SENSOPAD

SENSING ENDOMETRIOSIS ON PORTABLE AUXILIARY DEVICES

D1.3

Cell DNA Isolation

International Iberian Nanotechnology Laboratory

28/02/2025

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Project Information

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Abbreviations

Table 1: Abbreviations

WP	Work Package
ED	Endometriosis
sensoMFgFET	SENSOPAD Microfluidic graphene field effect transistor
µF	Microfluidic
gFET	Graphene Field Effect Transistor
D1.3	Deliverable 1.3
MF	Menstrual fluid
RBCs	Red blood cells
WBCs	White blood cells
KPI	Key performance indicator
PDMS	Poly(dimethylsiloxane)
CAD	Computer-aided design
GPTMS	(3-glycidoxypropyl)trimethoxysilane
LMW	Low molecular weight
MW	Molecular weight
MES	2-(N-morpholino)ethanesulfonic acid
DD	Deacetylation degree

Executive Summary

Work Package (WP) 1 is focused on the preliminary investigation of selection of materials and biological entities to be used along the project, as well as the fabrication of preliminary devices. Within it, **Task 1.3** is further focused on the selection of the sample processing workflow and reference sample preparation for successful isolation of cell DNA. **Deliverable 1.3,** presented herein, covers the advancements made to arrive at the selected sample processing workflow, as well as the experimental work performed to achieve the idealized cell DNA isolation. Preliminary results show that the selected strategy for DNA capture and recovery, using microfluidic devices with chitosan-functionalized micropillars, can be optimized to achieve the necessary isolation efficiency for downstream graphene sensing strategies.

1. Introduction

1.1 Context of Deliverable 1.3

The **SENSOPAD** project is built on the ground-breaking notion that not only the diagnosis of endometriosis (ED) can be done earlier than current practice, but that technology should be developed to empower users and streamline the diagnostic process. SENSOPAD's **Microfluidic graphene Field Effect Transistor (sensoMFgFET) device** looks to tackle these notions by bringing to the clinic a portable, benchtop point-of-care platform that incorporates a microfluidic (μF) system integrated with bio-conjugated graphene Field Effect Transistors (gFET), that will have the capability to capture specific DNA Single Nucleotide Polymorphisms (SNPs) from menstrual fluid, which serve as early indicators of ED. Based on these concepts, this report for **Deliverable 1.3 (D1.3)**, part of **Work Package 1 (WP1)**, describes the considerations that must be made when designing the sample processing workflow, given what is known about menstrual fluid characteristics. Moreover, the report covers the experimental work being performed to achieve idealized cell DNA isolation by exploring microfluidic devices with chitosan-functionalized micropillars.

1.2 Objectives

The goals for T1.3, and reported on this D1.3, are as follows:

- Identify menstrual fluid sample processing workflow for sensoMFgFET device;
- Design and fabrication of microfluidic devices for DNA isolation;
- Preliminary experiments for the optimization of DNA capture and recovery.

2. Identify menstrual fluid sample processing workflow for the sensoMFgFET device

Prior to the design of any µF module or experimental workflow, it is crucial to better understand the type(s) of sample(s) that the terminus of the project will use. In particular, it is fundamental to consider the specificities of menstrual fluid (MF) when compared to other typically used biological samples (e.g., cell line culture medium or peripheral blood). Moreover, there are key factors for sample processing using µF devices that must also be considered, namely sample volume, viscosity, expected cell/particle populations, as well as their heterogeneity in size/volume/morphology. All these must be considered before selecting the appropriate sample processing workflow for the sensoMFgFET device.

2.1 Composition of menstrual fluid

The clinical value of MF derives from its dual nature, being a composite fluid that can be roughly divided into two major fractions: fluid and tissue [1], with great variability between women and across the menstruation cycle. The “blood-like” fluid fraction can vary between 1.6% and 81.7%, with a mean of 36.1% [2], although the fluid fraction can go up to 50% in women with heavy menstrual flow [3].

