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Development of Electrospun Nanofiber Mesh for the Enhancement of DNA Recovery from Spermatozoa in Sexual Assault Evidence

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Development of Electrospun Nanofiber Mesh for the Enhancement of DNA Recovery from Spermatozoa in Sexual Assault Evidence

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Abstract

One out of every six American women has been the victim of an attempted or completed rape in her lifetime as well as one out of every thirty-three American men. Following a sexual assault, a forensic examination is performed during which probative deoxyribonucleic acid (DNA) evidence can be collected and preserved using a sexual assault kit (SAK). Over 150,000 untested SAKs currently exist in crime laboratories across the United States. Aside from the staggering number of untested SAKs, sexual assaults continue to occur. The untested SAKs need to be tested as do the SAKs collected from the sexual assaults that continue to happen because each SAK that remains untested contains possible DNA evidence that is not aiding in the identification of the suspects of these crimes.

Literature discusses both DNA analysis methods for testing SAKs and engineering products used in separation techniques. Current literature does not, however, discuss the application of engineering products used in separation techniques to the DNA analysis methods for testing SAKs. This lack of current literature provides the basis for and the necessity of this study.

The intent of this study was to enhance the efficiency of the current DNA extraction technique for sexual assault samples in order to expedite the analysis of SAKs and to ultimately work toward the goal of ridding the United States of its existing backlog of untested SAKs, preventing a backlog from happening ever again, and aiding in the identification of the suspects of these crimes. This research aimed to converge the fields of Engineering and Forensic Science by implementing an engineering product, electrospun nanofiber meshes (ENMs), into the traditional sexual assault sample DNA extraction technique, the differential extraction, in an attempt to physically separate sperm cells from epithelial cells based on their inherent size

differences. The engineering products that were implemented were the product of a process called electrospinning.

Three pilot protocols were designed and performed. The first two pilot protocols attempted to physically separate sperm cells from epithelial cells: the first by using an ENM made of polycaprolactone as a filter, and the second by using a polycaprolactone film as a nanosieve. The third pilot protocol also attempted to physically separate sperm cells from epithelial cells but by the incorporation of an ENM into a modified DNA analysis process. Extraction, quantification, amplification, and genetic analysis were all performed on simulated sexual assault samples. The resulting DNA profiles were interpreted and evaluated. Although the DNA profiles obtained were mixed profiles rather than single source profiles, this research was significant based on three positive discoveries. ENMs retain sperm cells; melting the ENMs during incubation does not inhibit PCR or any other aspect of downstream processing; and the resulting protocol merges the typical differential extraction protocol with a solid-phase DNA extraction, which has been automated, thus presenting potential to automate the developed protocol.

Future research should be conducted on each pilot protocol attempted in this study. This includes research to develop membranes that can withstand the heat of a laser, research to determine a way to adhere ENMs to the insides of microcentrifuge tubes, and research to design an automated electrospinning system capable of producing consistent, uniform ENMs. The pilot protocols used in this study prove that the potential to converge Engineering and Forensic Science via separation of sperm cells from epithelial cells based on their inherent size differences exists but that additional research is needed in order to successfully implement ENMs into forensic DNA analysis.

Table of Contents

List of Figures

List of Tables

Introduction

One out of every six American women has been the victim of an attempted or completed rape in her lifetime as well as one out of every thirty-three American men [1]. Following a sexual assault, a forensic examination is performed during which probative deoxyribonucleic acid (DNA) evidence can be collected and preserved using a sexual assault kit (SAK). In an effort to assist and expedite the examination of SAK evidence, a novel method for the analysis of SAK DNA evidence was developed and evaluated in this research project. More specifically, a means to physically separate sperm cells from epithelial cells based on the inherent size difference between the two cells types using electrospun nanofiber meshes (ENMs) was investigated. The separation of sperm cells from simulated victim epithelial cells was the focus of this study because sperm cells originate from seminal fluid, a biological component commonly present following a rape or sexual assault, and sperm cells contain DNA, which can be used to identify the individual from whom it originates.

The State of Sexual Assault Kit Examination in the United States

Tens of thousands of untested SAKs exist across the United States and are in the process of being inventoried and tested for biological and DNA evidence; meanwhile, sexual assaults continue to occur. End the Backlog, part of a national non-profit organization whose goal it is to rid the United States of its backlog of untested SAKs and to prevent a backlog from happening ever again, reports that over 150,000 untested sexual assault kits currently exist in crime laboratories across the United States [2]. End the Backlog has compiled a list of the inventory and reform status of the inventory of each state. The status of each state falls into one of the following three categories: completion of inventory as well as testing of all backlogged SAKs, in the process of testing, or have yet to take inventory [2]. Although there are differing opinions as

to the definitions of "untested" and "backlogged", it is important to note the general difference. The Department of Justice (DOJ) defines untested SAKs as those that have never been submitted to a laboratory for analysis, while backlogged SAKs are those that have been submitted but have yet to be tested thirty days after submission [3]. Aside from the staggering number of untested SAKs, sexual assaults continue to occur. Every 73 seconds, on average, another American is sexually assaulted [1]. In 2016 alone, approximately 320,000 known rapes/sexual assaults took place within the United States [4]. The untested SAKs need to be tested, and the SAKs collected from the sexual assaults that continue to happen need to be tested as each SAK that remains untested contains possible DNA evidence that could aid in the identification of the suspects of these crimes [5]. The intent of this study was to enhance the efficiency of the current DNA extraction technique for sexual assault samples in order to expedite the analysis of SAKs and to ultimately work toward the aforementioned goal of ridding the United States of its existing backlog of untested SAKs, preventing a backlog from happening ever again, and aiding in the identification of the suspects of these crimes.

