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Temperature and polymer crosslinking degree influence on drug transfer from α,β-polyasparthydrazide hydrogel to model membranes. A calorimetric study

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Abstract

A non-steroidal anti-inflammatory drug, diflunisal, has been chosen as drug model to be incorporated in α,β-polyasparthydrazide (PAHy) matrices to study the effect of polymer crosslinking degrees on the release processes from hydrogel ($X = 0.4$ and $X = 0.8$) to a model membrane represented by unilamellar vesicles of dipalmitoylphosphatidylcholine. The technique employed to monitor these processes was differential scanning calorimetry that appears to be particularly suitable to follow the transfer kinetics of a drug from a controlled release system to void biomembrane model. The drug release from the two PAHy hydrogels differently crosslinked by glutaraldehyde to the lipidic model was compared with that from the drug solid form, by examining the effects exerted on the thermotropic behaviour of unilamellar vesicles. The diflunisal (DFN) is able to interact with unilamellar vesicles by causing a decrease of the transitional (gel-to-liquid crystal phase transition) temperature characteristic of lipidic bilayer. The amount of DFN transferred and interacting with the dipalmitoylphosphatidylcholine (DPPC) unilamellar vesicles was quantified by comparing the effects caused on the thermodynamic parameters of bilayer (transitional temperature, T_m, and enthalpy variation, ΔH) with the effects obtained from increasing molar fractions of drug. The release kinetics of the drug from PAHy hydrogels were followed at different temperatures (25, 37 and 50°C) to determine the influence of temperature on the drug release and successive transfer at a biological membrane. Particularly, it appears evident that by increasing the polymer crosslinking degree the total amount of transferred drug and the release velocity are decreased. This behavior may be caused by the increase of the number of cruciate bonds in the hydrogels, which causes a free volume reduction obstructing the drug passing. The obtained results suggest that PAHy hydrogels constitute an innovative delivery system able to slightly release water-soluble drugs and to modulate their uptake by biomembrane.

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1. Introduction

The study and development of drug delivery systems has been, during the last few years, one of the most active fields of biopharmaceutical research. Hydrogel formulation and application in the controlled release of bioactive molecules have been one of the most recent applications in this field (Kamath and Park, 1993; Peppas and Khare, 1993; Dumitriu, 1994; Bettini et al., 1995; Colombo et al., 1996). Hydrogels are natural or synthetic crosslinked polymers able to adsorb water (usually $>20\%$ of their dry weight) and swell, reaching equilibrium volume and preserving their structural integrity because of permanent links among the polymer chains. Hydrogels, because of their low toxicity, good biocompatibility and ability to release the entrapped solutes when dispersed in the aqueous medium, are considered excellent 'carriers' for a variety of pharmacological agents ranging from small molecular weight compounds to macromolecules. Their use for controlled and prolonged drug release systems permits modulation of the drug release, with the release rate of entrapped solute depending on many factors such as chain mobility, crosslinking density, crystallinity degree, swelling degree, drug solubility in the polymer, etc. (Lee and Kim, 1991).

The preparation of new hydrogels obtained by chemical crosslinking of α,β-polyasparthydrazide (PAHy) by glutaraldehyde has been reported (Giammona et al., 1994a, 1995). These polymers shown interesting properties in drug controlled release (Giammona et al., 1996a) and studies on their biocompatibility have been carried out (Giammona et al., 1996b).

In this work, diflunisal has been chosen as model drug to be incorporated in PAHy matrices to study the effect of different polymer crosslinking degrees on the release processes. Diflunisal (DFN) is a non-steroidal anti-inflammatory drug poorly soluble in water and causing gastrolesivity when orally administered (Cotton and Hux, 1985). So to avoid gastrolesivity, the drug dispersion in a polymeric matrix should represent a good approach to obtain release while minimizing the dangerous side-effects.

Diflunisal release from PAHy differently crosslinked ($X=0.4$ and 0.8) to a model membrane represented by dipalmitoylphosphatidylcholine (DPPC) unilamellar vesicles (LUV) was monitored by differential scanning calorimetry (DSC). The DFN was able to interact with unilamellar vesicles by causing a decrease of the transitional (gel-to-liquid crystal phase transition) temperature characteristic of lipidic bilayer. In fact, the lipidic bilayer, when heated, shows a phase transition L_β–L_α characterized by a transitional temperature and an enthalpy variation. When a drug is dispersed in the ordered lipidic sea a destabilization of the lipid mosaic can occur causing a T_m depression, according with Van't'Hoff model; the validity of this model application has been verified for different classes of compounds (Lee, 1977; Suezaki et al., 1990).

