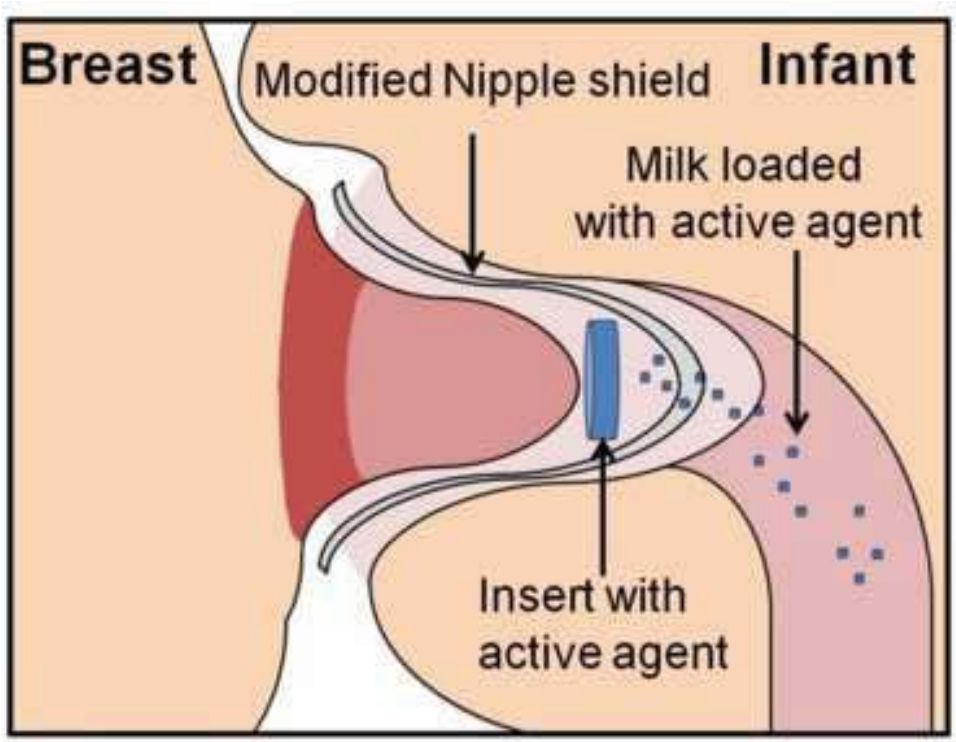
***Graphical Abstract**



A Nipple Shield Delivery System for Oral Drug Delivery to Breastfeeding Infants: Microbicide Delivery to Inactivate HIV

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ABSTRACT

A new drug delivery method for infants is presented which incorporates an active pharmaceutical ingredient (API)-loaded insert into a Nipple Shield Delivery System (NSDS). The API is released directly into milk during breastfeeding. This study investigates the feasibility of using the NSDS to deliver the microbicide sodium dodecyl sulfate (SDS), with the goal of preventing mother-to-child transmission (MTCT) of HIV during breastfeeding in low-resource settings, when there is no safer alternative for the infant but to breastfeed. SDS has been previously shown to effectively inactivate HIV in human milk. An apparatus was developed to simulate milk flow through and drug release from a NSDS. Using this apparatus milk was pulsed through a prototype device containing a non-woven fiber insert impregnated with SDS and the microbicide was rapidly released. The total SDS release from inserts ranged from 70-100% of the average 0.07 g load within 50 ml (the volume of a typical breastfeed). Human milk spiked with H9/HIV$_{IIIB}$ cells was also passed through the same set-up. Greater than 99% reduction of cell-associated HIV infectivity was achieved in the first 10 ml of milk. This proof of concept study demonstrates efficient drug delivery to breastfeeding infants is achievable using the NSDS.

KEYWORDS

Breastfeeding, Microbicide, Pediatric drug delivery, HIV, Mother-to-child transmission, MTCT, Sodium dodecyl sulfate, SDS, Breast milk

ABBREVIATIONS

MTCT, Mother-to-child-transmission (of HIV)
NSDS, Nipple shield delivery system
RLU, Relative luminescent units
SDS, Sodium dodecyl sulfate

1. INTRODUCTION

There is no single suitable drug and nutrient delivery method available for infants or young children (Kearns et al., 2003). In developing countries where medical infrastructure is often scarce, pediatric drug and nutrient delivery systems face numerous challenges in supply, stability, sterility, distribution, and dosing (Knoppert, 2009; WHO, 2010c). Liquid formulations are often the principal method of pediatric drug delivery, but are ill-adapted due to high-cost and lack of access to refrigeration or potable water for reconstitution (UNICEF and WHO, 2010). When liquid formulations are not available, a solid dosage form is often the only available method for administration of medicine. Many current medicines are only available in adult strength, so safe and accurate dosing for an infant is complicated (Pandolfini and Bonati, 2005; Stoltenberg et al., 2010). Additionally, liquid formulations can be unpalatable especially for young infants and may require undesirable toxic excipients, such as preservatives and solvents. There is a clear need for formulations that are appropriate, safe, and effective for children.

One clear example of the need for appropriate medicines to infants in developing countries is the prevention of mother-to-child transmission (MTCT) of HIV in breastfeeding. Of the approximately 500,000 infants per year who are infected with HIV from their mothers, it is estimated that 200,000 infants are infected through breastfeeding (Chasela et al., 2010), with 90% of MTCT occurring in Sub-Saharan Africa (UNAIDS, 2008). WHO policy on breastfeeding states that, '…when replacement feeding is acceptable, feasible, affordable, sustainable and safe, avoidance of all breastfeeding by HIV infected mothers is recommended...'(WHO, 2010b). This condition is often not met, and breastfeeding in low-resource settings has been shown to significantly increase infant survival (Brahmbhatt and Gray, 2003). In light of this, recent WHO guidelines recommend the continued use of oral anti-retroviral (ARV) drugs by the mother and/or the infant to prevent HIV transmission through breastfeeding (WHO, 2010a). However, widespread distribution of ARVs does not yet exist in Sub-Saharan Africa and ARV use can lead to side effects and resistant strains of the virus if infection still occurs (Zeh et al., 2011).

As an alternative approach, the administration of edible microbicides into expressed infected milk which is then delivered to the baby has been previously considered (Hartmann et al., 2006a). Sodium dodecyl (or lauryl) sulfate (SDS), an anionic surfactant, is a candidate for use as an edible microbicide with anti-HIV activity in human milk. It has been demonstrated that 0.1 – 1 wt% SDS rapidly kills sexually transmitted pathogens, including HIV in media (Howett et al., 2000, 1999; Krebs et al., 2000, 1999). A concentration of 0.1 wt% SDS has been demonstrated to rapidly inactivate cell free and cell-associated HIV in human milk (Hartmann et al., 2005; Tuaillon et al., 2009). This concentration is safe for infant use, based on a maximum acceptable infant oral exposure to SDS of 1 g/kg (of infant)/day and an biochemical analysis of the effect of SDS on milk content (Hartmann et al., 2006a, 2006b). Another benefit of SDS is its broad antiviral activity by solubilizing lipid membranes; therefore unlike many anti-viral compounds SDS is strain independent and unlikely to drive HIV mutation to a resistant form (Hartmann et al., 2006b).

Given that delivery of SDS during breastfeeding may be an effective method of reducing viral load in milk and preventing MTCT of HIV, we propose a new method to deliver SDS to infants during breastfeeding that also overcomes many of the general challenges associated with frequent drug delivery to infants. The concept is to incorporate a drug-impregnated insert into a nipple shield worn by a mother during breastfeeding (Fig. 1), where during suckling, a drug is released directly into the milk (Gerrard, 2011; Sokal et al., 2009). Nipple shields, typically a single molding of silicone, are available at low cost and are used to aid mothers and/or infants during breastfeeding, typically to reduce pain or nipple damage, or to assist latching on (Riordan, 2005). The NSDS would have an insert containing a dose of the API in dried form. In the studies reported in this publication, NSDS inserts were made from non-woven fiber, representing a flexible, high surface area support for drug incorporation. The mother would wear the NSDS as her child breastfeeds, and as milk passes through the insert the API would be released directly into the milk and pass to the infant. The insert could be placed inside the NSDS prior to each feed or the NSDS could be preloaded with the insert prior to the mother obtaining the device, and be entirely disposable after one use. Alternatively, the NSDS could be washed, disinfected, and reloaded with another insert for reuse.