The option of victims to forego testing, misperceptions about the benefits of DNA analysis, and lack of resources contribute to the number of untested SAKs. Agencies report that, from 2003-2007, an estimated 18% (27,595) of all unsolved rapes had untested forensic evidence [6]. Research into the reason for the high volume of untested evidence provides the following four potential explanations: 1) the victim chooses to not undergo forensic testing, 2) DNA is considered unnecessary by the investigator, 3) the investigator's perception that resources are limited, and 4) resources are truly insufficient to keep up with demand. If a victim does not want her/his SAK tested, the kit is not submitted for analysis. In some instances, DNA analysis is not considered necessary or helpful to the investigation of a reported crime by the investigator. This

occurs primarily when a suspect has not been identified in a case. The unfortunate side effect of this practice is that any DNA profile that would have been obtained from these "suspect-less" cases are not able to be submitted to the national database. In an effort to be frugal with a limited amount of testing supplies, police will often forego submission of a kit if their jurisdiction lacks or is perceived to lack sufficient resources. The lack of resources can be funding, staff, supplies or equipment. Campbell et. al (2017, p. 370) state that, "Resource scarcity may be creating a vicious cycle whereby police do not submit all SAKs for DNA analysis because crime labs cannot test all SAKs, and because DNA testing is a limited resource, law enforcement do not view it as a primary investigative tool." Lastly, a true lack of resources turns into the inability to test all SAKs [7]. While each of the provided reasons is justifiable, when considered in total, the travesty remains: SAKs not being tested, resulting in potential perpetrators not being identified.

Robotic Automation of the Differential DNA Extractions

In this pilot study, ENMs were engineered and evaluated for their potential to be used in the automated analysis of sexual assault samples typically associated with SAKs using robotic methods. A series of ENMs were engineered in-house and implemented into the traditional differential DNA extraction methods for the examination of sexual assault samples in an attempt to physically separate sperm cells from epithelial cells based on the inherent size differences of the two cell types. Current state-of-the-art technologies for the quantification, amplification, and analysis of DNA were also utilized to produce DNA profiling results that would be comparable to those presented in court.

The differential extraction of DNA from sexual assault evidence was originally established in 1985 by Peter Gill, Alec Jeffreys, and David Werret and remains virtually unchanged in present-day forensic science [8]. The differential DNA extraction technique relies

on the inherent differences between the strength of epithelial and sperm cellular membranes [9]. The technique results in the nearly complete separation of the sperm nuclei from that of the female cells, which ultimately results in the separation of the male DNA from the female DNA. In 1985, the successful application of the differential DNA extraction technique introduced the capability of positively identifying a suspect in a sexual assault case [8]. The same protocol as originally described is still used by the Federal Bureau of Investigation (FBI) Laboratory, as well as other forensic crime laboratories, to separate sperm cells from epithelial cells in sexual assault samples and is the current standard for sexual assault cases [10].

Differential extractions of epithelial and sperm cells employ a methodology comprised of multiple steps (Figure 1). Peter Gill, Alec Jeffreys, and David Werrett described the processes performed on a vaginal swab sample containing sperm cells. In their study, the sample was incubated in a cell lysis buffer composed of sodium dodecylsulfate (SDS) and proteinase K (Pro K). The mild incubation step preferentially lysed the female epithelial cells. Due to the cross-linked disulfide bridges in the cellular membranes of sperm, the sperm cells withstood the initial mild incubation and were subsequently separated from the female epithelial cells by centrifugation [11]. In the absence of female DNA, the sperm cells were then incubated in cell lysis buffer composed of SDS, Pro K and dithiothreitol (DTT). This much harsher lysis buffer resulted in lysing the sperm cells. The result was the separation of the DNA in the sperm nuclei from the DNA that was located in the female epithelial cells, providing two separate DNA profiles, and thus, the capability of positively identifying a suspect [8].

