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Development and characterisation of modified poloxamer 407 thermoresponsive depot systems containing cubosomes

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ABSTRACT

The purpose of this study is to develop a thermoresponsive sustained release delivery system combining phytantriol cubosomes and poloxamer 407 (P407). P407 undergoes thermoreversible gelation, where it exists as a free-flowing liquid at low temperature and gels upon heating. However, this polymer has the major draw back of fast erosion in aqueous environments which needs to be addressed. Three different concentrations of P407 (12%, 15% and 17% (w/v)) were formulated with various additives (methyl cellulose (MC), dextran, carrageenan and Pluronic-R (25R4)). The rheological characteristics and *in vitro* stability were investigated. The sol–gel transition temperature of P407 was lowered in the presence of the phytantriol cubosomes. The addition of MC and dextran did not affect the sol–gel transition temperature whereas 25R4 increased the gelation temperature. No transition was observed for the carrageenan formulations. The presence of 25R4 allowed the development of formulations that were free flowing liquid at working temperature (22 °C), gelled at body temperature (37 °C) and had improved stability in an aqueous environment.

Both rheological and *in vitro* stability studies suggested that cubosome-loaded 17% (w/v) P407 with 25R4 in 1:1 molar ratio may have a potential as sustained release delivery system.

1. Introduction

Particulate delivery systems have been extensively studied for protein drugs; especially for vaccine delivery as they effectively allow sub-unit antigens to be presented to antigen presenting cells and can protect antigens from rapid enzymatic degradation (Myschik et al., 2009). Cubosomes, a cubic phase lipid particulate system, have been shown to have a high efficiency in entrapping antigens as well as providing extended antigen release (Rizwan et al., 2009). Sustained release over an extended period of time may reduce the need for multiple vaccinations which will be a benefit in terms of reduced costs and increased patient compliance (Zhao and Leong, 1996; Myschik et al., 2008). Another potential mechanism for sustaining the release of vaccine antigen is to create a localised depot. Thermoresponsive systems have several advantages, including the possibility to formulate an *in situ* gelling controlled release system. Poloxamers are among the most commonly investigated thermoresponsive systems (Chun et al., 2005; Dumortier et al., 2006; Paavola et al., 1998; Ricci et al., 2005).

Poloxamer block copolymers were introduced in the late 1950s and have been used for many different pharmaceutical applications including parenteral, inhalation, ophthalmic and topical formulations (Dumortier et al., 2006). Poloxamer is a linear triblock polymer consisting of a polypropylene oxide block (PPO) between two polyethylene oxide blocks (PEO). These block copolymers have amphiphilic properties that are highly dependent on the length of the PPO and PEO chains. Poloxamer 407 (P407) has a molecular weight of approximately 12,600 (9840–14,600) and the lengths of PEO and PPO are 95–105 and 54–60 monomeric units respectively (Kabanov et al., 2002). Given that the PPO is less hydrophilic than the PEO, the copolymer easily self-assembles forming micelles. This process is both concentration and temperature dependent and is due to the dehydration of hydrophobic PPO blocks, which represents the very first step in the gelling process. The micelles are spherical with a dehydrated PPO core and an outer layer of hydrated PEO chains. The micellization is followed by gelation, which is attributed to the packing of the micelles, if the solution is sufficiently concentrated (Cabana et al., 1997; Habas et al., 2004; Hvidt

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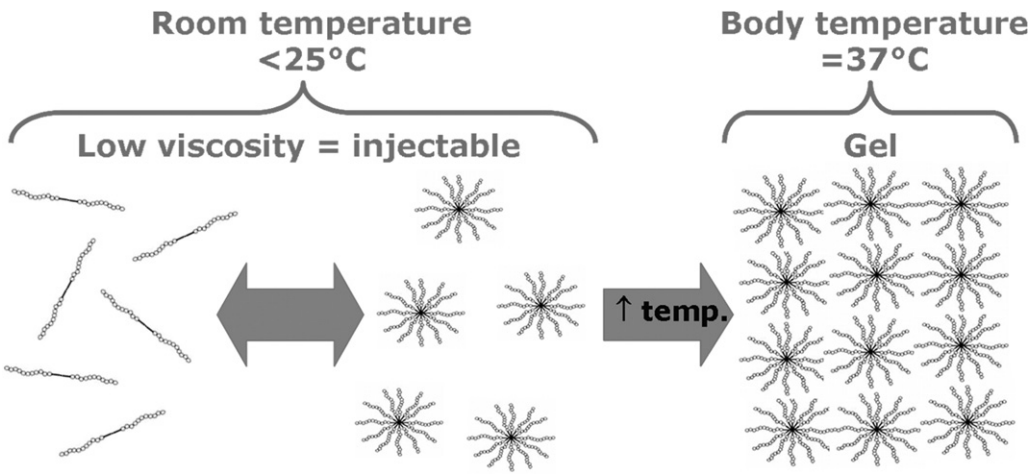

Fig. 1. Schematic diagram for the phase transition of P407 at <25 °C and when temperature increase to 37 °C. Adapted from Dumortier et al. (2006).

et al., 1994; Lenaerts et al., 1987; Miller and Drabik, 1984). Fig. 1 shows a schematic illustration of the sol–gel transformation of the poloxamer with increased temperature.