This study had two aims: firstly to determine the kinetics of drug release into milk from a NSDS insert during a pulsed flow that mimics breastfeeding; and secondly to establish whether the release of SDS from a NSDS into human milk can inactivate HIV within the fluid.

2. MATERIALS AND METHODS

2.1 Formulation of non-woven fiber inserts with SDS

To make the NSDS inserts, 10 mm diameter discs of a medical grade non-woven cellulosic (viscose) and polyester based fiber matrix with a 2.75 mm thickness and area density of 300 g/m^2 (Bathfelt, Texel, Québec, Canada) were soaked in a 30 wt% SDS (Reagent Plus > 98.5% purity, Sigma Aldrich, UK) solution at 60 °C for 10 seconds. They were then air dried at room temperature on a mesh. After 72 hours drying their weight stabilized with a final weight gain of 0.07 g (standard deviation 0.01 g, n = 13). This fiber grade was chosen because it is non-toxic, suitable for flow with low back pressure, and it is easy to load a compound such as SDS onto it.

2.2 Kinetics of SDS release into milk in simulated breastfeeding conditions

To simulate use of a NSDS to deliver SDS during breastfeeding, loaded inserts were placed in an O-ring (BS012 Viton ^{TM} O-ring, 3/8 " ID, UK) to seal them into a Sinnex filter holder (Millipore, MA, USA) (Fig. 2 a-c), or weighed amounts of SDS powder were placed directly into the holder (0.1 g). Sample fluids were passed through a peristaltic pump (Masterflex console drive, easy load 11 Masterflex L/S model 77200-50, Cole Palmer, UK), heated to 37 °C by passing through tubing in a water bath held at 42 °C, and then delivered through the SDS loaded device. Around 50 x 1 ml fractions per test were collected from the flow-through using a SuperFrac^{TM} fraction collector (GE Healthcare Sciences, UK) to reflect typical amounts of milk consumed in a feed (Kent et al., 2006). The milk reservoir was continuously stirred to prevent fat accumulating at the top inlet. Individual fractions were assayed in triplicate for SDS concentration using a colorimetric assay described below.

SDS concentration in milk fractions was measured using an adapted stains-all colorimetric assay (Rusconi et al., 2001). The assay relies on the shift in absorbance at 438 nm when the reagent dye, stains-all, is mixed with SDS. The stains-all reagent underwent a spectral shift when mixed with milk alone without SDS, presumably caused by interactions with lipids, proteins or components with surfactant-like properties in milk. However, a highly reproducible further spectral shift was seen when SDS was added. Thus, by diluting milk samples to a fixed ratio in water prior to testing, keeping the absorbance signal caused by milk alone constant, the absorbance at 438nm was still directly proportional to SDS concentration, allowing rapid and simple SDS measurement in milk (Fig. 3). A range of dilution factors were used to accurately detect concentrations of SDS in milk above 0.03 wt%. SDS concentration in test samples was calculated by comparison to calibration curves measured at the same sample dilution, using standard SDS solutions made in identical milk from a continuously stirred 5% wt/vol. (milk) SDS stock solution. Fluids used were: cow's milk, either pasteurized but not homogenized (Taste the Difference Jersey Milk, 5.2% fat, J.S. Sainsbury's, Cambridge, UK), pasteurized and homogenized (Whole milk, 3.6% fat, J.S. Sainsbury's, Cambridge, UK), or unpasteurized non-homogenized full-fat goat's milk (4% fat, Wobbly Bottom Farm, Hitchin, Hertfordshire, UK).

To make an assay solution sufficient to analyze 250 samples, 20 mg stains-all dye (Sigma-Aldrich, UK) was dissolved in 1 ml followed by a further 19 ml of 1:1 isopropanol:water, followed by dilution with water to a total volume of 380 ml plus the addition of 20 ml formamide and thorough mixing for 30 seconds. Unknown milk samples were thoroughly mixed and diluted to 1:2.5, 1:10 or 1:100 by volume with ultrapure MilliQ water. Triplicate 25 µL samples of each diluted fraction were mixed with 1000 µL stains-all stock solution followed by measurement of absorption at 438 nm. Triplicate standard samples were measured, mean absorbances plotted, and unknowns calculated typically using a linear regression (fig 3); in some cases 2nd or 3rd order polynomial curves were used outside the range of linearity with typical correlations of $R^2 > 0.998$.

2.3 Inactivating HIV in human milk with an SDS-loaded NSDS insert

To determine inactivation of cell-associated HIV by SDS released from the NSDS, human milk was spiked with H9/HIV_{IIIB} cells (provided by the NIH AIDS reagent program Cat. No. 400) to a final concentration of 2.6 x 10^5 cells/ml and was pumped through SDS impregnated NSDS inserts using a near identical setup to that used to measure SDS release kinetics, see section 2.2 (fraction collector: BioRad Model 2110, USA). Human milk samples were provided by the Mothers' Milk Bank, Valley Medical Centre (San Jose, California, USA). H9/HIV_{IIIB} cells are self replicating cells that express HIV (type-1 IIIB), and have been previously used to model cell-associated HIV (Lara et al., 2011; Yamaguchi et al., 2007). The cell content spiked in milk was based on previously reported concentrations of between 1 to 3255 infected cells per 10^4 in milk with typical total cell concentrations in the first few days of life to be 10^6 cells/ml (Nduati et al., 1995; Rousseau et al., 2004). Prior to being spiked in milk, the cells were centrifuged at 1500 RPM for 5 minutes and re-suspended in cell culture media to remove free virus. 5 ml milk fractions were assayed for HIV-infectivity using TZM-bl cells in triplicate (provided by the NIH AIDS reagent program Cat. No. 8129). TZM-bl cells are HeLa clones genetically engineered to express CCR5 and CD4 receptors and indicate HIV infectivity via a luciferase reporter (Montefiori, 2005). Infectivity values were calculated by comparing them with standard samples of known infectivity for concentrations of H9/HIV_{IIIB} cells in the same milk (Fig. 6a).

The concentrations of SDS released into early milk fractions, and the human milk itself, were both found to disrupt the TZM-bl reporter cells, preventing direct measurement of HIV infectivity in the fractions. Therefore, for all collected fractions, SDS and milk were separated from H9/HIV_{IIIB} cells 20 minutes after fraction collection, by 3 rounds of centrifugation and washing in cell culture medium and phosphate buffered saline (PBS). Preliminary experiments demonstrated this method removed sufficient human milk and SDS at all release concentrations to prevent direct disruption of TZM-bl cells with the donor milk used (data not shown). This protocol also prevented HIV inactivation by SDS following NSDS treatment, during subsequent sample incubation with TZM-bl cells, which would not be representative of the conditions encountered by cell-associated HIV passing through the NSDS in physiological breastfeeding conditions.

100-150 µL samples of milk fractions were diluted 1:10 (vol.) in cell culture media in a 96-well round bottomed plate (# 3799, Corning, USA), centrifuged (1500 RPM for 5 minutes at 15 °C) and washed twice in PBS, followed by centrifugation and re-suspension of washed H9/HIV_{IIIB} cells in 100 µL culture medium. Culture medium was based on Dulbecco's Modified Eagle Medium High Glucose (DMEM) (Invitrogen, USA) and 15% (vol.) fetal bovine serum (Invitrogen, USA). After washing, 25 µL of culture medium, 25 µL washed sample and 50 µL TZM-bl cells at 2 x 10^5 cells/ ml were added to flat bottomed 96-well plates and incubated for 2 days at 36.5 °C and 5% CO_2 (incubator: Sanyo, USA). Samples were re-suspended in culture medium and DEAE Dextran (30 µg/ml) was added to TZM-bl cells just prior to sample addition at 2 µL per 1 x 10^5 cells/ ml. A D-Luciferin potassium salt (Thermo Scientific, USA) reagent mixture was added and luminescence read using a GloMax® 96 Microplate Luminometer (ProMega, USA).

