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Controlled release of biomolecules from temperature-sensitive hydrogels prepared by radiation polymerization

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Abstract

Poly(acryloyl-L-proline methyl ester)-based hydrogels containing 1 and 5% of a crosslinking agent were studied as drug delivery systems. The drug loading properties were investigated by matrix incubation into solutions containing biomolecules with molecular weight ranging between 300 and 65 000 Da. The loading yield was found to depend on both the crosslinking degree and the molecular weight of the drug. In vitro release studies were carried out with both swollen and dry matrices loaded with gentamicin, isoniazid and insulin. Gentamicin and isoniazid were released by a bimodal Fickian diffusion with a remarkable burst that was found to depend on both matrix crosslinking degree and physical state. In vivo, the subcutaneous implantation into mice of the isoniazid loaded matrices allowed for an efficient drug release for 800 h. In vitro insulin was released from the swollen matrices for 1500 h by diffusional Fickian mechanism while the dry ones displayed a lag time followed by Fickian diffusion release. The subcutaneous implantation of the insulin-loaded matrices into diabetic mice induced a remarkable decrease in the glucose concentrations in blood. In particular, the dry 1% matrices were found to maintain a low glucose level for 700 h. © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

The influence of synthetic polymers in the development of delivery systems has been of paramount importance in biomedicine. This fact

promoted the rapid emergence during the past 2 decades of the controlled release technology as a new interdisciplinary science that offers different approaches to the delivery of bioactive agents. Such delivery systems present numerous advantages compared to conventional dosage forms including a higher effectiveness, lower toxicity and improved patient compliance [1,2].

Recently, a new class of hydrogels has been

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developed which undergo abrupt, reversible changes in volume in response to minor changes in the environment, such as solvent composition, temperature, pH, ionic strength, electric field, light intensity, pressure as well as specific chemical stimuli, i.e. glucose concentrations. Hydrogels undergoing volume transitions due to changes of the temperature have been termed ‘thermally responsive’ or ‘temperature sensitive’ hydrogels [3–6]. These types of hydrogels will suddenly shrink as the temperature increases above a critical temperature, which is approximately the lower critical solution temperature (LCST) of the corresponding linear polymer.

The release mechanisms of a drug entrapped in these hydrogels when the temperature is cycled above and below the LCST is determined by a cyclic swelling and deswelling similar to a physical ‘squeezing’ and water reabsorption of a sponge. In other words, a temperature-sensitive hydrogel works like a pump that sucks up the drug on cooling and squeezes it out on heating.

The advantage of using these hydrogels for the controlled release applications is the possibility of loading the drug at a low temperature at which the polymer matrix has a different porosity to that which occurs at a higher temperature when the drug is released. Moreover, the entrapment of drugs with different molecular weight gives useful information about the hydrogel loading properties.

Radiation is a technique advantageously used for the surface modification of polymeric biomaterial for an enhanced biocompatibility or when polymerisation has to be carried at low temperatures, as it is the case for special applications, for example immobilisation of biologically active species [7].

Radiation polymerisation and crosslinking are reactions that often take place concurrently during preparation of hydrogels [7,8]. By this technology, it is possible to control the crosslinking density, which, in turn, determines the hydrogel structure.

In our laboratories, thermosensitive hydrogels of acryloyl-L-proline methyl ester (A-ProOMe) have been synthesised by radiation-induced polymerisation, characterised using different techniques and used as matrices for a slow release of drugs [9–13]. It was found that their responsiveness can be easily modulated by copolymerisation of A-ProMe with hydrophilic or hydrophobic monomers: in the former

case the transition temperature increases while in the latter it decreases [9–11].

In this paper, we report the release both *in vitro* and *in vivo* of a few bioactive molecules with different molecular weights from poly(A-ProOMe) hydrogels obtained by radiation-induced polymerisation.

The study aimed at investigating the exploitation of these hydrogels for drug release systems, bearing in mind the proper formulation of biotechnological compounds such as proteins. The influence of the matrix as well as the biomolecule physicochemical properties on the drug release behaviour and the mechanism which underlies the drug release were analysed.