A major drawback of P407 gels as sustained release systems is the fast erosion of the gel that occurs in aqueous environments (Dumortier et al., 2006). This is due to the dilution of P407 copolymers upon contact with excess amount of solvent which causes the polymer to drop below the critical gellation concentration resulting in a loss of gel structure (Chun et al., 2005). A number of approaches have been used to improve the stability of P407 gels in aqueous environments. One of these approaches is to incorporate polymers that can support the system and thereby hinder the diffusion of P407 out of the system. The polymers can either support the network by scaffolding or by the formation of interconnections between the micelles in the network. Hydrophilic polymers interact with the PEO blocks through entanglements, facilitating scaffolding of the micelle network. Polymers that have been previously studied for this purpose are carrageenan (Liu et al., 2009), dextran (Paavola et al., 1998) and methyl cellulose (MC) (El-Kamel, 2002). The other type of additive is an interconnecting polymer, for example Pluronic-R (25R4), which has the reversed chemical structure of P407 (PPO–PEO–PPO) (Li et al., 2008). This polymer has been found to form intermicellear bridges between the core of the P407 micelles through interaction with the hydrophobic PPO blocks of the 25R4 (Li et al., 2008).

The aim of this study was to develop a sustained release delivery system, based on cubosomes, as a carrier that provides extended antigen release, loaded within P407 gels, giving a localized depot. This formulation should be injectable at working temperature (<25 °C), transform into a gel when heated to body temperature of 37 °C and form a stable gel in aqueous environments. The effect of P407 concentration and the presence of additives (carrageenan, dextran, MC and 25R4) on gel rheology and stability in aqueous environment were investigated.

2. Materials and methods

2.1. Materials

Poloxamer 407 (P407), MC (M_w = ~40,000), carrageenan (Commercial Grade Type I (product no: C1013-100G, Batch # 088K0178), containing predominantly kappa-carrageenan)) and dextran (M_w = 425,000–575,000) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phytantriol was purchased from A & E Connock (England), 1,2-Propanediol (Propylene glycol) was purchased from Merck (Germany). Poloxamer 407 (Lutrol® F127) for cubosomes preparation and Pluronic® 25R4 (M_w = 3600) were purchased from BASF (Ludwigshafen, Germany). Phosphate buffered Saline (PBS) was obtained from Oxoid (Hampshire, England). Milli-Q water used in this study was purified through a water purification system (Millipore Bedford MA, USA).

2.2. Methods

2.2.1. Preparation of poloxamer 407 gel

P407 were prepared by the cold method as described by Schmolka (1972). Three concentrations of P407, 12%, 15% and 17% (w/v), were prepared by dissolving the require amount of P407 in cold water and stirring at 4 °C for 24 h.

To prepare P407 gels with hydrophilic additives and/or cubosomes, MC (0.3%, w/v), dextran (0.2%, w/v), carrageenan (0.2%, w/v) or 25R4 (1:1 molar ratio to the P407 concentration used) were dissolved in cold water with P407. This was followed (when required) by the addition of cubosomes (20 mg/mL). The solution was then left to stir on a magnetic stirrer at 4 °C for at least 24 h to ensure homogenous solutions were obtained.

2.2.2. Preparation of cubosomes

Cubosomes were prepared by a liquid precursor method. Phytantriol and poloxamer 407 (Lutrol® F127) (lipid: polymer ratio of 6.7:1 w/w, in total 30% w/w) and propylene glycol (70% w/w) were dissolved in required amount of chloroform at 45 °C. The chloroform was evaporated under a stream of nitrogen and the resulting liquid precursor dispersed in 5 mL of MilliQ water. The samples were then vortex at high speed for 10 min and stored at ambient temperature until required. The final concentration of lipid in the dispersion is 20 mg/mL.

2.2.3. Rheological measurements

Oscillatory shear measurements were conducted on a TA AR-G2 rheometer (TA instruments) using a cone-and-plate geometry, with a cone angle of 1° and a diameter of 60 mm. The measuring device was equipped with a temperature unit (Peltier plate) that gives tight temperature control over an extended time. In order to prevent any evaporation of the sample, a tefflon ring was fastened on the peltier plate (hole-diameter of 64 mm), the sample applied and the cone lowered to measuring position. Thereafter a layer of low viscosity silicone oil was added onto the surface of the sample.

