), once properly scaled to comply with the plate counts (open rhombus dots). In the insert an example of the calibration signal. The dashed line represents the best fit trend according to a simple kinetic model for microbial growth (see text 3.3).

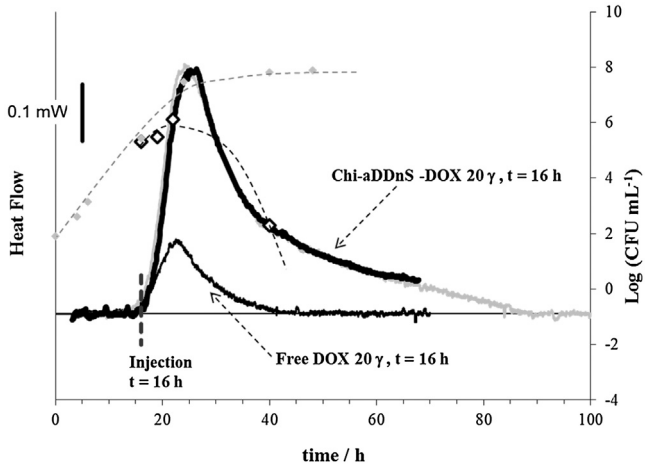


**Fig. 5.** ITC traces of a *L. helveticus* culture at 37 °C. Injection of 20 γ of free DOX (continuous thin black line) and 20 γ of chi-aDDnS –DOX (continuous thick black line) after 16-h incubation, namely when the calorimetric signal becomes detectable. Open rhombus dots are the corresponding plate counts in the case of free DOX injection. The traces obtained in the absence of DOX (gray line and dots) are the same as in Fig. 4 and are shown for comparison. Dashed lines represent only indicative trends of the plate counts.

### 3.3. Data modelling

To give a formal description of the collected evidence and quantify the (cell population)/(chi-aDDnS-DOX dose) ratio effect, a kinetic model of the microbial growth was tentatively attempted. Indeed, the growth of a microbial culture is often described with phenomenological kinetic models that split the overall process in some steps (Itoh and Takahashi, 1984; Beezer et al., 1990; Schiraldi, 1995; Fleming et al., 2010). Each step is represented like a chemical process with its own rate expressed with a linear differential equation. This approach generates some parameters (e.g., kinetic constants, reaction orders, etc.) which are adjustable in the fitting routine. Unless adequate information is available at the molecular level, the number of steps should be kept as low as possible not to compromise the overall reliability of the approach.

The simplest model that can be suggested for a microbial species whose metabolism coincides with the cell duplication as the result of the intake of substrate is:

$$N + S \xrightarrow{k_1} = 2N + \textit{catabolites}, \frac{dN}{dt} = k_1 \cdot N \cdot S \quad (1)$$

$$S = S_0 - \chi(N - N_0)$$

where $N$ and $S$ stand for the number of CFU and substrate amount (usually supposed unique and normalized in order to be dimensionless; the subscript "0" stands for starting value), respectively, $k_1$ is the kinetic constant and $\chi$ is a factor that accounts the substrate consumption per CFU. This model implies a growth that is limited only by the supply of substrate. The corresponding $N$ trend (integration of Eq. (1)) can be described with the expression:

$$N = \frac{A\ell^{Bt}}{1 + C\ell^{Bt}}$$

where

$$A = \frac{N_0}{S_0}(S_0 + \chi N_0),\ \mathrm{B} = \mathrm{k}_1\,(S_0 + \chi N_0) \text{ and } C = \chi\frac{N_0}{S_0}$$

This model does not reproduce the delay of the calorimetric response, related to the sensitivity of the instrument (see above) neither, in general, the presence of a peculiar lag time of the microbe selected. However, many solutions can be adopted for this issue, for example introducing a damping factor, $exp(-\tau/t)$, where τ can be used as a further adjustable parameter of the best fitting routine.

The presence of DOX obliges also to introduce adequate modification to account for the killing effect of the drug. For

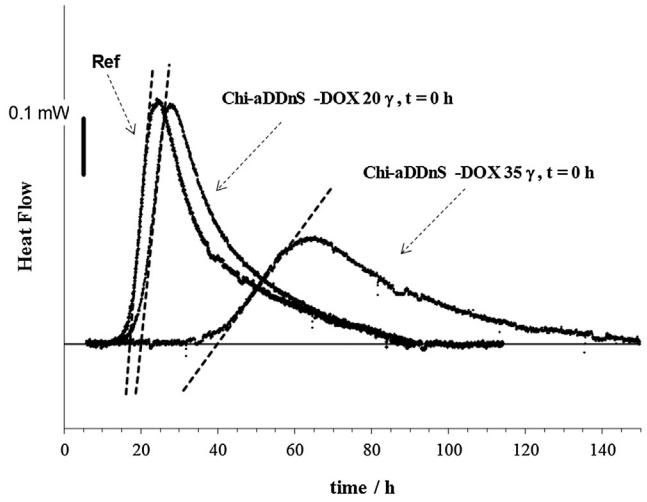


**Fig. 6.** Isothermal calorimetry traces of a *L. helveticus* culture at 37 °C. Reference (i.e. in the absence of DOX) and with initial (i.e. at t = 0) addition of 20 and 35 γ of doxorubicin in the chi-aDDnS-DOX form. Dashed lines represent the linear trend of the rising branch of the peaks (tangent lines).

