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Characterisation of protein stability in rod-insert vaginal rings

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ABSTRACT

A major goal in vaccine development is elimination of the 'cold chain', the transport and storage system for maintenance and distribution of the vaccine product. This is particularly pertinent to liquid formulation of vaccines. We have previously described the rod-insert vaginal ring (RiR) device, comprising an elastomeric body into which are inserted lyophilised, rod-shaped, solid drug dosage forms, and having potential for sustained mucosal delivery of biomacromolecules, such as HIV envelope protein-based vaccine candidates. Given the solid, lyophilised nature of these insert dosage forms, we hypothesised that antigen stability may be significantly increased compared with more conventional solubilised vaginal gel format. In this study, we prepared and tested vaginal ring devices fitted with lyophilised rod inserts containing the model antigen bovine serum albumin (BSA). Both the RiRs and the gels that were freeze-dried to prepare the inserts were evaluated for BSA stability using PAGE, turbidimetry, microbial load, MALDI-TOF and qualitative precipitate solubility measurements. When stored at 4 °C, but not when stored at 40 °C/75% RH, the RiR formulation offered protection against structural and conformational changes to BSA. The insert also retained matrix integrity and release characteristics. The results demonstrate that lypophilised gels can provide relative protection against degradation at lower temperatures compared to semi-solid gels. The major mechanism of degradation at 40 °C/75% RH was shown to be protein aggregation. Finally, in a preliminary study, we found that addition of trehalose to the formulation significantly reduces the rate of BSA degradation compared to the original formulation when stored at 40 °C/75% RH. Establishing the mechanism of degradation, and finding that degradation is decelerated in the presence of trehalose, will help inform further development of RiRs specifically and polymer based freeze-dried systems in general.

1. Introduction

A major goal in vaccine development is elimination of the 'cold chain', the transport and storage system for maintenance and distribution of the vaccine product. This is particularly pertinent to more conventional vaccine formulations, which are commonly developed in liquid form for parenteral administration of the antigen. Permeation-controlled vaginal ring devices, commonly fabricated from silicone elastomer or a thermoplastic polymer, are not useful for the delivery of biomacromolecules such as proteins, peptides and DNA, owing to permeability constraints. We have previously described the rod-insert vaginal ring (RiR) device, comprising an elastomeric body into which are inserted solid drug dosage units (Morrow et al., 2011). The inserts, in the form of directly compressed tablets or lyophilised gels that either dissolve or swell in contact with vaginal fluid, have been shown to successfully release biomacromolecules such as 2F5 (an anti-HIV antibody) and BSA. Given the solid nature of these insert dosage forms, antigen stability may be significantly increased compared with the corresponding gel formulation. We are particularly interested in the potential for using the RiR technology for vaginal administration of proteins for HIV prevention. Here, the protein could take the form of an HIV antigen (vaccine) or an HIV-specific monoclonal antibody (microbicide). The stability of proteins has been widely evaluated in PLGA matrices (Kang and Singh, 2003; Putney and Burke, 1998; Yang et al., 2008; Zhu et al., 2000) and in carbohydrate (sugar) based matrices (Andya et al., 2003; Chang et al., 1996b), but not in HPMC gels or their lyophilised forms, which are the subject of this work. Proteins are prone to various forms of chemical degradation, including hydrolysis, oxidation, disulphide exchange and β-elimination (Goolcharan et al., 2000). Physical instability is

Abbreviations: RiR, rod-insert vaginal ring(s); TAMC, total aerobic microbial count; TYMC, total yeast and mould count; NCTC, National collection of type cultures; NCTC, National collection of yeast cultures; MALDI-TOF, matrix-assisted laser desorption/ionisation-time of flight.

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manifested as changes in conformation, aggregation and surface adsorption (Andya et al., 2010; Brange, 2000; Chang et al., 1996a; Matthews et al., 2008) brought about by microbial activity, higher temperatures and moisture (increased molecular mobility). The aim of this study is to characterise the stability and degradation pathways for the model protein antigen BSA in HPMC gels and lyophilised HPMC gel dosage forms inserted into RiRs, in a bid to better understand the factors influencing protein stability in these novel systems.

2. Materials and methods

2.1. Materials

Condensation cure silicone elastomer MED-6382 was obtained from Nusil Technology, USA. Tetrapropoxysilane (TPOS), bovine serum albumin (>99%), stannous-2-ethylhexanaote, hydroxypropyl methyl cellulose (HPMC, 6 cps), sodium chloride, potassium hydroxide, D-glucose, urea, lactic acid, acetic acid, glycerol, hydrochloric acid, trifluoroacetic acid, acetonitrile, Tris hydrochloride, sodium hydroxide, calcium hydroxide, dithiothreitol (DTT), Tween 80 and dehydrated Sabouraud dextrose agar with chloramphenicol (50 mg/L) were obtained from Sigma–Aldrich, UK. Dehydrated media – peptone water, tryptic soya agar/broth and Sabouraud dextrose broth were obtained from Oxoid, UK. α,α-Trehalose dihydrate was obtained from Ferro Pfanstiel Laboratories Inc., USA. All materials required for gel electrophoresis were obtained from Invitrogen, UK. Water used for formulation and analysis was deionised using a Milli-Q® (Millipore, Ireland) system to a resistivity of 18.2 MΩ cm.

2.2. Preparation of rings

Human sized silicone elastomer vaginal rings, containing three cavities distributed equidistantly around the ring, were prepared by reaction injection moulding (T = 80 °C, ~4 min) of MED-6382 silicone elastomer mix using a temperature-controlled, laboratory-scale, ring-making machine fitted with specially designed vaginal ring injection moulds. The elastomer mix was prepared by mixing MED-6382 with 2.5% (w/w) TPOS using an overhead stirrer. Stannous-2-ethylhexanoate (0.5%, w/w) was added to the elastomer mix (30 g) and mixed using a DAC 150 FVZ-K Speedmixer™ (3000 rpm, 30 s). The final dimensions of the ring were: 5.8 cm outer diameter; 4.3 cm inner diameter; 0.76 cm cross-sectional diameter; and 3.0 mm cross-section diameter of cavity.