The rheology experiments were conducted within the linear viscoelastic regime, where the storage modulus (G′), the elastic response in the system, and loss modulus (G″), the viscous response

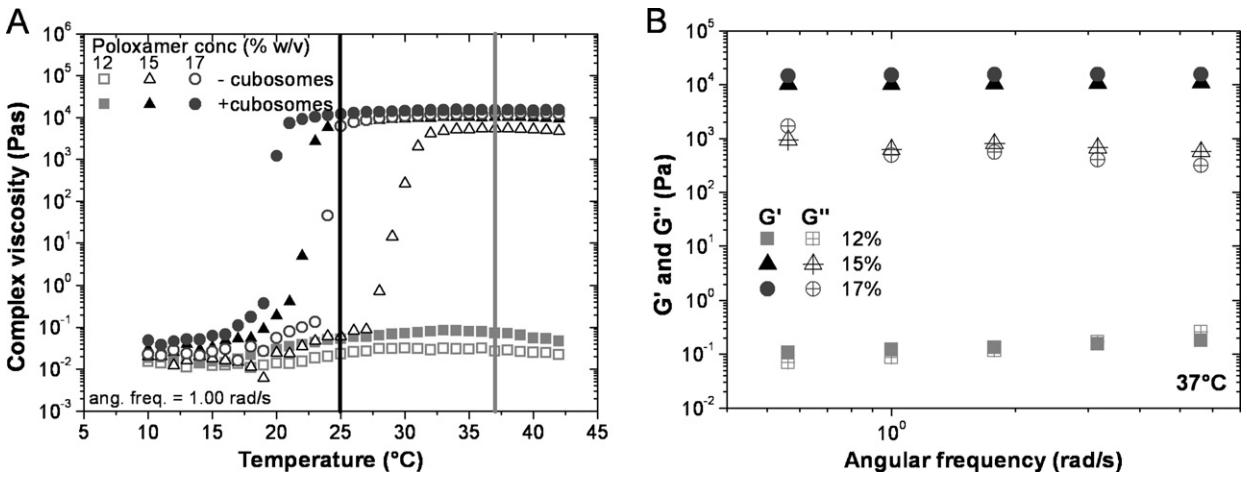

Fig. 2. (A) The sol–gel transition temperature of 12%, 15% and 17% (w/v) P407 in the presence or absence of cubosomes. (B) The frequency dependency of the dynamic moduli, G′ and G″, at body temperature for the systems containing cubosomes.

in the system, are independent of the oscillation torque. The sol–gel phase transition was followed by frequency sweep measurements from 0.6 to 6 rad/s at temperatures ranging 10–42 °C with 1 °C intervals and an equilibrium time of 1 min. The gelation time was scrutinized by time sweep measurement at 37 °C where the angular frequency was kept at 1.0 rad/s. The sample was placed on the peltier plate at 5 °C and thereafter the measurement was started. Thus, the first 4 points were measured during the temperature adjustment from 5 °C to 37 °C.

The complex viscosity ($|\eta^*|$) was calculated in order to depict the overall gelling profile of the poloxamer systems, thereby both G′ and G″ are taken into account (Eq. (1)):

$$|\eta*(\omega)| = \frac{\sqrt{G'(\omega)^2 + G''(\omega)^2}}{\omega} \quad (1)$$

where ω is the angular frequency.

The sol–gel transition temperature was determined from where the intercept of the baseline and a straight line fitted to the steep increase of the complex viscosity.

2.2.4. In vitro evaluation of P407 gel erosion

Vials (inner diameter = 13.5 mm) containing approximately 1 g of prepared solution were placed in a 37 °C water bath. After the formulations had transformed into gels, 1.5 mL of PBS (pH 7.4) pre-warmed to 37 °C was carefully layered over the gel surface. At predetermined intervals the entire release medium was removed and the weight of the vial and remaining gel recorded. The percentage of gel weight loss was calculated by dividing the change in the gel weight by the initial gel weight.

