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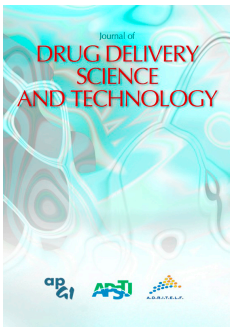

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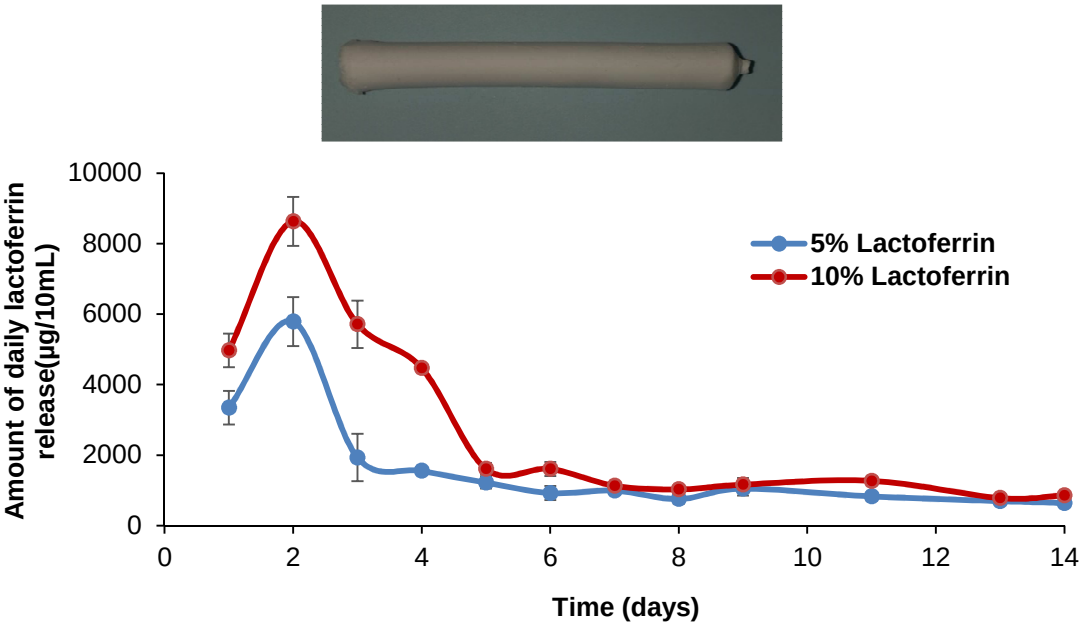

Evaluation of polycaprolactone matrices for sustained intravaginal delivery of a natural macromolecular microbicide, lactoferrin

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1. Abstract

Polycaprolactone (PCL) matrices incorporating lactoferrin as a natural macromolecular microbicide, were prepared by rapidly cooling a suspension of lactoferrin particulates in PCL solution to induce crystallisation and hardening of the polymer. Thermal analysis revealed a 7% decrease in crystallinity of the PCL phase for 10% lactoferrin-loaded matrices compared with lactoferrin-free matrices and a 41% decrease in hardness of lactoferrin -loaded matrices, indicating a major influence of lactoferrin through inhibition of PCL crystal nucleation and growth. Exposure of the matrices to simulated vaginal fluid (SVF) at 37°C resulted in rapid release of 13-14% of the lactoferrin content on day 1 and sustained delivery of the glycoprotein with high efficiency (90-95% of the content) over 14 days. SDS-PAGE analysis confirmed molecular weight preservation of the lactoferrin released from PCL matrices into SVF, indicating that it was not degraded during formulation and release. These findings recommend further investigations of PCL matrices as vaginal delivery systems for controlled release of macromolecular microbicides in the treatment and prevention of sexually transmitted infections.

2. Introduction

Vaginal drug delivery systems exist in a variety of forms including semi solids (gels and creams), tablets, films and ring-shaped inserts. Intravaginal rings (IVRs) were initially developed for contraceptive purposes and hormone replacement therapy and offer advantages over semi-solid formulations, including ease of insertion, avoidance of ‘messiness’ and long term, sustained drug delivery (1). More recently, IVRs have become the subject of intensive research for sustained delivery of small molecule microbicides that are effective against sexually transmitted infections (STIs) (2), including the non-nucleoside reverse transcriptase inhibitor (NNRTI) dapivirine (3) that inhibits HIV/AIDS transmission. Recent phase 3 clinical trials evaluating the safety and efficacy of an extended use vaginal ring containing dapivirine revealed that the incidence of HIV-1 infection was 31% lower (4) and 27% lower (5) in the dapivirine group than in the placebo group.