2. Experimental

2.1. Materials and methods

Bovine insulin, gentamicin, isoniazid, bovine serum albumin, ribonuclease, streptozotocin and the glucose Trinder kit were obtained from Sigma (St. Louis, MO, USA). The protein Bio-Rad DC1 assay kit was from Bio-Rad (Hercules, CA, USA). Superoxide dismutase was from DDI (Mountain View, CA, USA). The C₄ and C₁₈ reversed-phase columns were furnished by Vydac (Hesperia, USA). All the other reagents were purchased from Fluka (Buchs, Switzerland). The YPM medium and the agar were obtained from Difco.

The method of synthesis of acryloyl-L-proline-methyl ester monomer (A-ProOMe) has been previously described [9]. The crosslinking agent trimethylolpropane trimethacrylate (TMPTMA) was from Aldrich (Steinheim, Germany) and used as received.

The *in vivo* studies were carried out using male Balb/c mice weighting 25–27 g and fed *ad libitum* following the institutional European Guidelines.

2.2. Matrix preparation

Poly(acryloyl-L-proline-methyl ester) (poly(A-ProOMe)) hydrogels were obtained by radiation polymerisation of A-ProOMe at room temperature at a dose rate of 0.74 Gy/s and a total dose of 9.5 kGy

after flushing nitrogen. Cylindrical matrices were cut into disks of 6 mm diameter×2 mm height. After irradiation, the disks were repeatedly washed with cold water to remove the unreacted monomer. Subsequently, they were dried by lyophilisation.

2.3. Biomolecule loading

The matrices with 1 and 5% of TMPTMA, further referred to as 1 and 5% matrices, respectively, were extracted with 500 ml of distilled water at 4°C for 15 days; the water volume was changed every 2 days. The matrices were frozen, lyophilised, weighed and incubated for 10 days at 4°C with 200 µl/matrix of a 20 mg/ml solution of bovine serum albumin (BSA) or superoxide dismutase (SOD) or ribonuclease (RNAase) or insulin or gentamicin or isoniazid. Finally, the matrices were washed with 2 ml of water for 30 min. The final volume was accurately determined and the BSA, SOD, RNAase and insulin concentrations were estimated by the protein Bio-Rad DC₁ assay. Gentamicin was determined by UV at 335 nm after reaction with *o*-phthalaldehyde as reported in the literature [14] and isoniazid was determined by reversed-phase C₁₈ HPLC [15].

The biomolecule loading degree was determined on the basis of the results obtained with six 1% matrices and six 5% matrices and it was expressed as the percentage of the loaded biomolecule:matrix (w/w).

2.4. In vitro drug release

Lyophilised and nonlyophilised 1 and 5% matrices loaded with gentamicin, isoniazid and insulin were incubated at 37°C with 5 ml/matrix of 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2. At scheduled times, 1 ml of buffer was withdrawn and replaced by 1 ml of fresh buffer to maintain 5 ml of incubation volume. The gentamicin and isoniazid content in the withdrawn volumes was determined according to the methods reported above. Insulin was estimated by reversed-phase chromatography using a C₄ column isocratically eluted with water–acetonitrile–TFA (72.45:27.5:0.05, v/v).

The amount of released drug was calculated on the basis of the results obtained with five matrices for each sample set.

2.5. In vitro gentamicin loaded hydrogel activity

The 1 or 5% gentamicin loaded matrices were incubated in 25 ml of sterilised YPM medium containing 1% of *Acetobacter aceti* at 28°C. At scheduled times 100 µl of medium were taken and diluted to 10⁻⁶. The matrices were washed with sterile water and processed as reported above. The diluted medium was added to a 2% of agar plate and then incubated at 37°C. After 24 h the colony forming units (CFU) were determined.

The control was obtained following the same protocol without matrix incubation. The antibacterial activity was calculated as follows

$$\% \text{ of growth inhibition} = (1 - \text{CFU sample} / \text{CFU control}) \times 100$$

2.6. In vivo studies

2.6.1. In vivo isoniazid release

Isoniazid loaded lyophilised or nonlyophilised 1 and 5% matrices were subcutaneously implanted into mice. At scheduled times, the animals were anaesthetised with ethyl ether and bled. The blood samples were centrifuged at 3500 rpm and 25 µl of serum were added to 20 µl of PBS and 30 µl of acetonitrile. The samples were centrifuged and the isoniazid content in the solution was estimated by RP-C₁₈ column as reported above. The isoniazid concentration was determined on the basis of a titration curve obtained by addition of known amounts of drug to blood volumes that were processed as above.