3. RESULTS

3.1 Release of the edible microbicide SDS from NSDS inserts

The release of SDS from a NSDS insert in a mimicked breastfeeding simulation environment was studied using the apparatus outlined in section 2.2 and Fig. 2d. This was performed to provide evidence of the influence of the physiological variables within breastfeeding that could influence drug release from a NSDS. Preliminary experiments determined a suitable apparatus to mimic drug release from a drug-loaded NSDS insert. Conditions of milk flow through an NSDS insert resembling breastfeeding were achieved by maintaining the milk at 37 °C and using a peristaltic pump to produce pulsed flow to simulate the suction pressure created by a baby (Fig. 2d). During breastfeeding, pulse rate and volume vary greatly, so a pulse rate of 60/min with a volume of 0.07 ml per pulse was chosen that lies within the typical range of a feeding infant (Zoppou et al., 1997); this corresponds to a flow rate of 4.2 ml/min. Total feeds have been reported to have a mean of 76 g (std. dev. 12.6 g) and a range of 0-240 g per feed, i.e. mean 74 ml and range

0-233 ml per feed given a reported density of human milk of 1.03 g/ml (Kent et al., 2006). Test flow conditions were kept within these values.

The SDS insert formulation protocol was developed to produce an insert with total load of 0.7 g SDS, a sufficient quantity to release an effective microbicidal concentration to rapidly inactivate HIV in a feed (i.e. approximately 0.1%), while keeping the total SDS load within acceptable daily doses (Hartmann et al., 2005, 2006a, 2006b).

The spectrophotographic assaying method outlined in section 2.2 and Fig. 3 was used to detect SDS for all release tests. When flow conditions, insert loading, and milk type and batch were fixed, release kinetics in replicate experiments were highly reproducible confirming that the apparatus is suitable for release studies (Fig. 4, Fig. 5 and data not shown). Given the high variation in composition of human milk between individuals and even during feeds from the same individual (Daly et al., 1993; Kent et al., 2006), release studies were performed with commercial cow's and goat's milk which is available in bulk quantities with highly reproducible composition.

In all conditions tested, the majority (>70%) of SDS was released from non-woven inserts within 50 ml. A common release pattern presented itself: the highest amounts of SDS releasing into early fractions, followed by decreasing concentration over time, indicating approximately first order release kinetics. A model was fitted to the cumulative release data for each experiment to qualify this observation (see section 3.4).

3.2 Effect of flow conditions and temperature and insert form on release kinetics

The initial focus was to identify the principal release behavior of SDS from the non-woven fiber over a range of flow conditions. This was intended to examine the basic influence of fluid kinetics on release behavior, which may vary significantly from a feeding infant using the NSDS.

The effect of milk temperature upon release behavior was studied to provide evidence of the importance of fluid temperature for future laboratory studies. The release of SDS from the non-woven fiber insert into homogenized, pasteurized cow's milk at 16 °C (laboratory temperature), was similar to that detected at 37 °C (temperature of human milk) into homogenized, pasteurized cow's milk (Fig. 4a and b). Around 70-100% release was detected after 30 ml in all tests. This suggests that milk temperatures between 16 and 37 °C might not significantly influence SDS release rate from the non-woven fiber.

The influence of two types of flow conditions were compared between tests: the pulse rate (how quick the infant sucks) and the pulse volume (how much milk is extracted from the breast per suck); these were controlled by altering the size of tubing used by the peristaltic pump and the operating speed. Two test sets were run using non-homogenized pasteurized cow's milk; (1) maintaining the pulse rate at 60 pulses/min and varying pulse volume at 0.02, 0.07 and 0.45 ml/pulse and (2) maintaining the pulse volume at 0.07 ml/pulse and varying the pulse rate to 40, 60 and 80 pulses/min. The release results demonstrated that SDS was released into non-homogenized cow's milk at similar rates for all these flow rate conditions, with >50% of release of the disc's load after 20 ml for all tests (Fig. 4c and d). SDS concentrations of above 0.1 wt% SDS (previously reported to be highly anti-viral – see 1. Introduction) were seen for the tests in the first 20 ml of milk that passed through the non-woven fiber insert.

The influence of the non-woven fiber on SDS release was determined by comparison to SDS powder placed into the insert holder. 0.1 g of SDS powder was used per test. Similar release patterns were seen into milk as with non-woven fiber insert experiments (Fig. 4). Release from the flow chamber ranged from 40% to 70% after 50 ml for 16 °C pasteurized and homogenized cow's milk and 80% for 37 °C for the same milk source (Fig. 4e).

3.3 Effect of milk composition on release kinetics

The influence on release behavior due to milk composition was studied, using milk from different animal sources and with varying pasteurization and homogenization. Analysis of initial release behavior provided a suitable marker for the effect of different fluid types. Approximately 100% of the insert load had released into non-homogenized unpasteurized goat's milk within 10 ml, 70-90% for homogenized pasteurized cow's

milk and 30-60% into the non-homogenized pasteurized form, suggesting progressively slower release into these respective fluids (Fig. 5). The mean volume needed for 50% release of the SDS from the non-woven disc insert between these 3 fluids was also compared, and goat's milk (average 1.4 ml) induced significantly more rapid release than both homogenized pasteurized (5.1 ml) ($p < 0.05$) and non-homogenized pasteurized (16.3 ml) ($p < 0.1$) cow's milk (using unpaired two tailed t-tests). The difference in volume to 50% release into homogenized compared to non-homogenized cow's milk was not significant ($p > 0.05$). The observed difference in cow's and goat's milk release behavior indicates that milk composition significantly influences release kinetics

3.4 Modeling release behavior

For an initial model it was proposed that total drug release was dependent on the fraction of SDS released from the insert (Eq. (1)) for fixed flow and temperature conditions.

$$\frac{dM_r(q)}{dq} = k_2[k_1 - M_r(q)] \quad (1)$$

q: Volume of fluid passed through insert (ml)
$M_r(q)$: Mass fraction of SDS release (relative to initial insert load)
k_1: Constant
k_2: Constant (ml^{-1})

Integrating from the start of the test until a volume, q, has passed through the insert gives Eq. (2):

$$M_r(q) = k_1[1 - \exp(-k_2 q)] \quad (2)$$

Using Eq. (2) for each release test k_1 and k_2 were varied to optimize the least squares value using a computational non-linear regression analysis optimization algorithm (Tables 1. and 2.) (Software: Mathematica - Wolfram, IL USA).

The 1st order release kinetics model presented $R^2 > 0.969$ for all tests apart from one with the highest flow rates, with R^2 at 0.933 (Table 1.). This indicates that for most flow conditions the release behavior is well modeled by 1st order release kinetics. For non-woven fiber tests under the same flow conditions (Table 2.) the constant k_2 was noticeably higher in goat's milk (0.416-0.522) compared to non-homogenized cow's milk (0.141-0.181) to homogenized cow's milk (0.036-0.069). The mean k_2 values for each fluid were statistically different between all fluids ($p < 0.05$) using unpaired, two tailed t-tests. k_2, which indicates rate of release, was highest for the goat's milk, where SDS release was most rapid. k_1 reflects the total maximum release expected by 1st order release kinetics. Given the total cumulative release reaching 70-100% within 50 ml for most tests, k_1 values derived by regression analysis were found to be close to 1. Further tests are needed to expand the model and to determine which component(s) of milk influence the rate of release.

