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RESEARCH PAPER

Could Albumin Affect the Self-Assembling Properties of a Block Co-polymer System and Drug Release? An *In-Vitro* Study

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ABSTRACT

Purpose This work investigated the influence of a model protein, bovine serum albumin (BSA), on the properties of a thermogelling formulation intended for administration inside body compartments where there is high albumin content, as in the case of inflamed joints; it also explored the relation between the variation of these properties and release performance of methotrexate (MTX), a drug used to treat forms of arthritis and rheumatic conditions.

Methods The influence of BSA on the micellisation and gelation behaviour of Poloxamer 407, chosen as a model copolymer, was studied by differential scanning calorimetry (microDSC), dynamic light scattering (DLS), fluorescence spectroscopy and rheology studies. A release study of MTX loaded inside the hydrogel in presence and in absence of BSA was performed.

Results DLS and microDSC data revealed that the micellisation process was not affected by the protein, as demonstrated by unaltered micellar size and thermodynamic parameters. While the presence of BSA in the copolymer system reduced gel consistency, the hydrogel release performance was only slightly affected.

Conclusion Our results suggested that the kinetics of MTX release mainly depended on the presence of the thermogelling copolymer, although other mechanisms related to BSA could be involved. Finally, the study assessed the feasibility of using a thermogelling hydrogel for *in situ* drug administration in areas with the presence of high protein concentrations.

KEY WORDS BSA · methotrexate · poloxamer 407 · polymer-protein interaction · sustained release

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INTRODUCTION

Inflammatory arthritis is a chronic disease that markedly affects the quality of life of patients worldwide. The standard therapy is based on the association of several anti-inflammatory drugs administered orally, parenterally or through the intra-articular route.

The injection of drugs directly into the joints has proven effective (1), though it is reported that the drug rapidly disappears from the articular cavity (2,3). Thus, a prolonged-release system injected into the joints could offer a desirable alternative (4,5).

Among the different controlled release dosage forms proposed (3,6), thermogelling systems appear particularly promising. In fact, they can be administered as a flowable liquid and form a depot at the injection site at body temperature (7,8). Moreover, a thermogelling system could be more useful than solutions for intra-articular administration into the synovial cavity. For instance, hydrogels could improve the defect of lubrication that affects osteoarthritic joints due to the reduction of collagen synthesis by synoviocytes in inflamed sinovia (9).

A thermogelling systems for local delivery of methotrexate (MTX) into sinovia was developed by Miao *et al.* (4) using a poly(ε-caprolactone)-poly(ethyleneglycol)-poly(ε-caprolactone) (PCL-PEG-PCL copolymers) hydrogel. Alternative polymers that could be used to prepare thermogelling systems are di-block or tri-block PLGA/PEG copolymers, polyether modified poly(acrylic acid) and Poloxamers (10). Among them, Poloxamer 407 is the most commonly used and the best characterized.

This amphiphilic PEO-PPO-PEO triblock copolymer is able to self-aggregate into micelles or into gelled cubic phase according to concentration and temperature (11). Moreover, it has been shown that several additives, such as salts, surfactants and cosolvents, are able to influence these processes. A recent collection of references can be found in the literature (12).

The aim of this work was to evaluate the influence of BSA in a thermogelling formulation based on Poloxamer 407 used as model copolymer for a hydrogel-based controlled delivery system, for possible injection into sinovia.

The rationale for this investigation is based on the concern that high amounts of albumin at the administration site could modify the *in vivo* release of the drug. In fact, albumin accumulates in inflamed tissues, and particularly in the sinovia, as in the case of rheumatoid arthritis, due to an excess of albumin uptake *versus* the efflux across inflamed synovial membrane (13). The increased permeability of capillary vessels in the inflamed joints allows for a high rate of extravasation of macromolecules, such as proteins, into sinovia. In addition, in inflamed joints the proliferating synovial cells show high albumin consumption due to the increase of the metabolic turnover of proteins (14). Consequently, the high amount of albumin at the administration site could modify the *in vivo* technological properties of the hydrogel itself once it has been administered.

Moreover, since albumins act as protein carriers for drugs into physiological fluids, they have already been employed in different pharmaceutical formulations to target inflamed joints in mice with collagen-induced arthritis (15). In fact, albumins are able to bind drugs reversibly so that an equilibrium between bound and unbound drug exists both in sinovia and blood. Thus, it can be supposed that the amount of BSA in sinovia and blood circulation influences the relative concentration of drug in these two different body compartments. Recently, Sterner *et al.* observed that the presence of 2.5% (w/v) of BSA in the medium slowed down the release of poorly soluble peptides from a crystal suspension intended for intra-articular administration (16), while Li et. al showed that the presence of BSA can modulate the release of drugs from gelled microemulsions (17). Thus, it is possible that the same modulatory effect could also occur when thermogels are administrated.

Thus, in this study we sought to understand how the interaction between BSA, Poloxamer 407 and MTX affects the release performance of thermogelling systems, as this information is crucial for the development of a hydrogel for intra-articular drug administration, as well as for Poloxamer thermogelling systems designed to release peptides, proteins or combinations of them with small organic compounds.

MATERIALS AND METHODS

Materials

Poloxamer 407 (Lutrol F127, BASF AG, Ludwigshafen, DE), bovine serum albumin (≥96%) and methotrexate hydrate (>98%) (Sigma-Aldrich Co. Ltd, Gillingham, UK) were all used as received. Water was deionized and purified through a Sartorius arium® 611 Ultrapure Water Systems (Sartorius AG, Goettingen, DE).

Preparation of Hydrogels

Poloxamer 407 hydrogels were prepared for simple dispersion of the material in an adequate amount of degassed and deionized water, potassium phosphate buffer 100 or 200 mM pH 7.3, using the cold procedure (18). Samples were stored at 4°C for at least 24 h before starting the analysis. Poloxamer 407 hydrogels in presence of BSA were prepared from concentrated stock solutions of the polymer and protein in water or buffer phosphate 100 and 200 mM pH 7.3. Stock solutions were then mixed at appropriate volumes to give desired Poloxamer 407 and BSA concentrations.