IVRs offer advantages of targeted drug delivery at the point of entry of pathogens implicated in STIs and direct contact of the drug with infected cells. However, many of the newer biotech-derived microbicides under development for the treatment and prevention of STIs are macromolecular in nature and require new strategies to enhance the efficiency of vaginal delivery (6-8). Several monoclonal antibodies (mAbs) including enfuvirtide, have been reported to protect macaques from challenge with the simian/human immunodeficiency virus (SHIV) via IV, oral, rectal and vaginal routes. The mAbs were also found to be active against a wide variety of HIV isolates, thus offering major potential for controlling transmission (8). The HIV-1 envelope glycoprotein, CN54gp140, was formulated as a liposome gel to produce a vaccine against HIV-1 infection (9). Although an encapsulation efficiency of 35% was reported and the glycoprotein maintained antigenic activity above 80%, insertion and retention of the gel, coupled with the likelihood of inefficient exposure of the antigen to the host to induce an immune response remain a problem.

Silicone elastomer and polyethylene vinyl acetate (pEVA) are conventionally employed for manufacturing IVRs for delivery of small molecule therapeutics such as contraceptive steroids (10), but these polymers are almost impermeable to macromolecular drugs. In addition, curing of silicone elastomer is carried out at temperatures above 80^{o}C and pEVA is typically moulded at 140^{o}C, which raises concerns over thermal degradation of peptide/protein-based therapeutics. Consequently, IVRs which are designed for delivery of macromolecular entities commonly include the active in an integral pod or insert, which complicates manufacture of the device (11-13). Bovine serum albumin (BSA) (employed as a protein antigen) was incorporated in silicone elastomer IVRs in the form of an insert. Around 80% of the protein was released over a 12-week period *in vitro* and exhibited good protein stability (11). Similarly, a 10-pod insert IVR sustained release of antibody IgG *in vitro* at a level of 0.5-30 mg/day for 14 days with retained activity according to ELISA testing (12). A ‘rod-insert’ type vaginal ring for delivery of the anti-retroviral peptides T-1249 and JNJ54310516-AFP, has recently attracted interest (13). The broadly neutralising antibody VRCO1-N was included in pod-type IVRs for evaluation of pharmacokinetics and safety in macaques (6). Antibody release was sustained for up to 21 days resulting in levels in vaginal fluid in the range 10^{2}-10^{3} µg g^{-1} with no adverse safety indications. Although, designs such as these can provide alternative solutions to the delivery of challenging molecules their complex

design increases the cost of manufacture of the device.

Microporous polycaprolactone (PCL) matrices loaded with a bioactive compound can be produced without the use of high temperatures. Rapid cooling of bioactive particle suspensions in PCL solution has been investigated for intravaginal delivery of small molecule antiviral, antibacterial, antifungal and antiprotozoal drugs (14-17). PCL matrices have also been shown to be effective for sustaining release of gelatin *in vitro* as a model macromolecular biopharmaceutical (18). PCL matrices containing gelatin particles of size range 90–125 and 125–250 mm, respectively, displayed gradual and highly efficient release of around 90% of the protein phase in PBS over 21 days. Micro-CT analysis revealed formation of a highly macroporous structure following protein extraction (18). Enzymes have also been successfully loaded and released from PCL matrices, with activity shown to be retained at 100% for at least 11 days for collagenase (19). Lysozyme activity was 75–80% at 11 days, but catalase reduced to 10–20% at 5 days. The study indicated the potential of microporous PCL matrices for delivering bioactive protein and peptide macromolecules.

