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Wet sol–gel derived silica for controlled release of proteins

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Abstract

The potential of wet sol–gel derived silica gels as new matrices for the entrapment and sustained release of proteins was investigated. Model proteins, BSA, ribonuclease-A and avidin, with differing molecular weights and/or isoelectric points, were entrapped in two silica polymer formulations having different silica contents (4% and 12% wt/v). The conformational stability of the proteins after entrapment and their release after immersion into physiological conditions were measured. Circular dichroism analysis showed that protein conformation is maintained after entrapment and stability is enhanced. Protein-free formulations were injected intramuscularly into BALB/c mice to monitor the *in vivo* fate of the matrix, and the results showed that the gel is totally reabsorbed, without any apparent surrounding inflammation process. The time required for matrix bioerosion varied between one to three weeks, depending on its SiO_2 content. Erosion was also measured *in vitro* and the contribution of erosion and diffusion to the release of the embedded proteins was quantified. These data indicate that wet silica polymers obtained by the sol–gel route are promising matrices for the sustained release of protein drugs.

Keywords: Sol–gel; Silica; Controlled release; Protein drugs; Parenteral administration

1. Introduction

Silica gel obtained by the sol–gel technology is an inorganic polymer produced synthetically by controlled hydrolysis and condensation of alkoxysilanes. This material, originally developed for engineering applications, is currently also being studied as a polymer for the entrapment and sustained release of bioactive compounds [1,2].

As opposed to common glass, which is a viscous fluid obtained by high temperature fusion of SiO_2, sol–gel silica is an amorphous, porous material which is synthesised in mild conditions [3], compatible with the stability of most bioactive compounds. This fact allows its exploitation as a matrix for entrapping bioactive compounds, with several applications in biotechnology and biomedical sciences. The synthesis of sol–gel silica occurs from liquid precursors ('sol'), *via* the initial formation of 'wet' gels in which the inorganic network is surrounded by solvents which are later removed to yield a dry material (xerogel or aerogel) (Scheme 1).

Within the specific field of drug delivery, low molecular weight and non-protein high molecular weight bioactive compounds have been entrapped in sol–gel derived xerogels to yield slow release systems. In this context, hormones, antibiotics, anticancer drugs and heparins have successfully been entrapped with the final scope of their oral or local parenteral administration [2,4–8], and the non-toxicity and bioerodibility of the polymer after implant have been demonstrated [6]. Proteins can also easily be incorporated into these matrices and their folding has been demonstrated to be substantially unaffected by entrapment while their stability is increased [8,9]. However, it is commonly accepted that, whereas low molecular weight compounds can diffuse freely through the silica matrix, proteins embedded in silica xerogels remain firmly trapped within the polymer network and no leakage occurs. Indeed, protein entrapment by the sol–gel route is considered an alternative tool for protein immobilisation with all the known applications related to immobilised protein

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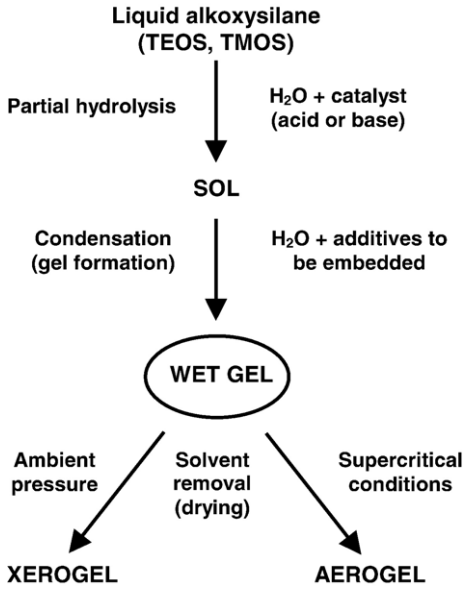
systems [10–20]. Nevertheless, some authors recently suggested using silica xerogels for sustained release of proteins (e.g., growth factors). Their pioneering works have provided a 'proof of concept', although very slow release and low overall protein recovery (less than 5%) were shown, in agreement with the immobilisation concept [21–25]. In all these works, proteins were embedded in either xerogels (dry) or partially dried gels, and the release kinetic was shown to depend on the degree of polymer syneresis following solvent removal which, as opposed to the case of organic polymers, is non-reversible.

Based on these results, we studied the use of sol–gel derived silica gels for sustained release of proteins by using the polymer in a still unexplored form, i.e., the wet conformation (Scheme 1). Unlike previous studies, we did not carry out solvent removal but used the gels in their wet form.

Two gel formulations, with different levels of polymer concentration, were loaded with model proteins avidin, bovine serum albumin (BSA) and ribonuclease-A and protein release was measured upon immersion in physiological buffer. The stability of the proteins in the formulations was examined by circular dichroism. Preliminary *in vivo* experiments were carried out in mice to evaluate the bioerodibility and local toxicity of the implanted material. The mechanism responsible for release, i.e., the relative contribution of diffusion and polymer erosion, was evaluated. The influence exerted on these processes by protein molecular weight and isoelectric point and by silica concentration was studied.

Results indicate that sustained release and quantitative protein recovery can be achieved within a time-frame that can be modulated by formulation parameters. These data, together with the fact that the embedded proteins are stable in time, hold promise for future developments.



Scheme 1.