For the release study, 7.5 mg of methotrexate were first dissolved in the minimum volume of potassium phosphate buffer (100 mM pH 7.3) and then added to the buffer-Poloxamer system.

Differential Scanning Calorimetry Measurements (mDSC)

The calorimetric studies were carried out with a micro DSC III (Setaram instrumentation, Caluire, FR) in 1 ml cells. The buffered solutions (100 and 200 mM) of BSA in the presence and in the absence of Poloxamer 407 (at 2.5% and 5% w/w) were analysed. BSA concentration was 15 mg/ml. Hydrogel samples (0.750 g) were loaded into the calorimetric cells at 5°C, left at this temperature for 20 min to obtain thermal equilibration and then heated at 0.25°C/min up to 90°C.

All samples were run in triplicates.

Dynamic Light Scattering Analysis (DLS)

DLS analyses were performed using a Malvern Zetasizer NanoS (Malvern Instruments, Worcestershire, UK) equipped with a back-scattered light detector operating at 173°. One milliliter of the samples was loaded inside disposable cuvettes and, after 180 s of thermal equilibration, analyses were performed between 5° and 25°C. The size distribution plots of intensity *versus* radius were characterized in terms of peak value (median value) and peak width (amplitude of the size distribution at the half-height of the peak value).

All analyses were performed at least in duplicate.

Steady-State Fluorescence Assay

Fluorescence spectra were collected at 25°C using a Hitachi 4500 spectrofluorimeter by exciting the samples at 280 nm and recording the emission in the range of 200–500 nm. A solution of 0.0652 mg/ml of BSA in 100 mM phosphate buffer was loaded inside a quartz cell (total volume 3 ml) and titrated with Poloxamer 407 20% w/w solution. Poloxamer 407 solution was added to the cuvette in an ice bath, gently mixed and then left in the sample holder of the instrument to equilibrate the temperature at 25°C. The amount of Poloxamer during the titration was in the range of 0.5%–10% w/w.

Moreover, in order to investigate the interaction between MTX (used for the release study) and BSA, quenching analyses were performed on BSA and MTX solutions in 100 mM buffer with or without the addition of Poloxamer 407 (10% w/w). The quenching Stern Volmer constants (K_{sv}) and the fractional maximum fluorescence intensity (f_a) were determined from the modified Stern-Volmer plot according to Lehrer equation:

$$\frac{F0}{F0-F} = \frac{1}{f_a} + \frac{1}{f_a \cdot K_{sv} \cdot [Q]} \quad (1)$$

where F_0 and F are the relative fluorescence of the fluorophore in absence and in presence of the quencher, respectively.

K_{sv} is related to the lifetime (τ_o) of the fluorophore and the collision constant (Kq) according to the Eq. 2.

$$K_{SV} = k_q \cdot \tau_0 \quad (2)$$

The binding constants (Ka) and the number of the binding sites (n) have also been calculated from F_0, F and the concentration of the quencher (Q) according to the following equation:

$$\mathrm{Log}\frac{F0-F}{F} = \mathrm{Log}\, K_a + n\mathrm{Log}[Q] \quad (3)$$

All signals were normalised with respect to the effective concentration of BSA in the cuvette after titration. Details on the quenching analysis can be found in Lakowicz (19).

Experimental Design and Rheological Analysis

The rheological behaviour of the BSA/Poloxamer 407 systems was studied through the Box-Behnken experimental design (2^3+3 central points). Further details about this experimental design can be found in the literature (20). The influence of three parameters (BSA and Poloxamer 407 concentrations, and the ionic strength of the phosphate buffer) on the viscosity at 8°, 25° and 35°C and on gelation temperature was investigated. The compositions of all samples analysed and prepared according to the experimental plan are reported in the supplementary materials (Table ST1).

The viscoelastic data G' (storage modulus), G" (loss modulus) and *tan δ* (phase angle) obtained from the rheological analysis were used to generate a mathematical model able to predict the responses for viscosity and gelation temperature values. All results were analysed through ANOVA and the parameters were considered statistically significant when p-value <0.05. The analysis of the results was performed with the statistical program MINITAB® Release 14.1 (Minitab, Ltd. UK).

Rheological analyses were performed using a stress control rheometer (Stress-Tech, Reologica, Lund, SE) equipped with cone-plate geometry (4/40) with a 150 μm gap operating in the oscillation mode.

Samples were analysed through temperature sweep tests in the temperature range of 5°–50°C at the rate of 1°C/min by applying a stress of 10 Pa at the frequency of 1 Hz.

Release Study

Dissolution studies were performed following the paddle over extraction cell method (21–23). All the experiments were carried out at 37°C using a USP dissolution apparatus 2 (AT7 smart, Sotax, CH) equipped with teflon enhancer cells (Agilent, USA) having a surface area of 4 cm^2 and mounting an 11 μm pore size filter paper (grade 1, Whatman, UK) as membrane.

A membrane with large pores allows the sample to remain in the enhancer cell during the experiments without interfering with the drug release.

A 1 g hydrogel sample was placed in the reservoir of the enhancer cell, and the membrane was placed on the cell cup and blocked with an o-ring; finally, the reservoir was closed with a screw cap.

The enhancer cell was left to equilibrate at 37°C for 10 min and then placed in the vessel containing 700 ml of potassium phosphate buffer 50 mM pH 7.3. The paddle was rotated at 50 rpm and its position was set 2 cm above the top of the membrane throughout the study. 1 ml was withdrawn at 0, 15, 30, 60, 120, 180, 240, 300, 360, 480 and 600 min. The absorbance of methotrexate released at each time-point was measured by UV-spectroscopy (UV 1800 spectrophotomer Shimadzu, JP) at the wavelength of 303 nm. BSA concentration in the samples was measured by Bradford colorimetric assay (24). All the experiments were run in triplicate.