3.5 HIV inactivation by a SDS loaded NSDS insert

For the final element of this proof of concept study the reduction of cell-associated HIV by SDS was studied using the same apparatus and test conditions as the release studies, but using human milk. Given the anti-viral concentrations of SDS found to release into various milk types in early fractions, it was predicted that similar release would be expected in human milk, and thus the NSDS should significantly reduce the amount of HIV infectivity at least in the first portion of milk passed through the insert.

It has been previously argued that cell-associated HIV may have the predominant role in MTCT of HIV in breastfeeding (Rousseau et al., 2004) so cell associated HIV was used in these virology studies. H9/HIV_{IIIB} cells were used as a model of cell-associated HIV. The cells were spiked into human milk to mimic milk from HIV positive mothers, and were then passed through SDS-loaded NSDS inserts at 60 pulses/min and 0.07 ml/pulse (used in release tests and typical of infant feeding conditions, see section 3.1). TZM-bl cells with luciferase reporter genes were used to measure the infectivity of H9/HIV_{IIIB} cells before and after the tests. Since exposure of TZM-bl cells to both human milk and SDS can artificially reduce the apparent infectivity of H9/HIV_{IIIB} cells, an assay was developed that allowed measurement of inactivation of cell-

associated HIV by the NSDS, whereby both human milk and SDS were removed 20 minutes after collection followed by measurement of HIV infectivity (see section 2.3).

When known doses of H9/HIV_{IIIB} cell samples were assayed with this method using TZM-bl cells, H9/HIV_{IIIB} cell concentrations between $0.26 \times 10^4 - 26 \times 10^4$ cells/ml were quantitatively detected, indicating a sensitive assay of HIV infectivity (Fig. 6a). Relative HIV infectivity levels in samples of NSDS-treated milk were then determined by comparing the measured luminescence from TZM-bl cells exposed to test samples with the calibration data in Fig. 6a. Using this method, it was found that treatment of HIV-spiked human milk with the NSDS SDS-loaded insert resulted in a significant reduction in the mean correlated infected cell content of 3 tests, compared to input cell content (Fig. 6b). Average infectious cell content reductions were significant at the following levels: More than 2 log reduction for 0-5 ml ($p < 0.0001$), 1.5 log reduction for 5-10 ml ($p < 0.0001$) and 0.6 log reduction for 10-15 ml ($p < 0.05$) (using paired single tailed t-tests). A 0.4 log reduction for 15-20 ml, 0.4 log reduction for 20-25 ml and 0.3 log reduction for 25-30 ml in mean infected cell content was observed but these reductions were not significant ($p > 0.05$). The individual infectious cell content in each volume fraction is illustrated in Fig. 6b. The small variation in reduction of infectivity between repeat tests is likely due biological variations in inactivation between tests, given the small variance observed between replicate HIV infectivity assays of individual fractions (Fig. 6b).

4. DISCUSSION

4.1 Drug release into milk from the NSDS

Parameters that are expected to influence release kinetics of an API from a NSDS are: drug form, support material/excipients, flow conditions and solvent type. For this study where flow conditions and milk type were changed the greatest variation in release behavior was seen between the differing milk types, with goat's milk producing the most rapid SDS release rate. Understanding in detail the effect of milk composition on release kinetics will be important for controlled release into human milk, which is known to have highly variable composition; for example during a typical feed, the fat content can increase by up to 3-fold (Daly et al., 1993).

In order to obtain consistent drug release between mothers despite their varying milk content, it may be necessary to produce an insert formulation that would allow for flow rate-independent release kinetics for various milk compositions possible in feeding. Further formulation methods should be considered for SDS and other APIs. They could include modifying or changing the current soaking impregnation method onto the fiber or using a different support material such as a rapidly disintegrating tablet or a soluble polymer film. Preliminary tests demonstrated that addition of hydroxymethyl propyl cellulose into the SDS insert during formulation may result in slower SDS release into milk and reduce the initial high release that appears to be highly composition dependent (unpublished observations). Alternatively, SDS or other APIs might be incorporated in the fiber during manufacturing, to further control release as seen in related studies (Cui et al., 2006).

The first-order cumulative release model presented fitted our observed data well, and the constants derived by regression analysis supported the observation that the majority of drug is released for most tests within 50 ml, and that milk composition significantly influences rate of release. However this simple model may not encompass all the factors influencing release from the non-woven fiber, especially at higher flow rates where the model fitted least well; further work is required to refine the model. We postulate that a combination of dissolution phenomena and solid and hydrated particulate release from the fibers govern SDS release from fibers.

4.2 Viral inactivation in human milk

There was a high inactivation of cell-associated virus in early fractions (0-10 ml) of human milk passed through the NSDS SDS-insert (> 99%), followed by a much smaller reduction in later fractions. The reported threshold of rapid HIV inactivation (> 0.1 wt%) also occurred within the first 10 ml of release for goat's milk but then rapidly decreased to below reported microbicidal concentrations (Hartmann et al.,

2006a, 2006b, 2005). This suggests that the initial high release behavior of SDS observed in goat's milk may also have occurred with human milk and therefore goat's milk may be a suitable mimic for use in NSDS release studies. Further work is needed to understand what components affect SDS release and dissolution, and how milk composition affects release kinetics before a suitability-of-use analysis can be made.

The importance of the release kinetics of SDS into HIV infected milk for prevention of MTCT can only be speculated about at this point. MTCT of HIV in breastfeeding is complex and incompletely understood. Transmission may occur either through the free virus or cell-associated virus, with possible sites of transmission including the mucosal surfaces of the tonsils and gut (Cavarelli and Scarlatti, 2011). Thus the SDS release rate into milk may alter transmission rates depending on where anti-viral concentrations of SDS in the digestive system reside during the feed. The proof of concept data in this paper should provide an indication of how cell-associated HIV could be inactivated by SDS in a physiological environment. *In vivo* SDS may act on both free virus and infected cells during their passage through the digestive system, and SDS released into early fractions may subsequently mix with infected milk consumed later in the feed. This would lead to a higher reduction of HIV infectivity than that seen in this simplified study. Further study will be required to better predict the effectiveness of a given NSDS microbicide formulation on preventing infection. One key advantage of SDS over other anti-viral compounds is its ability to rapidly inactivate HIV (Hartmann et al., 2005); this rapid inactivation may reduce viral load in infected milk before it even reaches proximal sites of infection such as the oral mucosal tissues of the infant.

The incorporation of immobilized microbicidal surfaces within the NSDS may also be a viable consideration for preventing MTCT of HIV specifically by inactivating virus during passage through the NSDS. For example, viral inactivation using copper-based fibers has also been considered in a breastfeeding device (Borkow et al., 2011, 2008). This could be combined with microbicide release to potentially increase viral inactivation using a NSDS.

4.3 Future uses of the NSDS

The acceptability of a NSDS to breastfeeding mothers must be carefully assessed prior to use. For its specific use in preventing MTCT of HIV during breastfeeding a study was conducted in Kenya. Mothers and stakeholders involved in deciding infant feeding practices were questioned, and gave positive feedback about the potential use of a NSDS to prevent HIV transmission in feeding (Israel-Ballard et al., 2010).

For any specific application, careful consideration will be needed to determine if a disposable single use device or a re-useable one, with a replaceable drug-loaded insert would be most suitable. Although a re-usable NSDS would be more sustainable and lower cost, in low-resource settings where sanitation equipment may be limited, the feasibility of ensuring hygienic device re-use will have to be carefully considered.

Aside from SDS delivery a wide range of individual or combinations of medicinal substances could be delivered to infants using the NSDS, including drugs such as antibiotics and antimalarials, or vitamins, nutrients and probiotics. Similar inserts could be incorporated into modified bottle teats, allowing equally effective drug delivery to infants fed with formula or expressed milk via a bottle.

