Inkjet Printing of Function-Spacer-Lipid (FSL) biomolecules

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ABSTRACT

FSL constructs (KODE Biotech Limited) consist of 3 parts: a functional head group (F), a spacer (S) and a lipid tail (L). The lipid tail acts as an anchor to localise the functional head group to a lipophilic surface, e.g. a cell membrane. In addition to satisfying the requirement for biocompatibility, the ability of the FSL constructs to disperse easily in water allows solutions of the constructs to be substituted for the ink in inkjet printer cartridges. FSL constructs can then be printed onto solid surfaces using a standard desktop printer and are being used to develop prototype diagnostic assays (Bovin *et al*, 2011). The constructs can be printed as words or in desired patterns. FSL constructs comprising ABO blood group antigens or biotin as the functional head groups (F) have been printed onto silica, nitrocellulose and paper. The printed constructs have then been used to identify antibodies in different biological samples. The inkjet printing of FSL constructs provides the advantage that multiple antibodies can be identified and quantified in the same reaction well. The inkjet printing of FSL constructs is proving to be a fast, inexpensive and flexible method of producing diagnostic assays.

INTRODUCTION

KODE™ Technology (KODE Biotech Limited) is a platform technology that allows antigens or other epitopes to be stably, easily and controllably incorporated into membranes by the use of FSL constructs (Frame *et al*., 2007; Henry, 2009; Heathcote *et al*., 2010). FSL constructs containing carbohydrates, peptides, fluorophores and biotin as the functional head group (F) are currently available (www.kodebiotech.com). Simple mixing of cells with a solution of these constructs allows the functional head group (F) to be localised to the cell membrane, creating modified cells referred to as "kodecytes". The amount of antigen localised to the cell surface is controllable. Kodecytes are currently used as control cells for blood grouping (Henry, 2009). Due to the novel properties of FSL constructs many other applications are possible. In particular, the dispersiblity of the constructs in water makes them ideal for use as "bio-ink" or "bio-glue" in inkjet printing.

Inkjet printing is an interesting and emerging technology in many different areas and is providing a fast, inexpensive and flexible method of delivering many different materials, including bio-molecules, to different surfaces. Its advantages of being precise and reliable have given it applications in electronics and optics (Calvert, 2001; Cooley *et al*., 2002), which have been extended to biomaterials and biomedical devices. The droplets can be in the picolitre to microlitre range making inkjet technology desirable for printing proteins, nucleic acids and cells in the fabrication of biosensors, microarrays, tissues and organs. Fluid micro-dispensing in this manner allows non-contact delivery of droplets into exact positions offering a precise tool for bio-surface and tissue engineering (Boland *et al*., 2007; Boland *et al*., 2006).

Standard inkjet printers have been employed to deliver various molecules for a range of applications. Both thermal and piezoelectric inkjet printers have been shown to successfully print proteins. Examples include immunoglobulins for ELISA plates (Lonini *et al*., 2008), multi-analyte patterning of biotinylated proteins and thiols on gold substrates to create biosensors, and HRP (horseradish peroxidase) enzyme patterning in microfluidic paper-based devices (Khan *et al*., 2010). These proteins remained active after printing and any degradation was shown to be insignificant, indicating shear or heat stresses did not damage the molecules in the process.

Cells have also been printed using inkjet technology. Cell patterning and printing offers the potential to produce 3D cellular structures in a layer-by-layer process with a goal of printing organs and tissues for transplantation or testing purposes. Organ printing has been defined as computer aided, inkjet 3D tissue engineering (Mironov *et al*., 2003) and the feasibility of printing viable cells has been demonstrated. Xu *et al*. (2005) used a thermal printer to deliver mammalian cells to hydrogels, which proliferated and differentiated indicating the cells survived the stress and heat of printing. This technology presents a method of simultaneously delivering a variety of cells along with growth factors and nutrients to allow cells to fuse and grow into desired structures.

The research reported here is focusing on the reactions in immunoassays of printed FSL constructs, the mechanism of immobilization, and onto what surfaces the FSL constructs can be printed. We are now determining if cells can be

attached to printed FSL constructs and then if kodecytes themselves can be printed.

I Materials and Methods

A. Printer Setup and Printing Solutions

An Epsom Stylus T21 piezoelectric inkjet printer was used to print the solution of FSL constructs (Fig 1). Epsom cartridges use a sponge to ensure a constant pressure of ink on the nozzles. Due to cleaning and the large volume required to refill these cartridges, sponge-less, refillable cartridges were obtained and were modified to allow small volumes (<1 ml) to be injected into the cartridge. Cartridges were filled by connecting a tube to a sealed chamber above the ink outlet. The cartridges were cleaned with 70% ethanol and DI water before adding printing solutions.

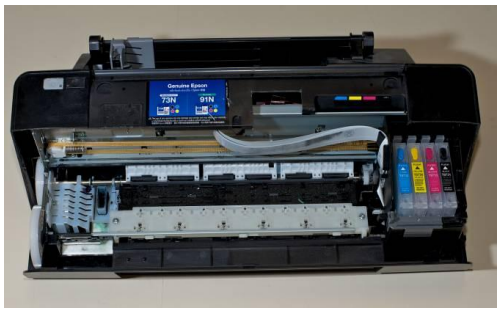

Fig. 1. Epsom Stylus T21 piezoelectric printer used to print FSL constructs.