Lactoferrin is an 80 kDa iron-binding glycoprotein of the transferrin family that is expressed in most biological fluids including mucosal secretions (vaginal, nasal, bronchial, intestinal), milk and colostrum (20, 21). Lactoferrin is a major component of the mammalian innate immune system and displays anti-oxidant, anti-inflammatory and anti-cancer activity. In particular, it has demonstrated microbicidal activity against a wide range of microorganisms implicated in sexually transmitted infection (22-25). The glycoprotein is bacteriostatic against iron-dependent bacteria due to its iron-binding property, which depletes the concentration of iron and consequently bacterial growth at sites of infection (22, 23). Antibacterial activity against gram-negative bacteria stems from electrostatic binding of lactoferrin's positive N-terminus with anionic moieties on lipopolysaccharide (LPS) resulting in deleterious changes in cell membrane permeability (24,25). Anti-fungal activity against *Candida albicans* is also considered to occur by direct interaction, resulting in altered permeability of the cell wall (22). Lactoferrin exerts antiviral activity *in vitro* by inhibiting HIV replication within infected cells (23) and by blocking entry of herpes simplex virus into host cells by binding to the virus particle or the glycosaminoglycan viral receptors on host cells (24). Sessa *et al* recently reported that lactoferrin could both interfere with *Chlamydia trachomatis* entry into epithelial cells *in vitro* and exert anti-inflammatory activity (25). Of particular note was the

authors' demonstration of a protective effect of lactoferrin against *C. trachomatis* infection *in vivo*, as evidenced by six out of seven pregnant women, who were asymptomatically infected by *C. trachomatis*, being negative for *C. trachomatis* after 30 days intravaginal administration of lactoferrin.

The mounting evidence in support of lactoferrin in a preventative and therapeutic role against a wide range of microrganisms stimulated the current evaluation of PCL matrices for sustained vaginal delivery of the glycoprotein as a natural macromolecular microbicide in the treatment of STIs.

3. Materials and methods

3.1 Materials

Polycaprolactone (PCL, MW 115,000 Da, Capa 650) was obtained from Solvay Interox (Warrington, UK). Bovine lactoferrin, native form (26) (from MG Nutritionals) was a gift from Dr. Nidhi Bansal (Lecturer, School of Agriculture and Food Science, University of Queensland). Sodium chloride, potassium hydroxide, calcium chloride, bovine serum albumin, glucose, glycerol, urea, lactic acid and acetic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Solvents (acetone, acetonitrile) were of analytical grade and purchased from Sigma-Aldrich, Australia. 2X Laemmli sample buffer, 12% SDS precast, 15 well gels were purchased from Bio-Rad (Gladesville, NSW, Australia). PageRuler plus pre-stained protein ladder was purchased from ThermoFisher Scientific (Scoresby, Vic, Australia).

3.2 Production of lactoferrin-loaded PCL matrices

Cylindrical PCL matrices loaded with lactoferrin were prepared according to a previously reported method (14). PCL pellets (1.5g) were dissolved in 10 mL acetone to produce a 15% w/v solution, by heating at 45°C for 45 min. Lactoferrin powder was dispersed in the PCL solution to produce protein loadings of 5% and 10% w/w with respect to the PCL content. The resulting suspension was poured into 3 mL polypropylene syringe barrels and cooled rapidly by immersion in methanol at -80^{o}C to cause crystallisation and hardening of PCL. After retention at -80^{o}C for 24 h the resulting lactoferrin-loaded PCL matrices were removed from the moulds and dried for 24 h under ambient conditions to evaporate solvent. The final matrices were in the form of cylinders of diameter 6.3 ± 0.2 mm and length 45.0 ± 3.0 mm.

3.3 Matrix morphology

The morphology of lactoferrin-loaded PCL samples was examined using a JEOL-6610LV scanning electron microscope (SEM) (Jeol Ltd., Tokyo, Japan). Samples were taken from the surface and interior of PCL matrices and attached to aluminium stubs prior to coating using an Eiko sputter coater automatic mounting press. Coated samples were imaged using an applied voltage of 10 kV.

3.4 Thermal analysis

Differential scanning calorimetry (DSC) (1 STARe DSC System, Mettler-Toledo Ltd., Victoria, Australia) was used to measure the glass transition temperature (Tg) and crystallinity of the PCL matrices. Samples of lactoferrin-free matrices and samples loaded with lactoferrin, respectively, were weighed and sealed in aluminium pans and heated over the temperature range 30-330°C at a rate of 10°C/min. The Tg and crystallinity of the PCL phase were obtained using the DSC software facility. The percentage crystallinity of the PCL phase was estimated using a heat of fusion of 139.5 J/g for the fully crystalline polymer (27).

3.5 Hardness testing of matrices

Values of hardness for lactoferrin-free matrices and lactoferrin-loaded matrices were obtained using a CT3 Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA). Cylindrical PCL mouldings were mounted horizontally and compressed locally at a speed of 0.1 mm/min to a depth of 2.0 mm using a 2.0 mm diameter, flat-ended, cylindrical probe (TA39). The hardness (or indentation resistance) of each sample was calculated based on the applied force measured at a depth of penetration of 2 mm. A pEVA IVR (Nuvaring®, Schering-Plough Pty limited, New South Wales, Australia) was subjected to the same test procedure for comparison.