2.3. Preparation of rod-inserts

HPMC (26.66 g) and buffered BSA solution (100 mL, 10 mg/mL, 20 mM Tris, 150 mM sodium chloride, pH 7.5) were added sequentially to a SpeedMixer™ container and mixed twice for 300 s at 3500 rpm. The resulting gel mixture was hydrated overnight at 2–8 °C followed by further mixing (300 s at 3500 rpm, twice). A 40 g gel sample was placed into smaller sealed SpeedMixer™ containers for use in the stability study. The gel was injected into pre-cut sections of medical-grade PVC tubing (Nalgene® metric, 7.0 mm length, 2.0 mm internal diameter, 4 mm outer diameter) using a disposable plastic syringe fitted with a micropipette tip. The gel-filled tubes were placed on a stainless steel tray and freeze-dried (adVantage freeze drier, VirTis, USA). The freeze drying method involved ramping to −60 °C and holding for 2 h, followed by primary drying at −30 °C for 15 h and finally ramping to +20 °C over 60 min and holding for 10 h (Morrow et al., 2011). The solid rods were then removed from the tubes, individually weighed, and those within a 10% deviation of the mean weight were selected for ring insertion. A single rod was inserted into each of the three cavities in a ring body (Fig. 1). The rings were packaged in pre-labelled semi-permeable paper-plastic ring pouches and heat-sealed using a PacSeal® impulse heat sealer.

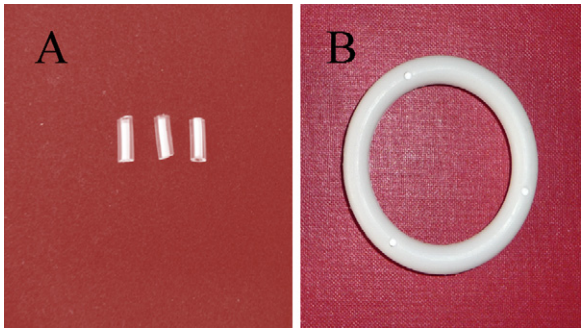

Fig. 1. (A) freeze dried inserts in PVC tubing; (B) freeze dried inserts placed into the silicone ring.

2.4. Stability study conditions and sampling

Gels and RiRs with three solid inserts were stored at 4 °C (2–8 °C) and 40 °C/75% RH. RiRs were stored within the semi-permeable paper-plastic ring pouches, while the gels were stored in screw capped plastic containers. At each stability timepoint (weeks 0, 3, 6 and 12), six rings and three 2 mL gel samples were removed from storage; the gel was sampled from different locations within the container. Samples were allowed to equilibrate to room temperature for 30 min before further testing.

2.5. Cyclic ring compression test

The force required to compress the rings by a distance of 5 mm was measured using the TA.XT2*plus* Texture Analyser (Stable Microsystems, UK) following the protocol described previously (Woolfson et al., 2006). Briefly, each ring (n = 4) was placed vertically in a holder fitted to the base plate of the Texture Analyser. A rectangular metal probe was used to compress the rings five times through a distance of 5 mm at a rate of 2 mm/s. Each ring was tested at three locations, corresponding to the position of the inserts. A trigger force of 0.04903 N was employed and the resulting force of compression was recorded in grams.

2.6. Thermogravimetric analysis

Rod portions (3–5 mg, n = 3) were heated in standard aluminium pans (TA instruments, USA) at 10 °C/min, from ambient temperature (approximately 20 °C) to 200 °C, on a TA Q500 instrument (TA instruments, USA). Universal Analysis software (TA instruments, USA) was used to calculate the weight change upon heating. Weight reduction up to 100 °C was used to calculate % free water.

2.7. BSA HPLC assay

Quantification of BSA assay was performed using reversed phase HPLC. Different wavelengths of absorbance in the UV region and fluorescence emission arise from different moieties within the protein molecule (Baudys and Kim, 2000). The most commonly used UV detection wavelength of 210 nm results mainly from the absorbance of the peptide bonds, with minor contribution from other amino acids, such as methionone and histidine. Due to the globular structure of BSA, only the surface of the protein is expected to interact with the stationary phase, and hence small changes, especially conformational, would not be detected. In order

to extend the stability characterisation, we used three additional wavelengths to probe the integrity of different parts of the protein; 280 nm which targets absorbance from aromatic residues, fluorescence at 275/307 nm, and 295/348 nm which targets fluorescent aromatic residues especially tryptophan and tyrosine (Baudys and Kim, 2000). It is well known that tryptophan fluorescence is extremely sensitive to conformational changes (Lakshmikanth and Krishnamoorthy, 1999) and serves as an extremely sensitive indicator of instability.

Gel sample (~20 mg) or one rod insert was dissolved in distilled water (1 mL and 5 mL, respectively, $n \geq 3$). Analysis was performed by HPLC using a Jupiter 5 μm C5 300 Å column (4.6 mm i.d. × 250 mm length; Phenomenex, UK) fitted to a Waters® Alliance® HPLC system with Waters® 2475 multi λ fluorescence and Waters® 2489 UV visible detectors. The analysis method was adapted from a previously described method (Umrethia et al., 2010). Elution was carried out using 0.1% (v/v) TFA in water (A) and 0.1% (v/v) TFA in acetonitrile (B) applying a gradient A/B from 95:5 to 35:65 in 20 min, total run time 30 min, UV detection at 210 nm and 280 nm, fluorescence detection using λ_{ex} 295 nm/λ_{em} 348 nm and λ_{ex} 275 nm/λ_{em} 307 nm; flow rate 1.0 mL/min; injection volume 100 μL. BSA retention time was ~16.6 min.

2.8. Native PAGE

BSA (control), gels or inserts were dissolved in distilled water and adjusted to a nominal BSA concentration of 50 μg/mL. Each sample was mixed 1:1 with 2× native Tris glycine loading buffer and 10 μL loaded into a Novex® (4–20%) Tris glycine electrophoresis gel. NativeMark™ marker was used as a molecular weight marker. The gel was run at 125 V until completion in a Tris glycine native electrophoresis buffer. The gel was stained with SimplyBlue™ stain according to the manufacturer's instructions. The gel was scanned using Biospectrum® multi-imaging system (UVP, UK) documentation system.