2.6.2. In vivo insulin release

Diabetes was induced in 24 mice by intraperitoneous administration of 130 mg/kg of streptozotocin. After 5, 7 and 9 days from streptozotocin administration, the animals were anaesthetised by ethyl ether and 20 µl of blood was taken by the retrobulbar site. The blood was centrifuged at 3500 rpm for 5 min and the glucose level in the diluted serum (1/10 in distilled water) was determined by the glucose Trinder Kit. Insulin loaded lyophilised or nonlyophilised 1 and 5% matrices were subcutaneously implanted into the diabetic mice and the animals were bled at scheduled times and the glucose level in the serum was determined as reported above.

3. Results and discussion

To exploit the peculiarity of the temperature sensitive poly(A-ProOMe) matrices as drug delivery systems, hydrogels containing 1 and 5% of TMPTMA as crosslinking agent were prepared by radiation polymerisation [13].

3.1. Drug loading studies

The hydrogel loading properties were investigated by matrix incubation at 4°C into solutions containing biomolecules of different molecular weight and structural complexity. At this temperature the hydrogel swelling was estimated to be about 74 and 63% (swollen hydrogel volume/dry hydrogel volume, %) for the 1 and the 5% matrices, respectively and, therefore, high drug loading can be obtained [13].

The data reported in Fig. 1 show that the drug loading yield depends upon the matrix crosslinking degree and the drug molecular weight. These two parameters are, in fact, related to the pore hydrogel size and the drug size that control both the absorption volume and the drug diffusion into the matrix.

The linear correlation between drug $\log M_w$ and loading yield obtained with both matrices (1 and 5%) can be ascribed to the reduced drug diffusion into the swollen matrix as the biomolecule molecular weight and structural complexity increase. On the other side, the lower drug loading obtained with the 5% matrices relative to the 1% hydrogels is due to the more compact polymeric structure of the former that limits the absorption of the drug solution. The lack of high M_w protein loading (SOD and BSA) obtained with the 5% matrices can be related to the

small pore size of the hydrogel. As SOD is a globular protein with M_w of 31 000 Da, we can suppose that in the case of the 5% matrices the hydrogel pore size is smaller than the hydrodynamic volume of such protein. Instead, in the case of 1% matrices, even proteins with M_w up to 65 000 Da, such as BSA, can be entrapped in significant amounts, thus showing that a low level of crosslinking can provide a suitable formulation for high-molecular-weight biomolecules, such as biotechnological products, proteins and oligonucleotides.

3.2. Low-molecular-weight drug release

The drug release from nonbiodegradable cross-linked hydrogels is usually controlled by a combination of mechanisms [16]. In the first stage, the drug release from temperature sensitive hydrogels in the swollen state is determined by the matrix shrinking and the Fickian diffusion throughout the soaked hydrogel, while the release from dry hydrogels is determined by Fickian diffusion, the boundary layer and the drug dissolution rate [17]. In both cases, when the hydrogel has reached the swelling equilibrium, the drug is released by Fickian diffusion through the swollen matrix.

In order to evaluate the influence of the matrix physical state on the release mechanism and kinetics, *in vitro* studies were carried out with both lyophilised and swollen poly(A-ProOMe) 1 and 5% matrices. In this study, three drug models were used: gentamicin, isoniazid and insulin. These drugs were selected because of their therapeutic relevance: gentamicin in infectious diseases, isoniazid is used in the treatment of tuberculosis and insulin is the drug of

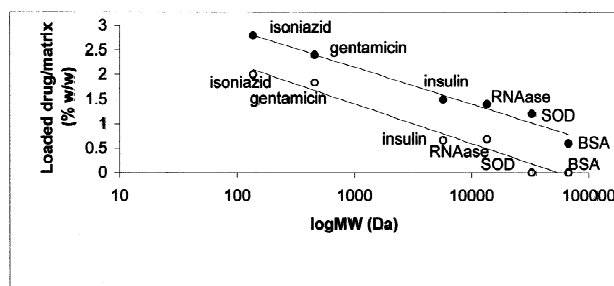


Fig. 1. Drug loading profiles obtained with 1% matrices (●) and 5% matrices (○). Each point represents the mean value obtained with five matrices.

choice in diabetes treatment. Furthermore, the data reported above demonstrate that these drugs could be loaded efficiently in the matrices at a level suitable for a sustained drug delivery.