The drug release from the two PAHy hydrogels differently crosslinked was compared with the drug transfer from the solid form to the lipidic model by examining the effects exerted by the drug on the thermotropic behavior of unilamellar vesicles. The amount of DFN transferred and interacting with the DPPC unilamellar vesicles was quantified by comparing the effects caused on the thermodynamic parameters of bilayer (transitional temperature, T_m, and enthalpy variation, ΔH) with the effects obtained when increasing molar fractions of drug were dispersed in the DPPC vesicles.

The aim of the present work was to examine, by DSC, the suitability of employing the studied hydrogels as carriers for prolonged release of poorly soluble drugs, and the modulating effects exerted by the polymer crosslinking degrees as well as by the temperatures, chosen to follow the

transfer process. The DSC technique appears to be particularly suitable to follow the transfer kinetics of a drug from a controlled release system to a site mimicking a biomembrane. So it should offer a promising approach for studying drug release in in vivo studies as compared to the classical in vitro studies (Castelli et al., 1997).

2. Materials and methods

2.1. Materials

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine was obtained from Fluka Chemical (Switzerland). The lipid solution was chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentration was determined by phosphorous analysis (Bartlett, 1959).

α,β-Polyasparthydrazide was prepared and purified as previously reported (Giammona et al., 1994b).

Diflunisal (2-4-difluoro-4-hydroxy [1,1-biphenyl]-3-carboxylic acid) was provided by Sigma (USA).

2.2. PAHy crosslinking

PAHy crosslinking was performed using glutaraldehyde (50% v/v aqueous solution) as crosslinker and acetic acid as catalyst. First, 500 mg of PAHy were dissolved in 11 ml of distilled water, then 8 ml of 10 vol.% acetic acid were added. This solution was kept at 0°C using an ice bath, then a sufficient amount of 50 vol.% glutaraldehyde was added to give the desired crosslinking ratio, (X), expressed in mol crosslinking agent per mol PAHy repeating unit. In particular, 277.5 and 555 μl of glutaraldehyde were added to obtain the two different PAHy crosslinked (respectively, $X = 0.4$ and $X = 0.8$). The reaction mixture was kept at room temperature for 12 h and heated at 60°C for 6 h to promote crosslinking. After completion of the reaction, the crosslinked materials were filtered and washed several times with distilled water until washing medium pH was 7. Then, they were dried to constant weight under vacuum in the presence of P_2O_5 at 25°C. The obtained powder was ground and the particle fractions between 20 and 90 μm were separated by sieving and were stored in a dryer containing P_2O_5 (Giammona et al., 1996a). Microparticles of PAHy networks were immersed in a concentrated ethanolic solution of diflunisal and left to soak for 5 days at room temperature. The microparticles were filtered, rapidly washed with ethanol and dried to constant

Table 1
Drug partition coefficient between crosslinked PAHy and Tris buffer at different temperatures

	25°C	37°C	50°C
PAHy $X = 0.4$	0.30	0.20	0.055
PAHy $X = 0.8$	0.18	0.08	0.025

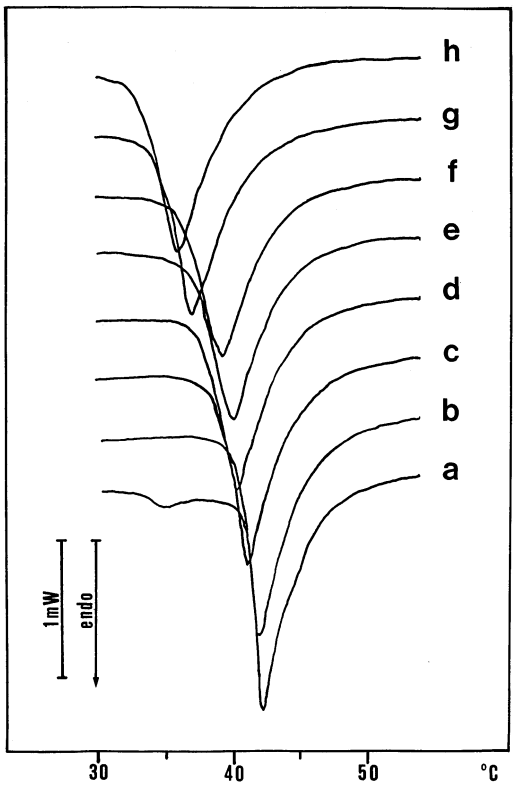

Fig. 1. Calorimetric curves of the LUV in presence of increasing molar fraction of DFN: a = 0.0, b = 0.02, c = 0.04, d = 0.06, e = 0.09, f = 0.12, g = 0.18, h = 0.24