2.9. Circular dichroism

Circular dichroism has been used to estimate the secondary structure of proteins in formulations (Andya et al., 2003; Arakawa and Kita, 2000). At the end of the study period, gels and rods ($n = 3$) were dissolved in water to a nominal BSA concentration of 0.2 μM. The actual concentrations were measured using the previously described HPLC method. BSA in 50% ethanol was used as a positive denatured control. The spectra were recorded using a Jasco J 815 Spectropolarimeter (Jasco, UK) at a scanning speed of 200 nm min^{-1} and the data was obtained at a resolution of 0.1 nm.

2.10. Turbidimetry

At the end of the study, one rod insert or equivalent amount of gel was dissolved in water (5 mL, $n = 3$), and the turbidity was measured using a Varian Cary 50 (Varian Inc., USA) spectrophotometer at 560 nm. The samples were then centrifuged at 14,000 rpm (~21,000 × g Sigma 3K30 laboratory centrifuge, Sigma, UK) and the turbidity (absorbance) was measured in the supernatant. The absorbance was subtracted from the turbidity of each sample.

2.11. Characterisation of insoluble aggregates

Pellets of insoluble material from the centrifuged samples that showed a high turbidity were re-suspended in water (1 mL) and centrifuged at 14,000 rpm (~21,000 × g) for 30 min. This was repeated three times to ensure that there was no soluble BSA in the sample. This washed precipitate was used for analysing its solubility in 100 μL of 5 M urea, 6 M GuHCl, and 50 mM DTT with/without heating at 70 °C for 10 min. The solubility was judged subjectively.

The washed pellet was used to carry out a MALDI-TOF (mass) characterisation. The pellet was re-suspended in a solution containing sinapinic acid (in 60% TFA and 40% acetonitrile), before spotting on a plate and then analysed using MALDI Micromax® with time of flight analyser (Waters, USA). Washed pellets ($n = 3$) derived from additional inserts were re-suspended in 20 μL of LDS loading buffer with 50 mM DTT, heated at 70 °C for 20 min, and run on a NuPAGE Novex® bis-Tris (4–12%) gel, according to the manufacturer's instructions and using SDS-MOPS buffer and NuPAGE® antioxidant. The gel was stained using Simply Blue™ stain and documented using Biospectrum® multi-imaging (UVP, UK) documentation system.

2.12. Total aerobic microbial count (TAMC)

A gel or rod insert sample (10 mg, $n = 3$) was dissolved in sterile peptone water (2.5 mL) to a dilution of 1:250. Part of this solution was further diluted with sterile peptone water to a dilution of 1:1250. Plates were prepared in duplicate using 1 mL of the 1:250 mL dilution and 10 mL of molten tryptic soya agar (TSA). Similar plates were prepared using the 1:1250 dilution. Once set, all plates were inverted and incubated for 16 h at 37 °C after which time the number of colonies present was counted on a Stuart® SC6 colony counter. As a positive control, the 1:250 dilutions of all the samples were inoculated with suspensions of *Escherichia coli* (NCTC 10418), *Staphylococcus aureus* (NCTC 10788) or spores of *Bacillus subtilis* (NCTC 10073). *E. coli* and *S. aureus* were grown overnight in tryptic soya broth in an orbital incubator at 37 °C. Cultures were standardised using optical density measurements (550 nm) and standard growth curves, such that the final concentration in the sample would be ~100 CFU/mL. *B. subtilis* spores of a known concentration were diluted so that this final concentration would also be achieved. Plates were prepared as for the test samples in TSA and incubated overnight at 37 °C for 16 h.

2.13. Total yeast and mould count (TYMC)

TYMC was performed in a similar manner to TAMC, using Sabouraud dextrose agar with added chloramphenicol (SBDc) for plating the samples instead of TSA and using an incubation temperature of 25 °C for 5 days. Presence of high dextrose concentrations, a broad spectrum antibiotic (chloramphenicol) and lower incubation temperatures provided a selective condition for the growth of fungi over bacteria. As a positive control, the 1:250 dilutions of the samples were inoculated with viable *Candida albicans* (NCYC 610), grown overnight in Sabouraud dextrose broth at 25 °C and adjusted to give the same final concentration of 100 CFU/mL. Plates were incubated for 24 h at 25 °C before counting.

2.14. In vitro release studies

Three RiR systems, each containing ~500 μg BSA, were placed into 250 mL circular Schott-Duran® bottles and immersed in simulated vaginal fluid (SVF, 20 mL) containing no mucin or BSA components (Owen and Katz, 1999). The bottles were placed in a controlled temperature rotary shaker at 60 rpm at 37 °C. 1 mL samples of the SVF were taken at 0, 15, 30, 60, 120, 240 and 480 min. The aliquot was replaced using 1 mL of pre-warmed SVF at 37 °C. The samples were immediately transferred to a refrigerator at 2–8 °C and analysed by RP-HPLC within 24 h.

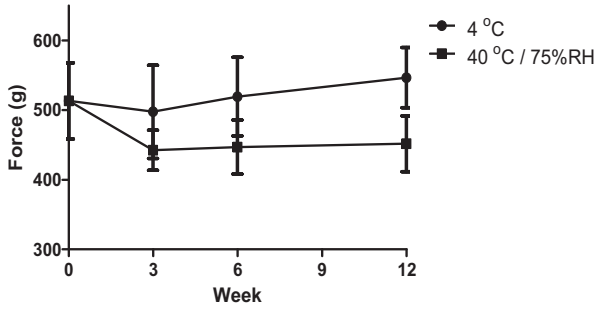

Fig. 2. Mean force ± SD (n = 3 for week 0 and n = 4 for all other time points) required to compress RiRs by 5 mm as a function of stability storage time.

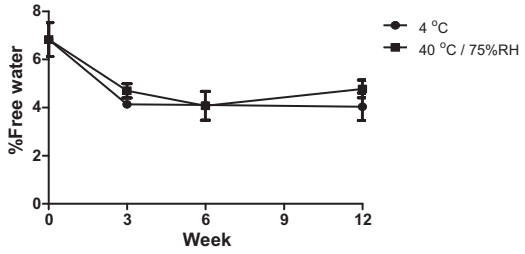

Fig. 3. Mean percentage free water ± SD (n = 3) measured in the rod inserts measured by using TGA over 12 weeks stability storage.