Using a NSDS to deliver agents other than microbicides will generally require simple direct API release rather than potentially sustained release, with the primary focus to ensure full dose release within a typical feed. Taste, solubility and the effect of the formulation on the nutrition value of the milk would be primary considerations, and alternative insert forms such as tablets should be considered.

Potential advantages of the NSDS over other infant drug delivery routes and devices include ease of use and precise dosing compared with drops or spoon-fed liquids. Alternatives to parenteral delivery are particularly important for frequently administered drugs because of the burden on trained healthcare workers, the risks associated with needle use, and to avoid pain associated with injecting infants. For some oral APIs, milk may mask taste, improving acceptability for the infant. Furthermore, for labile APIs a dried

formulation offers improved stability over liquid formulations. Drug administration during breastfeeding may also increase the bioavailability of some drugs (Charkoftaki et al., 2010).

Additional benefits of the NSDS in low-resource healthcare settings include simplicity, low cost production, a low level of training needed for correct dosing, potential for a single-use disposable device avoiding requirement for sterilization, and a robust dry formulation for thermostable distribution. Most importantly, with reducing MTCT of HIV, the NSDS is designed to be compatible with breastfeeding, which is often the safest method of infant feeding even when the mother is infected (Brahmbhatt and Gray, 2003).

5. CONCLUSION

A sustained release of the edible microbicide SDS into HIV infected milk during breastfeeding from a NSDS placed over the mother's breast, is proposed to be an effective method for oral delivery of microbicides to prevent MTCT of HIV. This study has demonstrated that a NSDS can deliver SDS into milk from a non-woven fiber insert at non-toxic microbicidal concentrations. It has also demonstrated that SDS release using the NSDS is capable of rapidly inactivating significant amounts of cell-associated HIV in human milk. The NSDS is especially valuable for use in developing countries where no safer alternative to breastfeeding exists. Future work is needed to fully understand the effects of milk composition on release kinetics. Modifying the non-woven fiber composition, the addition of cellulose based compounds onto the fiber, or the addition of microbicides and cellulose in fiber construction, may enable controlled release patterns. With better understanding of the sites of transmission in breastfeeding these methods could be adapted to maximize reduction of MTCT of HIV.

ACKNOWLEDGMENTS AND ASSOCIATIONS

We are grateful to the Bill and Melinda Gates Foundation, the Clinton Foundation (Clinton Global Initiative), the UK EPSRC, Cambridge University and King's College (Cambridge University), Pembroke College (Cambridge University) - UC Berkeley Exchange, and the International Design Development Summit for financial support and advice. We thank Wobbly Bottom Farm, Hertfordshire for their supply of goat's milk and Pauline Sakamoto of the Milk Bank, Santa Clara Valley Medical Centre (San Jose, California, USA) for coordinating use of human milk samples. We thank the JustMilk team including Geoff Galgon, Elizabeth Kneen, Ryan Hubbard, Tombo Banda (inventors), Arron Rodrigues of EWHCambridge, Krishnaa Mahbubani, Yucy Fang, Samantha Gooneratne and David McNally of the Bioscience Engineering Group, Department of Chemical Engineering and Biotechnology, University of Cambridge, UK, Peter Patiris, Leo Oceguera and Haynes Sheppard of the California Department of Public Health, Richmond and David Jenkins of FHI 360 for helpful discussions. Stephen Gerrard and David Sokal are inventors of the nipple shield delivery system (patent pending: US 12/536,219, PCT/US10/44589).

410 **Tables:**

Table 1.

Insert	Pasteurized	Homogenized	Fluid Temp (° C)	Pulse Rate (pulses/min)	Pulse Volume (ml/pulse)	Total release (/initial load)	k_1	k_2 (ml^{-1})	R^2	Graph Ref
Fiber	x	x	16	60	0.07	0.81	0.800	0.139	0.990	4a
Fiber	x	x	16	60	0.07	0.77	0.750	0.157	0.969	
Fiber	x	x	37	60	0.07	0.80	0.794	0.185	0.984	4b
Fiber	x	x	37	60	0.07	0.86	0.870	0.141	0.988	
Fiber	x		37	60	0.02	0.83	0.824	0.156	0.984	4c
Fiber	x		37	60	0.07	1.14	1.180	0.069	0.994	
Fiber	x		37	60	0.45	0.87	0.803	0.124	0.933	
Fiber	x		37	80	0.07	0.97	0.933	0.097	0.971	4d
Fiber	x		37	60	0.07	1.14	1.180	0.069	0.994	
Fiber	x		37	40	0.07	0.90	0.879	0.252	0.987	
Flow Cell	x	x	16	60	0.07	0.46	0.439	0.098	0.992	4e
Flow Cell	x	x	16	60	0.07	0.70	0.666	0.075	0.984	
Flow Cell	x	x	37	60	0.07	0.93	0.900	0.057	0.991	

Summary of SDS release experiments using cow's milk with varying flow conditions. Fitted model parameters to a first-order release kinetic model according to Equ. (2) also displayed.

Table 2.

Milk	Pasteurized	Homogenized	Total release (/initial load)	k_1	k_2 (ml^{-1})	R^2	Graph Ref
Cow	x	x	0.80	0.794	0.185	0.984	5a
Cow	x	x	0.86	0.870	0.141	0.988	
Cow	x	x	1.04	1.026	0.183	0.994	
Cow	x		1.14	1.180	0.069	0.994	5b
Cow	x		0.79	0.906	0.036	0.984	
Cow	x		0.76	0.753	0.065	0.988	
Goat			1.07	1.057	0.416	0.978	5c
Goat			1.14	1.149	0.522	0.989	
Goat			1.02	1.030	0.452	0.995	

Summary of SDS release experiments using cow's and goat's milk for constant flow conditions.
Fluid temperature 37° C, 60 pulses/min, 0.07 ml/pulse and SDS-fibre insert. Fitted model parameters to a first-order release kinetic model according to Equ. (2) also displayed.

Figure legends:

Graphical Abstract:

Cross sectional diagram of milk leaving breast passing through nipple shield delivery system insert.

Fig. 1. Nipple shield delivery system for oral drug delivery to breastfeeding infants

(Images provided courtesy of http://justmilk.org) (a) Non-woven fiber inserts. (b) Demonstration of blister pack containing replaceable inserts. (c) A modified silicone nipple shield adapted to hold inserts in place during breastfeeding (prototype, not for clinical use).

Fig. 2. Methods for studying SDS release into milk in pulsed flow conditions

(a) The fiber insert sealed into the housing within an o-ring. (b) The assembled housing. (c) SDS-impregnated non-woven fiber insert housed within an o-ring. (d) Diagram of rig used to deliver pulsed flows of milk through the filter housing and collect fractions to be measured for SDS content/cell associated HIV infectivity.

Fig. 3. Simple, rapid measurement of SDS concentration in milk using stains-all dye

The absorbance at 438nm was measured for known concentrations of SDS dissolved either in (a) water or milk subsequently diluted in (b) 1:10 water dilution or (c) 1:100 water dilution. A clear linear relationship between absorbance and SDS concentration is apparent for each fixed dilution ration allowing accurate measurement of SDS release into milk over a range of concentrations. Data representative of >20 experiments; fresh standard curves were prepared for every release experiment using the same batch and type of milk tested to determine SDS concentrations. The standard error of repeat measurements is displayed.

Fig. 4. Effect of SDS form, temperature and flow on release kinetics

Pasturised cow's milk was flowed through SDS loaded onto non-woven fibre discs (a-d) or SDS powder (e) and SDS concentration determined. (a, b) The effect of temperature on release at a flow rate of 4.3 ml/min and pulse rate 60 pulses/min was determined. (c) The effect on release of varying pulse volume at a fixed pulse rate of 60 pulses/min was determined. (d) The effect of varying pulse rate for a fixed pulse volume of 0.07 ml/min was determined. (e) The release of SDS in powder form at 16°C and 37°C at a flow rate of 4.3 ml/min and pulse rate 60 pulses/min was measured. Data displayed as (i) concentration of SDS in individual collected 1 ml fractions and (ii) cumulative SDS release relative to input SDS load. In all cases, each set of symbols represents an individual release experiment, with the mean of triplicate measurements of SDS concentrations for each fraction shown.