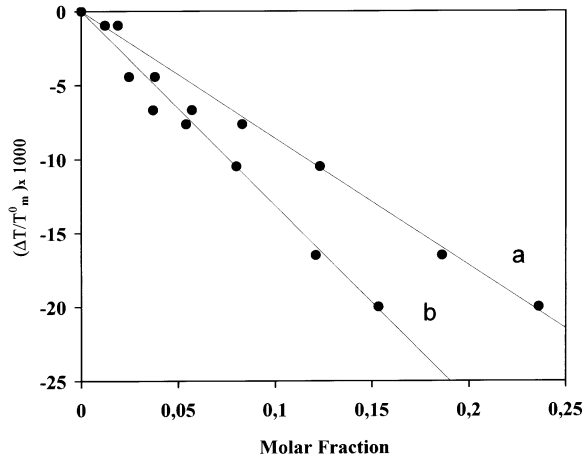

Fig. 2. Calibration curve relating the depression of DPPC lipid bilayer transition temperature to increasing concentration of DFN present in: (a) the aqueous lipid dispersion; or (b) effectively dissolved in the lipid matrix.

weight. The amount of drug loaded on the microparticles was determined by extraction with ethanol, the samples being assayed by UV spectrophotometry at 252 nm and the absorbance being converted into concentration using an $E^{1\%}$ value of 541.23. The drug loading was found to be 2.9% w/w for $X = 0.8$ and 2.58% w/w for $X = 0.4$.

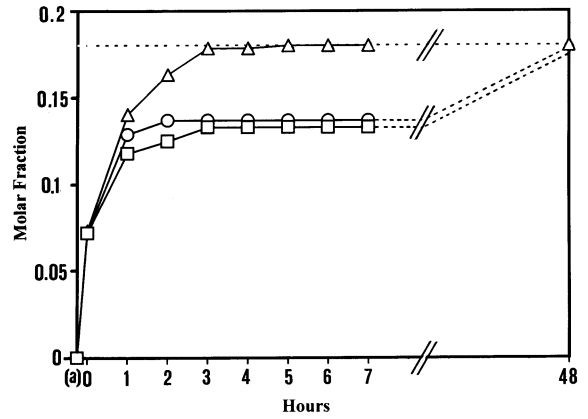

Fig. 3. Release kinetics curves of free DFN transfer to DPPC unilamellar vesicles at three different temperatures 25°C (□), 37°C (○) and 50°C (△) for increasing incubation times (0–7 h; 48 h); (a) represents the pure DPPC, and (- - -) the theoretical amount of drug to be released.

2.3. Swelling studies

Swelling of PAHy microparticles was studied in Tris buffer at pH 7.4, using a Leica Quantimet 500 image-processing and an analysis system equipped with a Leica Wild 3B stereomicroscope. This image processor calculated the particle area and converted it to an equivalent circle diameter. The apparatus calculated, also, the roundness index that was always less than 1.3. Using the method previously proposed (Robert et al., 1987), we observed that microparticle diameter increased until equilibrium was reached.

Equilibrium swelling ratio Q was determined from the relationship:

$$Q = [(d_\infty - d_0)/d_0] \times 100$$

where d_∞ and d_0 are, respectively, the diameters of swollen and dry microparticles.

The obtained results show, respectively, for $PAHy_{0.4}$ and $PAHy_{0.8}$, an increase in the particles size diameters of 6 and 3%, with respect to the dry polymers.

2.4. Drug partition between polymer matrix and Tris buffer

The determination of drug partition coefficient between crosslinked PAHy and Tris buffer at pH 7.4 was performed using the following procedure.