Methotrexate (MTX) Activity Assay

The efficacy of MTX released from the Poloxamer and BSA/Poloxamer hydrogel formulations was evaluated in terms of dihydrofolate reductase (DHFR) residual activity, since MTX acts as a competitive inhibitor of this enzyme. MTX activity was determined by UV analysis (spectrophotometer UV-1800, Shimadzu, JP) by monitoring the decreasing absorbance of NADPH at 340 nm. The reaction mixtures containing the MTX aliquots stored after release (diluted 1:100 in potassium phosphate buffer 50 mM, pH 7.3), DHFR enzyme and the cofactor NADPH (80 μM) were equilibrated for 5 min at 30°C. Then the substrate dihydrofolate (40 μM) was added to start the reaction (final volume 1 ml). The decrease of the absorbance at 340 nm, due to the oxidation of NADPH, was registered over the time and related to the enzymatic activity.

RESULTS

Differential Scanning Calorimetry (DSC) and Dynamic Light Scattering (DLS)

Calorimetric and dynamic light scattering measurements were performed to analyse the influence of BSA on the micellisation process of Poloxamer 407. Micellization of Poloxamer 407 is a self-assembly process that occurs as consequence of the desolvation of polymer unimers in function of temperature and polymer concentration (25). In water at some specific conditions, unimers of the polymers are forced to self-organize into micelles before arranging themselves into a tridimensional network. Thus, micellisation could be considered an intermediate process leading to gel formation.

For this reason, factors that affect micellisation could also influence the gelification of Poloxamer 407. Micellisation is an endothermic transition and can be directly followed by calorimetric measurement. On the other hand, the formation of micelles leads to an increase of size, which can be evaluated through dynamic light scattering measurements.

In order to study the effect of BSA on the micellisation process of Poloxamer 407, it was necessary to perform a preliminary characterization of pure BSA solutions in terms of mDSC and DLS behaviours. We performed a mDSC study on BSA at the concentration of 15 mg/ml both in phosphate buffer 100 and 200 mM. Calorimetric scans did not reveal differences in the protein thermal behaviour in presence of the two different buffers. It can be observed from the thermograms (Fig. 1b) that the protein underwent a transition at about 63°C with an enthalpy of 8.56 J/g in 100 mM phosphate buffer and 7.79 J/g in 200 mM phosphate buffer. The broadness of the peak suggested that denaturation of BSA involves the presence of intermediate states, as reported in the literature (26). Figure 1a shows that traces of Poloxamer at 2.5% and 5% w/w (buffer phosphate 100 mM) with and without BSA are superimposable in the range of the temperatures at which micellisation occurs. This means that BSA does not influence the micellization of Poloxamer 407 neither CMT nor enthalpy of the process are altered, as reported in Table 1.

On the contrary, BSA denaturation seemed to be affected by the polymer at the analyzed concentrations. In fact, although the transition related to BSA denaturation remained centred at around 63°C, the enthalpy decreased from 8.5 to around 5 (J/g). Therefore, micelles of Poloxamer 407 do not influence BSA stability in terms of denaturation temperature but in the presence of the copolymer a decrease of energy requested for this transition occurs. Figure 1b reports the effect of buffer concentration (phosphate buffer 100 and 200 mM) on poloxamer 407 micellisation and BSA denaturation. Results obtained for samples in presence of BSA were in agreement with those reported in a previous work studying how different ionic strengths of buffer affect the micellisation of Poloxamer 407 (12). In fact, it can be noted that by increasing the ionic strength of the buffer, CMT decreases.

All the calorimetric data (CMT and enthalpy) are reported in Table 1.

In order to confirm the results obtained from mDSC analysis, dynamic light scattering measurements were also performed. Figure 2 shows the particle size distribution for the samples of Poloxamer 407 2.5% with or without BSA in 100 mM phosphate buffer at three different temperatures (15°–20°–25°C) in comparison to alone BSA (15 mg/ml) signal. The size distribution of BSA over the three analysed temperatures did not change and the peak showed its maximum at about 4.5 nm, referred to the hydrodynamic radius of the protein. The 2.5% Poloxamer 407 sample denoted, by increasing the temperature, the trend already observed in a previous work (12). Typically, the single peak referred to the unimers below CMT (15°C) converts two distinct peaks (one related to the unimers and another to the micelles) when the micellisation process occurs (20°C). Then, once all unimers self-assemble into micelles, the presence of a monomodal distribution trace distribution indicates that the micellisation is complete (25°C).

In the case of the Poloxamer 407/BSA sample, a single size distribution of the species at each operating temperature can be noticed. In analysing the curves, it was noted that the bandwidth of the size distribution itself became larger as temperature increased from 15° to 25°C, denoting an increase of the size polydispersity in function of the temperature. Nevertheless, also at 25°C, when the micellisation process could be considered at the end, it seemed that the value of the peak was not strongly affected by the presence of micelles. In fact, there was no notable increase of size up to the typical micellar radius (Table 2).