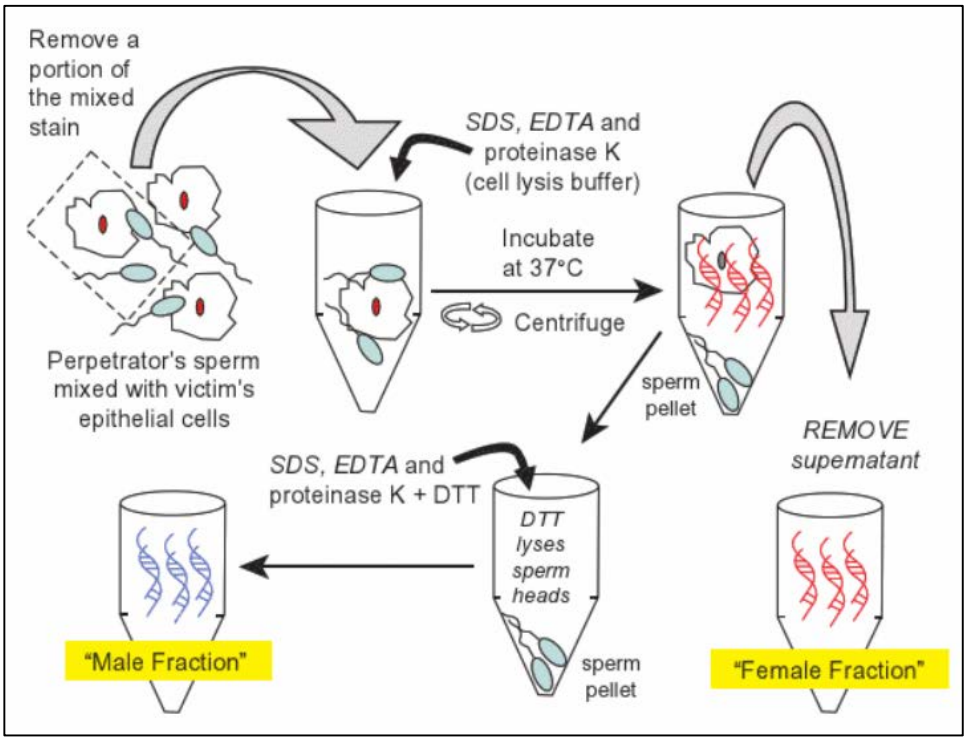

Figure 1: Diagram of differential DNA extraction. The differential DNA extraction process is used to separate DNA from sperm and epithelial cells. A cutting of sexual assault evidence is incubated in a cell lysis buffer composed of SDS and Pro K; this lyses the epithelial cells without harming the sperm cells. The sample is centrifuged, which causes the intact sperm to pellet. The supernatant, containing the epithelial DNA, is removed. The sperm cells are incubated in cell lysis buffer composed of SDS, Pro K and DTT. This lyses the sperm cells and is the "male fraction". From Butler (2011) [12].

Modified versions of the differential extraction have been published over the last thirty years. The original differential extraction publication describes a protocol that washes the sperm pellet one time to remove remaining epithelial DNA [8]. One of the modified versions was published in 1991 by Wiegand et. al. This version is called a mild preferential lysis. Instead of separating sperm cells from epithelial cells, this method aims to reduce the amount of epithelial DNA via multiple wash steps, meanwhile the sperm DNA remains relatively constant. The researchers' claim was that this would help in samples with a low sperm count by eliminating the further loss of sperm DNA caused by differential extraction [13]. The advantage of using additional wash steps over the one wash step used in a differential extraction is that multiple washes physically remove more of the unwanted epithelial DNA; however, multiple washes also

remove some of the sperm, are tedious, and, like differential extractions, are difficult to automate [10]. Therefore, this modification is not adequate to meet the high demand of the untested SAK backlog.

A common occurrence in sexual assault samples is that the epithelial cells of the victim are present in much higher quantity than the sperm cells of the perpetrator. Studies have shown that when the ratio of the minor to major contributor exceeds 1:10-1:20, there is not enough of the minor contributor to be detected. Because the ratio of sexual assault samples often exceeds this range, differential extraction is performed to separate the cells prior to DNA analysis. However, differential extraction has been shown to be capable of losing fifty to sixty-four percent of epithelial DNA and ninety-four to ninety-eight percent of sperm DNA [14]. It is, therefore, crucial that as much DNA be preserved as possible before beginning analysis. Researchers state that alternative cell-separation techniques are needed in order to combat this loss [14].

Multiple factors contribute to the success, or lack thereof, of the overall analysis of a sexual assault sample. The productiveness of a differential extraction is dependent upon the following two factors: the percentage of sperm DNA recovered from a sexual assault sample and the percentage of epithelial DNA that remains in the sperm fraction after the extraction is complete [15]. An ideal result is a sperm fraction that contains no epithelial DNA. Any non-sperm DNA remaining in a sperm fraction extract can result in a mixed DNA sample, which complicates interpretation due to the high sensitivity of current DNA typing systems [9, 15]. At the time that differential extractions were first described in 1985, the DNA typing method used required approximately 5µg of DNA for successful DNA typing. As DNA typing methods have progressed, the amount of DNA required for successful DNA typing has decreased. The

sensitivity of today's DNA typing methods is such that only approximately 100pg, or 0.0001µg, of DNA is required for success [9]. Sexual assault samples containing a much higher quantity of epithelial cells than sperm cells can still be of great value if the sperm cells are able to be successfully isolated from the epithelial cells. The result of this capability has led to a need for improved separation results when limited sperm DNA is present in a sexual assault sample [9].

Automated Techniques for the Analysis of Sexual Assault Evidence

Modern and automated DNA extraction methods exist for many forensic sample types including, but not limited to, body fluid swabs and stains, bone, tissue, teeth, gum, and cigarette butts; sexual assault samples, however, lack an automated method of DNA extraction [16-19]. Of all extraction methods, the differential extraction is the only original method to have remained unchanged [20]. Other extraction methods have successfully advanced to the point of automation. One such automated technique is solid-phase DNA extraction method. The solid-phase DNA extraction methodology lyses all of the cells in the evidence sample using chaotropic salts. The DNA from the lysed cells can then be bound to magnetic silica particles. Once bound, the DNA can be washed and eluted by altering the magnetic field and chemical treatments to which the sample is exposed. The DNA remains, while other material, such as proteins, contaminants, and inhibitors, are washed away. The purified DNA is then eluted off the beads and into a clean sample tube. In 2008, Applied Biosystems released the PrepFiler™ Forensic DNA Extraction Kit. The PrepFiler™ Kit employed solid-phase DNA extraction and was validated as a method which results in high quality DNA from biological samples that is suitable for subsequent analysis [16]. PrepFiler, in combination with the Tecan Freedom EVO® 150 automated liquid-handling workstation, compose the HID EVOlution™ Extraction System. This system can extract up to 96 samples typically in less than 2.5 hours [21]. A single manual

differential extraction, on the other hand, takes hours to complete. A system capable of accomplishing a fully automated differential DNA extraction would have the potential to dramatically reduce the nationwide backlog of SAKs by streamlining the workflow and ultimately reducing the number of hours required to complete the extraction by removing the manual component and allowing for the simultaneous processing of multiple samples.