Aliquots of crosslinked PAHy (50 mg) with $X = 0.4$ and $X = 0.8$ were dispersed in a suitable volume of Tris buffer at pH 7.4 containing a known amount of diflunisal and kept to equilibrate at 25, 37 and 50°C for 48 h. After this time, the samples were filtered and the amount of drug was determined by UV spectrophotometry for the liquid phase ($E^{1\%}$ 252 nm = 1040) and exhaustive extraction followed by UV analysis for the solid phase. Each experiment was carried out in triplicate and the results, reported in Table 1, agreed with each other within 4% error.

2.5. Liposomes preparation

DPPC multilamellar liposomes were prepared in the absence of drug, at a temperature above the phase transition, starting from a chloroform/

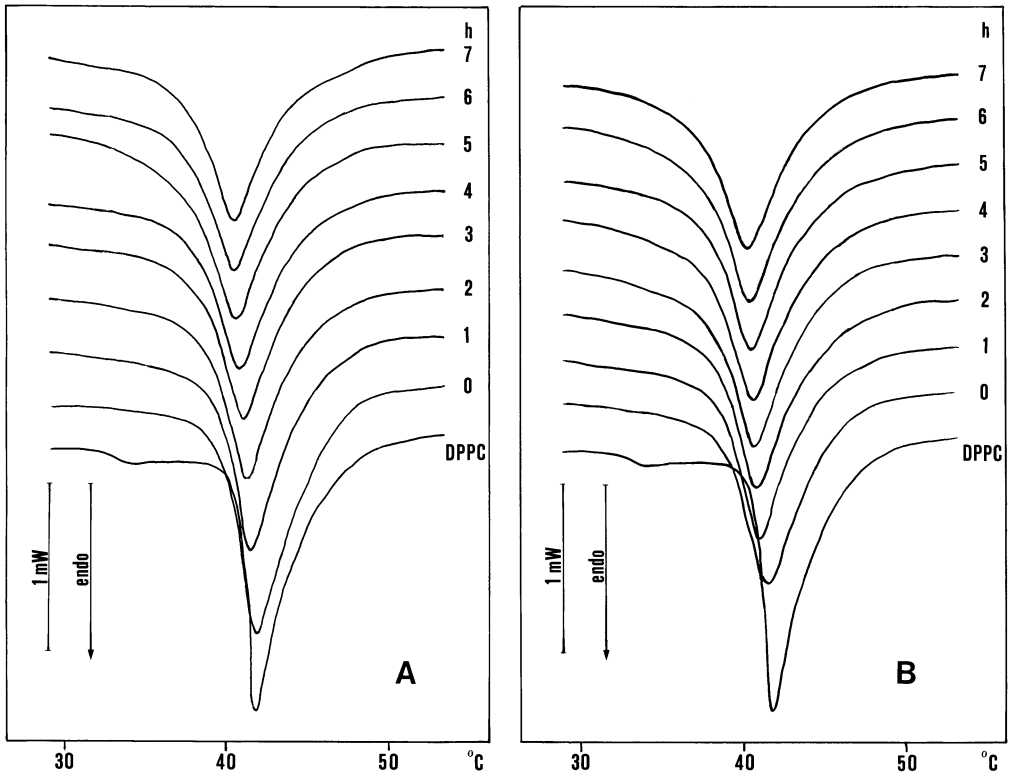

Fig. 4. Calorimetric curves of the DFN release from PAHy crosslinked (A = 0.4, B = 0.8) to DPPC unilamellar vesicles for increasing incubation times (0–7 h) at a temperature of 25°C.

methanol (1:1 v/v) lipid stock solution. The solvents were removed under a nitrogen stream in a rotavapor. The resulting film was kept overnight under vacuum to remove the residual solvents.

Then 50 mM Tris buffer (pH 7.4) was added to the dried lipidic film, and it was heated at 50°C and vortexed three times for 1 min. The samples were shaken for 1 h in a water bath at 50°C to homogenize the liposomes.

Afterwards, the obtained multilumellar vesicles (ULV) were submitted to extrusion through polycarbonate membranes (Nucleopore, Pleasanton, CA, USA), obtaining large unilamellar vesicles of defined diameter (100 nm) (Hope et al., 1985).

2.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were carried out using a Mettler TA3000 system equipped with a DSC-30 cell and TC10 processor. The scan rate employed was 2°C/min, and the temperature range was 10–50°C with a sensitivity of 1.72 mW. The DSC pans employed were 150-μl aluminum pans sealed after being filled with the samples. The reference pan was filled with Tris buffer solution. Temperature calibration was made using indium, stearic acid and *n*-octane. Indium was employed to calibrate the transitional enthalpies (ΔH). Enthalpy changes were calculated from peak areas using the integration program of Mettler processor.