2. Methods and materials

2.1. Reagents and instrumentation

Tetramethylorthosilicate (TMOS) was obtained from Acros Organics (New Jersey, USA). Bovine Serum Albumin (BSA) and ribonuclease-A were purchased from Sigma (St Louis, Mo, USA); avidin was obtained from Belovo (Belgium), and Fluorescein 5-isothiocyanate was purchased from Molecular Probes (Eugene, Or, USA). All other chemicals were of analytical or reagent grade and were purchased from Sigma.

Fluorescence measurements were made on a Jasco FP-6200 spectrometer and UV–VIS on a Varian Cary50 UV spectrometer. Circular Dichroism (CD) spectra were recorded on Jasco J-810 or J-710 spectropolarimeters. High Performance Liquid Chromatography (HPLC) analyses were performed on a Shimadzu LC10AVP with a Phenomenex C18 column.

2.2. Fluorescent labelling of proteins

All proteins used in release experiments (BSA, avidin and ribonuclease-A) were fluorescently labelled with an amine group-specific fluorescent reagent, fluoresceinisothiocyanate (FITC). 50 μl of FITC solution in dimethylsulfoxide (DMSO) (2.84 mg/ml) was slowly added to 1 ml of protein solution (5 mg/ml) in 10 mM phosphate, 150 mM NaCl, pH 7.4 buffer (PBS) and the reaction was incubated overnight at 4 °C in the dark. NH_4Cl was later added to a final concentration of 50 mM, and the solution was incubated for 2 h at 4 °C. The FITC–protein was purified by gel filtration (NAP-5 column, Pharmacia Biotech). Protein concentration and the fluorescein/protein ratio were estimated by measuring absorbance at 280 nm and 495 nm, according to standard procedures. These conditions gave fluorescein/protein ratios of 2, 2.2 and 0.3 for BSA, avidin and ribonuclease-A, respectively.

2.3. Sol–gel silica matrix polymerisation and protein entrapment

Partial hydrolysis of the liquid alkoxysilane precursor TMOS was initially promoted by acid treatment: TMOS and HCl were mixed at room temperature for 30 min (Si:HCl:H_2O molar ratios = $1:6\times10^{-5}$:1.25). This solution was then added, under gentle vortex, to the FITC–protein solution in TRIS buffer (trishydroxymethylaminomethane 10 mM, NaCl 150 mM, pH 7.4). This 'sol' was immediately transferred into a polyethylene cylinder (diameter = 1 cm) until gelling occurred. The gel was then extruded with a piston into 10 ml of TRIS buffer and rinsed three times with the same buffer to extract the methanol liberated by the condensation reaction. This procedure gave rise to quantitative entrapment of proteins in the gel as confirmed by fluorescence analysis of the washes which did not reveal any significant amounts of protein. Gels (0.5 ml) were preserved until further experiments in a closed vial at 4 °C, surrounded by a minimal amount of buffer to prevent drying. Matrices were loaded at a final concentration of 500 μg FITC–protein/ml of gel. Two formulations with differing concentrations of silica in the gel, 4% and 12% (wt/vol), were studied. Protein-free gels were prepared for *in vivo* experiments by using the same method and the protein solution was replaced with TRIS buffer

Table 1
Reagents and their quantities used for gel preparation

% SiO_2 wt/v	4	12
μl of TMOS	100	300
μl of HCl 2.5 mM	15	46
μl FITC–protein solution (1 mg/ml)	500	500
μl of TRIS buffer	385	154
R(H_2O/Si)	73.5	19

only. The amounts of reagents employed for the two formulations are shown in Table 1.

2.4. Circular dichrosim experiments and thermal stability of entrapped proteins

The effect of entrapment on protein conformation was verified by CD measurements on formulations immediately after their preparation which were then compared with solutions at the same protein concentration. 4% and 12% wt/v SiO_2 gels were prepared as described above and loaded with proteins (either fluorescein modified or non-modified). Wet gels were manually crushed with a glass rod and suspended in 10 mM phosphate buffer, pH 7.4. Far-UV wavelength CD spectra were recorded at room temperature (25 °C). Data were collected from 195 to 260 nm, at 0.2 nm intervals with 20 nm/min scan speed, 2 nm bandwidth, and 16 s response: CD values are expressed as molar residue ellipticity in units of deg cm^2 $dmol^{-1}$. In order to normalise ellipticity values to molar units, the protein concentration in each suspended sample was calculated from its UV absorbance at 280 nm. Protein concentration in these slurries was between 0.15 and 0.3 mg/ml, depending on gel preparation and its degree of packing in the quartz cuvette.

The thermal stability of proteins embedded in the gels was monitored by t-melt analysis. T-melt curves were obtained by measuring CD in far-UV. CD spectra (0.5 nm resolution, 1 nm bandwidth) were recorded at 10 °C intervals at temperature ranging from 20 °C to 96 °C and a heating rate of 30 °C/h. Thermal denaturation curves were derived from the change in the molar residue ellipticity value, measured at 222 nm for BSA and ribonuclease-A and at 213 nm for avidin. For comparison, the same experiments were performed on proteins in solution (0.2 mg/ml in 10 mM phosphate buffer, pH 7.4).

Thermal denaturation was also monitored by incubating crushed gel samples at 50 °C together with the minimum amount of buffer to prevent drying. At scheduled times – and for a total period of one week – gels were transferred to a quartz cuvette and analysed by far-UV CD and protein α-helix contents were calculated.