Three FSL constructs were obtained (KODE Biotech Limited):

'FSL-A' with a blood group A trisaccharide epitope GalNAca3(Fuca2)Galb (FSL-A(GALNa3[Fa2]GALb)-SA1-L1, cat # 421604);

'FSL-B' with a blood group B trisaccharide epitope Gala3(Fuca2)Galb (FSL-B(GAL3[Fa2]GALb)-SA1-L1, cat # 199283); and

'FSL-biotin' with a single biotin moiety as the functional head group (F) (FSL-CONJ(1Biotin)-SC2-L1, cat # 187786).

Solutions containing 1mg/ml concentration of each FSL construct were prepared for printing in phosphate buffered saline (PBS), pH 7.2. The dye bromophenol blue was included in the solution at a concentration of 0.5% so the printed area could be observed. The functional moiety (F) of the FSL constructs was also printed, e.g. the A trisaccharide (F) of FSL-A.

Patterns were designed using standard graphics software. The colour management of the printer was adjusted to print the desired solution from the correct cartridge. Most desktop printers will mix inks from different cartridges to produce the majority of colours. It was important to be able to print specifically from one cartridge or multiple cartridges containing different solutions of FSL constructs.

The printer was primed by simulating the printing of blocks of colour after inserting the cartridge. This removed air bubbles and pulled the printing solution through to the print head. Epsom printheads are located inside the printer and not on the cartridge as with other printers. It is important to ensure the solution has flowed though the system to the print head. Printing surfaces were threaded through the printer using the paper feeder. All surfaces were flexible enough to be able to be printed on in this way. After printing the surfaces were dried and stored at RT.

For some assays 3mm clear acrylic plates with 32 holes were attached to the printed surfaces to create reaction wells. These plates were designed to fit the footprint of half a microwell plate. This enabled the fabricated microwell plates to be compatible with automatic equipment used for microwell ELISAs (Enzyme-Linked Immunosorbent Assay). The acrylic plates were produced by laser cutting 32 wells using the same template produced for printing the FSL constructs (Fig 2). This enabled exact matching of the printed areas and the holes to create 32 discrete reaction wells on one plate. The printed surface was washed in deionised (DI) water for 30 minutes, air dried, and the acrylic plate then glued to the printed surface using multipurpose spray glue. The blank wells are then ready for immunostaining/testing.

Fig. 2. Acrylic plate cut with 32 holes for reaction wells.

B. Printing Surfaces

Surfaces printed onto include silica thin layer chromatography (TLC) plates (silica 60), nitrocellulose (0.2µm pore size), standard office printer paper, a variety of coated papers, fabrics and transparent overhead projector plastic.

C. Immunostaining

Surfaces were washed with DI water for 30 minutes to remove the printing dye and air dried. The surface appeared blank at this stage. Surfaces were then blocked with bovine serum albumin (BSA) for 1 hour at room temperature (RT) to prevent non-specific binding. The BSA was removed and the surface incubated with antibody or binding molecule at

the required concentration for 1 hour at RT. For blood group antigens this was monoclonal or polyclonal antibodies. For biotin this was streptavidin conjugated to alkaline phosphatase. The surfaces were then washed 6 times in PBS to remove unbound molecules followed by incubation with a 1 in 400 dilution in BSA of the secondary antibody or binding molecule conjugated to alkaline phosphatase for 30 minutes at RT. For blood group antigens the secondary antibody was species-specific anti-Ig. For biotin, no secondary molecule was used as a conjugate was used in the primary binding. The surfaces were washed again 6 times in PBS and once in tris substrate buffer pH 9.5. A 1 in 50 dilution of the chromogenic substrate NBT/BCIP (nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche)) in substrate buffer (pH 9.5) was then flooded over the surfaces for 15-30 minutes. The alkaline phosphatase converts the substrate from a yellow to a dark purple which is easily readable. The areas where the antibodies or binding molecules have attached are clearly visible. Membranes were washed in DI water to stop the reaction.

II. Results

A. Blood Group Antigen FSLs

FSL-A and FSL-B have been printed onto different surfaces in a variety of designs. When printed alongside A trisaccharide and B trisaccharide only the FSL molecule was detected. This indicates that printing in the form of the FSL construct is required to localise these molecules to the surface and that the trisaccharides on their own were washed away during the immunostaining process. Fig 3 shows a format where the FSL-A was printed with the well coordinate. The positive reactions are easily identified and traceable as they read the location of the positive reaction. Fig 4 shows a well where FSL-A has been printed in a ladder format in increasing amounts from 0 to 100% using the grey scale on the printer. The numbers printed alongside are assigned to each band of the ladder and would indicate at which point the reaction stops, i.e. how much antibody is in the sample. The option to include as a positive control the printing of a construct that would react with all serums is available. The numbers presented in these results were printed in font size 6 and are easily readable with the naked eye.