2.15. Preliminary evaluation of protein protectants

As a preliminary study to prevent protein degradation, inserts where either 10% of HPMC was replaced with α,α-trehalose dihydrate or 2.5% of HPMC was replaced with Tween 80 were prepared. These inserts were prepared as described previously (Section 2.3). The rods were exposed without the rings (to accelerate degradation) but within paper-plastic pouches to either 4 °C or 40 °C/75% RH for three weeks and analysed for BSA content by HPLC (UV detection at 210 nm). Inserts containing no protectants (standard formulation) served as control.

2.16. Statistical analysis

One-way ANOVA (with Tukey post hoc test) or two-way ANOVA (with Bonferroni's post hoc test) was used for analysing the results. The tests were carried out using the Graphpad® Prism software. Confidence limits of 95% were used to determine statistical significance.

3. Results

3.1. Appearance

Gels and RiR stored at 4 °C did not show any significant changes in appearance, while the gel stored at 40 °C/75% RH showed a strong and gradual increase in brownish discolouration. Similar discolouration, but to a very mild degree, was evident on the inserts in the RiR stored at the higher temperature. None of the rod insert samples displayed cracks or fissures.

3.2. Cyclic ring compression test

The effect of storage time and condition on the RiR compression force is presented in Fig. 2. No significant differences ($p < 0.05$) in the compression forces were observed with storage time. A small difference is observed between the storage conditions at week 12; the trend suggests that this difference might become increasingly significant at even longer storage times.

3.3. Thermogravimetric analysis

The percentage free water in the sample in the inserts was estimated using TGA. A significant reduction ($p \leq 0.001$) in the free water content was measured during the course of storage (Fig. 3).

3.4. BSA HPLC assay

The HPLC method was validated for use at all four wavelengths in the range of analysis. UV detection at 210 nm was found to be the least sensitive while the fluorescence detection at 295/348 nm was found to be the most sensitive.

Figs. 4 and 5 show degradation profiles for the formulations stored at different conditions using the various detection wavelengths. Fluorescence based detections show higher levels of degradation due to their ability to detect conformational changes as opposed to the solely structural changes picked up by UV detection.

Based on the 210 nm detection wavelength, the RiR stored at 4 °C retained 88% of the initial BSA content at the end of 12 weeks and the degradation was not statistically significant ($p < 0.05$) compared to a statistically significant reduction in the assay value of all other formulations (to less than 37% of the initial content at the end of 12 weeks). Similar results were obtained at 280 nm detection.

Based on the 295/348 nm degradation profile, the gel formulations were significantly ($p < 0.05$) degraded to less than 19% initial content at the end of 12 weeks. The RiR formulation stored at 40/75% RH was also degraded significantly ($p < 0.05$) to less than 11% initial content at the end of 12 weeks. In contrast, the RiR formulation at 4 °C was relatively protected from degradation ($p < 0.05$) compared to other formulations, with a mean assay content of 68% after 12 weeks. Similar results were obtained at fluorescence detection of 275/307 nm. Analysis of RiRs at the end of 12 weeks at 4 °C showed very high variability in assay content, further confirmed with additional samples (n = 3), and probably arising due to the presence of localised areas of degradation within the rod inserts.

Protection against BSA degradation for the RiR formulation stored at 4 °C was clearly evident at all four wavelengths. However, the fluorescence method showed a higher level of degradation compared to the UV method, probably owing to its ability to detect more subtle conformational changes.

Figs. 4 and 5 show the first order kinetic order plots for BSA degradation, which were capable of modelling at least 72% ($R^2 \geq 0.72$) of the degradation when studied at Flr 295/348 nm and at least 89% ($R^2 \geq 0.89$) when studied at Flr 275/307 nm. The zero order model was less successful (lower R^2 value than for first order) at modelling the degradation kinetics. The first order degradation rates for all the formulations at different wavelengths are presented in Table 1. The rates of degradation were greatest for the formulations stored at 40 °C/75% RH. Also, the RiR formulation stored at 4 °C has a significantly lower rate of degradation than either the gels or RiR stored at 40 °C/75% RH at any wavelength.

3.5. Native PAGE

Native PAGE gel electrophoresis of the samples showed clear BSA bands upon preparation (week 0), indicating no BSA degradation during preparation (Fig. 6). Loss of native protein in formulations stored at 40 °C/75% RH was indicated by almost complete loss of the 66 kDa band by the end of six weeks. These results correlate with degradation rates determined by HPLC analysis. The rest of the PAGE gel was also scanned for the presence of soluble