2.7. DFN-LUV interaction

DPPC unilamellar liposomes were submitted to DSC analysis in the presence of an increasing

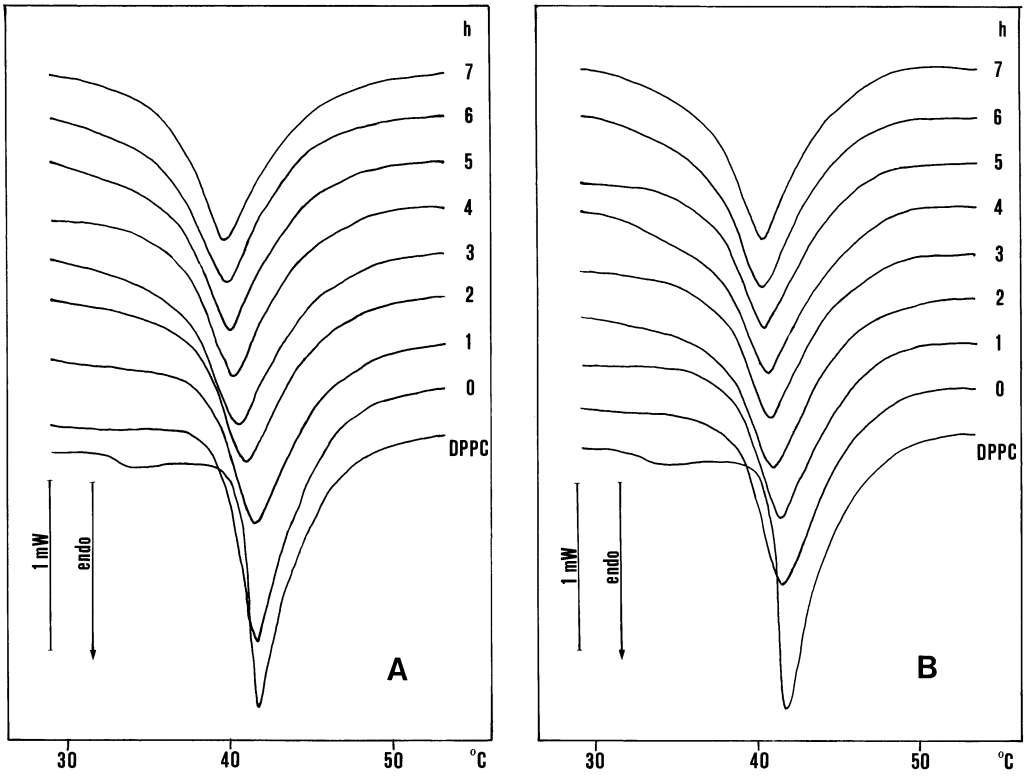

Fig. 5. Calorimetric curves of the DFN release from PAHy crosslinked (A = 0.4, B = 0.8) to DPPC unilamellar vesicles for increasing incubation times (0–7 h) at a temperature of 37°C.

amount of DFN, after a prolonged incubation time, 1 h under gently shaking.

By the calorimetric curves, reported in Fig. 1, it is evident that the DFN interacts with the liposomes causing a shift of the DSC curves towards lower temperatures. The $\Delta T/T^{\circ}_{m}$ values ($\Delta T = T_m - T^{\circ}_{m}$, where T°_{m} and T_m are the transitional temperatures (K) of pure DPPC and DPPC liposomes loaded with DFN, respectively) were plotted versus drug molar fractions (Fig. 2, plot a). The following equation was obtained by data linear regression: $Y = -0.73 - 82.97X$; $r = 0.993$. This plot will constitute the correlation between the increasing drug molar fractions and the effect exerted on the lipidic transition, and will be employed to follow the drug release kinetics by obtaining the amount of drug interacting with the model membranes by the effect (T_m depression) exerted by the drug release.