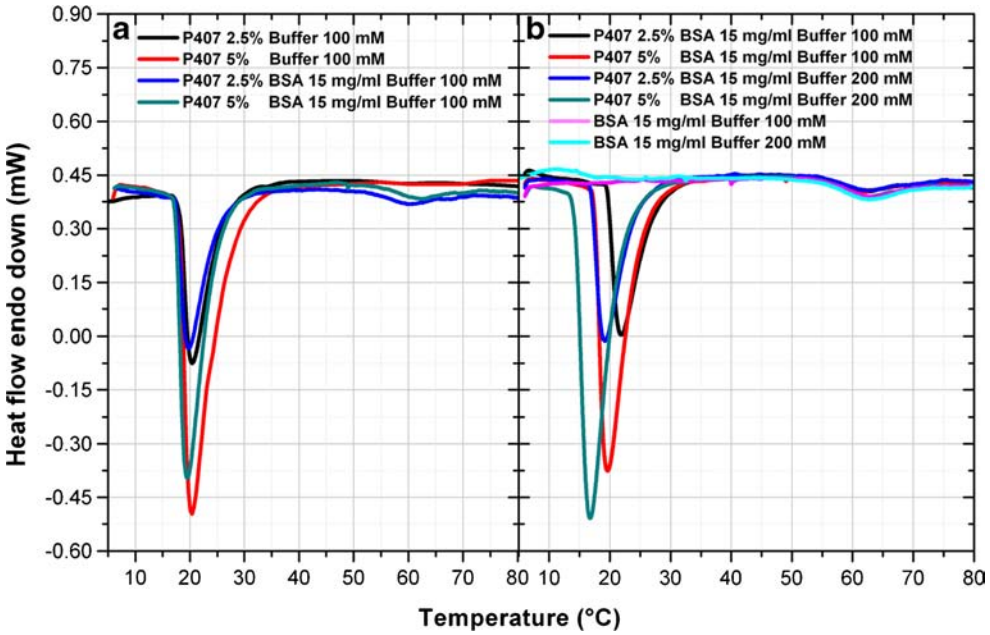

Fig. 1 (**a**) MicroDSC traces of poloxamer 407 disssolved in potassium phosphate buffer (100 mM pH 7.3) in presence and in absence of BSA. (**b**) Effect of the ionic strength of phosphate buffer (100 and 200 mM) on microDSC traces of poloxamer 407 in presence of BSA (15 mg/ml).

Fluorescence Study

To further investigate the influence of Poloxamer 407 in its different aggregation states on the microenvironment around BSA, a spectrofluorimetric study was performed.

Figure 3 shows the emission spectra at 25°C of BSA alone and after titration with a dispersion of Poloxamer 407 when excited at 280 nm. The intrinsic fluorescence emission of BSA is related to the energy transition of the two tryptophans (Trp-134 and Trp-214) and tyrosine residues in the protein., The wavelength of maximum BSA emission was 348 nm, when Poloxamer 407 concentration increased up to 10%, the intensity of the BSA emission decreased and a blue shift occurred from 348 to 336 nm (Fig. 3).

Thus, Poloxamer 407 is able to increase the hydrophobicity of the local environment around the fluorophores of the protein. The main peak of BSA at 5% of Poloxamer was accompanied by a slight shoulder at 310 nm that become markedly evident at 10% of Poloxamer. This change in BSA fluorescence behavior is indicative of an increase of hydrophobicity in the microenvironment around the tryptophan residues, induced by the presence of micelles (27).

In order to study the binding interaction of MTX with BSA in buffer and in presence of the copolymer, quenching studies were performed. Data obtained from quenching studies were analysed using the Lehrer equation as reported in a previous study investigating the interaction between BSA and MTX (28). Table 3 reports the values of the Stern Volmer (K_{SV}) and bimolecular quenching (Kq) constants, calculated using experimental data shown in Fig. 4a.

Both in buffer and 10% Poloxamer 407 dispersion, the calculated Kq was found higher than 10^{10} M^{-1} s^{-1}, the maximum limit value for the collisional or dynamic quenching. Thus, the results suggest that static quenching predominates over dynamic quenching, also in presence of the copolymer. In the case of static quenching the equation:

Table 1 Thermodynamic Parameters of Poloxamer 407 Micellisation and BSA Denaturation Process in Phosphate Buffer 100 mM Determined by mDSC Measurements

Samples	CMT of poloxamer 407		Denaturation of BSA	
	Peak (°C)	Enthalpy (J/g of solution[a])	Peak (°C)	Enthalpy (J/g of protein[a])
P407 2.5%	21.7 ± 0.50	0.77 ± 0.03	–	–
P407 5%	19.9 ± 0.45	1.44 ± 0.02	–	–
P407 2.5% BSA 15 mg/ml	21.9 ± 0.40	0.80 ± 0.06	62.48 ± 0.71	5.09 ± 0.15
P407 5% BSA 15 mg /ml	19.7 ± 0.46	1.63 ± 0.11	62.20 ± 0.63	4.95 ± 0.12
BSA 15 mg/ml			62.61 ± 0.43	8.56 ± 0.08

[a] Enthalpy values for BSA and Poloxamer 407 are reported in two different units for a better comparison with data from the literature

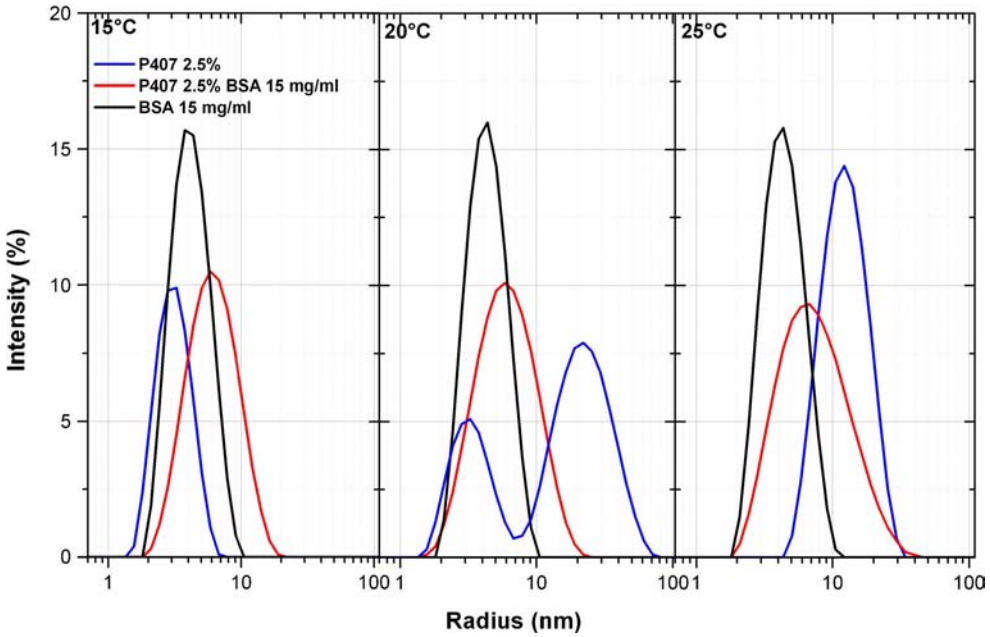

Fig. 2 DLS signals of BSA alone (15 mg/ml) and P407 2.5% samples in presence and absence of the protein at three different temperature (15°, 20° and 25°C).