This fluid fraction is largely similar to peripheral blood, containing plasma, red blood cells (RBCs), white blood cells (WBCs), platelets, and dissolved proteins. RBCs are the dominant cell type, although with a lower concentration than in venous blood (a few million per mL), and they must be dealt with during µF processing. As for WBCs, they comprise a diverse population, including lymphocytes (roughly 7–10 µm in diameter) such as T cells and natural killer cells, monocytes (15–20 µm), neutrophils (~12–15 µm), and other granulocytes. In MF, immune cells often constitute 70–90% of the viable nucleated cells in the fluid fraction, reflecting a robust inflammatory and immune regulatory milieu [4].

As for the tissue fraction, it consists of a mixture of cervicovaginal mucus, secreted factors, and fragments of the endometrial lining, derived from the functional layer shed during menstruation. These fragments (which range from tens of micrometers in diameter to several millimeters) are composed of extracellular components and multiple cell types, including glandular epithelial cells (10–20 µm), stromal fibroblasts (15 to 30 µm), and a small (approximately 0.01–0.1% of the total nucleated cells) but functionally significant subset of mesenchymal stem cells (15-25 µm) [4,5]. This poses challenges for efficient µF sample processing, requiring methods that can efficiently handle a wide range of particle sizes.

Finally, while the total volume of menstrual blood collected over a menstrual cycle typically ranges from 30 to 80 mL, many research protocols rely on collecting smaller aliquots (e.g., 5–10 mL per donation). Reported processing protocols include serial passages of MF through 100 µm and 70 µm cell strainers and dissociation of retained cellular clumps, resulting in 2.6 million nucleated cells per mL from the fluid fraction. **Table 2** provides a short summary of the key MF characteristics identified.

Table 2: Key MF characteristics for sample processing workflow selection and cell DNA isolation and recovery

Fluid fraction	~36% of MF (possibly varying between 1.6% to 82%); composed of RBCs, WBCs (lymphocytes, T cells, natural killer cells, monocytes, neutrophils and other granulocytes), platelets, dissolved proteins
Tissue fraction	~64% of MF; composed of cervicovaginal mucus, secreted factors, and fragments of the endometrial lining containing, for example, glandular epithelial cells, stromal fibroblasts, and mesenchymal stem cells
Volume	30 to 80 mL (but highly person and menstrual cycle stage dependent)
Cell concentration	2.6 million nucleated cells per mL from the fluid fraction
Genomic DNA available	15,600 ng/mL from the fluid fraction (assuming 2.6 M cells/mL)

2.2 Implications for microfluidic sample processing

The unique composition of MF presents several challenges for developing diagnostic and analytical technologies, especially µF platforms tailored for its processing. Standard µF devices are typically optimized for processing simpler samples (e.g. cell re-suspended in saline buffers), although there are multiple examples of devices capable of processing samples such as peripheral blood - a relatively homogeneous suspension of single cells with well-defined viscosity and particulate size. However, as presented above, MF exhibits several characteristics that necessitate sample processing and device design modifications.

To start, the presence of a high percentage tissue fraction substantially increases the likelihood of particle aggregation and clogging of devices. Menstrual samples could be pre-processed using 70-100 µm cell strainers to mitigate the challenges posed by tissue clumps. The clump fraction could then be dissociated to retrieve nucleated cells, ensuring µF compatibility of the treated fraction. Complementarily, on-chip filtration should be included in the initial stages of the sensoMFgFET device. These stages can use micro-sized sieves capable of trapping any remaining large clump without the risk of fouling the overall device.

Another aspect to consider is the variability in viscosity found in MFs that will affect flow behavior in microchannels. As such, sample processing must consider pre-processing steps (such as dilution or enzymatic degradation of mucus) or even incorporate adaptive flow controls to arrive at a standard viscosity across samples.