3.6 *In vitro* release of lactoferrin from PCL matrices

The release behaviour of lactoferrin-loaded PCL matrices was investigated by immersing samples in simulated vaginal fluid (SVF) as the release medium. The ends of cylindrical mouldings were sealed using 5% w/v PCL solution to confine release of lactoferrin to the cylinder surface and thus mimic the release behaviour of an IVR. Samples were immersed in 10 mL of SVF and retained in an incubator at 37°C for 14 days. The volume of SVF was based on the

maximum volume of vaginal fluid produced by healthy, non-pregnant, pre-menopausal women in one day (28). SVF was produced using the method reported by Owen and Katz (28) and contained 3.51g NaCl, 1.40g KOH, 0.222g $Ca(OH)_2$, 0.018g bovine serum albumin, 2.00g lactic acid, 1.00g acetic acid, 0.16g glycerol, 0.4g urea and 5.0g glucose in 1L of distilled water. The pH was adjusted to 4.2 using 10% HCl to simulate the pH condition of vaginal fluid. The complete release medium was collected daily and replaced with 10 mL SVF. Release samples were stored at -18°C prior to testing. The concentration of lactoferrin in the collected medium was analyzed using a Shimadzu HPLC system equipped with binary pump and SPD-M20A photodiode array detector. Separation was carried out using an Agilent Extend C-18 column (2.1 x 150 mm, 5 µm). Two mobile phases were used, water (eluent A) and 95% acetonitrile (eluent B), both with 0.1% (v/v) trifluoroacetic acid added.

Time (min)	%Solvent A	%Solvent B
0-15	85-30	15-70
15-20	30	70
20-30	30-85	70-15

Gradient elution was used, with eluent B starting at 15%, increasing to 70% at 15 minutes and holding for 5 minutes. The sample injection volume was 50 µl and quantification of lactoferrin was performed at 276 nm by comparison with a standard curve (concentration range 10-60 µg/ml (R^2 = 0.9988)). Each experiment was performed in triplicate.

3.7 SDS-PAGE analysis of lactoferrin released from PCL matrices

PCL matrices loaded with 5% and 10% w/w lactoferrin and incubated in SVF (10mL) at 37°C for 14 days. The incubation media were collected and analysed using SDS-PAGE to determine the structural stability (based on molecular weight) of lactoferrin released from the matrices over this time scale. Aliquots (5 µL) of the release media containing lactoferrin were mixed with 5 µL of sample buffer (4.5 µL of 2x Laemmli sample buffer and 0.5 µL of beta mercaptoethanol). Aliquots of the mixture (5 µL) were added to separate wells in a 12% SDS-PAGE gel and electrophoresis was performed according to Laemmli (29).