$$\mathrm{Log}\frac{F_0 - F}{F} = \mathrm{Log}\,K_a + n\mathrm{Log}[Q] \quad (3)$$

can be applied to calculate the binding constant (Ka) and the number of the binding sites (n). The fitting of the experimental data with Eq. 3 is reported in Fig. 4b. From the results reported in Table 2 it can be noted that a decrease of the value of Ka occurs in the presence of the copolymer, which means that MTX has less affinity for BSA in Poloxamer 407 10% dispersion compared to the buffer solution without Poloxamer. However, the number of binding sites remained around 1.

Thus, it can be deduced that the presence of the copolymer does not affect the position of the interactions of MTX molecules on BSA but only the strength of these interactions. The decrease of affinity may mean that the quencher (MTX) has less access to the fluorophore (the tryptophan residue of BSA), as demonstrated by the decrease of f_a in presence of the copolymer.

Table 2 Peak and Peak Width of the DLS Raw Signals for the Poloxamer 407 2.5% Systems in Potassium Phosphate Buffer 100 mM pH 7.3 with or without BSA. In the Table, Results from Monomodal Distribution Traces at 15°C and 25°C are Reported Since only at These Temperatures were Stable Unimers and Micelles Respectively Present (12)

Samples	Temperature	Peak (nm)	Peak width (nm)
BSA	15°C	4.497 ± 0.02	1.470 ± 0.28
P407		3.101 ± 0.02	0.844 ± 0.14
P407/BSA		6.477 ± 0.03	3.070 ± 0.13
BSA	25°C	4.581 ± 0.05	1.582 ± 0.36
P407		12.93 ± 0.05	4.846 ± 0.74
P407/BSA		8.560 ± 0.07	5.486 ± 0.18

Experimental Design Results and Rheological Analysis

The experimental design (Box-Behnken 2^3+3) was useful to characterize these systems in terms of rheological behaviour in order to understand how much the concentration of BSA, Poloxamer 407 and buffer could affect the gelling point and the viscosity of the final samples.

The experimental design results (Table ST2 in the supplementary materials) show that the thermogelling properties and the sample consistency around the gel point of the analysed samples are dependent on Poloxamer 407 and BSA concentrations. The two parameters act in opposite directions: an increase of copolymer concentration lowers the gel point while an increase of BSA brings about the opposite effect. Yet, when the BSA concentration was 2.5% or higher and the Poloxamer amount lower than 20.5%, partially gelled systems were observed, as previously stated both for BSA-Poloxamer 407 systems (29) and in presence of other additives (30).

The experimental design made it possible to select the optimal concentrations of all the components in order to obtain a suitable release system, which would maintain a good viscosity to control drug release *in situ* in the presence of the endogenous proteins.: The system with such features, resulted to be Poloxamer 20.5% w/w in 100 mM potassium phosphate buffer. In the selected system, the presence of BSA exerts a peculiar effect. At 25°C, the presence of 2.5% w/w of BSA lowers the elastic modulus (G') 10 fold compared to the same system without the protein (Fig. 5). Moreover, the presence of BSA prevents the formation of a real gel from a rheological point of view. In fact, the phase angle does not decrease below 45° but remains at about 60°–65° (partially gelling behaviour).

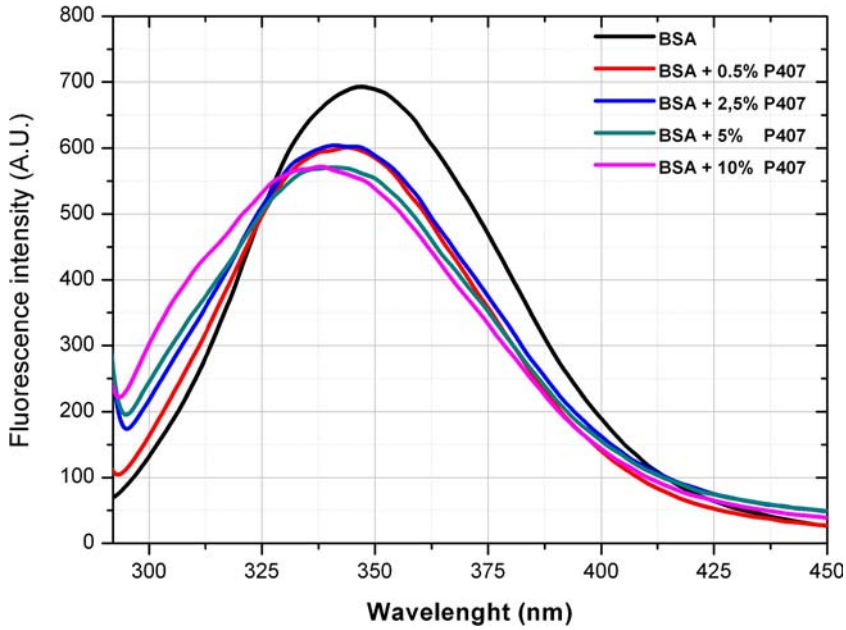

Fig. 3 Effect of the increasing concentration of P407 on the intrinsic fluorescence emission of BSA at 25°C.

The rheological parameters of these systems were compared with those loaded with 7.5 mg of methotrexate in order to investigate the influence of the drug on the system consistency. There was no remarkable difference between the systems loaded with the drug and the controls without MTX. Consequently, the presence of the drug does not alter the internal reorganization of the polymer chains in the desolvation conditions leading to the formation of a real gel (in the case of systems without BSA) or a partially gelling system (such as in the presence of BSA).