2.3. Statistical analysis

For comparison between different formulations, one-way analysis of variance (ANOVA) was applied using SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). The student's paired *t*-test was used to analyze differences between means. *P* values of < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Effect of poloxamer 407 concentration

The aim of this study was to develop a cubosome-loaded biocompatible sustained release system using P407 which was a free flowing solution at working temperature (<25 °C), which gelled upon an increase to body temperature (37 °C) and had an extended stability over unmodified P407 gels. One possibility investigated for increasing the stability of the gels was to increase the P407 concentration. Three different concentration of P407 (12%, 15% and 17% w/v), with and without cubosomes were investigated and the rheological results are shown in Fig. 2. The concentrations chosen were those that could potentially meet the criteria of being free flowing solutions at room temperature and gels at 37 °C. It was found that the sol–gel transition temperature was dependent on the concentration of the P407. At 25 °C in the absence of the cubosomes, the 12 and 15% P407 formulations were solutions, while the phase transition had already started for the 17% formulation. Upon heating to 37 °C the 12% P407 remained in a solution phase while the 15 and 17% P407 formulations formed gels. The concentrations of P407 investigated (12–17%) are lower than those used in previous animal studies where the P407 concentrations used were in the range of 20–35% (Dumortier et al., 2006). However, when P407 concentrations of 20–35% are used, the formulations are not free-flowing liquids at working temperature and their high viscosity can make them difficult to manipulate and inject. Therefore in this study we choose to use lower P407 concentrations (12–17%) as one of the criteria was that the formulations should be free-flowing solutions at working temperature (20 °C).

The addition of cubosomes to the P407 resulted in increased complex viscosity of the gel at 37 °C and lower of the transition temperature for the 15% and 17% P407 formulations by 5 °C, as shown in Fig. 2A. Consequently, the gelation of these formulations occurs at 22 °C and 19 °C for the 15% and 17% P407, respectively, thus the formulations may not be free flowing at working temperature. The presence of cubosomes also caused a slight increase in the complex viscosity for the 12% P407 formulation, thus a soft gel was formed (G′ = G″) at increased temperatures, in contrast to the 12% P407 alone which did not reach the sol–gel transition throughout the temperature range studied. The gel formation of the 12% P407 formulation containing cubosomes was also confirmed during the erosion studies. The frequency dependency of the dynamic moduli, G′ and G″, for the systems containing cubosomes at 37 °C is depicted in Fig. 2B. At body temperature the G′ is dominating for the 15% and the 17% formulations indicating a well developed gel, whereas for the 12% formulation the G′ and G″ are similar over the frequency range measured, confirming that only an immature gel is formed. The cubosomes utilized in this study are dispersions of the phytantriol bulk cubic phase. The dispersed particles are stabilised through the addition of P407 where the hydrophobic PPO portion is presumed to adsorb to the surface of cubosomes while

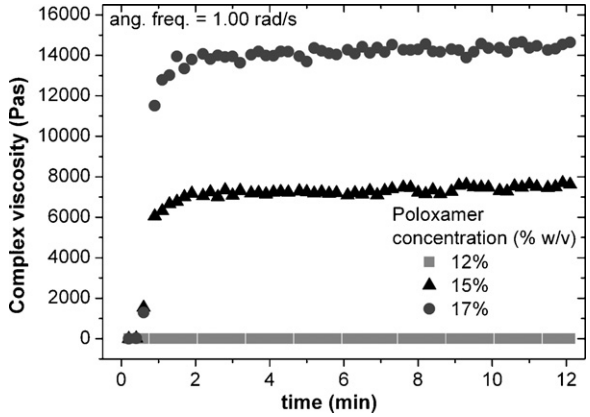

Fig. 3. The sol–gel transition time of 12%, 15% and 17% (w/v) P407 in the presence of cubosomes.

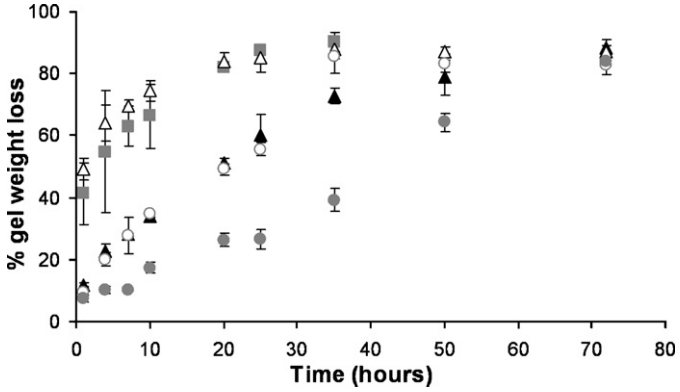

Fig. 4. *In vitro* erosion profile of P407 gels with or without cubosomes at 37 °C: (■) 12% P407 with cubosomes; (△) 15% P407; (▲) 15% P407 with cubosomes; (○) 17% P407; (●) 17% P407 with cubosomes. Data is the mean ± SD of three independent experiments.

the hydrophilic PEO chains extend out into the aqueous environment to provide steric shielding (Yang et al., 2004). The presence of P407 on the surface of the cubosomes increases the apparent concentration of polymer in the solutions and thereby a higher complex viscosity of the gels at 37 °C is detected, and a lower sol–gel transition temperature for the 15% and 17% P407 solutions is observed, where the micelles become closely packed forming a gel network. Similar effects have been reported when introducing inorganic salts to the P407 system which have been described as "salting-out effects". This phenomenon has been ascribed to the ability of the salts to reduce the water activity by hydrogen bonding to the water and thereby increasing the effective aqueous concentration of the polymer (Ananthapadmanabhan and Goddard, 1987; Malmsten and Lindman, 1992; Pandit and Kisaka, 1996). Thus, the addition of cubosomes might also result in decreased water activity leading to an increased effective concentration of P407 and thereby lowering of the sol–gel transition temperature of the system.