Two of the reasons for SAK backlogs are the time and cost requirements of the differential extraction process [22]. Not only are time and cost issues with this process but so is the difficulty associated with its automation. Current automated systems are designed to reach to the bottom of tubes to ensure retrieval of all contents. In the case of a differential extraction or mild preferential lysis, a pellet of sperm is formed and remains at the bottom of the tube (Figure 1). If an automated system were to remove the pellet with the epithelial DNA, the process would be ineffective. Several general requirements exist for an automated system to be successful. The first is that an automated system must generate high quality DNA in greater quantity than a manual extraction. The second is that an automated system needs to be able to process all types of samples commonly encountered by forensic scientists. The final requirement is that the contamination risks of an automated system must not be higher than those of manual extraction methods [23]. A study performed on the Biomek® 2000 Laboratory Automation Workstation tested whether the Biomek® system met the requirements. When performing differential extractions, the samples the Biomek® system extracted did provide more sperm DNA data than a manual extraction when the semen dilutions were 1:100 or greater. The conclusion was that the system outperforms manual extractions in sensitivity and sperm DNA yield in instances of “very dilute” semen. However, the Biomek® system could only complete the extraction after the sperm cells had already been separated from non-sperm cells, meaning the Biomek® system is only

partially automated [23]. Although these conclusions were made regarding the Biomek® system specifically, the conclusions apply to all systems that have achieved a level of automation for sexual assault samples; the level of automation is partial at best. Without automation, advanced techniques are required to perform either differential extractions or mild preferential lyses manually. Decisions that require understanding of the process must be made in order to garner the greatest outcome, meaning specific education and training is required. Time, cost, automation restrictions, and personnel requirements are all complications of differential extractions and contribute to a continually growing national case backlog.

Physical Separation of Sperm Cells from Epithelial Cells

The aim of the current research is to expand upon the methodology for the physical separation of sperm cells from epithelial cells in sexual assault samples. In 1998, a method was suggested for achieving separation that is entirely different than the chemical separations in a differential extraction [24]. The research determined that filtration can be used to physically separate sperm cells from epithelial cells. The method used nylon mesh filters and gravity to capitalize on the size differences of the two cell types. The research found that membranes with average pore sizes from 5-10µm allowed sperm cells to pass through while simultaneously keeping the epithelial cells from doing so. More specifically, when using a 10µm pore size filter, approximately 73% of sperm cells passed through the membrane along with about 1-2% of intact epithelial cells and a “small number” of nuclei from spontaneously lysed epithelial cells. Using membranes with average pore sizes larger than 10µm resulted in increasing numbers of epithelial cells passing through the membrane along with the sperm cells. Using centrifugation or mild vacuum filtration is an option to replace gravity; however, the research found that these options tend to result in a smaller percentage of epithelial cell retention and an increase in the number of

epithelial cells crossing the filter. This is presumably due to the malleability of the cell membranes in combination with the centrifugal force, resulting in the cells being forced through the filter. Therefore, Chen et. al (1998) did not use either option, instead opting for gravity filtration. Ultimately, the conclusion was that filtration is a rapid, effective technique for separating sperm cells from epithelial cells and that the resulting filtered sample is sufficiently pure to be followed by the successful performance of PCR-based analytical methods "with little apparent cross contamination" [24].

Chen et al. (1998) included a comparison discussion between Gill's differential lysis method and Wiegand's modified preferential lysis method versus the gravity filtration method. The disadvantage of both Gill and Wiegand's methods is that the separation of sperm cell DNA from epithelial cell DNA is not as efficient as desired, often resulting in cross contamination. The preferential lysis method requires multiple washes; this tends to reduce the efficiency of sperm recovery but decreasing the number of washes tends to result in an excess of epithelial cells in the sperm fraction. The use of the gravity filtration method previously described yields verifiably better separation rates using a much simpler method: separation based on morphological differences of the two cell types. Furthermore, the speed and simplicity of the filtration method lends itself to being useful in laboratories that process high volumes of sexual assault evidence, but the conversion to this method has not occurred because it is not as effective as the chemical-based separation method [24].

Application of ENMs to Forensic Science

This pilot study aimed to converge the fields of Engineering and Forensic Science by implementing an engineering product, ENMs, into the traditional sexual assault sample DNA extraction technique, the differential extraction, in an attempt to physically separate sperm cells

from epithelial cells based on their inherent size differences. The engineering products that were implemented were the product of a process called electrospinning.

Electrospinning is a technique that produces nanofibers of different materials in various fibrous formats that have applications in healthcare, biotechnology and environmental engineering, defense and security, and energy storage and generation [25]. The process of electrospinning begins by charging a suspended droplet of polymer solution to a high voltage. Once that voltage level overcomes the force of surface tension, fine jets of liquid shoot out toward a collector. The jet stretches and lengthens as it shoots; meanwhile, the solvent evaporates. When the jet reaches the collector, the jet results in an interconnected web of small fibers (Figure 2, Image A). The properties of the nanofiber membranes – weight, porosity, strength – are determined by the polymer solution used and the voltage applied to create them [26, 27].