3.8 Statistics

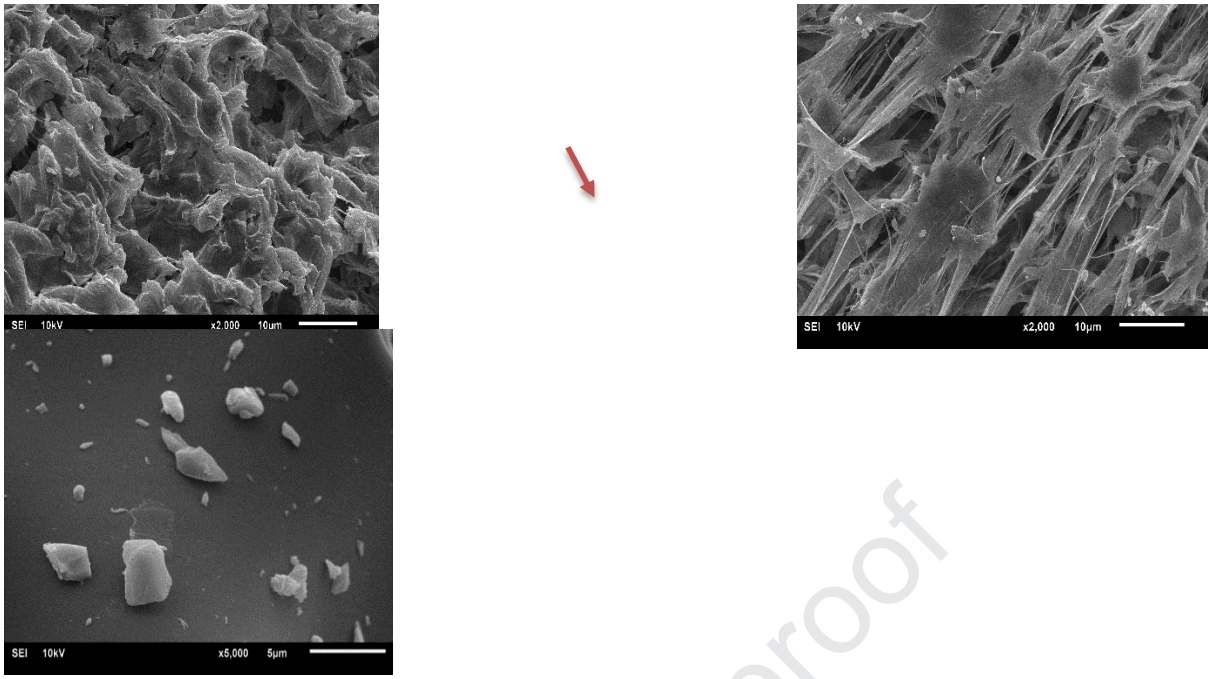
Significant differences between the thermal properties and hardness of the matrices were evaluated using one-way ANOVA with a Tukey multiple comparisons test ($p<0.05$).

4. Results and discussion

4.1 Production and morphology of the PCL matrices

PCL matrices containing lactoferrin were produced successfully by rapidly cooling suspensions of lactoferrin particles dispersed in PCL solution in acetone. Cooling of the suspension at -80^{o}C resulted in rapid crystallisation of the PCL phase, which counteracted sedimentation of the Lf particles. Previous investigations of the rapid cooling technique revealed a uniform dispersion of protein particles in the resulting PCL matrix (18). The LF-loaded PCL matrices could be produced in the form of IVRs, for example, by solvent bonding or thermal bonding of rods or by direct moulding to shape in suitably constructed moulds. The matrix production method involving crystallisation of polymer solutions, solvent evaporation and drying is time consuming and disadvantageous for production scale up. However, its simplicity and low cost lends itself to development of insertable, vaginal delivery systems for macromolecular actives for a range of clinical applications including vaccines and cervical cancer.

The Lf-loaded matrices exhibited a microporous morphology featuring interconnected pores and channels. The pore size in the case of blank PCL matrices ranged from 3-5 µm (Figure 1a) and increased to 6-10 µm when lactoferrin was incorporated (Fig 1b). The lactoferrin powder used for matrix formation was made up of particulates ranging in size from 0.5-3 µm (Figure 1c). Traces of lactoferrin particles were in evidence on the matrix surface (Fig 1b, arrowed).



a b c

Figure 1: Scanning electron micrographs of a) interior of blank PCL matrix, b) interior of PCL matrix loaded with 5% w/w lactoferrin, and c) lactoferrin particles

4.2 Thermal behaviour and hardness of matrices

A reduction in crystallinity of around 7% occurred when lactoferrin was incorporated in PCL matrices at a level of 10% w/w but there was no statistically significant difference between 0, 5, and 10% loading. (Table 1). The reduction in crystallinity suggests that the lactoferrin particles are inhibiting PCL nucleation and crystal growth. Similar effects were recorded previously for doxycycline which caused a decrease in crystallinity of PCL matrices of around 5% on increasing the drug concentration from 5% to 15% w/w (14). The glass transition temperature (Tg) of PCL was found to increase by approximately 10^{o}C on inclusion of lactoferrin particles in the PCL matrix (Table 1) indicating that the polymer chain mobility was restricted by the presence of lactoferrin, possibly due to adhesion between PCL chains and the lactoferrin particle surface.