The sol–gel transition time for the P407 formulations containing cubosomes are shown in Fig. 3. The complex viscosity for 15% and 17% P407 increased sharply and a gel was formed within 2 min, with 17% P407 formulations having the highest complex viscosity. However, for the 12% P407 formulation containing cubosomes the complex viscosity only increased from 0.014 Pas to 0.055 Pas in 2 min. This modest increase, which cannot be seen in the Fig. 3, is not sufficient for the gel to be considered for use as a thermoresponsive vaccine delivery system. For all formulations the increase in complex viscosity or the gellation occurred within 2 min, regardless of the P407 concentration. This fast sol–gel transition time is important in the formation of a depot system for sustained release of antigen. During the injection of the P407 solution into the body at 37 °C, the faster the gel transformation occurs the smaller the risk of the gel being diluted with physiological fluid at the site of application (Charrueau et al., 2001). Furthermore, this reduces the risk of burst release from the gel precursor which has the associated risk of dose dumping.

The stability of 12%, 15% and 17% P407 formulations in the presence or absence of cubosomes was investigated *in vitro*, see Fig. 4. The stability of formulations with 12% P407 alone was not studied as, consistent with the rheology results, no gel formation was observed upon incubation at 37 °C. The erosion of the gels decreased significantly in 15% and 17% formulations containing cubosomes as compared to formulations of 15% or 17% P407 alone ($p < 0.05$). The stability of the 17% P407 formulation containing cubosomes was significantly higher as compared to 12% P407 containing cubosomes ($p < 0.0001$), 15% P407 alone ($p < 0.0001$), 15% P407 containing cubosomes ($p = 0.0411$) and 17% P407 alone ($p = 0.0400$). 50% weight loss was reached at approximately 41 h for the 17% formulation containing cubosomes as compared to 20 h for the 17% formulation without cubosomes, see Fig. 4. The presence of cubosomes increased the stability of P407 gels but this was also dependent on the concentration of P407. This is in good agreement with the rheological results where increased complex viscosity is detected with both increased concentration of the polymer and the presence of cubosomes, indicating a tighter network that is more resistant to the erosion of the polymers. This *in vitro* stability data is encouraging, however animal experiments are needed to investigate this further and establish the biological relevance of the increased stability on immune responses to the vaccines.

3.2. Effect of additives on gelation and stability of cubosome-containing 12 and 15% P407 gels

We examined the effect of the addition of four different polymers on the rheological properties, shown in Fig. 5, and the erosion of these gels in an aqueous environment, shown in Fig. 6, (the 17% P407 preparation will be covered in greater detail in the next section). Addition of MC and dextran to the 12% P407 containing formulations resulted in a 10-fold increase in the complex viscosity for the gels obtained, see Fig. 5A. The presence of 25R4 resulted in 1000-fold increase in complex viscosity at 37 °C, however, the gel did not reach a plateau within the temperature interval studied. These results indicate an immature gel at 37 °C which might affect stability in the body. Despite the improvement of the complex viscosity for the gels obtained, the G′ is similar to the G″ (data not shown) and thereby the gels were not expected to resist erosion. The addition of carrageenan caused an increase in complex viscosity at lower temperatures, which is not suitable for an injectable formulation. Additionally the complex viscosity decreased from 32 °C to 37 °C, indicating an unstable gel. This is likely due to the thermal behaviour of carrageenan as it is known to form a solid gel at lower temperatures while at high temperatures it exists as a solution (Yuguchi et al., 2002). The stability of 12% P407 formulations, shown in Fig. 6A, was slightly enhanced in the presence of MC, dextran and 25R4 as compared to P407 alone, thus the 10-fold versus the 1000-fold increase in complex viscosity gave similar erosion, most likely due to the incomplete network of the system containing 25R4 making that more fragile and thereby counteracting the complex viscosity increase. The addition of carrageenan seemed to increase the erosion of the gel, see Fig. 6A. However these differences were not statistically significant. Based on the results obtained from 12% P407 rheology and stability studies, no further studies on carrageenan as an additive were performed.