The time course profiles shown in Fig. 2A indicate that gentamicin is released from all the matrices by a bimodal behaviour: a fast release at the very beginning followed by a slow release thereafter. When the sample temperature goes from 4° to 37°C, the diffusion coefficient changes with the temperature. The configuration of the polymer chain also changes during the variation of phase that lasts at least 6 h and, as a consequence, a change of porosity occurs that interferes with the diffusional processes. When the hydrogel reaches equilibrium, the release can be considered as the Fickian type. It should be borne in mind that the kinetics of release could be further complicated by the formation of a polymeric ‘skin’ on the gel surface that induces an incomplete dehydration of the hydrogel. The lower release rate observed with 5% matrices during the first 4 h, as compared with the 1% ones, can be attributable to the slower drug diffusion because of the higher crosslinking degree of this hydrogel and the lower drug loading yield.

The dramatic decrease in the diffusion rate observed after 24 h indicates that in a few hours the hydrogel shrinking reaches a critical point corresponding to a remarkable alteration of the polymeric structure. The similar behaviour of the two matrices, despite their differences in the swelling degree at 37°C, the very slow and incomplete drug release,

seem to indicate that at this point some interaction of the drug with the polymeric structure takes place.

The 1 and 5% dry matrices display comparable gentamicin release profiles with a burst during the first 24 h followed by a slow drug release. Also in this case the drug was released by Fickian diffusion indicating that the hydrogel swelling does not control the release process.

In vitro studies carried out by incubation of the gentamicin loaded matrices with *Acetobacter aceti* demonstrated that the drug was released in the active form. Under the experimental conditions, both 1 and 5% matrices displayed 96–98% of bacterial growth inhibition during the initial 24 h that decreased to 88% after 72 h. This activity lasted for over 120 h.

The isoniazid in vitro release profiles reported in Fig. 3 do not show any significant difference between the 1 and 5% matrices. Again the drug is released from the swollen and dry matrices with an initial Fickian diffusion burst indicating that neither the hydrogel shrinking in the case of the swollen matrices nor its swelling in the case of the dry ones control the drug release. After 24 h, a negligible drug release was observed probably because of strong drug–polymer interactions that seem to be enhanced by the lyophilisation process.

The Fickian behaviour obtained in all cases with gentamicin and isoniazid show that when drugs entrapped in the hydrogels possess a low molecular size as compared to the matrix pore, any control due to the physical change of the matrix structure (shrinking or swelling) fails. Therefore, in this case