The resistance of embedded proteins to protease digestion was monitored by mixing protein-loaded gels (4% and 12% SiO_2) with a pronase solution in 10 mM phosphate, pH 7.4 (pronase:protein studied = 1:100 w/w). Samples were incubated at 37 °C and CD spectra were recorded at scheduled times. For comparison, proteins in solution (0.2 mg/ml in 10 mM phosphate buffer, pH 7.4) were treated and analysed in similar fashion.

2.5. Protein release and matrix erosion

Release assays were performed in two experimental conditions, differing in the amount of medium employed. In condition 1 (*sink* for silica), the release buffer was sufficient to allow free dissolution of all the SiO_2 in the gel, on the basis of the 2 mM concentration at saturation measured experimentally. Condition 2 (*non-sink*) was designed in order to reach silica saturation (and thus to stop the erosion process) within 2–3 h of immersion.

2.5.1. Sink conditions for silica erosion (buffer-to-gel ratio = 1000)

A USP dissolution apparatus (Sotax, Basel, Switzerland) was used. The assay was performed at 37 °C with paddle rotation speed of 60 rpm. Gels (500 μl) were immersed into 500 ml of sterile TRIS buffer and aliquots of supernatant (3 ml) were removed at scheduled times for protein and silicic acid determination and replaced with fresh buffer.

2.5.2. Non-sink conditions for silica erosion (buffer-to-gel ratio = 30)

Gels (500 μl) were immersed in 15 ml of sterile TRIS buffer in closed vials. Samples were kept at 37 °C in a continuously shaking water bath. At scheduled times, 0.15 ml aliquots of the supernatant were removed for protein and silicic acid content analysis.

FITC–protein concentration in release samples was quantified by measuring fluorescence (λex. 495 nm, λem. 525 nm), according to a calibration curve measured in parallel. Fluorescent data were confirmed by RP-HPLC (Phenomenex C-18, eluted with a H_2O/CH_3CN (+0.05%TFA) gradient).

2.6. Matrix erosion in vitro

All release samples were analysed for silica content. Silica concentration was determined using the molybdenum-blue colorimetric test [26]. Briefly, to 1 ml of sample, 40 μl of the molybdate reagent (12.7 g of $(NH_4)_6MoO_{24}\cdot 4H_2O$ in 100 ml H_2O, added to 100 ml of 4.5 M H_2SO_4) is added, mixed, and allowed to stand for 15 min. 40 μl of oxalic acid solution (10 g/100 ml H_2O) is then added, followed immediately by 40 μl of ascorbic acid solution (2.8 g/100 ml H_2O). The blue silicomolybdenum complex forms within 40 min and was quantified spectrophotometrically at 810 nm. A calibration curve was obtained in the same way using standard solutions (0–30 μM).

2.7. Mice and histological analysis

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Calco, Como, Italy) and housed in a Specific Pathogen Free (SPF) animal facility. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies. To assess the biological fate of the gel upon subcutaneous (s.c.) injection, formulations were crushed with a glass rod and suspended in equal volumes of