The reinforcing effect of particulate fillers on polymers and elastomers is well known, resulting in increases of strength, stiffness (or modulus) and hardness with increasing content or volume fraction of the filler. We have previously reported increased hardness of PCL matrices following inclusion of the small

molecule antiviral, tenofovir (15), and the antibacterial, doxycycline (14), but a decrease in hardness for metronidazole (30). In the present study, the hardness of PCL matrices (1380 mN/mm^2) decreased significantly to 810 mN/mm^2 when lactoferrin particulates were incorporated (Table 1), which is much lower than the value measured for Nuvaring® pEVA IVR (9280 mN/mm^2). Intravaginal devices produced from lactoferrin-loaded matrices would thus be expected to increase user acceptance on insertion and residence in the vagina and to reduce the risk of irritation and subsequent inflammation of the vaginal epithelia. However, investigations of the flexural properties of Lf-loaded PCL matrices in the form of IVRs are essential to provide supporting information for an assessment of insertion and retention characteristics.

Particulate reinforcement theory is based on the condition of inextensible or non-deformable particulates (31), which may be considered to be met in the case of inorganic small molecule, crystalline microbicides such as tenofovir and doxycycline. However, biopolymers including lactoferrin are commonly classed as low stiffness, extensible materials and their reinforcing effect on a PCL matrix, of similar mechanical characteristics, would be expected to be negligible. Instead, the substantial softening (41%) of lactoferrin-loaded matrices, indicates that the glycoprotein particulates exert a major influence through their inhibition of PCL crystal nucleation and growth.

Table 1: Thermal properties and hardness of PCL matrices incorporating lactoferrin. Mean ± standard error of three replicate experiments are given; values within a column sharing the same superscript letter are not significantly different ($p<0.05$).

Lactoferrin loading (%w/w)	**Glass transition temperature (°C)**	**Crystallinity (%)**	**Hardness (mN/mm^2)**
0	-66.5 ± 1.2^a	82.3 ± 2.3^a	1382 ± 132^a
5	-55.2 ± 0.8^b	82.1 ± 4.6^a	1097 ± 87^b
10	-55.9 ± 0.9^b	76.7 ± 3.3^a	810 ± 62^c

4.3 *In vitro* release of lactoferrin from PCL matrices

Rapid release of around 14% of the lactoferrin content occurred from both 5%

and 10% loaded PCL matrices during the first 24 h of exposure to SVF (Figure 2a). Thereafter, lactoferrin was gradually liberated from the matrices, resulting in highly efficient release of 90-95% of the lactoferrin content over the 14-day test period. The higher release observed for the 10% lactoferrin-loaded matrices reflects the diffusion-controlled release mechanism which predominates in matrix-type drug delivery systems comprised of a dispersion of drug particles in a polymer matrix (32, 33). Drug diffusion from the matrix is controlled in major part by the concentration gradient between the matrix and surrounding fluid. Gradual release of lactoferrin macromolecules is expected through fluid-filled, interconnected pores and channels in the microporous PCL matrix and may be facilitated by micro-cracking effects in the 10% lactoferrin-loaded material caused by the higher concentration of lactoferrin particulates (19).

Chlamydia trachomatis is responsible for the most common STI and may lead to severe health problems including pelvic inflammatory disease and ectopic pregnancy (25). Sessa *et al* found that pre-incubation of human epithelial cells (HeLa-229) with lactoferrin at a concentration of 100µg/mL prior to infection with *C. trachomatis* or the addition of lactoferrin to *C. trachomatis* at the moment of infection, strongly inhibited bacteria uptake into host cells (25). In the present study, the minimum weight of lactoferrin released per day from cylindrical matrix samples into SVF (10 mL) was measured as 750µg. (Figure 3), resulting in a concentration of 75µg/mL. The cylindrical test samples used in this study (45mm length) correspond to approximately one third the linear length of an IVR and the turnover rate of vaginal fluid is approximately 8 mL/day (28). Therefore, the concentration of lactoferrin produced in vaginal fluid *in vivo* by a PCL matrix in the form of an IVR, may be expected to exceed, by a factor of 2 the lactoferrin concentration of 100µg/mL which was found by Sessa *et al*. to inhibit *C. trachomatis* entry into human mucosal epithelial cells *in vitro*. This basic modelling approach neglects the changes in volume and biochemistry of vaginal fluid over time (for example, pH), which may influence lactoferrin release kinetics and local concentration, but the example illustrates the potential of PCL matrices for maintaining high levels of lactoferrin in vaginal fluid at protective concentrations over extended periods of time.