The volume of samples to be processed must also be carefully considered. Since the sensoMFgFET device aims to detect specific SNPs from genomic DNA with a graphene sensor, enough genomic material must be obtained for successful detection. A classic estimation of DNA amount per nucleated cell situates it at 6 picograms per cell. Assuming 2.6 million nucleated cells per mL of the fluid fraction alone [6], at least 15,600 nanograms of DNA could be retrieved per mL. Allowing for a nucleated cell sorting efficiency of 75% (*KPI-5*) and a DNA extraction efficiency of 50% (*KPI-6*), it could be estimated that our goal is to retrieve approximately 5,850 ng per mL of the original MF sample. Volume-wise, literature examples typically report the processing of samples between 1 and 5 mL (from a larger MF

sample typically within 30-80 mL). Besides this starting value, extra volumes must be considered from processes such as tissue enzymatic de-complexing, RBC lysis agent, and cell sorting buffer. This means that the final sensoMFgFET device must be able to accommodate total volumes of up to 10 mL or more. This can be achieved by multiplexing some of the modules, which increases the total flowthrough of the device and reduces the risks associated with a specific module being clogged/affected by the sample components.

Regarding cell type composition, as previously planned in the project's original proposal, it will handle the major background of RBCs by including an RBC lysing module based on μF mixing of the sample with an RBC-specific lysis agent. This can be efficiently performed with designs such as the "herringbone device", which is capable of mixing samples at a high flow rate (100s μL/min) in a fast manner (a few minutes) [7]. As for the remaining cells, the main goal is to isolate as many nucleated cells as possible for downstream graphene sensor processing. Given the cell types and typical sizes reported above, the cell isolation module should thus aim to sort out all particles >5 μm in diameter. This size-based isolation can be performed in a label-free manner with techniques such as "deterministic lateral displacement", which can be tailored to separate and sort out particles of specific size ranges simply by altering design features (such as the micro post diameter, gap, and angle of shift between different columns of micro-posts) [8]. After the cell isolate is obtained, another module for cell lysis will be incorporated, followed by the module to perform cell DNA isolation. The upcoming **Deliverable 1.5** will further discuss in detail the work being performed on the design and simulation of the various μF modules mentioned previously, while the upcoming section of this report will focus on the experimental work performed until now on the optimization of the DNA isolation stage of the sensoMFgFET device.

3. Design and fabrication of microfluidic devices for DNA isolation

Within the sensoMFgFET device, the DNA isolation is the last µF module before the integrated gFET graphene sensor. As previously mentioned, its aim is to isolate, capture and recover genomic DNA for SNPs detection, and, as per KPI-6, must perform those tasks aiming for a DNA extraction efficiency of 50%. Also as previously mentioned, the device might have to deal with 1000s of nanograms of DNA per sample, with DNA possibly being found with different lengths or clustered in different forms (depending on the cell lysis module chosen process and outcomes). Thus, the device must be optimized to not only capture the DNA with high efficiency but to then be able to release it and allow for efficient recovery of the same. Not only that but the sample volumes that might have to be processed could be on the mL scale, thus the design and approach chosen must be scalable, multiplex-able while meeting the criteria set by KPI-6.

Some of the most common techniques for DNA capture are based on microscale solid-phase extraction. These are based on binding DNA to a solid phase, typically due to charge differences between the negatively charged DNA and the positively charged solid phase, followed by washing of unbound material, and the elution of DNA from the solid phase. While silica (in the form of beads or membranes, for example) has been usually the most frequently selected material, it requires the use of reagents that could hinder downstream processing of the recovered DNA [9]. Alternatively, we have selected chitosan as the solid-phase extraction material. Chitosan is a natural polysaccharide that is positively charged at low pH, thus promoting electrostatic interaction with the negatively charged DNA, leading to DNA capture[10]. However, at higher pH values, it returns to an uncharged state, resulting in the release of DNA. This provides a good control over the µF DNA isolation process, as µF devices are capable of easily swapping the fluids and buffers flushing the microchannels. Indeed, we have previously worked on a similar system, from which the design and protocols reported herein take inspiration from [11].

When designing the device, the priority was to optimize the surface-area to volume ratio, thus effectively increasing the amount of chitosan that could be functionalized inside the device. To achieve this, an array of hundreds of micropillars, evenly distributed across the device, was selected. At the same time, if a device has too large features the chance for unwanted clogging increases. Moreover, as the resistance inside the device also increases, the flowrates that can be used without compromising the stability of the device proportionally reduce, reducing the overall volume throughput of the device.