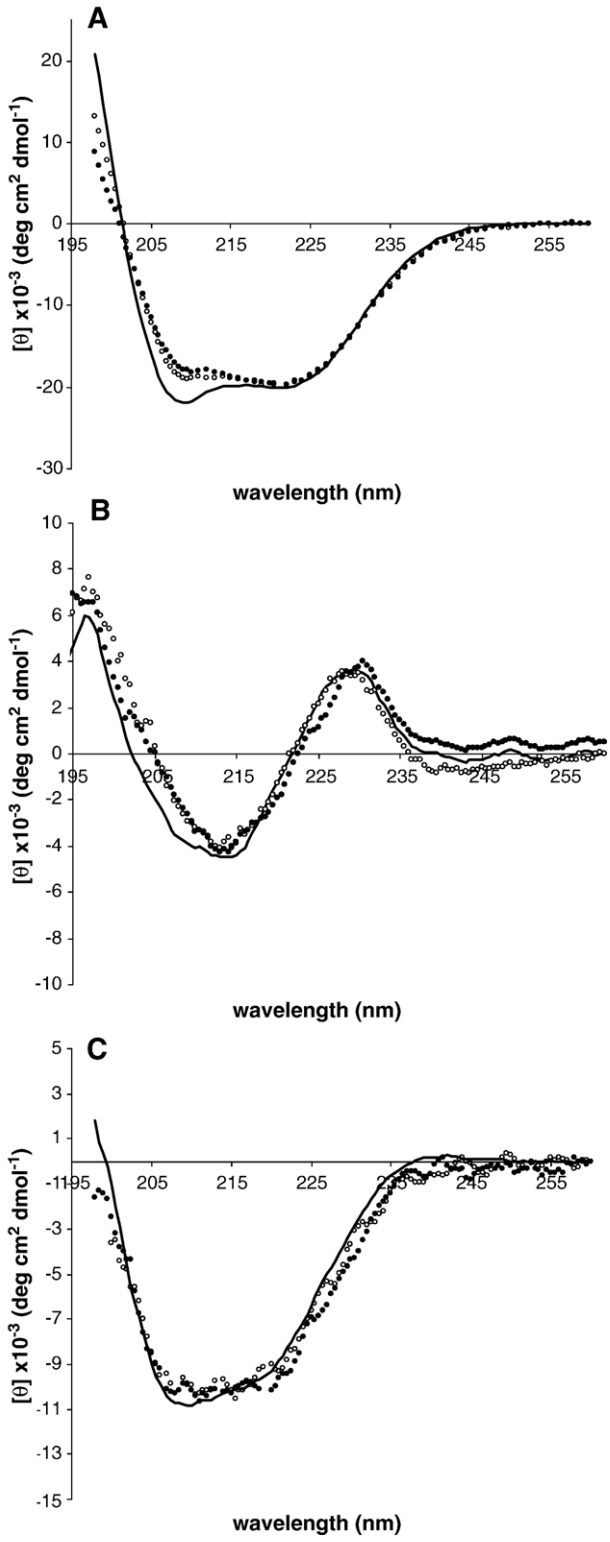


Fig. 1. Far-UV circular dichroism spectra of BSA (a), egg-white avidin (b) and ribonuclease-A (c), in solution (full line) and upon entrapment into two wet gel formulations differing in silica contents 4% wt/v (o) and 12% wt/v (●). Gels were analysed immediately after preparation upon crushing and extraction of methanol liberated after gel formation.

sterile PBS buffer. Aliquots of the suspension corresponding to 100 μl of wet gel were injected s.c. in the left flank, with a 5 ml plastic syringe and an 18-gauge needle. All subcutaneous inoculations were carried out in mice that had been previously shaved. This allowed us to examine gel distribution and potential spreading away from the injection site, at least macroscopically. Immediately after inoculation, a circular area

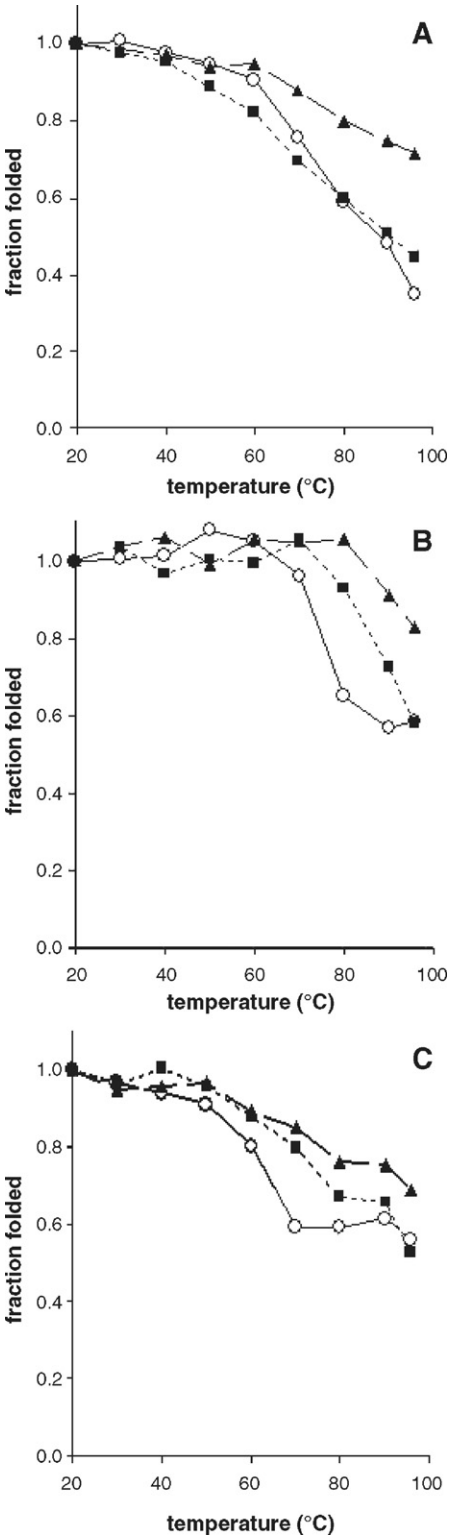


Fig. 2. Thermal denaturation profile (t-melt) of BSA (a), egg-white avidin (b) and ribonuclease-A (c) in solution (o) and upon entrapment in 4% (■) and 12% (▲) SiO_2 wt/v wet gel formulations.

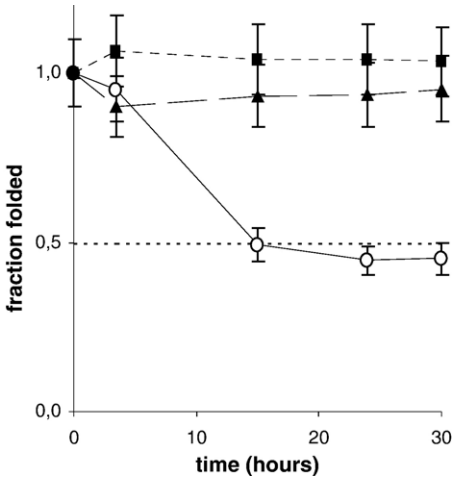


Fig. 3. Kinetics of protease digestion (with pronase) of egg-white avidin in solution (O) and entrapped in 4% (■) and 12% (▲) SiO_2 wt/v wet gel formulations.

with a diameter of 1 cm with the gel bleb at the centre was labelled with a skin marker, in order to follow the movements of injected material. At various time-points thereafter (24 h, 4, 11 and 19 days), the animals were sacrificed and fragments of their abdominal wall were collected for morphological analysis. All samples were collected by cutting a specimen of about 1 cm^2 with the gel bleb at the centre. Each specimen included the cutis and the entire abdominal wall, so that the gel bleb was sandwiched in the middle and could be removed completely. After inclusion, this approach allowed sectioning the entire area occupied by the gel. For histological evaluation, tissue samples were fixed in 4% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with haematoxylin and eosin (H&E staining).