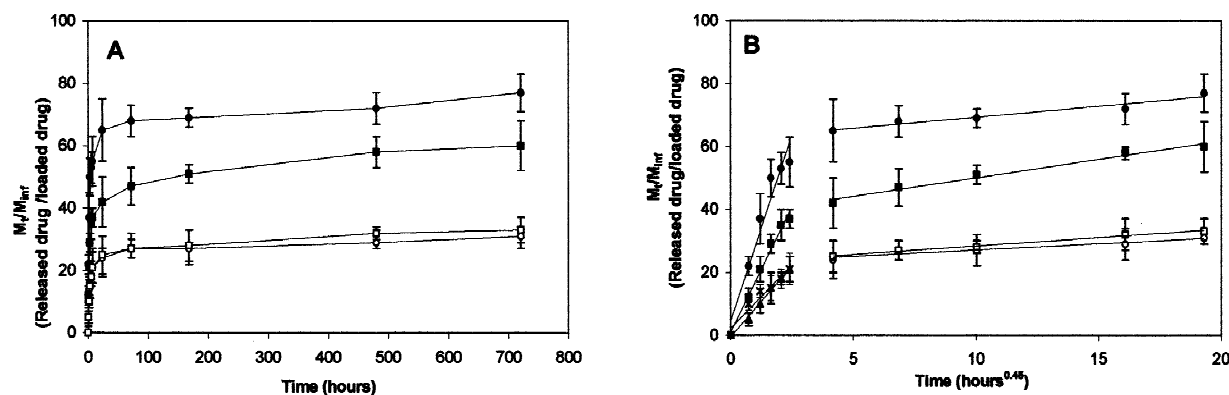


Fig. 2. In vitro gentamicin cumulative release profile: 1% (●) and 5% (■) swollen matrices; 1% (○) and 5% (□) dry matrices. Each point represents the mean value obtained with five matrices.

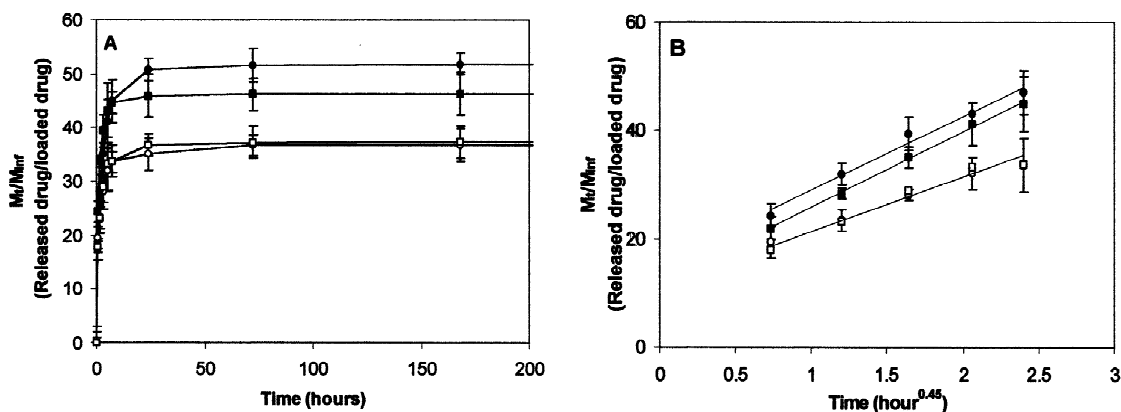


Fig. 3. In vitro isoniazid release profile: 1% (●) and 5% (■) swollen matrices; 1% (○) and 5% (□) dry matrices. Each point represents the mean value obtained with five matrices.

the use of thermal sensitive hydrogels as drug delivery systems seems questionable.

In order to evaluate the in vivo performance of these hydrogels as drug delivery systems the isoniazid loaded matrices were subcutaneously implanted into mice and the drug concentration in the blood was estimated.

The in vivo isoniazid profiles reported in Fig. 4 show a similar behaviour for the 1 and 5% matrices. Similar results were also obtained with the dry matrices (not reported data). Unexpectedly, both 1 and 5% hydrogels can provide for an efficient isoniazid delivery to blood for over 800 h despite the

fact that the in vitro studies did not show any significant release after 24–72 h.

3.3. Insulin release

The potential application of the poly(A-ProOMe) matrices in the protein delivery was investigated using insulin as a protein model.

The in vitro insulin release profiles reported in Fig. 5 indicate that in all cases the hormone release during the first stage was controlled by a combination of Fickian diffusion, hydrogel shrinking or swelling and drug dissolution, since neither a pseudo

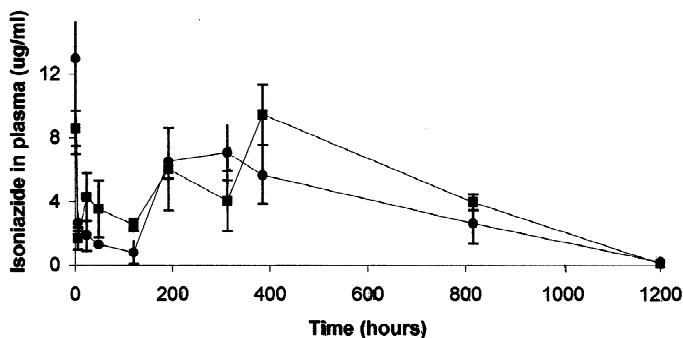


Fig. 4. In vivo isoniazid release time course: 1% (●) and 5% (■) swollen matrices. Each point represents the mean value obtained with seven matrices.