3.1 Protocols for functionalization & DNA capture and recovery

The preparation of the fabricated devices for DNA capture and recovery experiments starts with the surface activation of the PDMS material using oxygen plasma. This standard process in microfluidics not only allows for the irreversible bonding between the device and a glass substrate, but also results in the PDMS surfaces being activated and becoming prone to functionalization with further materials. As such, the first step of the device functionalization protocol is to flush ethanol through in order to increase the wettability of the PDMS surface and improve its hydrophilicity. After this, GPTMS is added to work as a cross-linker between the PDMS surface and the chitosan polymer being tested. A washing step with ethanol is then performed to remove GPTMS excess from the device, which is followed by the chitosan

functionalization step. A NaCl solution is then flushed in to remove unbounded chitosan, leaving the device ready to be tested.

With the device fully functionalized, the DNA capture and recovery protocol starts by a conditioning step using a MES buffer which protonates the chitosan within the device, resulting in its surface charge now being positive. Following this, the DNA sample being tested runs through the device for the capture stage. The DNA tested included a short and a long DNA model, with ~230 bp and ~48,500 bp in average. After DNA has been captured, the MES buffer is used in a washing step to remove unbounded DNA and assess the capability of the device for DNA retention. Finally, DNA recovery is achieved, as previously mentioned, by increasing the pH conditions inside the device, effectively deprotonating the chitosan, rendering it electrically neutral, and thus permitting the captured DNA to be released and eluted out of the device. This recovery step is done using a Tris-HCl buffer. The experimental setup used for both device functionalization and the DNA capture and recovery experiments can be seen on **Figure 1**.

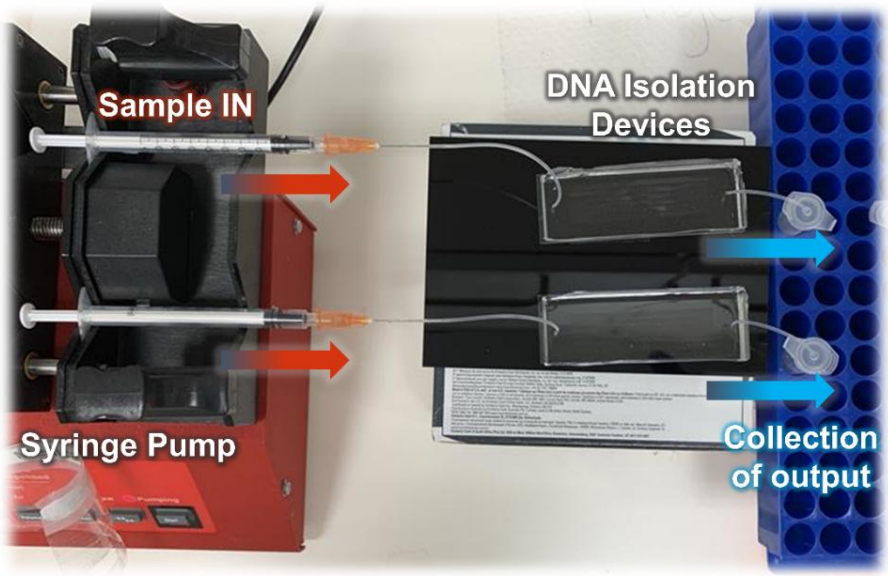

Figure 1: Photo of experimental setup of DNA isolation module.

3.2 DNA capture & recovery assessment

The preliminary experiments for optimizing DNA capture and recovery from the µF module focused on assessing the influence of three main components: the type of chitosan used, the flow rate applied to the capture step, and the type of DNA tested. Previous reports have shown that longer polymer chains in relatively high MW chitosan can more easily capture DNA once the initial electrostatic interaction has happened [13]. Moreover, the so-called deacetylation degree (DD) of a chitosan also seemed to play a role, as reported in a different work [10].

3.3 Opportunities for optimization

Although the current iteration of the cell DNA isolation module already seems to provide with positive results, there are a plethora of options for protocol optimization. These will be explored as part of WP5 (M3-M18), specifically on Task 5.2, dedicated to "*Optimization and testing of the sample preparation microfluidic modules with biological surrogates*".

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