3. Results

3.1. Conformation and stability of embedded proteins

Silica polymers are UV-transparent and the conformation of entrapped proteins can be monitored by circular dichroism. Fig. 1 shows the far-UV CD spectra of BSA, avidin and ribonuclease-A in solution and embedded in 4% and 12% SiO_2 gel formulations. The spectra almost overlap indicating that entrapment does not affect protein secondary structure to any significant extent.

Fig. 2 displays the t-melt curves of BSA, avidin and ribonuclease-A in solution and entrapped in 4% and 12% SiO_2 formulations. In the tested experimental conditions, unfolding of proteins in solution began at around 50 °C for BSA and ribonuclease-A and 70 °C for avidin. Overall, all gel-embedded proteins displayed higher stability than those in solution. Thermal denaturation occurred at a slower rate and stability increased with gel SiO_2 content. Loss of conformation in gel-embedded proteins may partly be due to trace amounts of alcohol in the gel. Alcohol is released upon siloxane condensation and, despite extensive washings performed, trace amounts of it might still be present and accelerate protein unfolding. In order to eliminate this risk, other siloxane precursors which do not liberate alcohol (e.g. diglycerilsiloxane—DGS) [27–29]) may be used to enhance the stability of the entrapped biomolecule further.

It is important to note that denaturation profiles measured by the t-melt analysis closely depend on experimental conditions. We therefore performed a further experiment by measuring unfolding kinetics of a selected protein (BSA), in solution and embedded in the 4% gel, kept constantly at 50 °C for one week. Even if t-melt data did not show any significant difference between the solution and gel-embedded sample at this temperature, long-term incubation revealed the significantly higher stability of the gel-embedded sample: after 5 days of incubation, BSA in solution totally lost its original conformation but the gel-embedded protein kept almost 90% of its original α-helix content. The increased stability of gel-embedded proteins was further tested by adding an external protease. The results (Fig. 3) clearly demonstrate the protective effect of the gel:avidin embedded in both 4% and 12% gels was totally unaffected by the presence of pronase, as opposed to the protein in solution, which was quickly degraded.

3.2. Matrix erosion and in vivo data

Fig. 4 shows the silica dissolution profiles of the two formulations, analysed in sink and non-sink conditions. The

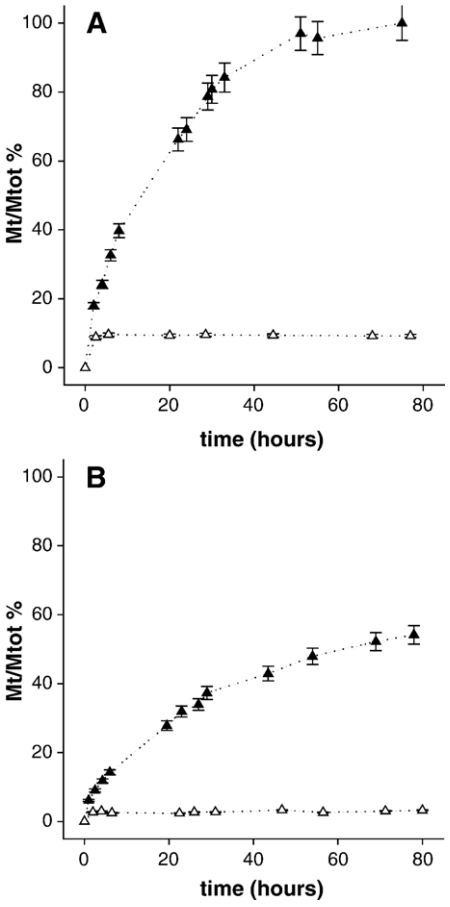


Fig. 4. Silica matrix dissolution in *sink* (▲) and *non-sink* (Δ) conditions. Formulation containing a) 4% wt/v SiO_2; b) 12% wt/v SiO_2.