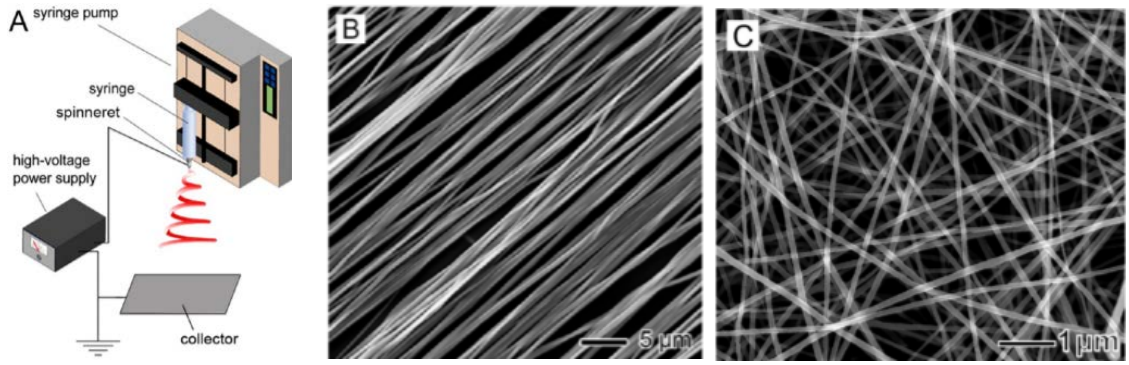

Figure 2: A depiction of the process and pictures of the product of electrospinning. (A) Schematic illustration of a typical setup for electrospinning. A typical setup includes a syringe pump, syringe, spinneret, high-voltage power supply, and a collector. (B) A scanning electron microscope image of nanofibers aligned along a single axis. Nanofibers aligned along a single axis can result when the collector is mechanical, magnetic, or electrostatic. (C) A scanning electron microscope image of nanofibers with no directional order. Nanofibers with no directional order result when the collector is a conductive substrate. From Xue et. al (2017) [28].

Different variations to the process can be implemented depending on whether the application for which the nanofiber membranes will be used requires aligned nanofibers or not.

When the collector is a conductive substrate, the electrospun nanofibers are collected as a nonwoven mat with no directional order (Figure 2, Image C). Should the application require the nanofibers to be in a well-defined arrangement, mechanical or magnetic means can be employed. An example of the mechanical approach is the use of a rotating mandrel as the collector. The rotating mandrel arranges the fibers along the direction of rotation. Magnetic nanoparticles can instead be added to the polymer solution which, when electrospun in a magnetic field, will align along a single axis (Figure 2, Image B) [29].

The process of electrospinning is capable of creating solid continuous fibers of material with diameter in the micro- to nanometer range. Electrospinning is a concept that began in 1934 when Anton Formhals obtained and published a series of patents which described an experimental setup that used an electrostatic force to produce polymer filaments. In 1971, Peter K. Baumgarten was the first to electrospin acrylic fibers to a diameter in the nanometer range. Baumgarten's nanofibers were 500-1100nm in diameter. Since then, research on electrospinning has continued to progress; the diameter of the nanofibers has decreased as the potential applications of nanofibers has increased [27]. As technology has advanced, nanofiber applications have been discovered in a rising number of fields. This research project explores the potential of nanofibers applied to forensic molecular biology.

Fiber dimension and morphology, porosity, surface structure, and chain conformation and crystallinity are features and properties of ENMs that are unique compared to structures produced by other techniques. The diameter of a fiber is influenced by the concentration of the solution used to make the fiber. The stronger the concentration of the polymer, the larger the diameter of the resulting fiber. The diameter of a fiber is also influenced by the voltage applied to the spinneret. The higher the voltage applied, the more fluid ejected, the larger the fiber

diameter. Also, the higher the conductivity of the polymer solution, the smaller the fiber diameter. There are two types of identifiable pores in ENMs. There are pores on/within each fiber, and there are pores between the fibers of a nanofibrous membrane, the latter of the two crucial to this pilot study. The pores of nanofiber membranes are relatively large and fully interconnected, resulting in a three-dimensional framework. Pore size is the determining factor for what can permeate through a nanofiber membrane, versus the porosity, which is simply a measure of the flow across the membrane [27]. Determining the pore size of the ENMs to be used in this project was the first step taken in the methodology. A 2017 study on water treatment and ultrafiltration found that by increasing the number of nanofibers composed of single-wall carbon nanotube/polyaniline (SWCNT/PANI) within a membrane and by applying external electric potential, the pore sizes/selectivity and permeability of the membrane was able to be controlled and manipulated [30]. The surface structure of electrospun nanofibers is dependent upon the electrospinning parameters used – polymer concentration, voltage, and conductivity. Generally speaking, the surface structure is smooth, and the cross-sectional shape is circular. However, using a low concentration polymer solution or a high electrical potential will result in rougher nanofibers. Using a high molecular weight and high concentration polymer will result in a flat nanofiber. This is because the evaporation of the solvent decreases as the solution viscosity increases. The result is wet fibers that are flattened by the impact when they reach the collector. As far as the chain conformation and crystallinity of nanofibers, the polymer chains within the nanofibers are noncrystalline. Although that is the case, they are highly oriented. The direction of orientation reflects the direction in which the nanofibers are elongated as they are electrospun due to the force of the electrospinning jet; the polymer molecules orient themselves in the

direction of elongation, ultimately meaning the resulting nanofibers are oriented in the direction of elongation [27].

In order to move nanofiber membrane synthesis and application beyond its current state to use in commercial and industrial settings, Lim (2017) states that producing nanofibers via electrospinning needs to be updated. Electrospinning has several drawbacks including low throughput, high voltage, and a requirement for specialized equipment. ENMs also generally possess low mechanical strength due to their low crystallinity and random orientation and alignment. It is imperative to move beyond the current electrospinning method to more innovative variations of electrospinning [30].