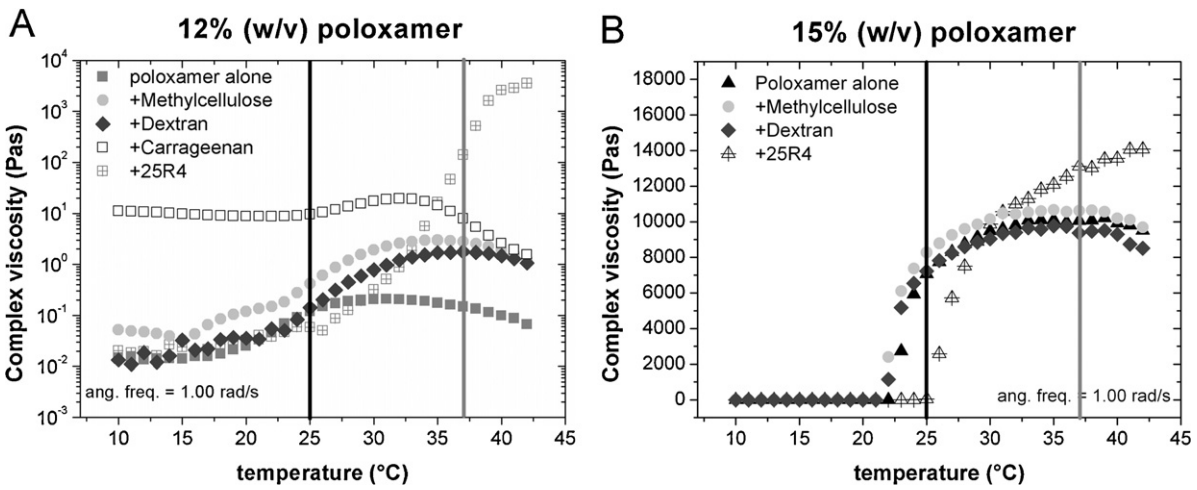

Fig. 5. Complex viscosity of 12% P407 (A) and 15% P407 (B) with various additives (MC, dextran, carrageenan, 25R4) in the presence of cubosomes as a function of temperature.

The hydrophilic polymers, MC and dextran, did not affect the rheological behaviour of the 15% P407 cubosome-containing formulations. Fig. 5B shows that similar sol–gel transition temperatures were observed and improvement was neither seen in the dynamic moduli nor in the complex viscosity of the final gel. Similarly the stability of 15% P407 gels was not enhanced by the addition of MC and dextran as shown in Fig. 6B. The scaffolding by the hydrophilic polymers for the 12% P407 might have been enough to support the unstable gel that was formed, however, as the gel strength increases with increasing P407 concentration, this support becomes unnecessary. However the addition of 25R4 increased the phase transition temperature to approximately 25 °C, giving a free flowing liquid at working temperature, and increased the complex viscosity at 37 °C, see Fig. 5B. This is in contrast to results reported by Li et al. (2008) where the addition of 25R4 to pure P407 in aqueous solutions resulted in a lowering of the sol–gel transition temperature. However, the addition of cubosomes, which already lowers the sol–gel transition in the current system, complicates this system. The rheological behaviour of the 15% P407 formulation containing the 25R4 is promising compared to the unmodified 15% P407 formulation or to P407 formulations containing the hydrophilic polymers, as the increase in the G′ and thus the complex viscosity indicates a stronger gel that may potentially resist erosion. The rheology results were reflected in the gel stability, as the formulation containing the 25R4 additive had significantly enhanced gel stability as compared to the unmodified 15% P407 formulation ($p = 0.0267$), see Fig. 6B.

3.3. Effect of 25R4 on gelation and stability of cubosome-containing 12, 15 and 17% P407 gels

As the addition of 25R4 to the cubosome-containing 12% and 15% P407 gels produced the most promising results, the effect of increasing P407 concentration, in combination with the addition of 25R4 (the additive that was shown to be the most promising in stabilising the gel in the above mentioned studies) was examined in more detail. The influence of the addition of 25R4 on the three concentrations of cubosome-containing poloxamer formulations is depicted in Fig. 7A. In general, the addition of 25R4 increased the complex viscosity of the final gel for all the formulations and this effect seems to be most pronounced for the 15% P407 formulation. The frequency dependency of the dynamic moduli, G′ and G″, for the systems containing cubosomes and 25R4 at 37 °C is depicted in Fig. 7B. At body temperature the G′ is dominating for the 15% and the 17% formulations as for the systems without the 25R4. A parallel increase in the G′ and G″ is observed for the 12% system, however, the elastic and the viscous response is similar indicating again a poorly structured, immature gel.

Enhancement of the sol–gel transition temperature is detected for the 17% P407 formulation containing 25R4, similar to the 15% P407 (discussed earlier), however the transition still starts below 25 °C, or more precisely at 22 °C.