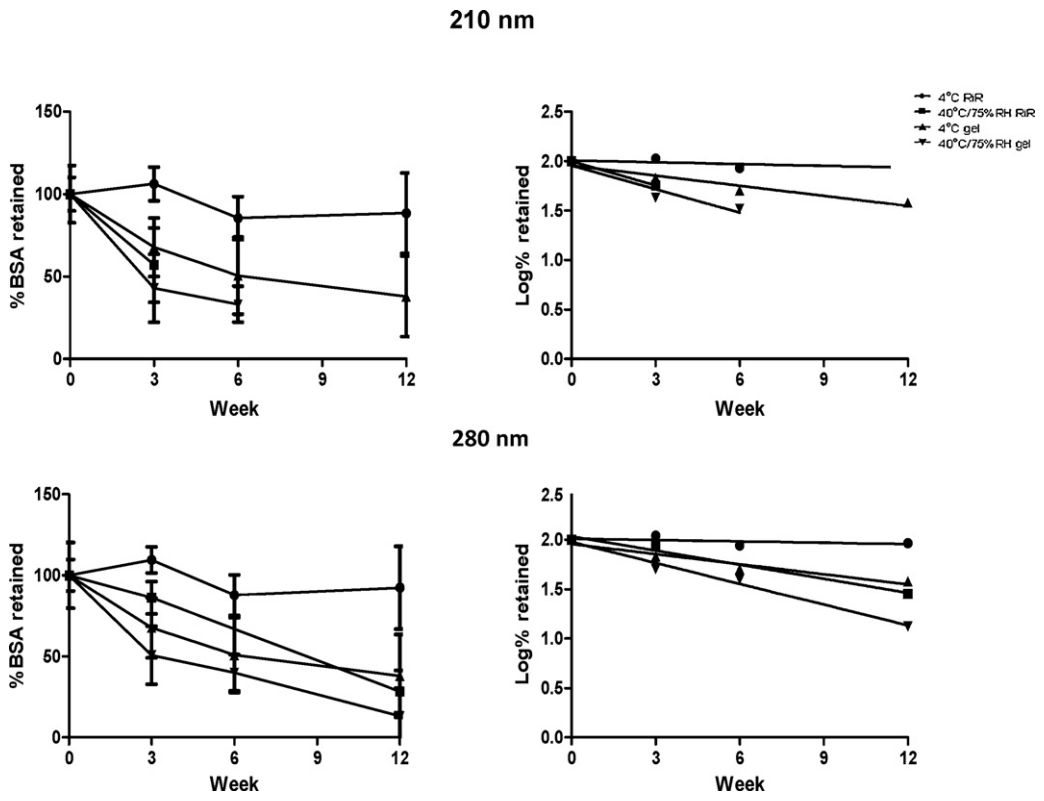

Fig. 4. Degradation profiles of formulations (left) and first order degradation plots (right) from UV detection HPLC at 210 nm and 280 nm ($n = 3$). Points below the limit of quantification of HPLC method were not considered. Error bars show SD.

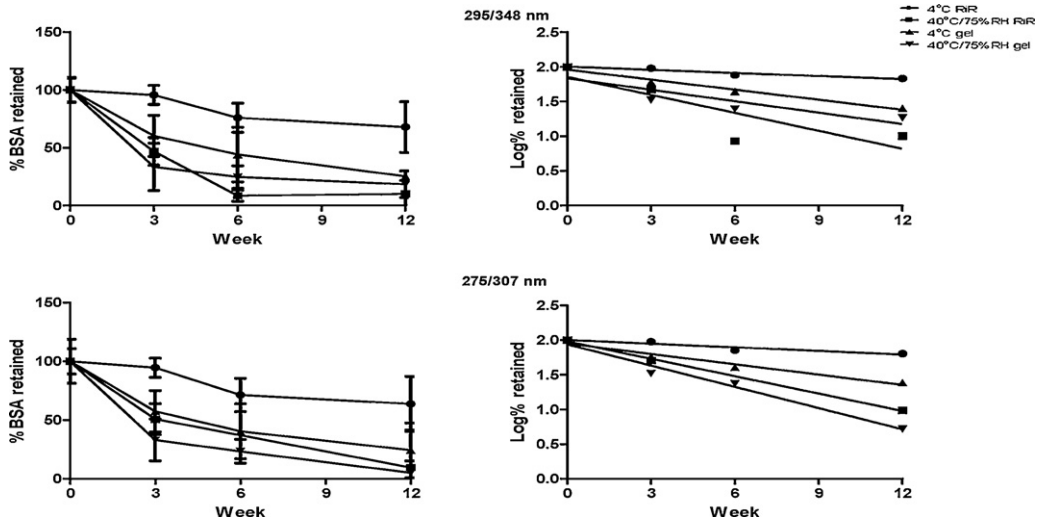

Fig. 5. Degradation profiles of formulations (left) and first order degradation plots (right) from fluorescence detection HPLC at 295/348 nm and 275/307 nm ($n = 3$). Points below the limit of quantification of HPLC method were not considered. Error bars show SD.

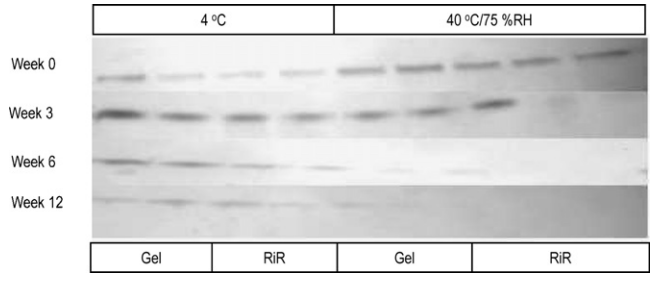

Fig. 6. Native PAGE bands of BSA at about 66 kDa, stained using SimplyBlue™ stain.

aggregates, which were either not detected or were detected at levels no greater than present in a pure sample of BSA.

3.6. Circular dichroism

Although circular dichroism (CD) was performed on all the samples (Fig. 7), the measured concentrations of BSA in the RiRs stored at 40 °C/75% RH were below the limit of detection. BSA diluted in 50% ethanol (ETOH) was used as a known sample of denatured protein (Liu et al., 2010). The CD spectra showed minor perturbations in

Table 1
The rate of BSA degradation studied at different wavelengths and corresponding correlation coefficients for 1st order degradation.

Formulation/condition	1st order R^2	Mean degradation rate (week^{-1}) (±SD)
Flr 295/348 nm		
4 °C RiR	0.9148	−0.01496 (0.003227)
40 °C/75% RH RiR	0.7236	−0.08642 (0.037690)
4 °C gel	0.9789	−0.04825 (0.005013)
40 °C/75% gel	0.7729	−0.05492 (0.021050)
Flr 275/307 nm		
4 °C RiR	0.8989	−0.01743 (0.004134)
40 °C/75% RH RiR	0.9982	−0.08360 (0.003576)
4 °C gel	0.9642	−0.04955 (0.006756)
40 °C/75% gel	0.9780	−0.10200 (0.010820)
210 nm		
4 °C RiR	0.4739	−0.00598 (0.004457)
40 °C/75% RH RiR	Not determined since some points are below LOQ	
4 °C gel	0.9408	−0.03407 (0.006042)
40 °C/75% gel	0.9135	−0.07982 (0.024570)
280 nm		
4 °C RiR	0.3146	−0.00460 (0.004802)
40 °C/75% RH RiR	0.9821	−0.04739 (0.003390)
4 °C gel	0.9409	−0.03390 (0.006008)
40 °C/75% gel	0.9836	−0.07038 (0.064290)

secondary structure for samples stored at 4 °C, while the gel stored at 40 °C/75% RH showed a very heavy perturbation.