ENMs have been either established for use or are continuing to be researched in the following areas: healthcare, biotechnology and environmental engineering, defense and security, and energy storage and generation [25]. The success of ENMs within these fields, particularly size exclusions in biotechnology and environmental engineering, provided a basis for the research and application of ENMs to molecular biology within forensic science in this pilot study. Within healthcare, nanofibers can mimic the extracellular matrices (ECM) of tissues and organs. Studies on the interactions of cells with nanofibers have shown that cells adhere and proliferate well when cultured on polymer nanofibers. Because of these properties, nanofibers are well suited to tissue engineering. ENMs can be engineered to provide growth factors, drugs, and therapeutics for the purpose of stimulating tissue regeneration. The suggestion has been made that further research will result in the ability to successfully engineer these cell-nanofiber interactions into viable options for tissue and organ repair, regeneration, and transplant [25]. Khandaker et. al (2017) demonstrated that bone and tissue growth around titanium alloy implants, one of the most widely used implants for orthopedic and orthodontic surgeries, is

significantly improved by coating the implants with a nanofiber mesh composed of collagen-poly-Ɛ-caprolactone. Faster healing times and a reduction in the number of revision surgeries required due to implant loosening are possible with implementation of nanofiber mesh on implants [31]. The incorporation of collagen-poly-Ɛ-caprolactone into the ENMs lends itself to the possibility of successful incorporation of other components, such as antibodies, into ENMs as well. When forensic DNA analysis advances to a level at which antibodies can be used to distinguish between the DNA of more than one individual, those antibodies should theoretically be able to be incorporated into the ENMs. This would result in a separation device capable of distinguishing between multiple characteristics, which would make ENMs of tremendous value to the field of forensic science. This concept would essentially be equivalent to an affinity membrane, which is another application of nanofiber membranes discussed in the literature. Ramakrishna et. al (2006, p. 46) state that, affinity membranes "…selectively capture specific target molecules by immobilizing a specific capturing agent (or ligand) onto the membrane surface." Nanofiber membranes as affinity membranes could be used in protein purification and toxin removal in organic waste and heavy metal removal from water [25]. The success already achieved combined with the remaining potential for ENMs in both tissue engineering and as affinity membranes is grounds for continued research in healthcare and forensic science.

Within the fields of biotechnology and environmental engineering, membranes have been formed for size exclusion purposes, such as particulate removal from air and wastewater [25, 32]. The demonstrated success of membranes in these fields provided a basis for the research and application of ENMs to molecular biology within forensic science in this pilot study. Electrospun membranes can successfully remove particles 3-10μm in size from aqueous solution [25]. In doing so, ENMs essentially act as filters separating two distinct phases. The

main function of a membrane as a filter is to discriminate species it comes into contact with in one phase (feed) and transport them across to the other (permeate) [33]. Research has used ENMs to separate 1, 2, and 10µm polystyrene particles out of water. The pores of the membranes used ranged in size from 4µm to 10.6µm. The separation rate achieved for the 10µm particles was ninety-six percent, meaning the electrospun membranes were successful in rejecting ninety-six percent of the particles from permeating. Compared to the 10µm particles, the separation rate for the 5µm particles was lower at ninety-one percent. This is to be expected, however, because the size range of the pores in the membrane included pores larger than 5µm. This naturally allowed some of the 5µm particles to permeate. Finally, the separation rate for the 1µm particles, did not correlate with any of the data obtained previously. The separation rate for the 1µm particles was ninety-eight percent. The cause for this was determined to be a layer of particle coagulation on the surface of the ENM. Because the particles were so small, the particles were able to pack close together, reducing the effective pore size, and ultimately keeping the majority of the particles from permeating. This was the reason for the unusually high separation factor [33]. These results provided a basis for the experimentation performed for this pilot study, which applied the concept of particulate removal to the separation of sperm cells from sexual assault samples. Sperm cells (approximately 3x5µm) are within the size range of the particles shown to have been successfully filtered. Whereas the research discussed here used ENMs to separate 1, 2, and 10µm polystyrene particles out of water, this pilot study used ENMs to separate 3x5µm sperm cells out of sexual assault samples.

The nanofiber market has been projected to one day be a one billion dollar industry [34]. Industries including food, pharmaceutical, biotechnology, medical, and electronics are projected to be heavily involved in the rising market. Should the application of ENMs to forensic

molecular biology prove beneficial, forensic science could one day be added to the list of industries contributing to the success of the nanofiber market. Ramakrishna et al. (2006) assert that mass production of nanofibers could easily lead electrospinning to be "one of the most significant nanotechnologies of this century." Conversely authors B. Kim and I. Kim (2011) state that despite the potential of nanofibers, their application has been held back by their poor mechanical properties and the difficulty of their mass production. The authors, however, expect emerging innovative applications to be enhanced due to the overall progress made with nanofiber membranes [35]. In a 2015 article, Gajanan Bhat discusses the recent technology advancements within nanofiber production. The study found that, thus far, the success of electrospun nanofibers has not lived up to its potential. Bhat notes this is particularly true for filtration applications, which he says will likely be the largest application for electrospun nanofibers. Bhat blames this on issues related to solvent handling, high voltage, environmental and safety issues, productivity, accuracy and reproducibility at higher production rates, and cost of production. In spite of these findings, the projection of a one-billion-dollar nanofiber industry still stands [34]. This pilot study aimed to enhance the application of ENMs to filtration within molecular biology in forensic science, the specific area that Bhat stated had not lived up to its potential. One of the issues stated was accuracy and reproducibility, which turned out to be, interestingly, one of the issues this pilot study encountered. Despite current shortcomings, there is potential that ENMs within the field of forensic molecular biology could be a contributor to the success of the nanofiber industry.