As previously mentioned a fast sol–gel transition is an important factor for the effective delivery of a sustained release vaccine formulation. As shown in Fig. 8, a sharp increase in complex viscosity

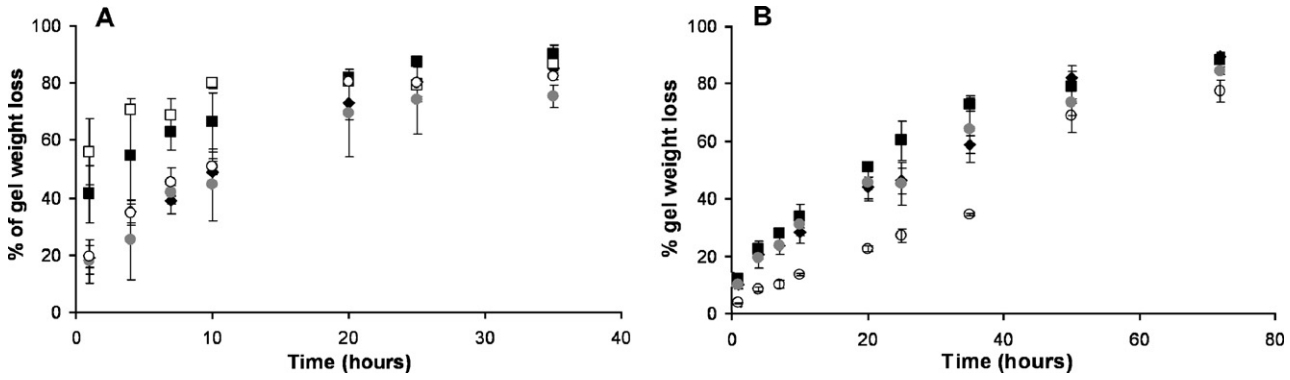

Fig. 6. *In vitro* erosion profile of unmodified 12% (A) and 15% (B) P407 gels containing cubosomes (■) or modified through the inclusion of dextran (♦), MC (●), carrageenan (□), or 25R4 (○). Data is the mean ± SD of three independent experiments.

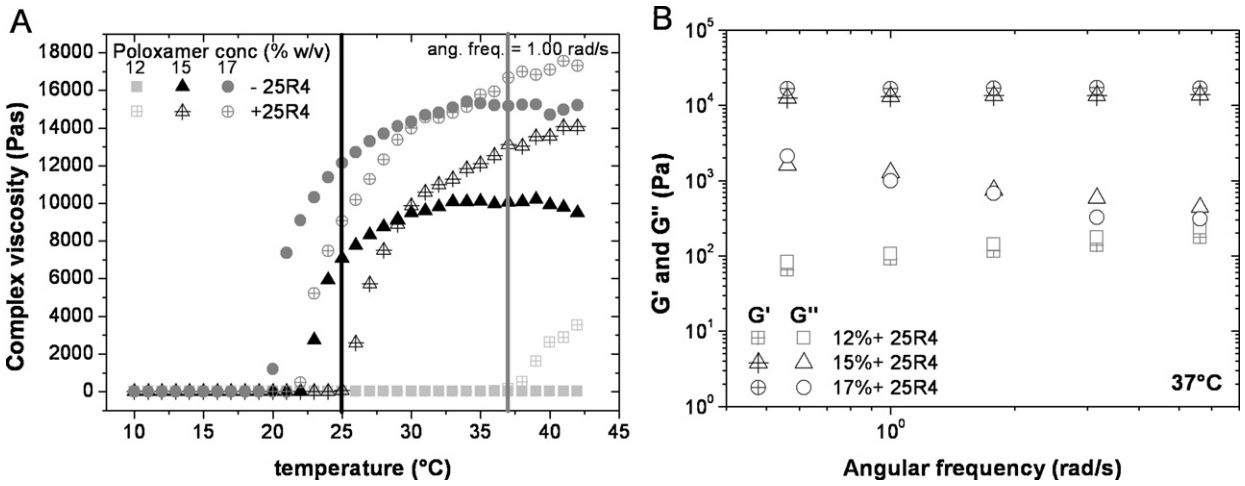

Fig. 7. (A) Complex viscosity as a function of temperature for 12%, 15% and 17% P407 with or without 25R4 (1:1) in the presence of cubosomes. (B) The frequency dependency of the dynamic moduli, G′ and G″, at body temperature for the systems containing cubosomes and 25R4.

was detected in 15% and 17% cubosome-containing P407 formulations, with or without the addition of 25R4, where the complex viscosity reached plateau within 2 min. This suggests the fast formation of a solid gel. From these rheology results it appears that cubosome-containing 15% P407 and 17% P407 with 25R4 meet the criteria of being free flowing liquids at room temperature (<22 °C), having a fast-gellation and of being a solid gel (with complex viscosity >10,000 Pas (angular frequency = 1.00 rad/s)) at body temperature of 37 °C. The 15% and 17% P407 formulations without the 25R4 were disregarded as potential formulations whereas the 15% P407 did not show a sufficient increase in the complex viscosity in the measurement of gelation time and the 17% P407 showed sol–gel transition below 20 °C and would therefore be at risk of geling before injection.