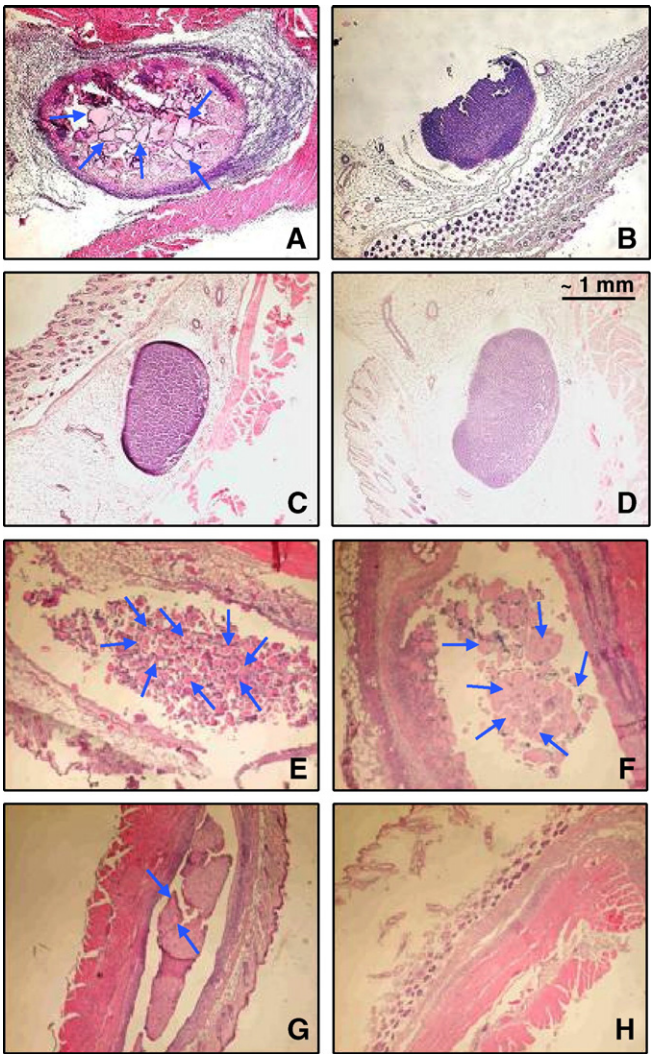


Fig. 5. *In vivo* fate of a crushed sample of 4% (a–d) and 12% (e–h) SiO_2 formulations. Histological analysis was carried out on cutaneous samples at different time-points after s.c. injection. Photographs: effects after A, E) 24 h; B, F) 4 days; C, G) 11 days; D, H) 19 days (H&E staining; original magnification 5×). Pictures are centred on inoculation site and show the entire area occupied by the gel.

speed of erosion was strongly affected by the amount of buffer used in the assay. In non-sink conditions, matrix dissolution stopped within the first 2 h of immersion and the gels remained in their initial shape indefinitely. Instead, erosion proceeded quite fast in sink conditions. Significant differences were observed between the two formulations: after 4 days of immersion, the 4% SiO_2 formulation was totally dissolved, but the 12% SiO_2 had lost about 50% of its initial silica content. The dissolution data fit well with a power law behaviour in time with exponent 0.5. This suggests that silica erosion depends on a diffusion process, probably due to the ability of the aqueous media to diffuse through the matrix. According to the fit, the estimated time for total erosion was 70 h and 250 h for the 4% wt/v and 12% wt/v, respectively.

It is important to note that sink conditions most probably mirror the situation *in vivo* in which silica saturation cannot be reached, and the results of *in vivo* experiments do support this hypothesis. The injection of 100 μl of wet gel typically produced a 3–5 mM round bleb which progressively shrank in the following 5–10 days until apparent complete macroscopic disappearance. Gel inoculation was not followed by a wheal and flare reaction as in type I hypersensitivity disorders and no spreading was observed from the injection site. Fig. 5 shows the injection site as it appeared at days 1, 4, 11 and 19 after administration of crushed 4% wt/v and 12% wt/v SiO_2 formulations. Images at low magnification give a panoramic view of the whole area containing the complete gel mass. The 4% gel was clearly visible only 24 h after injection. Already at day 4, there was almost a complete shrinkage and a complete substitution by a cell infiltrate that persisted over the next days (a–d). On the other hand, reabsorption of the 12% gel took longer, as amorphous material was still found at the injection site for at least two weeks and disappeared only at the last time-point tested (e–h). These results fit the *in vitro* experiments in sink conditions (Fig. 4), confirming that the sink environment mimics the *in vivo* environment. According to these data, we conclude that the 4% material is almost completely reabsorbed within 3–4 days and that the 12% material predictably requires longer, between 10 and 15 days.

It is noteworthy that the speed of reabsorption of a polymer implant is related to the surface area exposed by the polymer to external media. The fact that the *in vivo* data, obtained using a crushed material, are similar to the *in vitro* sink ones, obtained with a cylinder-shaped gel of a much larger size, indicates that material dissolution occurs both on the visible external surface and inside the matrix. We may therefore assume that the size of the crushed beads does not significantly affect the time for reabsorption as long as body fluids can circulate freely around and within the implant.

From a histological point of view, the 4% gel appeared to be heavily infiltrated by a few granulocytes and high numbers of mononuclear cells resembling small lymphocytes. Macrophages were almost absent, and no signs of inflammatory events in the subcutaneous tissues immediately surrounding the gel deposit were observed at any time-point tested. Conversely, the 12% gel induced an initial cell infiltrate, mainly represented by granulocytes which were subsequently replaced by a many macrophages and a few lymphocytes.

3.3. Protein release

Protein release from both 4% and 12% SiO_2 formulations was investigated in sink (Fig. 6) and non-sink (Fig. 7) conditions. Non-sink data were also plotted against the square root of time, according to the Fick equation [30] and the slopes of the resulting linear curves (indents in Fig. 7) are listed in Table 2. In non-sink conditions, the release occurs in the absence of matrix erosion so that the contribution of diffusion can be measured and differences due to protein–matrix interactions can be observed. Protein release in both sink and non-sink conditions was inversely related to the gel SiO_2 content, independent of the protein in question.