In Vitro Methotrexate Release Study

The dissolution profiles and the kinetics of MTX *in vitro* released from the system with 20.5% Poloxamer 407 and 100 mM phosphate buffer in the presence and absence of BSA (2.5%) are reported in Fig. 6. Figure 6b shows how the rate of drug released reaches a maximum at around 30–60 min and then constantly decreases over the time. According to the results of Moore *et al.* (31) and Anderson *et al.* (32), who studied the mechanism of drug release from Poloxamer gel employing a similar apparatus, the dissolution profiles can be attributed to both drug diffusion and hydrogel erosion. The two formulations analysed showed similar drug release profiles up to 6 h. After that time, the profile started to diverge. Particularly, in the formulation containing 2.5% of BSA, a slower MTX release was observed and there was around 10% less drug in the dissolution medium at the end of the experiment. The differences observed at the last two time-points resulted to be statistically significant after the comparison of the release profiles using a two-sample t-test (minimum level of significance $p<0.05$). This behaviour occurs even though the presence of the protein reduces gel consistency, as observed from rheological analyses.

Figure 6 also reports the release profiles of BSA. It can be observed that the protein release rate was slower after 3 h in comparison with MTX and, at the end of the study, less protein was released (approximately 75% compared to around 85% for MTX). Thus, there is a correlation between the release of BSA and MTX. In fact, after 3 h the MTX in the BSA formulation began to be released more slowly.

In Vitro Enzymatic Activity Test

The activity of MTX released from the two systems, mentioned above in the release study, was evaluated by measuring the inhibitory effect of the drug against the enzyme dihydrofolate reductase, its biological target. This experiment was performed to investigate whether BSA-MTX binding could affect *in vitro* activity of dihydrofolate reductase in presence of the drug.

Figure 7 shows the decrease of DHFR activity due to MTX released at different timepoints.

Table 3 Quenching and Binding Parameters of BSA and MTX in Presence and Absence of Poloxamer 407 10%

	K_{SV} (M^{-1})	f_a	K_q (M^{-1} s^{-1})	K_a (M^{-1})	n
MTX-BSA	1.32×10^{7}	0.34	1.34×10^{15}	2.79×10^{6}	1.29
MTX-BSA-Pol407 (10%)	8.79×10^{6}	0.14	8.97×10^{14}	1.18×10^{4}	0.88

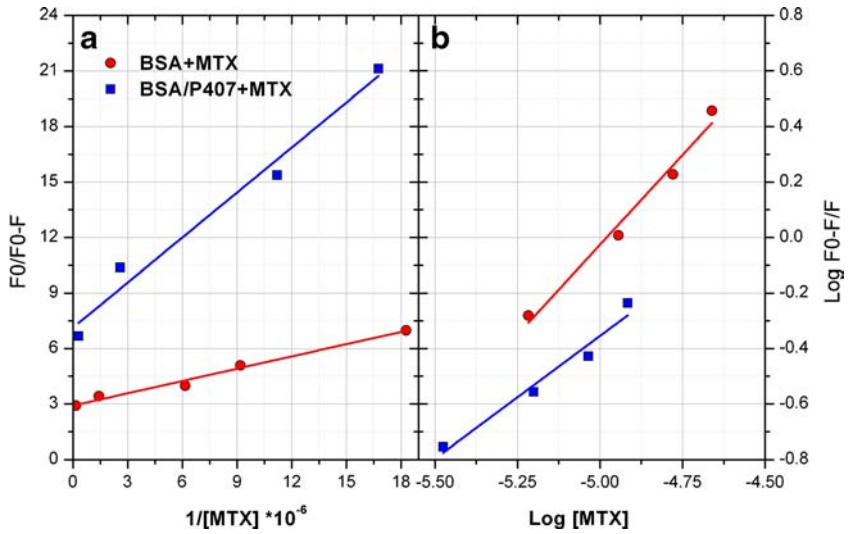

Fig. 4 (**a**) Modified Stern-Volmer plot at 25°C for BSA-MTX and BSA/P407-MTX systems (**b**) Plot of Log(F_0-F)/F *versus* Log[MTX] at 25°C for BSA and BSA/P407 systems.

It can be noted that the amount of MTX released 40 min after the beginning of the experiment is sufficient to completely determine the loss of enzymatic activity in both systems. The two curves are comparable and have a sigmoid shape. This suggests that the inhibitory effect of MTX is maximal even at very low concentrations and causes a large decrease of dihydrofolate reductase activity.

DISCUS?SION

Though Poloxamer 407 is a well-characterised thermogelling triblock copolymer that has been used for sustained release of several drugs and biotechnological products (33,34), to date no complete investigation has been conducted to determine how the presence of a protein would affect the physical transition of this copolymer.

It would be interesting to examine how the properties of the hydrogel might be influenced by biological fluid that contains macromolecules such as proteins, because a polymer could probably give much more interactions with another macromolecule with respect to a small molecule as conventional drugs (35).

This study evaluated the effect of the model protein, bovine serum albumin, on the micellisation and gelation of Poloxamer 407. Micellisation of Poloxamer 407 in presence of BSA was studied through two techniques, microDSC and