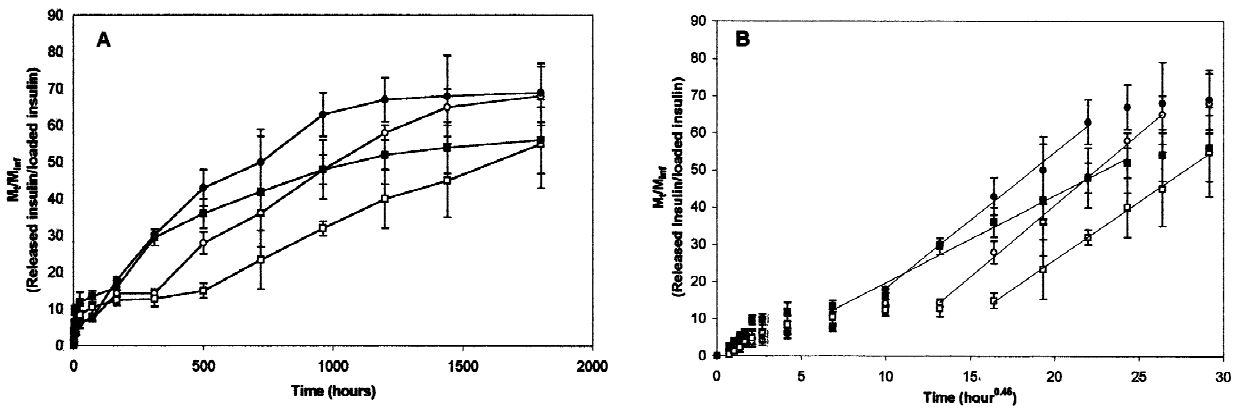


Fig. 5. In vitro insulin release profile: 1% (●) and 5% (■) swollen matrices; 1% (○) and 5% (□) dry matrices. Each point represents the mean value obtained with seven matrices.

first order nor a zero order kinetic were found. The release by Fickian diffusion occurs after about 50 h with the swollen matrices and 200 h with the dry ones. The delay in the diffusion release observed with the dry matrices is probably due to the slow dissolution of the hormone in the soaked hydrogels.

The slower release obtained with the 5% matrices

relative to the 1% ones reflects the lower diffusion of high-molecular-weight molecules as the crosslinking degree increases.

Fig. 6 reports the glycaemic profiles obtained by subcutaneous implantation into diabetic mice of the insulin loaded matrices. Soon after implantation, the swollen matrices induced a more rapid reduction in