Fig. 9 shows the effect of 25R4 on P407 gel stability in an aqueous environment. The stability of cubosome-containing 17% P407 gels with 25R4 was significantly enhanced as compared to that of unmodified cubosome-containing 17% P407 gels ($p = 0.0434$). The increase mediated through the inclusion of 25R4 is likely due to the formation of intermicellar bridges where the end blocks of the 25R4 chain can form a bridge between two P407 micelles (Li et al., 2008). Interestingly the 17% P407 25R4 formulation had an improved stability compared to the 15% formulation at early time points but by 72 h there was no difference in gel stability. This delay in the erosion may be due to the scaffolding of the system by 25R4 that gives strength to resist diffusion of water into the system, as well as the diffusion of the polymers out of the system, however, eventually the system will weaken and the erosion increases.

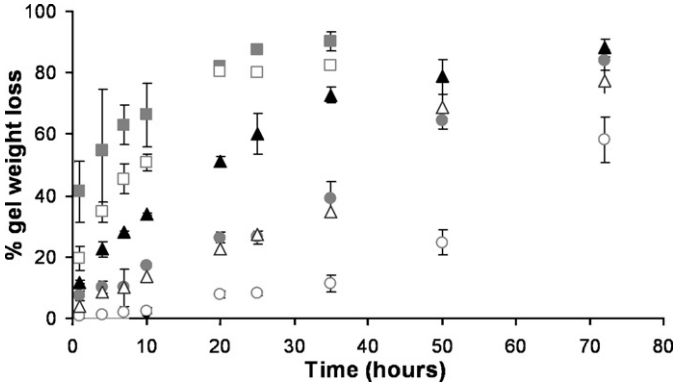

Fig. 9. Comparison of *in vitro* erosion profile of three different concentrations of P407 gel loaded with cubosomes and with or without 25R4: 12% (w/v) P407 alone (■) and with 25R4 (□) versus 15% (w/v) P407 gel alone (▲) and with 25R4 (△) and 17% (w/v) P407 gel alone (●) and with 25R4 (○). Data is the mean ± SD of three independent experiments.

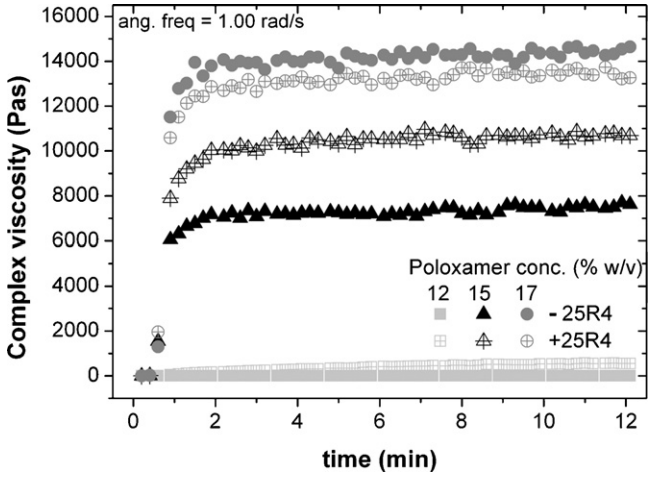

Fig. 8. The time of gelation depicted as the complex viscosity versus time for 12%, 15% and 17% P407 with or without 25R4 (1:1) in the presence of cubosomes.

4. Conclusion

The aim of this study was to utilize P407 for the development of a sustained release thermogelling system containing cubosomes. The required characteristics for the formulation were that it was a free flowing liquid at room temperature, that gelled quickly upon heating to 37 °C and that it had improved aqueous resistance to erosion as compared to unmodified P407 gels. Formulations were modified through the addition of hydrophilic or amphiphilic polymers and by increasing the concentration of P407. Only the amphiphilic polymer, 25R4, improved the rheological characteristics of the gel and stability, most likely through formation of intermicellar bridges between P407 micelles thereby supporting the gel network. Increasing P407 concentration increased the G′ and thus the complex viscosity of the gels at body temperature meaning a more solid gel was formed, but also increased viscosity at working temperatures and lowered the sol–gel transition temperature thus increasing the risk of gelation during preparation prior to injection. However the cubosome-containing 25R4 modified 17% P407 formulation was still free flowing in a temperature range suitable for

preparation and will therefore progress into *in vivo* studies where the effect of the increased gel stability on antigen vaccine release will be examined.

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