2.8. Drug partition between lipid and aqueous phases

To determine the real amount of drug transferred on the model membrane, three samples of unilamellar vesicles loaded with different DFN molar fractions (0.06, 0.12, 0.18) were prepared. The samples were incubated and centrifuged using a Beckam model J2-21 centrifuge at $37800 \times g$ for 25 min at controlled temperature. Afterwards the supernatant was separated from the lipidic pellets, which were dried and lyophilized. The amount of drug present in the two fractions (aqueous and lipidic) was detected using UV spectroscopy at 252 nm. These data were employed to modify curve a obtaining plot b in Fig. 2 ($Y = -0.73 - 127.60X$; $r = 0.992$) which represents the effect exerted by the real molar fraction of drug present in the membrane, and can be employed to calculate the drug uptake by void liposomes.

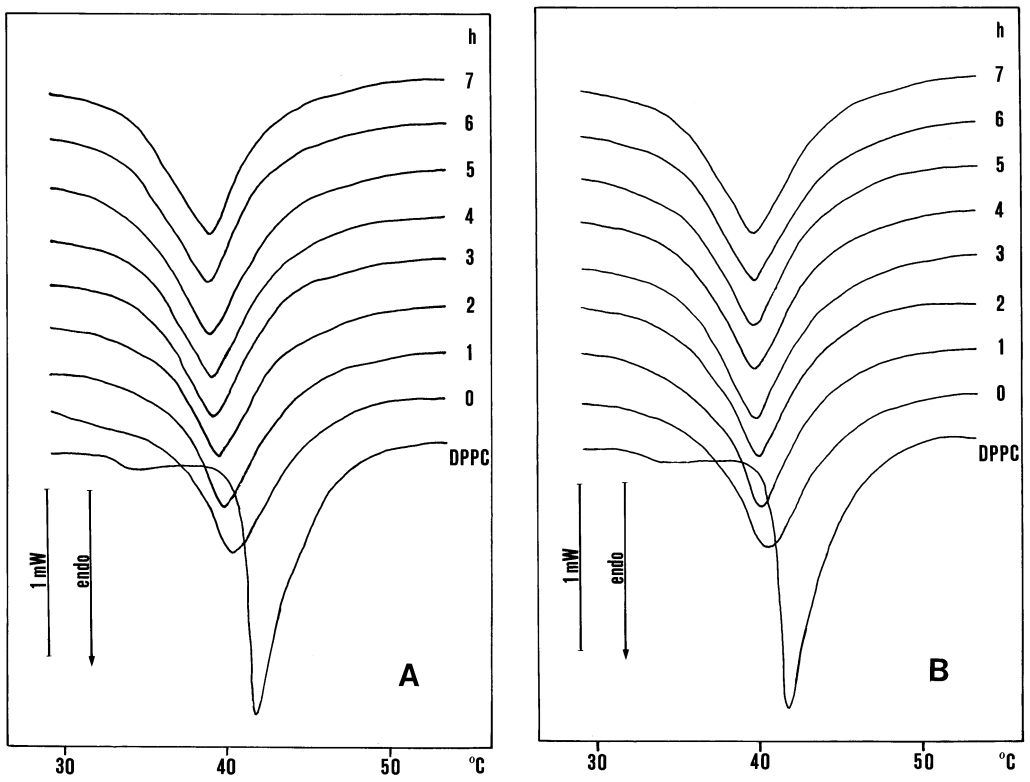

Fig. 6. Calorimetric curves of the DFN release from PAHy crosslinked (A = 0.4, B = 0.8) to DPPC unilamellar vesicles for increasing incubation times (0–7 h) at a temperature of 50°C.

2.9. Release experiments monitored by DSC

Free powdered diflunisal or diflunisal loaded PAHy hydrogels, were added to DPPC unilamellar vesicles in known amounts to obtain the same molar fraction (0.18) of drug, free or dispersed in the polymer with respect to lipid. The release kinetics were followed at different temperatures (25, 37 and 50°C) for every sample to evaluate its influence on the drug transfer at the membrane. The samples, loaded in DSC pans, were analyzed immediately after preparation by the following procedure: (1) a first scan (from 10 to 50°C) to detect the drug release, bringing the sample to 50°C; (2) an isothermal incubation period of 1 h at different temperatures (25, 37 or 50°C) in accordance with the temperature selected; (3) a fast cooling scan of the sample to 10°C; and (4) subsequent scan from 10 to 50°C to detect the drug released after the incubation period.

This procedure was repeated several times until no further drug release was observed.