Fig. 5. Effect of milk type on SDS release kinetics

The release of SDS from loaded non-woven fibre discs during pulsed flow into (a) homogenised pasturised cow's milk, (b) non-homogenised cow's milk and (c) non-homogenised unpasturised goat's milk was measured with a flow rate of 4.3ml/min and pulse rate of 60/min. Data are displayed as concentration of SDS in collected 1 ml fractions (i) and cumulative SDS release relative to input disc load (ii). In all cases, each set of symbols represents an individual release experiment, with the mean of triplicate measurements of SDS concentrations for each fraction shown.

Fig. 6. Reduction in HIV infectivity in human milk after flow through SDS-loaded NSDS insert

(a) Calibration curve used to determine H9/HIV_{IIIB} cell content in milk; TZM-bl reporter cells were infected with a range of H9/HIV_{IIIB} cell concentrations in milk and assayed for infection by luminescence reporter activity (relative luminescent units, RLU). (b) TZM-bl cell infection by H9/HIV_{IIIB} cells in milk was measured after passage of the milk plus cells through SDS-containing non-woven fiber inserts. Reporter activity (infectivity) is plotted as the equivalent number of H9/HIV_{IIIB} cells, calculated using the calibration assay shown in (a). 3 repeat experiments were performed and individual data plotted for all experiments; all used a fluid flow rate of 4.3 ml/min and pulse rate of 60 pulses/min, and 5ml aliquots were collected to measure infectivity. The standard error between repeat measurements is displayed for all tests. Average reduction in HIV infectivity was significant with $p < 0.0001$ (**) or $p < 0.05$ (*) based on paired t-tests.

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Figure 1a
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Figure 1b
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Figure 1c
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Figure 2a
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Figure 2b
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Figure 2c
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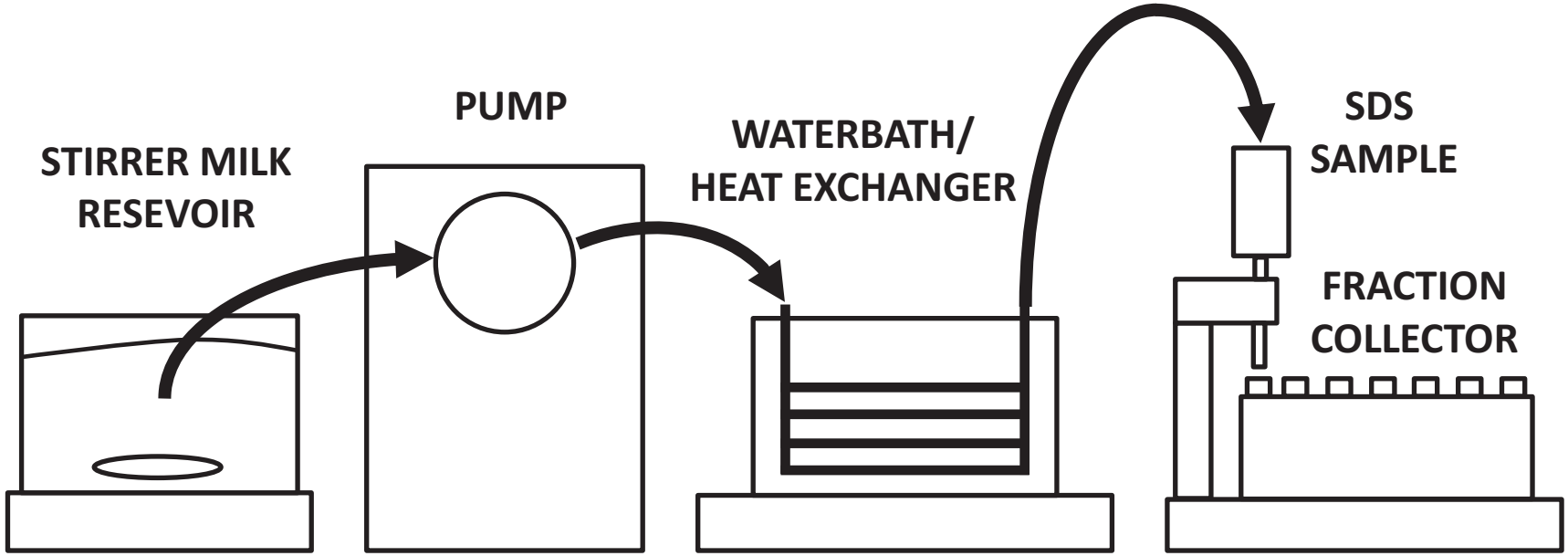
Figure 2d


STIRRER MILK
RESEVOIR
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HEAT EXCHANGER
SDS
SAMPLE
FRACTION
COLLECTOR

Figure 3-6 - Resubmission

Fig. 3.