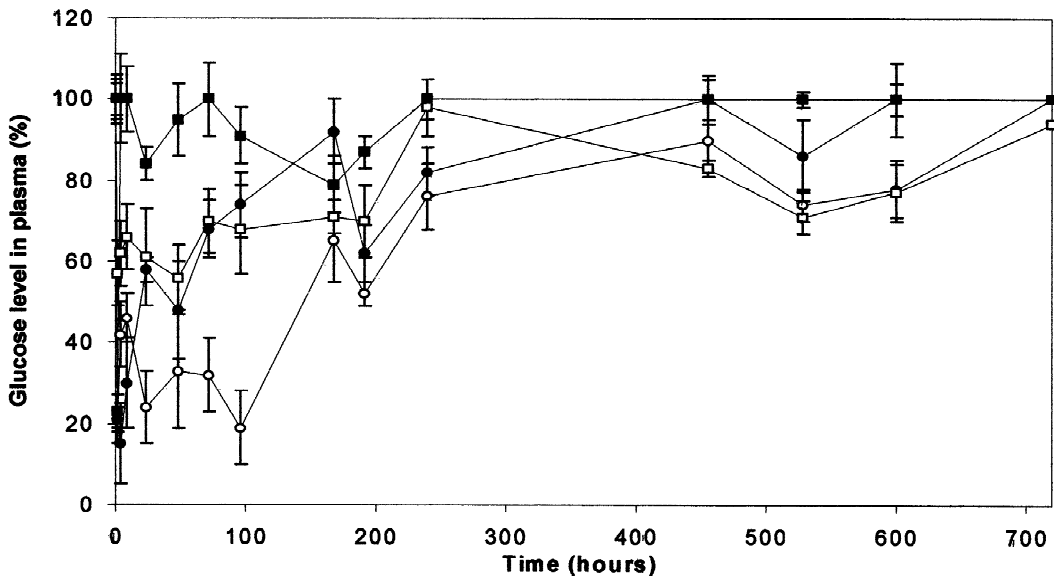


Fig. 6. Hypoglycaemic time courses: 1% (●) and 5% (■) swollen matrices; 1% (○) and 5% (□) dry matrices.

glycaemia than the dry ones. However, after the initial burst effect they displayed a lower and shorter pharmacological activity. Instead only a limited hypoglycaemic effect was obtained with the 5% matrices probably because of the low drug loading.

Interestingly, the lyophilised 1% matrices induced a remarkable glycaemia reduction (about 80%) from the second hour from implantation which was maintained for about 100 h. The lyophilised 5% matrices reduced the glucose concentration to about 50% and that was maintained for 50 h. In both cases the starting glucose concentration was slowly restored in 700 h.

The differences observed in the pharmacological performance of the dry and swollen matrices reflect the uneven drug release mechanism and kinetics. In the case of the swollen matrices the hydrogel shrinking after implantation probably induces a massive drug release that reflects in a rapid reduction in glycaemia. After the rapid release the drug could be too slowly released to guarantee a prolonged pharmacological effect. The lower activity observed with the 5% matrices seems in good agreement with the slower protein release observed after 14 h from *in vitro* incubation. On the other hand, the results obtained with the dry matrices seem to indicate that the hydrogel swelling provides for a more suitable control of the drug release kinetic that on one side prevents the too rapid and remarkable glycaemic decrease and, on the other one, allows for a sustained pharmacological effect.

After the *in vivo* studies the matrices were explanted and no fibroblast, connective tissue or exudates were found. Furthermore the matrix implantation did not provoke any tissue damage. These features underline the high local biocompatibility of these materials.

4. Conclusions

Temperature sensitive polymers have been recently investigated as materials for preparation of sustained drug delivery systems. Their structural modification by temperature changes confers them advantageous properties over the 'classical' hydrogels, namely easy elimination of toxic chemical agents used during their fabrication, drug loading under

mild conditions, temperature control of the drug release.

The data reported in the present work demonstrate that poly(A-ProOMe) based hydrogels can be successfully used for the preparation of sustained drug delivery systems. Biomolecules of different molecular weights and structural complexity can be suitably incorporated into the hydrogels by swelling to equilibrium in a suitable drug solution at low temperature that allows for the maintenance of the drug biological activity as demonstrated by the *in vitro* and *in vivo* results obtained with the gentamicin- and insulin-loaded matrices.

The loading yield can be modulated by using different amounts of crosslinking agent that affects on one hand the absorbed volume and on the other the diffusion of the molecules into the hydrogel. In particular, the significant incorporation of amounts of peptides and proteins into matrices with a low crosslinking degree makes these systems particularly useful for the delivery of biotechnological products.

Hydrogel shrinking or swelling was found to play a relevant role in high-molecular-weight drug release. Indeed, in the case of insulin the release was found to be affected by the temperature sensitive properties of the materials. However, we found that the shrinking effect on drug release depends strictly on the physicochemical properties of the drug. Low-molecular-weight drugs were released by Fickian diffusion throughout the swollen or the dry matrices indicating that the temperature sensitive properties of these properties do not control the drug release. The structural changes of the matrix due to the shrinking or swelling process can be reflected in strong drug-matrix interactions that can deeply influence the release rate. It was interesting to note that the lyophilisation process can enhance this effect.

Finally, *in vivo* studies demonstrated that these materials possess high local biocompatibility, a prerequisite of essential importance in the development of devices for pharmaceutical applications.

Acknowledgements

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