3.7. Turbidimetric detection of insoluble aggregates

The baseline corrected turbidites for samples after 12 weeks are presented in Fig. 8. Gel samples stored at 4 °C and RiRs at 40 °C/75% RH showed significant amounts of turbidity compared to other formulations, indicating the formation of insoluble aggregates. RiRs stored at 4 °C did not exhibit any significant turbidity.

3.8. TAMC and TYMC

It was important to establish that turbidity in the samples was not due to microbial growth. Samples were analysed for total aerobic microbial count (TAMC) as well as total yeast and mould count (TYMC). All the samples were seeded with ~100 CFU of viable Gram positive bacteria (*S. aureus*), Gram negative bacteria (*E. coli*), yeast (*C. albicans*) and viable spores of *B. subtilis.* All these control microorganisms grew ≥75 CFU indicating no inherent growth inhibiting properties in any of the formulations.

The only formulation to show any significant levels of microbial load was the gel stored at 40 °C/75% RH (Fig. 9); most of

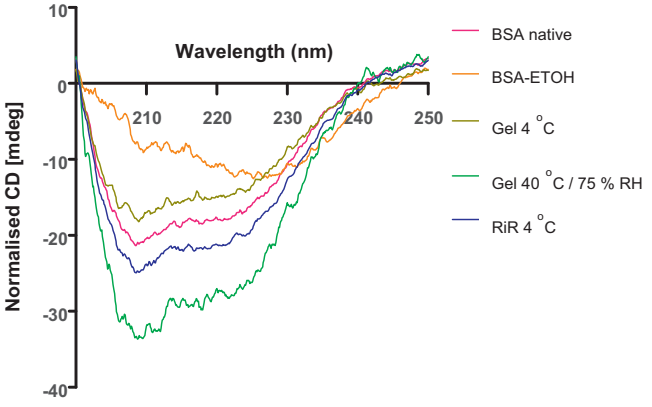

Fig. 7. Representative baseline corrected and normalised CD spectra of BSA of various samples in the far UV range.

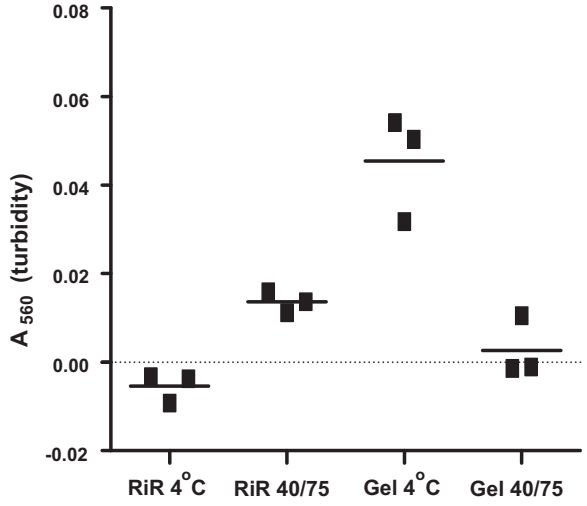

Fig. 8. Baseline corrected turbidity of formulations ($n = 3$) at the end of 12 weeks. The turbidity of the solutions of RiRs stored at 40 °C/75% RH and gel stored at 4 °C were also visually found to increase over the weeks of stability study.

this growth was mould. The other formulations were re-plated at a lower dilution to detect microbial growth, but no formulation exhibited >250 CFU/mg microbial contamination. This confirms that the observed turbidity is due to insoluble aggregates rather than microbial growth, and establishes the microbial stability of the freeze-dried dosage forms.

3.9. Characterisation of insoluble aggregates

The washed pellets of the insoluble aggregates were slightly soluble in 5 M urea and 6 M GuHCl and partially soluble in a 50 mM DTT solution (improved by heating at 70 °C, 10 min). Partial solubility in urea and GuHCl indicated extensive non-covalent chemical

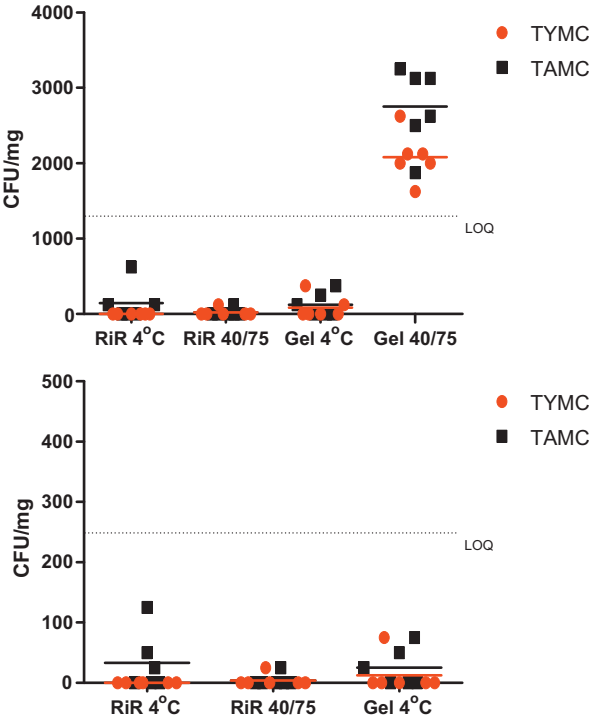

Fig. 9. Microbial load on formulations ($n = 3$, in duplicates), at a dilution of 1:1250 (top) and at a dilution of 1:250 (LOQ: limit of quantification).