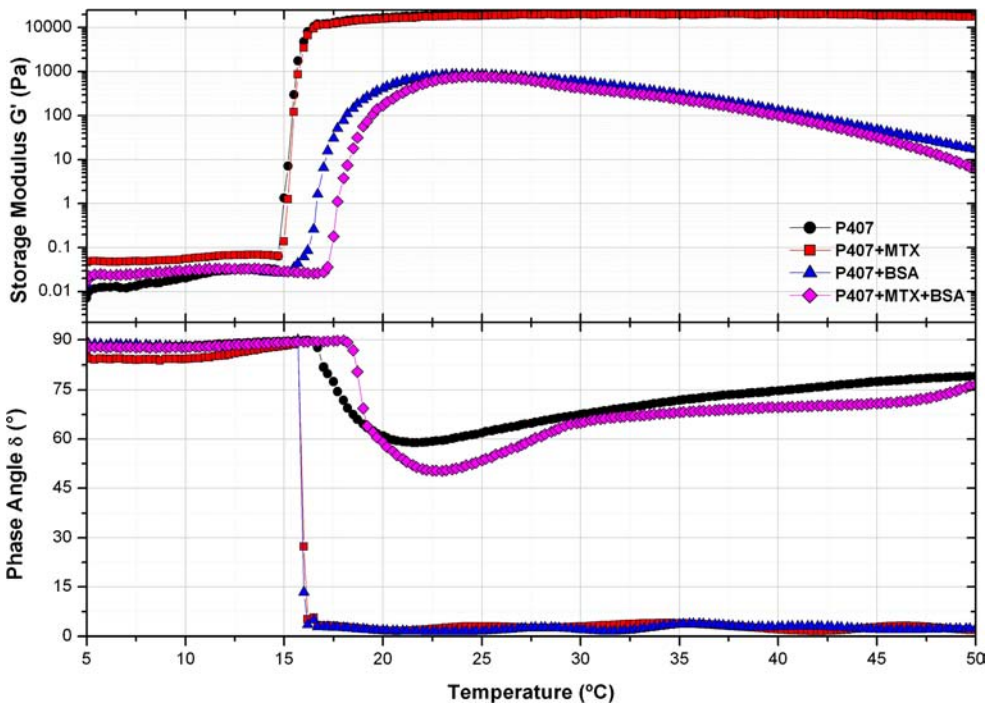

Fig. 5 Effect of temperature on the elastic modulus (G') (**a**) and phase degree (**b**) of the two systems selected for the release study.

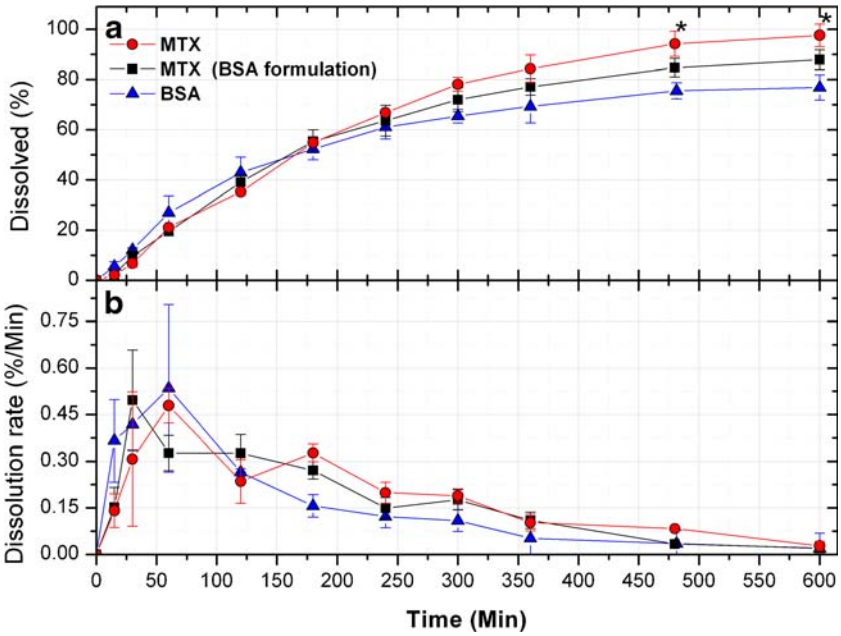

Fig. 6 Amount (%) (**a**) and dissolution rate (%/min) (**b**) of the methotrexate and BSA released from the hydrogels over time. The presence of asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicates the statistically differences (two samples t-test) in the percentage of MTX released between the two formulations.

dynamic light scattering, which are used to monitor different parameters of the aggregation of copolymer unimers into micelles, thus allowing characterisation of the phenomenon.

In particular, microDSC analyzes the exchange of energy associated with the transitions. DLS, instead, makes it possible to follow the particle size evolution related to the copolymer and protein during the aggregation process. As shown in Table 1, the presence of BSA has no effect on the thermodynamic parameters of CMC and enthalpy of micellisation.

On the other hand, the enthalpy associated with the thermal denaturation of BSA decreases in the presence of the copolymer. Typically, micellisation transition can be indicated in DLS raw curves (Fig. 2) by two peaks, one that refers to unimers and another to the population of copolymer involved in the aggregation process, in function of temperature. The end of the micellisation can be considered the temperature at which the signal related to the population of unimers disappears and one single peak related to micelles can be noted. In fact, for Poloxamer 407 2.5% in 100 mM phosphate buffer both in presence and in absence of BSA, the calculated CMT are superimposable at around 22°C (Table 1). The CMT of the sample without BSA is confirmed by the fact that at 25°C there is only one peak, suggesting that micellisation is complete. When BSA is present, a single larger peak appears at all three temperatures (15°, 20°, 25°C). This peak reflects the presence of a single size population that can be attributed to both polymer and protein.

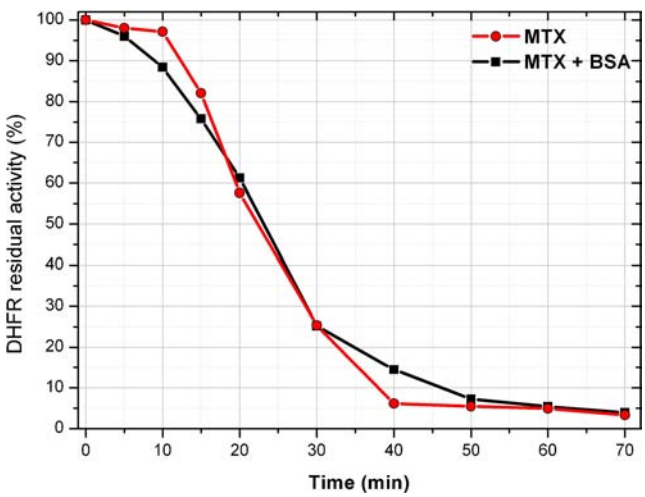

Fig. 7 Residual activity against DHFR of the methotrexate released from hydrogels over time.