In non-sink conditions, significant differences between the three proteins were shown at both SiO_2 concentrations, and the

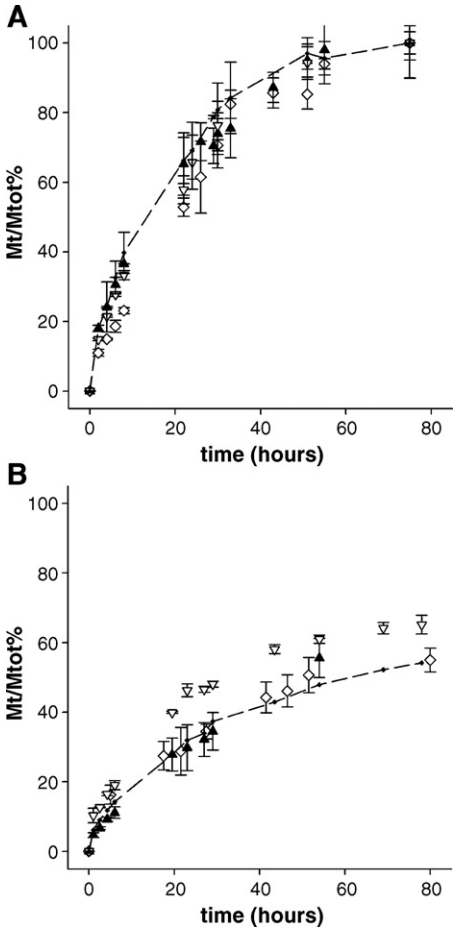


Fig. 6. FITC–protein release from 4% (a) and 12% (b) SiO_2 formulations analysed in *sink* conditions. ◊ BSA; ▲ ribonuclease-A; ▽ avidin; dotted line: silica dissolution.

rate of diffusion always followed the order avidin > ribonuclease-A > BSA (Table 2). The differences between individual proteins were more pronounced at the higher SiO_2 content. The different release behaviour cannot be explained on the basis of molecular weight or isoelectric point (pI). In fact, avidin and BSA have similar molecular weights (64,000 and 66,000 Da, respectively) and have opposite pI (10.5 and 4.8), whereas ribonuclease-A is smaller and has similar pI as avidin (MW 13,700, pI = 9.6). All tested proteins are globular so that a similar dependence of their molecular size on MW can be expected. The X-ray data for avidin indicate that the molecule measures 7 × 7 × 8 nm [31]. Data on BSA were not available, but its human counterpart (HSA) measures 8 × 8 × 3 nm [32]. Ribonuclease-A has a much lower MW and is therefore much smaller. This means that neither protein size nor charge are driving elements in determining the interaction with the negatively charged matrix. It seems that each protein has a different strength of interaction with the gel, but as the key elements involved are still unclear, a general rule cannot be defined. The interaction probably depends on the combination of hydrophilic and hydrophobic forces and steric interactions so that every protein will behave differently.

When release was monitored in sink conditions, protein release mimicked matrix erosion in all the 4% SiO_2 formulations, independent of the protein in question. In the case of the 12% SiO_2 formulation, only BSA and ribonuclease-A release mimicked the erosion process, whereas avidin release was faster.

It is interesting to note that the data of Table 2 can be used to estimate the time required for total release of each protein eased in the absence of erosion (time for matrix exhaustion, t-ex). Theoretically, by comparing this value with the time required for erosion in sink conditions, it is possible to predict whether the sink release will follow the erosion process or if it will occur at a faster rate. For example, the diffusional t-ex values

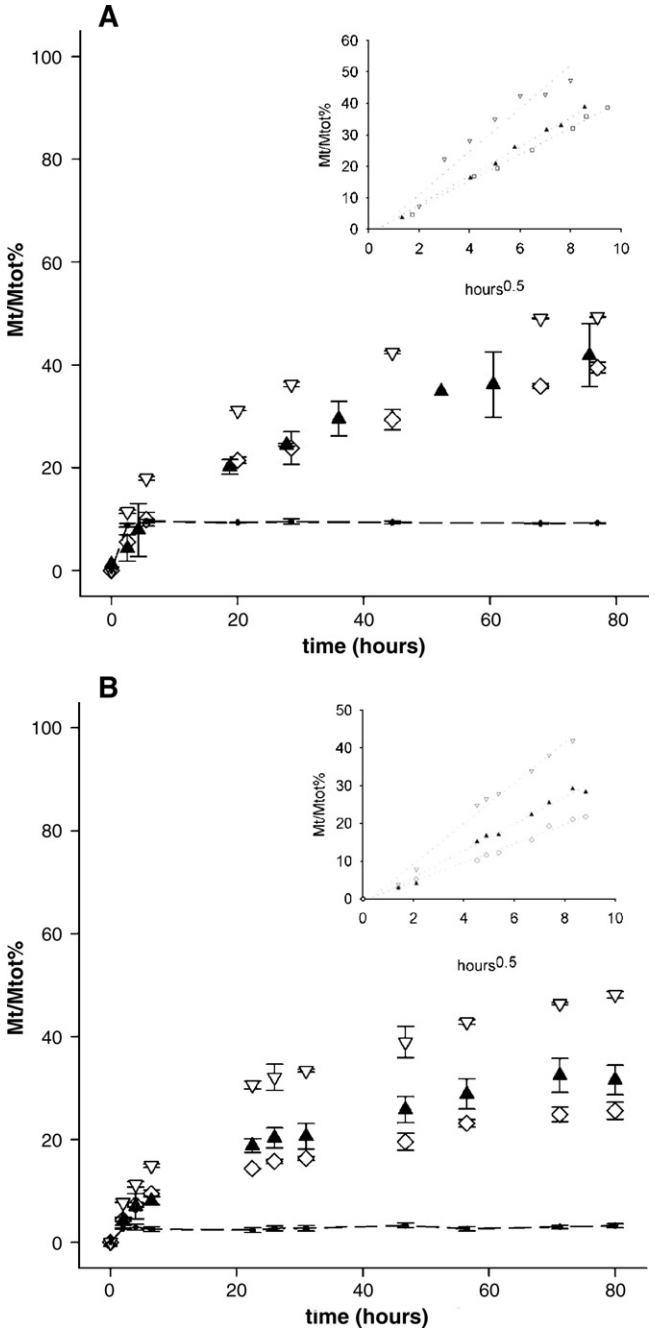


Fig. 7. FITC–protein release from 4% (a) and 12% (b) SiO_2 formulation analysed *non-sink* conditions. ◊ BSA; ▲ ribonuclease-A; ▽ avidin; dotted line: silica dissolution.