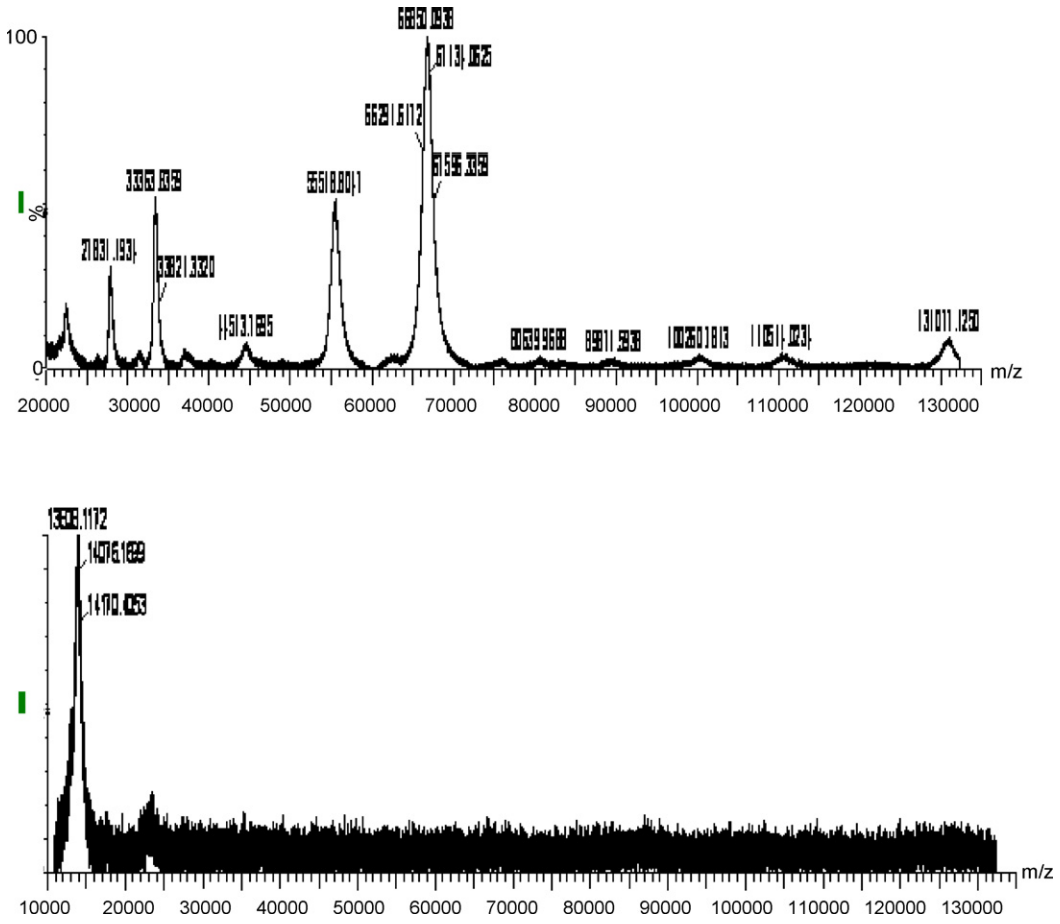

Fig. 10. MALDI-TOF spectrum of BSA from a freshly made formulation (top), with the main BSA peak around 66 kDa while the pellet of insoluble material shows no BSA peak (bottom).

and structural changes and partial solubility in DTT indicated possibility of S-crosslinked BSA molecules. Thus, the aggregates were a mixture of non-covalent and covalently (S-linked) linked BSA molecules.

The possibility of BSA being physically entrapped in an insoluble pellet of degraded HPMC was also evaluated. The pellet was subjected to MALDI-TOF analysis (Fig. 10). Matrix assisted laser desorption ionisation could not detect any BSA in the pellet indicating that any insoluble BSA was chemically reacted and could not be vaporised into ions. SDS PAGE on pellets dissolved on heating in loading buffer containing 50 mM DTT yielded BSA (approximately 66 kDa band) (Fig. 11), supporting the hypothesis of a S-crosslinked insoluble degradation product as a major part of the insoluble aggregate.

3.10. In vitro release in SVF

The influence of storage time on the *in vitro* release of BSA from RiRs into SVF for rings stored at 4 °C is presented in Fig. 12. No

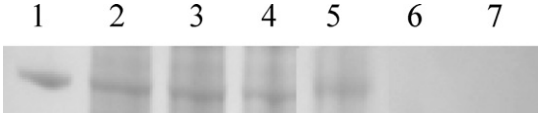

Fig. 11. BSA bands (1: standard, 2/3: from pellets of gel stored at 4 °C, 4/5: from pellets of RiR stored at 40 °C/75% RH, 6: supernatant from gel pellet, 7: supernatant from RiR pellet) showing that BSA could be recovered after reducing the pellet with DTT. Absence of BSA in supernatant validates washing steps. The bands are slightly below the standard indicating changes in the primary structure.

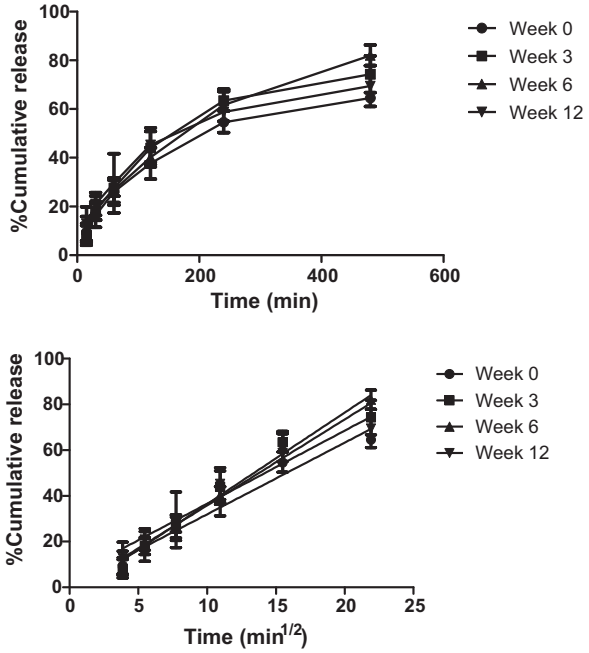

Fig. 12. Mean percentage cumulative release of BSA (±SD) from RiR stored at 4 °C plotted against time (top) and against square root time (bottom). The release rate was essentially unchanged over the stability period of 12 weeks.