Due to the limits of this technique, it is not simple to explain the micellisation of Poloxamer 407 as a kinetic process in the presence of BSA. In fact, through DLS, it is not possible to discriminate between the peak of the copolymer and that of the protein. However, it can be noted that at 15°C, when micellisation of Poloxamer 407 has not yet started, this single large peak of the system containing BSA is slightly shifted with respect to the signal of the unimers of Poloxamer 407 and BSA alone. At 20° and 25°C, the presence of the signals related to the micellisation process does not allow comparison between the peak of the unimers and the peak of the Poloxamer 407 system containing BSA. As a consequence, using DLS, in the presence of the protein it is difficult to follow the kinetics of the aggregation process. However, from the fluorescence studies, the presence of an isosbestic point centred at 330 nm, as reported in Fig. 4, denotes the presence of two different fluorescence populations at the equilibrium. Since Poloxamer 407 is not a fluorescent copolymer, it does not emit when excited at 280 nm. The only fluorophore in the

system is BSA. Thus, it can be supposed that the two species at equilibrium could be free BSA and BSA that interacts with the copolymer. By considering the spectrum of BSA in presence of an increasing concentration of the copolymer (SF 1 reported in the supplementary materials) at 35°C, one can note that the wavelength of the isosbestic point (324 nm) is not significantly blue shifted and the wavelength of the shoulder (310 nm) is the same observed at 25°C. At 10°C, instead, there is no isosbestic point and the shoulder at 310 nm appears only in the presence of 10% of Poloxamer 407 (SF 2, reported in the supplementary materials). In fact, this is the concentration of copolymer at which the presence of micelles could be considered negligible at 10°C. Thus, it can be assumed that BSA interacts both with unimers and micelles depending on temperature and poloxamer concentration. The micellisation process at 25 and 35°C is rather complete (as it results from mDSC and DLS data) and BSA fluorescence spectra exhibit a trend in which Trp emission is modified according to the interaction with micelles (36), while at 10°C the presence of a considerable fraction of unimers make the system more heterogeneous. Micellisation of Poloxamer 407 is a dynamic process that evolves continuously by the self-assembling of unimers into micelles and the disassembling of micelles to form again unimers in a sort of aggregation-disaggregation process (12). Therefore, a small fraction of unimers always remains available for the interaction with BSA at any temperature. The hypothesis about the existence of this interaction between BSA and copolymer finds further confirmation in microDSC data. In fact, less energy is associated with the thermal denaturation of the protein, suggesting that the presence of the copolymer is able to cause a change in the intra- and intermolecular interactions in the protein itself and protein/copolymer system from which an enthalpy/entropy compensation in the energetic of protein unfolding occurred (no changes in BSA denaturation temperature Tm were observed, see Table 1).

On the contrary, rheological analysis indicated that BSA strongly influences the gelation process of Poloxamer 407. Interestingly, in the samples selected by the experimental design and analysed by temperature sweep, an inhibition of thermogelation or the presence of a pseudo-gelled system characterized by low value of viscosity occurs in function of the concentration of BSA and copolymer. A possible explanation is that the size of BSA causes steric hindrance during the reorganization of micelles into the gelled network.

Moreover, the experimental design proved useful in confirming the results obtained from microDSC and DLS. Briefly, from ANOVA results of the experimental design reported in the supplementary materials, it can be deduced that buffer concentration primarily influences the micellisation of Poloxamer 407, whereas the presence of BSA has more effects on the copolymer gelation. MTX, a drug able to bind to albumin (28), was selected as model drug to investigate whether the effect of BSA on gelation of Poloxamer 407 could effectively influence the drug release. From the results of our release studies, it can be deduced that the protein influences the MTX release kinetics, slowing the drug efflux from the hydrogel only after the polymeric matrix has partially degraded.

Similar dissolution results were also found when BSA was studied in gelled microemulsions (17). According to the authors, this result was due to reversible drug-protein binding, demonstrated through fluorescence studies, although it was not clear why such interaction affects the drug release. Actually, this behaviour could only be explained by demonstrating that the drug is bound to the protein, and the rate of protein released is the limiting factor in the dissolution analysis. This fact can be confirmed by measuring the BSA released at each time point in MTX-BSA formulation. The lower amount of BSA released after 3 h could be correlated to the ability of the protein to bind and retain the drug inside the hydrogel. Moreover, our *in vitro* assay demonstrated that in the presence of BSA, MTX released from the Poloxamer hydrogel maintained the same inhibitory effect toward DHFR compared to the system without the protein. Thus, although the interactions of MTX with BSA modify the dissolution kinetics, they do not alter the drug activity after its release. The sigmoidal trend of DHFR residual activity showed a total inhibitory effect of MTX at very low concentrations, both in presence and absence of BSA (Fig. 7), denoting that low doses of the drug are required to obtain a pharmacological effect *in vitro*. The optimization of the therapeutical regimen by suitable dosage forms could allow to increase binding selectivity towards a specific target and minimize aspecific interactions, enhancing bioavailability.

CONCLUSION

This study investigated the interaction between BSA and Poloxamer 407, chosen as a model thermogelling copolymer in order to evaluate the influence of a protein inside a hydrogel intended for the controlled release of MTX into sinovia. To this end, a preliminary characterization of the system containing BSA was carried out, highlighting that the protein exerts greater influence on the Poloxamer gelation than on the micellisation process. Despite a decrease of gel consistency, the lower amount of MTX released in presence of the protein demonstrated the modulatory effect of BSA during the release, which could be related to some mechanisms of drug release different from drug molecular diffusion or hydrogel erosion in which albumin itself is involved. It has been demonstrated from both fluorescence and enzymatic activity studies that the presence of poloxamer 407 did not modify the BSA ability to bind methotrexate and eventually the inhibitory effect of the drug with respect to its molecular target

(DHFR). Finally, this study confirms the potential application of thermogelling systems for *in situ* drug administration in inflamed body compartments enriched in proteins.

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