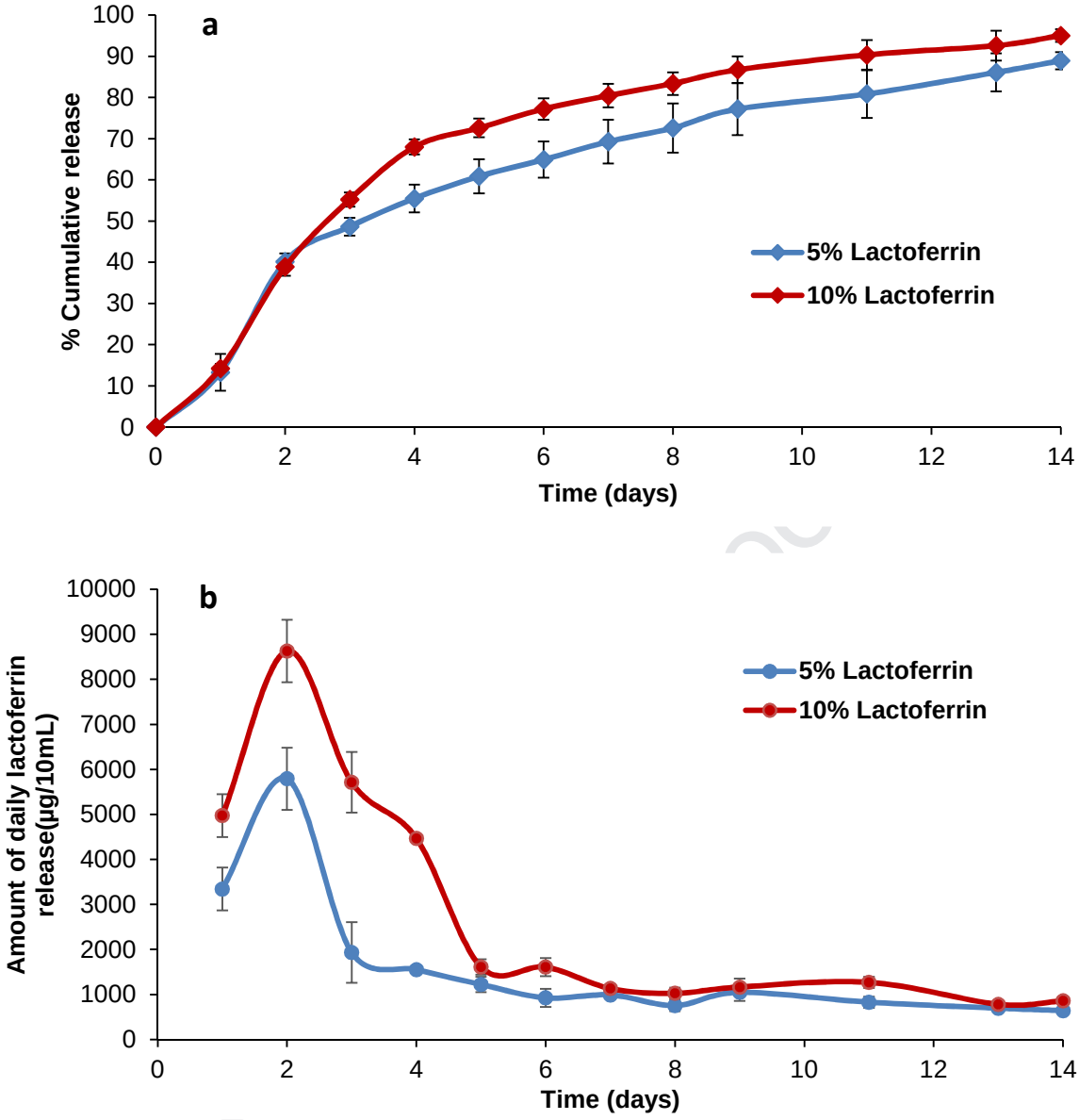


Figure 2: Cumulative release of lactoferrin (a) and weight of lactoferrin released daily (b) from PCL matrices into SVF at 37°C. PCL matrices contained 5 or 10% w/w lactoferrin. Values are the mean ± standard error of three replicate experiments.

A clinical study by Sessa *et al* (25) also revealed that prolonged intravaginal delivery of lyophilised lactoferrin over 30 days as a fast dissolving tablet formulation (100 mg every 8h) results in a protective effect against infection by *C. trachomatis*, in that six out of seven pregnant women asymptomatically infected with the bacteria, tested negative after the 30-day treatment. Intravaginal insertion was carried out three times a day, which may have been necessitated by a high leakage rate of dissolved lactoferrin. Lf-loaded PCL matrices offer opportunities for replacing the multiple dosing regimen by a

single administration, sustained delivery formulation, resulting in increased convenience, lower dosing and improved compliance.