Fig 5 shows FSL-A and FSL-B printed onto paper and reacted with polyclonal human O serum, which contains antibodies to A and B antigens. Both the A (FSL-A) and B (FSL-B) were visible in the well, showing the antibodies in the serum had bound to the immobilised antigens, and hence were detected using the colorimetric system. The unprinted area surrounding the printed letters acts as a negative control and the background signal, hence each well has the possibility of including a positive and negative control.

Fig 6 shows the structure of FSL-A printed, using FLS-A as the ink, onto paper and immunostained using monoclonal anti-A, representing the flexibility inkjet printing offers.

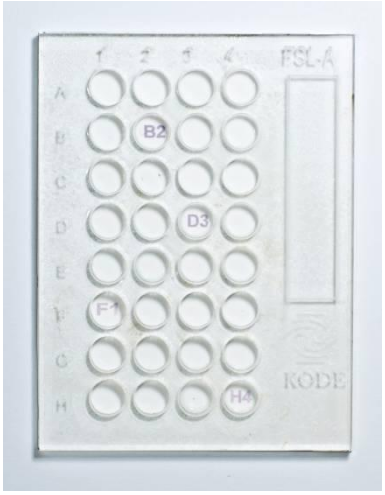

Fig. 3. The well coordinate printed using FSL-A at a concentration of 1mg/ml onto silica, attached to an acrylic plate and 4 wells immunostained with monoclonal anti-A at decreasing concentrations.

Fig. 4. FSL-A printed onto silica in a ladder format in decreasing amounts, attached to an acrylic plate and immunostained with monoclonal anti-A.

Fig. 5. FSL-A and FSL-B printed onto paper at a concentration of 1mg/ml, attached to an acrylic plate and immunostained with polyclonal serum.

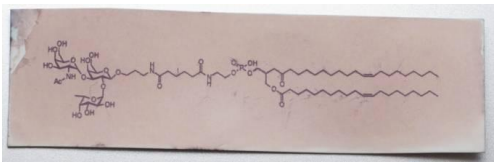

Fig. 6. The structure of FSL-A printed onto a coated paper with FSL-A, at a concentration of 1mg/ml.

B. Other FSL constructs

FSL-biotin has been printed onto various surfaces. Biotin is a water soluble vitamin and is used widely in biological applications due to its high affinity for the tetrameric protein avidin. Being able to immobilise biotin to different surfaces allows the opportunity to attach a wide variety of molecule to these surfaces through the avidin-biotin bond, one of the strongest known in non-covalent protein-ligand attachments.

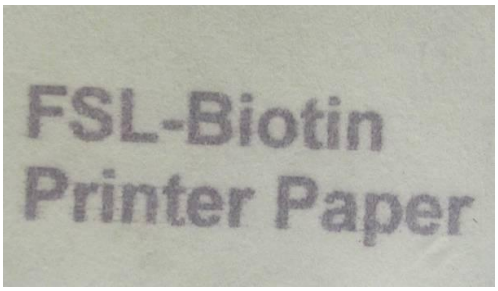

Fig. 7. FSL-biotin printed at a concentration of 1mg/ml onto standard printer paper, stained with streptavidin conjugated to alkaline phosphatase, and NBT/BCIP substrate.

III. Discussion

This research is focused on the inkjet printing of FSL constructs for use in the fabrication of microarrays and diagnostics assays, and eventually for the immobilization of living cells (Henry, 2011). FSL constructs permit multiple antigens and epitopes to be localised to surfaces using the same methodology. It is the lipid tail (L) of the FSL constructs that enables the functional head groups (F) to be localised to the surfaces. A and B trisaccharides lacking this lipid tail are not detected in the assays employed here. It is assumed the trisaccharides are washed away during the blocking and washing steps of the assays.

Inkjet printing of blood group antigens and peptides of blood transferable diseases creates a fast, inexpensive and flexible method of developing diagnostic assays. The format of these assays can range from wells in an acrylic plate, compatible with automation equipment, to dipstick assays easily used in developing countries. All assays are carried out at room temperature and don't require any sophisticated equipment. The flexible, digital control inherent in inkjet printing means FSL constructs can be printed as words, barcodes, or patterns of any shape or size to create bioactive areas capable of detecting multiple antibodies in biological samples. As well as blood group assays, this technique can be extended to diseases where multiple antigens can be printed in the words of each disease, creating a multiplex assay capable of detecting many different antigens at the same time with a very small amount of blood.

The technology can potentially allow any antigen to be localised to a surface and therefore has great potential for developing biological assays and diagnostics tests. There is also potential to immobilise cells and/or kodecytes onto surfaces printed with FSLs to create layers of cells. These could be the initial steps in the construction of 3D structures, printing multiple cell layers which will fuse together (Henry, 2011).

IV. Conclusion and Future Work

Inkjet printing of FSL constructs is fast, precise and versatile making it highly suited for bio-assay development, multi-analyte tests and tissue engineering.

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