2.10. PAHy effect on DPPC liposomes

To ensure that the effect exerted by drug-PAHy systems on DPPC liposomes was assigned only to the drug without any polymer interference, the eventual interaction of polymer, crosslinked or not, with lipidic model membranes was investigated using the same experimental conditions. DSC analysis of DPPC liposomes in the presence of an amount of polymer free of drug (PAHy, $PAHy_{0.4}$ and $PAHy_{0.8}$), similar to that employed in the DFN release experiments, was carried out for increasing incubation times (1–7 h) and for different temperatures (25, 37 and 50°C).

The results (data not reported) demonstrate that as no polymer effects are exerted on thermotropic behavior of liposomes, all variations

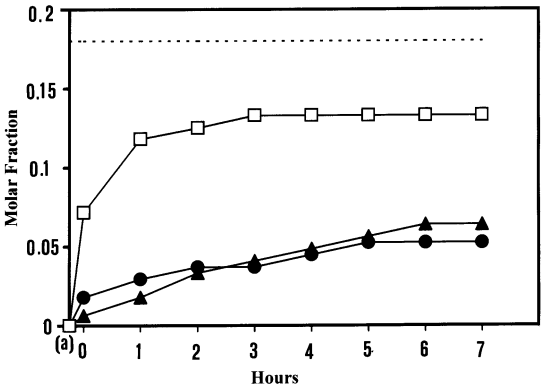

Fig. 7. Drug release from free DFN (□), $PAHy_{0.4}$ (▲) and $PAHy_{0.8}$ (●) at a temperature of 25°C; (a) represents the pure DPPC and (- - -) the theoretical amount of drug to be released.

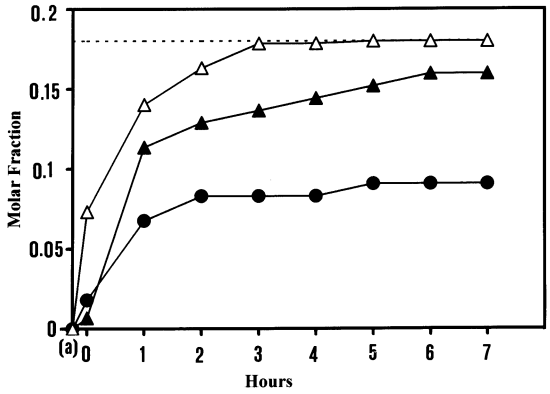

Fig. 9. Drug release from free DFN (△), $PAHy_{0.4}$ (▲) and $PAHy_{0.8}$ (●) at a temperature of 50°C; (a) represents the pure DPPC and (- - -) the theoretical amount of drug to be released.

observed during the drug release from PAHy are to be attributed only to the drug.

3. Results and discussion

3.1. Free drug-DPPC liposomes interaction

The DFN interaction with DPPC unilamellar vesicles was studied at three different temperatures (25, 37 and 50°C) to better clarify the influence of temperature on the drug dissolution process and on the following migration to liposomial bilayer. The NSAID-liposomes interaction has been previously studied and a 'fluidifying' effect, caused by the intercalation of drug molecules in the ordered lipidic structure was suggested (Castelli et al., 1989, 1990, 1992, 1994, 1996). This effect is revealed by the decrease in the transitional temperature of the lipid bilayer.

Fig. 3 shows the data of the free DFN transfer to DPPC LUV, obtained from the calorimetric curves, for increasing incubation times (0–7 h) at 25, 37 and 50°C, respectively.

These curves are successively compared with the drug release from the studied hydrogels at different crosslinking degrees. The different behavior in the DFN transfer to liposomes should be explained by the influence of temperature on the phospholipid phase transition and drug solubility (37°C, 0.032 mg/ml; 50°C, 0.050 mg/ml) (Cotton and Hux, 1985). In fact, at 50°C, where either a high drug solubility or LUV existence in the disordered state permitting a faster drug permeation are observed, the drug transfer is higher with respect to lower temperatures, thus reaching the theoretical amount transferable quickly compared to that obtained at lower temperatures only after 48 h; it was possible to obtain the same value obtainable at 50°C because of the slower kinetics (dotted line).