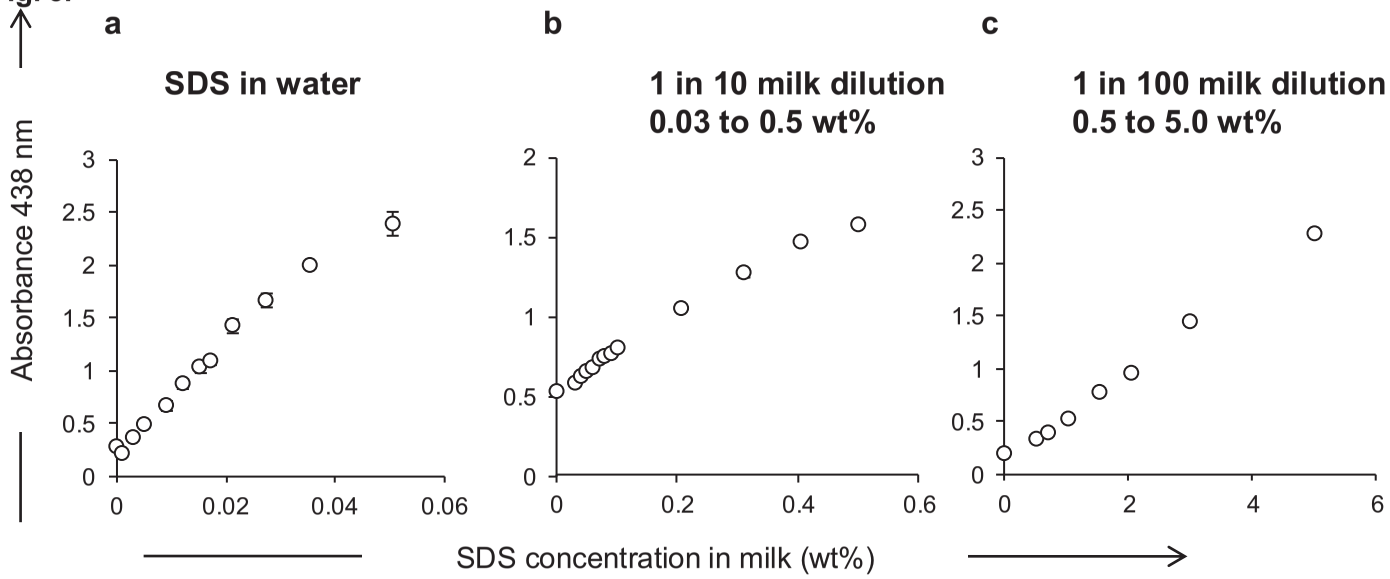
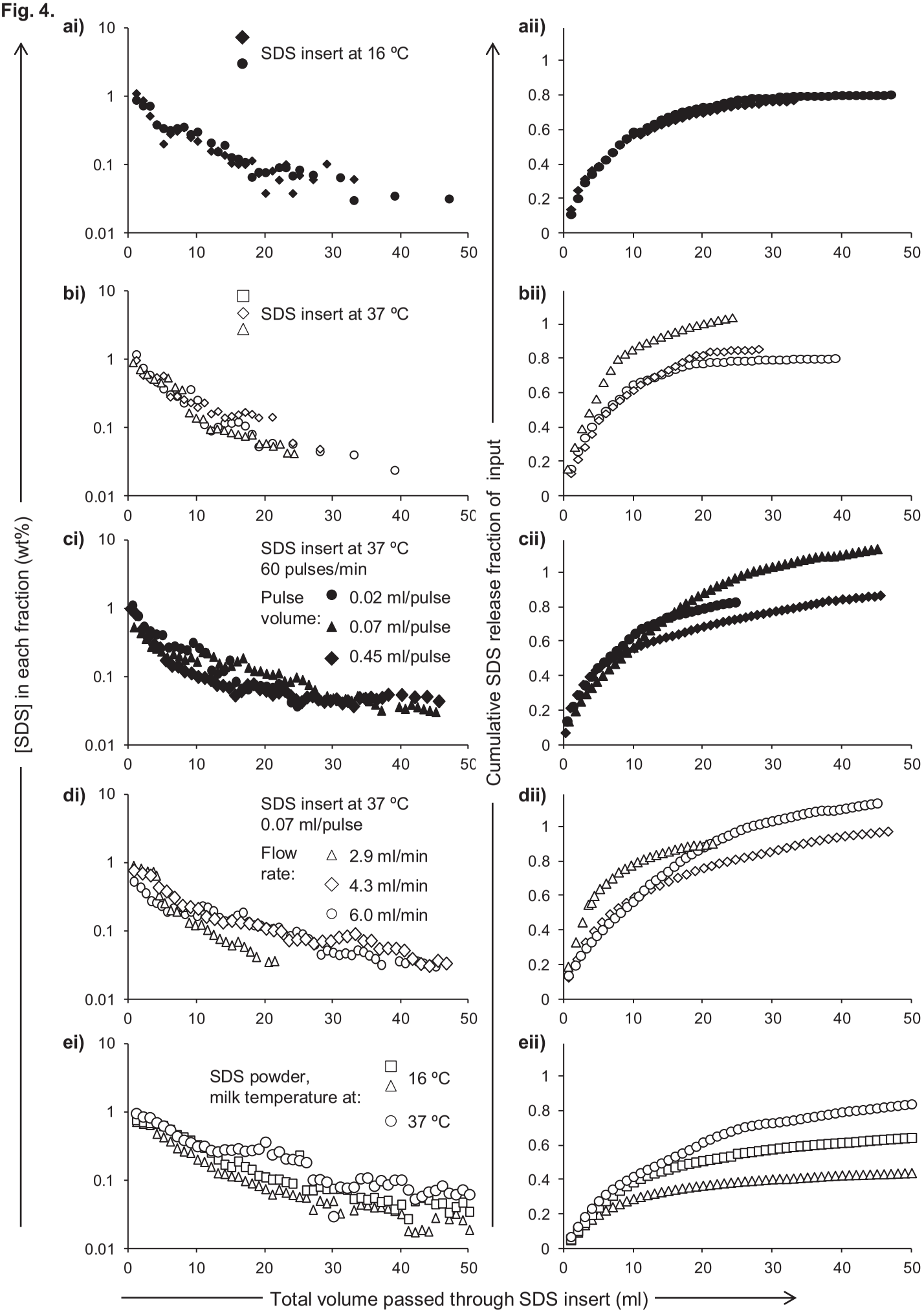


Fig. 4.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SDS insert at 16 ºC
aii)
bi)
SDS insert at 37 ºC
bii)
ci)
SDS insert at 37 ºC
60 pulses/min
Pulse volume:
0.02 ml/pulse
0.07 ml/pulse
0.45 ml/pulse
cii)
di)
SDS insert at 37 ºC
0.07 ml/pulse
Flow rate:
2.9 ml/min
4.3 ml/min
6.0 ml/min
dii)
ei)
SDS powder, milk temperature at:
16 ºC
37 ºC
eii)
[SDS] in each fraction (wt%)
Cumulative SDS release fraction of input
Total volume passed through SDS insert (ml)

Fig. 5.

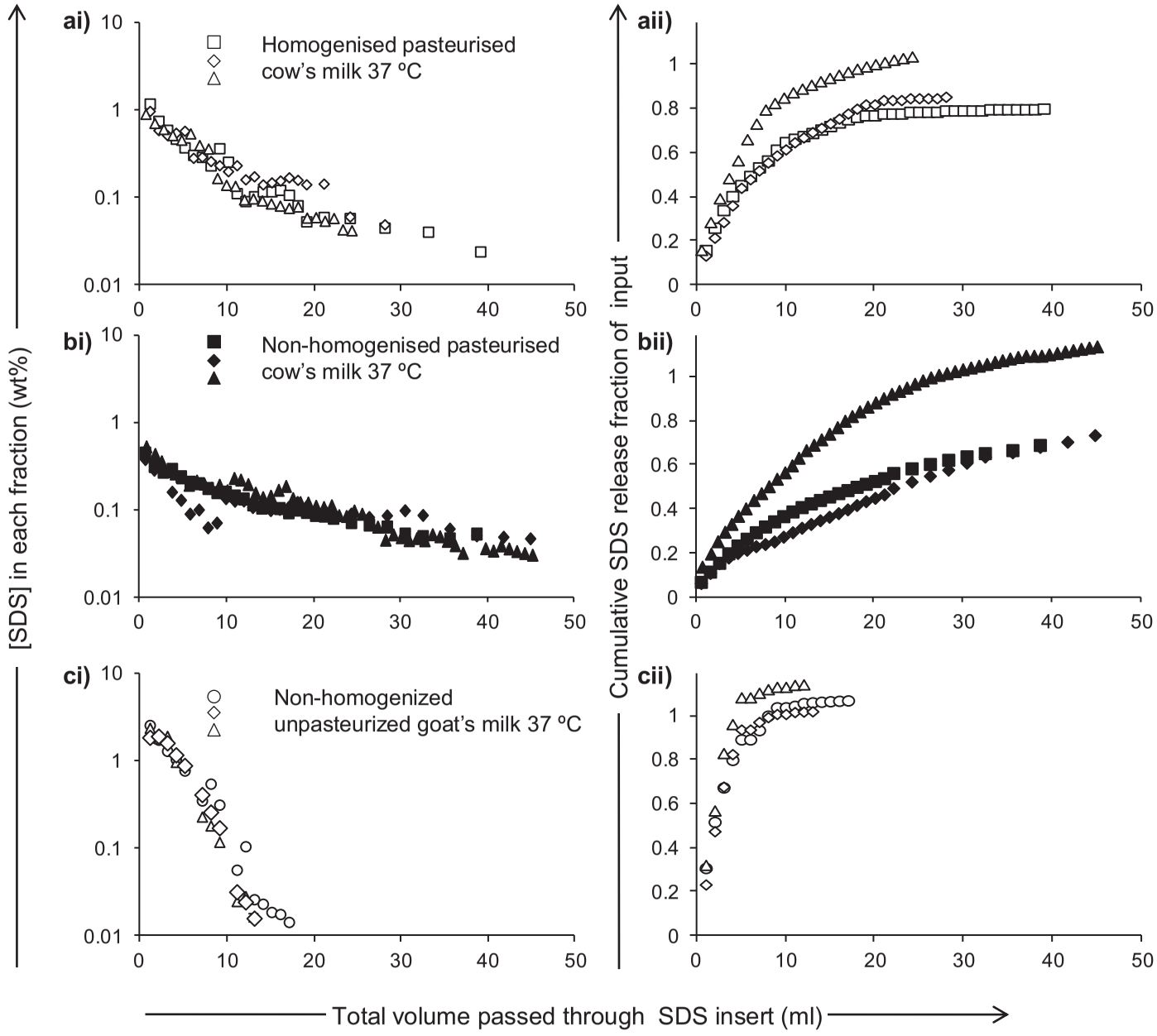
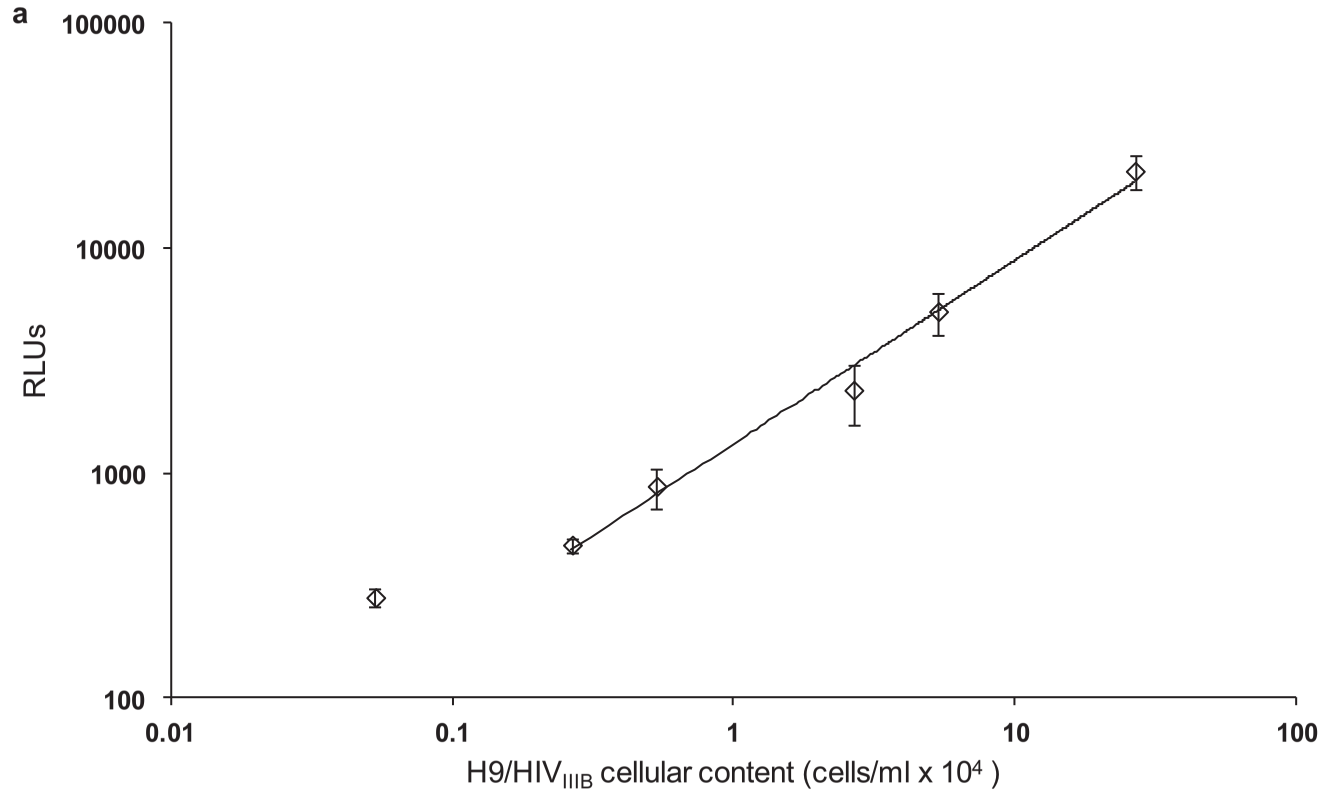