We have previously formulated sustained-release PCL matrices loaded with drug combinations for *in vitro* assessment of their anti-viral activity (tenofovir and nevirapine) against HIV (15) and their anti-bacterial activity (metronidazole and doxycycline) against pelvic inflammatory disease (34). Lactoferrin has demonstrated synergy against HIV when combined with the anti-viral AZT analogue, zidovudine (23) and has been reported to reduce the minimum inhibitory concentration of anti-fungal drugs including clotrimazole and fluconazole against *C. albicans* (35). Thus, an investigation of intravaginal PCL matrices loaded with a combination of macromolecular and small molecule microbicides presents a logical and potentially useful progression of the current study.

4.4 **SDS-PAGE analysis**

Lactoferrin is an 80 kDa iron-binding glycoprotein of the transferrin family that is expressed in most biological fluids. SDS-PAGE analysis revealed that lactoferrin maintained its molecular size during matrix production and release into SVF over 14 days. The major protein band of lactoferrin at 80kDa was observed for the lactoferrin standard and for lactoferrin released from PCL matrices (Figure 3) together with a minor band at approximately 14 kDa which is assigned to α-lactalbumin (36). No additional bands were detected, indicating an absence of protein degradation. However, further studies are required to confirm preservation of the protein 3-D structure and the bioactivity of lactoferrin released from PCL matrices.The antibacterial activity of lactoferrin decreased by 96% against *C. sakazakii* during heat treatment at 72°C for 15 min and 85°C for 10 min (37), while heat treatment above 85^{o}C adversely affected the antibacterial activity of bovine lactoferrin against the food-borne bacteria *Escherichia coli, Salmonella enteritidis* and *Listeria monocytogenes* (38). Thus, the avoidance of high temperatures during PCL matrix formulation provides a decided advantage when incorporating macromolecular microbicides. Furthermore, lysozyme and collagenase have been successfully incorporated into PCL matrices and released into PBS over 11 days with retained activity in excess of 75% of the non-formulated enzymes (18). Thus, lactoferrin activity is expected to be retained when loaded and released from PCL matrices using the method described herein.

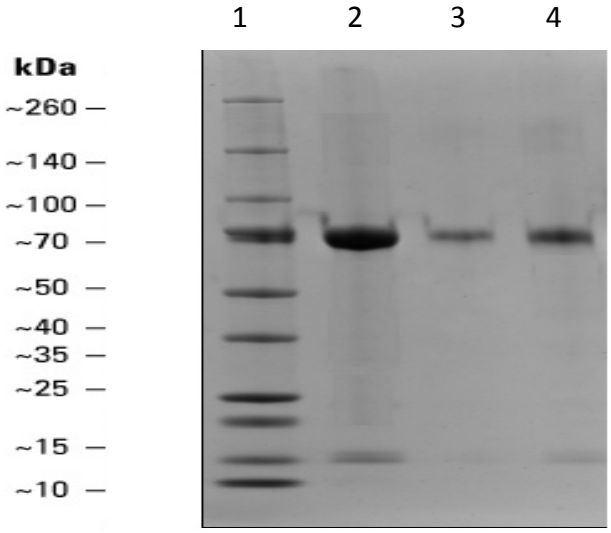


Figure 3: SDS-PAGE analysis of lactoferrin (80 kDa): Lane 1) Molecular weight biomarker ladder. Lane 2) lactoferrin standard. Lane 3) Lactoferrin released from 5% w/w-loaded PCL matrix and Lane 4) Lactoferrin released from 10% w/w –loaded PCL matrix into simulated vaginal fluid at 37^{o}C over 14 days.

5. Conclusion

Lactoferrin was incorporated successfully in PCL matrices by rapidly cooling dispersions of lactoferrin particulates in PCL solution. Highly efficient delivery of 90-95% of the lactoferrin content occurred over 14 days in SVF. Lactoferrin was released from the matrices over 14 days without affecting glycoprotein integrity on the basis of SDS-PAGE analysis of molecular weight. These findings recommend further investigations of PCL matrices for vaginal delivery of lactoferrin and other macromolecular microbicides, either alone or in combination with conventional small molecule drugs, for the treatment and prevention of STIs.

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Evaluation of polycaprolactone matrices for sustained intravaginal delivery of a natural macromolecular microbicide, lactoferrin

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