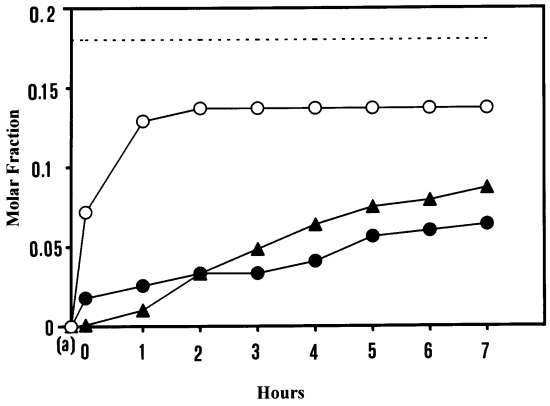

Fig. 8. Drug release from free DFN (○), $PAHy_{0.4}$ (▲) and $PAHy_{0.8}$ (●) at a temperature of 37°C; (a) represents the pure DPPC and (- - -) the theoretical amount of drug to be released.

3.2. Temperature and polymer crosslinking degree influence on drug release

The calorimetric curves of the DFN release at the three examined temperatures for the two degrees of PAHy crosslinked (A: $X = 0.4$; B: $X = 0.8$) are reported in Figs. 4–6. The release kinetic plots obtained from the calorimetric curves are reported in Figs. 7–9.

The drug release from $PAHy_{0.4}$ and $PAHy_{0.8}$ at 25°C (Fig. 7) and 37°C (Fig. 8) appears to be similar but lower with respect to the free drug. Small differences can be observed only for short times, when the sample with higher crosslinking degree seems to release the DFN faster with respect to the sample with the lower crosslinking degree. This behavior should be caused by impregnation process. The drug should penetrate $PAHy_{0.4}$ better than $PAHy_{0.8}$, because DFN could be impeded in penetrating the three-dimensional matrix by the high crosslinking degree and the consequent lower swelling capacity. Further confirmations comes from the drug partition coefficient between polymeric matrix and Tris buffer (0.3 for $X = 0.4$ vs 0.18 for $X = 0.8$ at 25°C, and 0.2 for $X = 0.4$ vs 0.08 for $X = 0.8$ at 37°C). In consequence, the initial drug release (first two incubation periods) from $PAHy_{0.8}$ appears faster than that from $PAHy_{0.4}$.

This process is evident looking at the calorimetric curves (Fig. 4A,B and Fig. 5A,B) where the calorimetric curve at time 0 (without incubation period) peak for the DFN released from $PAHy_{0.8}$ is smaller and broader than the sharper $PAHy_{0.4}$, an indication of a deeper interaction between DFN released by $PAHy_{0.8}$ and phospholipid bilayer. After 2-h incubation, the amount of drug released from both polymers (0.4 and 0.8) seems to be the same because of the smoothing effect due to the lower swelling of $PAHy_{0.8}$, with respect to $PAHy_{0.4}$.

Looking at Fig. 8 it appears evident that the trend of DFN release at 37°C is similar to that observed at 25°C, even though the different crosslinking degree causes a separation of the two kinetics curves. This behavior could be due to the higher temperature (37°C), which influences the drug solubility and migration, and the phospholipid phase which is near to the disordered phase.

By incubating the sample at 50°C it is evident (Fig. 9) that the release of DFN from $PAHy_{0.4}$ exceeds that observable for $PAHy_{0.8}$ for nearly all incubation times. Both release curves are higher than those observed for the lower temperatures. This may be explained by considering that the lipidic bilayer is in a liquid crystal phase, able to accept the DFN released by the polymer. The release kinetics become dependent only on the crosslinking degree, decreasing by increasing the polymeric reticulation, while the acceptor system remains in a fully disordered state. This behavior can be caused: (1) by the increase of the number of crosslinks in the PAHy hydrogels, which causes a 'free volume' reduction obstructing the drug diffusion; and (2) by the increase in temperature determining an increase in the DFN solubility. As shown in Fig. 6, just after the first hour of incubation the peak immediately becomes large and remains this shape for longer incubation times.

4. Conclusions

The reported results suggest that PAHy hydrogels represent a valid device for sustained drug release, promoting their uptake by biomembrane. The examined sample seems to be able to reach a release modulated by crosslinking degree (only for high temperature acting both on solubility and membrane disordered state). The DSC technique allows study of the release and diffusion of the drug from the polymer to the site mimicking a biological membrane, as the polymer properties (kind, crosslinking degree, swelling etc.) as well as temperature (acting on drug solubility and bilayer lipidic capacity to adsorb the drug) are able to influence the drug release kinetics and the related uptake kinetics of the membrane model.

Such a technique, enabling the evaluation of the release kinetics, can be proposed as a valid alternative to classical dissolution in vitro studies, to determine the amount of drug really transferred to a model membrane.

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