Purpose of Study

Previous research has demonstrated that nanofiber membranes are capable of removing particles – similar in size to sperm cells – from solution via filtration [25, 32, 33]. There are

potential applications for this technology in forensic molecular biology, specifically the application of nanofiber technology to the removal of sperm cells from sexual assault samples via filtration. This research project was designed to explore this application. The goal was to apply ENMs to forensic molecular biology by developing an ENM with proper dimensions and adequate strength to separate sperm cells from epithelial cells based on size, not chemical composition, without lysing the cells in the process. Due to the difference in dimensions between sperm cells and epithelial cells, the hypothesis was that properly designed ENMs could successfully separate male and female DNA via a physical separation mechanism that would filter sperm cells from epithelial cells in sexual assault samples, ultimately leading to two separate DNA profiles – one from the perpetrator and one from the victim. The head of a human sperm cell is approximately 5µm by 3µm with an approximately 41µm tail. The vagina is lined with a 0.2mm thick layer of squamous epithelial cells that is composed of the following four layers with the following approximate epithelial cell diameter dimensions: superficial, 50-60µm; intermediate, 30-50µm; parabasal, 20µm; and basal, 12-14µm [36]. This study aimed to bolster the physical separation method Chen et al. (1998) demonstrated by using ENMs in place of nylon mesh filters because ENMs have the potential to double as both filters and affinity membranes. ENMs allow for the transport of sperm across the membrane, while acting as a barrier to keep the epithelial cells from permeating [33]. Successfully accomplishing this task would lead to improved results in samples containing low sperm quantity by increasing the amount of sperm initially recovered. Successful accomplishment would also introduce a new preliminary step to a differential extraction that would lead to cleaner extraction results and, therefore, cleaner profiles for a DNA analyst to interpret. Complete separation of sperm cells from epithelial cells would eliminate the subjectivity associated with current analysis and

interpretation due to the carryover between the male and female fractions and complications from improper minor to major contributor ratios.

The successful development of a forensically relevant ENM filtration mechanism would not only contribute to the field today but would also pave the way for further contributions as science continues to advance, specifically within the realm of affinity membranes. As previously defined by Ramakrishna et. al (2006, p. 46), affinity membranes "…selectively capture specific target molecules by immobilizing a specific capturing agent (or ligand) into the membrane surface". Using the electrospinning technique, nanoparticles can be spun into ENMs. This introduces specific molecular recognition capabilities into ENMs, thus ultimately resulting in affinity ENMs. Yoshimatsu et. al (2008, p. 1209) state that the qualities of nanoparticles "…should make them an ideal affinity matrix for sample preparation in trace analysis". The specific application used to test this statement was whether nanoparticles spun into an ENM could detect/extract trace amounts of propranolol in tap water samples. The results showed that the affinity nanofiber membrane could indeed extract propranolol from the water samples. The researchers projected that affinity nanofiber membranes will lend themselves to additional complex applications that are yet to be explored [37].

During this study, as results were obtained, the purpose of the research shifted from using ENMs to physically separate epithelial cells from sperm cells via filtration to implementing ENMs into the current differential extraction process. The exact application was determined by means of trial and error through a series of experiments. The overarching goal was to contribute to the development of an objective method for the analysis and interpretation of sexual assault samples that would give ENMs relevance within the field of forensic science. This, in turn,

would assist in determining the perpetrators in the hundreds of thousands of sexual assaults that occur annually.

The goal of this literature review was to show the potential application of electrospun nanofibers in forensic science and to demonstrate a gap in the literature regarding the use of ENMs in forensic molecular biology. The idea that they could aid in the separation of sperm cells from epithelial cells and aid in processing the thousands of untested and backlogged SAKs in the United States, is potentially ground-breaking and most certainly worth pursuing.

Methodology

All ENMs used throughout this research project were spun by the Nano Biology Research Lab at the University of Central Oklahoma. This research project was approved by exempt review under **45 CFR 46.101 (b) (4)** by the University of Central Oklahoma Institutional Review Board on December 8, 2017.

Pilot Protocol: Physical Separation via ENM Filtration

Polycaprolactone was used as the polymer to create ENMs for this study because it is biocompatible, nontoxic, has been documented in the literature as a polymer that is effectively electrospun, and has been used successfully on a recurring basis by the Nano Biology Research Lab at the University of Central Oklahoma [27, 31, 32]. ENMs made of polycaprolactone and composed of 6, 12, 24, 36, 48, and 60 layers were electrospun onto acrylic molds (Figure 2, Images A and C). This was done by collecting a single layer of unidirectional electrospun nanofibers followed by collecting an additional single layer of unidirectional electrospun nanofibers perpendicular to the first. This process continued until the specified number of layers was collected. The purpose of collecting each layer perpendicular to the previous one was to establish clearly delineated pores that could be measured. Samples were prepared to have differing numbers of layers as a means to establish, first, a baseline of what the pore sizes were and, second, how they changed as additional layers were added. Each sample was measured; the measurements were used to determine how many layers of nanofibers would be needed to meet the 10μm pore size specifications needed for the separation. Ten micrometers was the goal pore size because it would theoretically be small enough to allow sperm cells to pass through the pores while keeping epithelial cells from doing so. As the number of layers increased, the size of the pores established by the additional layers was expected to be precise, controllable, and