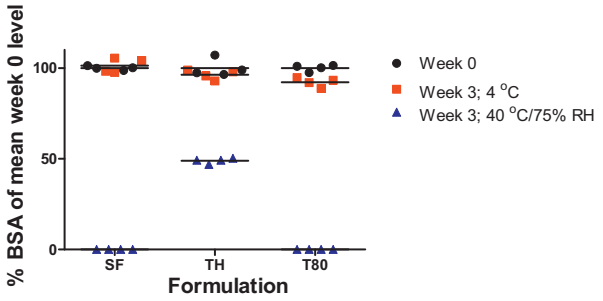

Fig. 13. % BSA in the formulation compared to mean week 0 level when stored at either 4 °C or 40 °C/75% RH ($n = 4$). SF – standard formulation (no protectant); TH – formulation with 10% HPMC replaced with trehalose; T80 – formulation with 2.5% HPMC replaced with Tween 80. Addition of trehalose protects significantly against BSA degradation at 40 °C/75% RH. The levels of SF and T80 at week 3, 40 °C/75% RH are arbitrarily shown at zero, since the levels were either below quantification limits or the HPLC peaks were split/broadened due to degradation, preventing a meaningful integration.

statistically significant difference was found in cumulative release except at 480 min, week 6. Release was also performed on the rings stored at 40 °C/75% RH, but due to extensive degradation, amounts in the release study were below the limit of quantification.

Release data were fitted to zero order (% release vs. time), first order (log% retained vs. time) and Higuchi (% release vs. root time). The data were best modelled by Higuchi kinetics with $R^2 \geq 0.9597$ for all curves. Zero order and first order kinetics were less successful at modelling the data (R^2 values > 0.97). Plots of % release vs. root time (Fig. 12), showed that the release kinetics are essentially unchanged, indicating that there has been no significant change in the matrix over the period of the stability study.

3.11. Preliminary evaluation of protein protectants

Studies up to this point clearly demonstrated that BSA was degrading in the formulation by means of aggregation. In order to evaluate if Tween 80 or trehalose could provide any protection to the protein, we examined formulations containing either 10% HPMC replaced with trehalose or 2.5% HPMC replaced with Tween 80 for stability by exposing them to 4 °C or 40 °C/75% RH for a period of three weeks and analysing the BSA content with HPLC (UV detection at 210 nm). It was found that the formulation containing trehalose provided a significant protection against BSA degradation compared to the standard formulation or the formulation containing Tween 80 when stored under accelerated conditions (40 °C/75% RH; Fig. 13).

4. Discussion

RiR formulations stored at 4 °C showed no change in physical appearance or mechanical strength. By contrast, formulations stored at 40 °C/75% RH showed a yellowish discolouration, but no significant change in mechanical strength. The amount of structurally intact BSA indicated by UV detection HPLC showed that RiRs stored at 4 °C were relatively protected from structural degradation, while all other formulations were extensively degraded at the end of 12 weeks. Similarly, probing with fluorescence showed that RiRs stored at 4 °C, despite some loss of conformational stability, were significantly protected against such changes compared with other formulations. These differences are confirmed in the native gel electrophoresis and circular dichroism studies. Clearly, high molecular mobility (gel formulation and higher temperature) is a driver for the protein degradation process. In the absence of microbial degradation, intermolecular S-bond linkages seems to be an important pathway for degradation leading to be formation of insoluble aggregates. It is important to comment on the denaturation and loss of protein within the gel formulations. Standard solutions of BSA supplied with the BCA protein kits are routinely stored at room temperature for prolonged time periods without degradation of BSA. Similarly, gp140 buffer solutions also demonstrate good stability (Donnelly et al., 2011). However, BSA and gp140 showed rapid degradation in HPMC and RSV gels, respectively (Donnelly et al., 2011). It is likely that this degradation effect observed in gel formulations is due to entangling of the protein molecules with the polymeric molecules in the gel network coupled with the relatively high molecular mobility environment afforded by the liquid component of the gel. The process of entanglement within the gels could lead physically to change in protein conformation seeding the further aggregation process. Excipients such as Tween 80 and hydroxypropyl β-cyclodextrins have been shown to reduce aggregation of BSA (Arakawa and Kita, 2000; Kang and Singh, 2003) even at elevated temperatures. Such excipients added to the RiR might help to stabilise the formulation even under accelerated storage conditions. This formed the rationale for the preliminary evaluation of Tween 80 as a protectant in the formulation.

During the first three weeks of storage there was a significant reduction in free moisture content. Removal of free moisture may change conformational properties of the protein (Brange, 2000), which could explain the conformational effects observed for RiR stored at 4 °C. It has now been established that increased molecular mobility (storage > T_g) is not the primary cause of degradation, since it can occur even when a formulation is stored below T_g if damage is seeded during the freeze drying step. It is possible that addition of sugars, which can effectively replace water (Andya et al., 2003; Brange, 2000) during the freeze drying process by forming hydrogen bonds with the protein, may help to prevent this change in conformation (Andya et al., 2003). Thus, addition of sugars which can remain amorphous such as sucrose and trehalose to the RiR formulations might help to prevent this change in conformation preventing degradation of the protein. This formed the rationale for the preliminary evaluation of trehalose as a protectant. Finally, the release properties of the RiR stored at 4 °C for 12 weeks remain essentially unchanged with the release best modelled by Higuchi kinetics.

As a preliminary study to improve the formulation, we prepared inserts where either 10% of HPMC was replaced with trehalose or 2.5% of HPMC was replaced with Tween 80. It was found that the formulation containing trehalose provided a significant protection against BSA degradation compared to the standard formulation or the formulation containing Tween 80 when stored under accelerated conditions. It seems likely from this study that inclusion of a lyoprotectant like trehalose is capable of decelerating degradation, possibly by preventing seeding of denaturation changes during freeze-drying, thus providing a framework for further development of RiRs.

5. Conclusions

This study investigated the stability of BSA-loaded HPMC gels and BSA-loaded lyophilised gels inserted into silicone rings from four important perspectives: (i) mechanical strength, (ii) protein stability, (iii) microbial stability, and (iv) release properties. When stored at 4 °C (but not when stored at 40 °C/75% RH), the RiR offers relative protection against structural and conformational changes to the protein, retains matrix integrity, and maintains release characteristics. The results clearly demonstrate that lypophilised gels can protect against degradation at lower temperatures and that additional stabilisers such as sugars may be indicated to improve long-term stability.

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