example, in the simplest case we must add a further step, namely

$$N + DOX \xrightarrow{k_2} deadcell$$

However, the tentative fitting using the above model was not satisfactory (lack to fit the late part of the calorimetric trace, see Fig. 4 dashed line) even in the simplest case of the growth without the presence of DOX (reference). Indeed the second part of the calorimetric trace shows a significant difference in the growth rate profile. Possibly further steps should be included in the kinetic model (e.g. to account for effects of catabolites, modification of the substrate uptake mechanism and/or kinetics as a consequence of the changed *N/S* ratio, pH changes, solution density etc. (Fleming et al., 2010; Arioli et al., 2010)), but this would have the simple effect of increasing the number of adjustable parameters at the price of losing the reliability of the interpretation that would require more detailed information about the real growth mechanism.

On the other hand, this paper focuses on the use of microbes as biosensors in order to quantify the overall effect of the drug carrier in systems where the drug release out of target is low and not to the peculiarities of the microbes. It therefore seemed reasonable to avoid the use of arbitrary multi-step kinetic models and simply accept an empirical description that allows estimation of a couple of parameters that are strictly bound to the experimental evidence and depend on the initial (cell population)/(chi-aDDnS-DOX dose ratio), namely, the slope of the rising branch of the peak (see *dashed* lines in Fig. 6) that reflects the acceleration of the microbial growth and the delay of the onset of the ITC signal (time quantified here by the intersection of the *dashed* lines with the signal base line, see Fig. 6). The results are reported in Table 3.

In order to obtain parameters independent of the kind and sensitivity of the instrument used, the data of Table 3 were converted in percentage variation with respect to the reference

**Table 3**
Onset delay and slope of the rising branch of the ITC records from L. helveticus culture at 37 °C in different conditions: free, in the presence of chi-aDDnS –DOX 20 and 35 γ.

| | DOX free | chi-aDDnS-DOX 20 γ | chi-aDDnS –DOX 35 γ |
|---|---|---|---|
| Onset delay/h | 17 ± 1 | 20 ± 1 | 40 ± 2 |
| Slope/mW $h^{-1}$ | 80 E-03 | 65 E-03 | 10 E-03 |

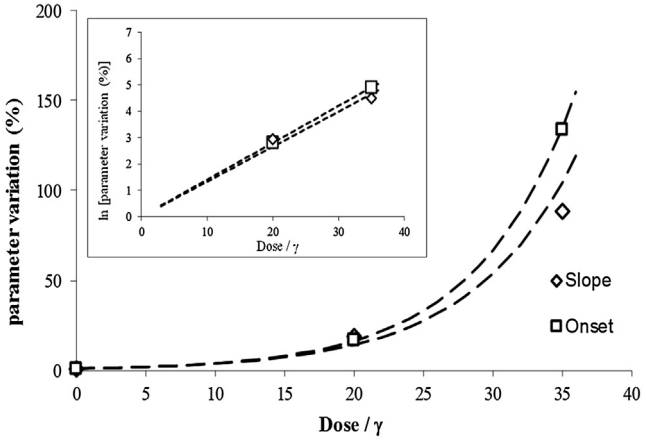


**Fig. 7.** Percentage variation with respect to the reference of the onset delay and slope of the rising branch of the ITC records from *L. helveticus* in initial 100 CFU culture at 37 °C in the presence of chi-aDDnS –DOX 20 and 35 γ. In the insert, the same graph in the logarithmic scale.

(Fig. 7). This approach allows definition of parameters of practical use for analogous calorimetric investigations.

The changes observed either for the rising branch slope or for the onset delay are well described with a simple exponential function of the chi-aDDnS-DOX dose (straight line in the logarithmic format, see the insert). The two parameters selected seem correlated and their trend in function of the dose may be used as index to quantify the sealing effect produced by the encapsulation of the drug in a chi-aDDnS-DOX system and to compare results from similar investigations dealing with the same drug inserted in other supramolecular bodies.

## 4. Conclusions

*Lactobacillus helveticus* cultures can be used as biosensors to check the effectiveness of the sealing effect of Doxorubicin encapsulated in a chimeric advanced Drug Delivery nano-System. Isothermal calorimetry is a reliable experimental technique to monitor the microbial growth providing a profile of the whole process with a single experiment permitting the discrimination of drug release and dose effects. The method proposed allows simple, well reproducible tests that may be used in alternative and/or complementary manner with respect to the traditional drug release assays, to obtain objective parameters, directly drawn from the record that quantify the drug sealing effect of the carrier. The peculiar results for the DOX drug carrier system investigated indicate also that, the use of hydrophilic dendrimers in this case, produces high sealing effect that is promising in terms of the intestinal flora protection.

## Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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