Table 2
Protein release rates d(Mt/Mtot%)/d(h)$^{0.5}$ measured for all formulations

Formulation (%SiO_2)	d(Mt/Mtot%)/d(h)$^{0.5}$		
	BSA	Ribonuclease-A	Avidin
4	4.18±0.13	4.60±0.13	6.85±0.68
12	2.52±0.08	3.54±0.14	5.36±0.21

calculated for the 4% wt/v gel are approximately 213, 472 and 572 h for avidin, ribonuclease-A and BSA respectively. Considering that in sink conditions total dissolution of this formulation occurs in about 70 h, erosion is clearly the driving element responsible for release and, therefore, in these conditions, no significant difference should be recorded in the release of the three proteins. Similarly, the diffusional t-ex values calculated for the 12% formulation are 348, 798 and 1574 h for avidin, ribonuclease-A and BSA respectively, whereas matrix erosion was estimated to occur in about 250 h. In this case, the difference in the diffusion behaviour of the three proteins is more significant. However, as in the 12% gel erosion is faster than diffusion for all tested proteins. In this formulation too, release may be expected to be mainly dictated by erosion. Only in the case of avidin was the diffusional t-ex of the same order of magnitude as that of erosion. In this case the contribution of diffusion is probably significant, and may explain why, in avidin, release in sink conditions is faster than the erosion process.

4. Discussion

Overall, our data indicate that wet silica gel obtained by the sol–gel method is a promising polymer for the entrapment and sustained release of proteins:

1) The thermal stability data are in good agreement with those already described in the literature [1,8,33,34] and indicate that entrapped proteins not only maintain their original folding but are also more stable compared with those in solution. Although slow denaturation may occur after long-term incubation of gels in physiological conditions, our data predict that a significant fraction of embedded proteins will remain in their folded conformation within the time-frame required for total release. It must be pointed out that partial protein unfolding may induce an immune reaction. This issue, the importance of which may have a different impact depending on the protein studied, will have to be addressed in future works. Embedding not only protects proteins from thermal degradation but also impedes access to proteases. This is a further advantage when considering *in vivo* applications.
2) Gels are easily eroded, as demonstrated by both *in vitro* and *in vivo* experiments. Erosion occurs through the formation of silicic acid, in a similar way to that identified for the dry gels. Although the absence of local and systemic toxicity has been demonstrated for dry materials [35], additional studies will be needed to evaluate these new more erodible polymers. Further *in vivo* experiments are currently in progress and will be reported elsewhere. The fact that wet material dissolves much faster than classical xerogels may explain why no fibroid capsule forms at the site of injection. This lack is indeed a positive aspect, because it allows for a continuous release of the silica-embedded proteins and, at the same time, permits multiple injections in the absence of collateral side-effects or granuloma formation.
3) Our *in vivo* experiments, although still preliminary, did not show any inflammatory response or macrophage recruitment around the site of injection of the 4% gel, thus suggesting that the material is well tolerated and does not induce immune system stimulation. Also of interest is the fact that the gels appeared to be infiltrated by cells different from classical macrophages. Conversely, the 12% gel induced a cell infiltrate closely resembling that observed during an inflammatory response against a foreign material, with a first influx of polymorphonuclear cells, followed by a chronic phase with macrophages and lymphocytes. The specific impact of infiltrating cells in the induction of an immune response will require further dedicated experiments aimed at comparative evaluation of the immunogenicity of proteins loaded in these wet silica matrices and other delivery systems.
4) Embedded proteins are quantitatively released both through diffusion and erosion. Our experimental protocol distinguished and quantified both erosion and diffusion processes. It was found that the sink conditions mimic *in vivo* behaviour well. It appears that, in these conditions, matrix erosion is the major driving force responsible for release, especially for the fast-eroding 4% formulation. Diffusion, the rate of which depends on the individual properties of each protein influencing physico-chemical interactions with the matrix, is more important when the gel erodes slowly, as in the case of the formulation with higher SiO_2 content. It is important to note that matrix erosion and protein–matrix interactions may be modulated by introducing some organic elements into the polymer, for example, by substituting part of the tetralkoxysilane with trialkoxysilanes monoalkyl-substituted [36].

5. Conclusions

Wet sol–gel derived silica is a highly promising material for parenteral administration and sustained release of protein compounds. Embedded proteins maintain their original conformation and are stable to both thermal denaturation and protease degradation. Gels are highly erodible, as demonstrated by both *in vitro* and *in vivo* experiments. No local or systemic toxicity was observed after subcutaneous administration in mice.

The formulations investigated here displayed sustained release and quantitative recovery of the embedded proteins within a time-frame that may be modulated by formulation parameters. Protein release was demonstrated to occur upon both a diffusion process and matrix erosion. The relevance of each of these two phenomena varies with each formulation. At low silica concentration, erosion is the driving element; at higher concentration differences between individual proteins are observed. The possibility of modulating in several ways the matrix composition and concentration theoretically allows

infinite combinations, to suit individual formulation requirements better.

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