ai)
Homogenised pasteurised cow's milk 37 ºC
aii)
bi)
Non-homogenised pasteurised cow's milk 37 ºC
bii)
ci)
Non-homogenized unpasteurized goat's milk 37 ºC
cii)
[SDS] in each fraction (wt%)
Cumulative SDS release fraction of input
Total volume passed through SDS insert (ml)

Fig. 6.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100
RLUs
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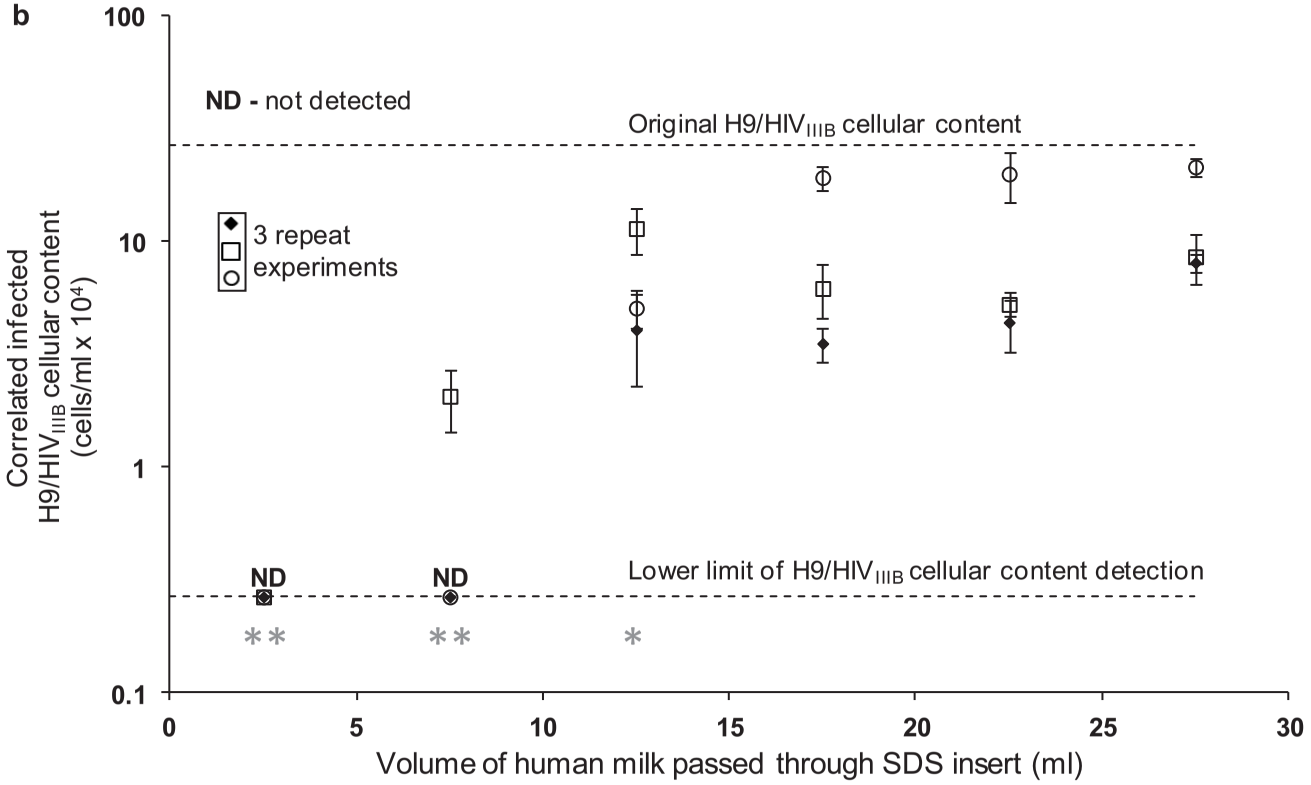
0.1
1
10
100
H9/HIV$_{IIIB}$ cellular content (cells/ml x 10^4)


b
100
10
1
0.1
ND - not detected
Original H9/HIV$_{IIIB}$ cellular content
3 repeat experiments
Correlated infected H9/HIV$_{IIIB}$ cellular content (cells/ml x 10^4)
ND
ND
Lower limit of H9/HIV$_{IIIB}$ cellular content detection
**
**
*
0
5
10
15
20
25
30
Volume of human milk passed through SDS insert (ml)