repeatable. Each prepared acrylic mold was mounted onto an aluminum hub using carbon tape. Microscopic examination was used to analyze and image the alignment of the ENMs for use in subsequent measurement of the pore sizes. Each mounted sample was imaged using a Hitachi TM3000 scanning electron microscope (SEM). SEMs are reliable instruments for aiding in pore size determination [27]. Each image was imported into Photoshop, which was used to visually identify the pores to be measured. The pores were selected with the intent to include a representative sampling of the various pore sizes of each ENM. Subsequently, each image was imported into a scientific image analysis program called Image J, which was used to measure each of the delineated pores [38]. A slurry of deionized water and magnesium oxide (MgO) was prepared to mimic sperm cells suspended in a solution containing a sexual assault sample. MgO is a microparticle with a known median size of 2.778μm [39]. MgO was selected because its median size of 2.778μm is comparable to the 3μm width of a sperm cell. The slurry was pipetted onto each of the ENMs in order to simulate a sexual assault sample being pipetted onto each of the ENMs. The MgO microparticles represented sperm cells, and the deionized water represented buffer added in the processing of a sexual assault sample. The intent behind this experiment was to determine whether or not ENMs would allow MgO to flow through their pores. Testing the capabilities of ENMs using this method was a cost-effective mechanism for obtaining preliminary results as to whether or not ENMs would potentially allow sperm cells in a sexual assault sample to flow through their pores. If ENMs allowed MgO to flow through their pores, testing whether or not ENMs would allow sperm cells to flow through their pores would be the next phase of the experiment since MgO and sperm cells are comparable in size. Success with MgO would be an indication for potential success with sperm cells.

Pilot Protocol: Physical Separation via Polycaprolactone Film

Membranes or films, also referred to as sieves or nanosieves, are used for fluid separation in the field of engineering. These nanosieves can be constructed with cylindrical pores of a uniform size down to below 10nm in diameter (Figure 3). Permeability, strength, and selectivity are three characteristics for which the design process accounts. Nanosieves are able to achieve a high throughput, possess the strength to withstand flux without breaking, and have the ability to separate the desired component from the fluid [40]. Polycaprolactone films were produced in this pilot study with the intent to achieve a nanosieve product with the specific ability to separate sperm cells from epithelial cells in sexual assault samples. Polycaprolactone was used because it is biocompatible, nontoxic, and has been used successfully on a recurring basis by the Nano Biology Research Lab at the University of Central Oklahoma [27, 31, 32]. The polycaprolactone films were produced by sonicating 0.5g of polycaprolactone with 5g of acetone for thirty minutes, pouring the resulting solution onto a slide, then using a spin coater to achieve a smooth film. Once the film had dried, a laser was used in an attempt to drill 10μm holes through the film. This did not work as the film surrounding the laser-cut holes melted away, resulting in holes that were neither the desired size nor the desired shape. Figure 3 illustrates the intent behind this experiment.

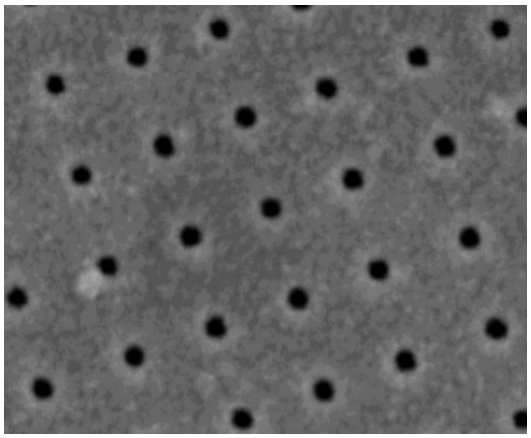

Figure 3: SEM image of a nanosieve. This image is of a nanosieve constructed with cylindrical pores with a consistent size of 25 nanometers in diameter. From Tong et al. (2004) [40]. Polycaprolactone films were produced in this pilot study with the intent to achieve a nanosieve product with the specific ability to separate sperm cells from epithelial cells in sexual assault samples.

Sperm Capture via ENM within Microcentrifuge Tube

Quantitation of Spermatozoa

In order to determine whether sperm cell retention was possible using ENMs, an experiment first had to be developed. The goal was to quantify the number of sperm cells present in a semen sample. The hypothesis was that this could be done by dyeing the sperm cells in a semen sample, viewing them under a fluorescence microscope, and hand counting them. The first step taken in an attempt to achieve this was using the Click-iT™ EdU Cell Proliferation Kit for Imaging [41]. Two microliters of neat semen were added to a six-layered ENM. The full imaging kit protocol was followed. The following steps were performed in order to fixate and permeabilize the cells: the sample was washed with 1,000µl of 3% BSA in PBS two times with a five-minute room-temperature incubation following each wash. One thousand microliters of 0.5% Triton X-100 in PBS were added and allowed to incubate for ten minutes at room temperature. Fifty microliters of prepared Click-iT™ stock solution were added and incubated at room temperature for thirty minutes while being protected from light. (Note: the sample was protected from light while all subsequent steps were performed.) The Click-iT™ stock solution was removed. The sample was washed with 1,000µl of 3% BSA in PBS and incubated at room temperature for five minutes. At this point, the DNA staining portion of the imaging kit protocol was performed to achieve nuclear staining. The sample was washed with 1,000µl of PBS and incubated at room temperature for five minutes. Fifty microliters of prepared Hoechst solution (1:2000 in PBS for a concentration of 5µg/mL) were added and incubated for fifteen minutes. Two more 1X PBS washes were performed with five-minute room-temperature incubations following each wash. At this point, the sample was ready to be viewed under a fluorescence microscope. When viewed under a fluorescence microscope, the sperm cells did exhibit fluorescence.

Since the full Click-iT™ EdU Cell Proliferation protocol proved successful, the next step taken was eliminating the proliferation portion of the protocol since proliferation was not necessary to the goal of this research project. This was accomplished by attempting only the nuclear DNA staining portion of the protocol. If this were to work, the sample dyeing time would be greatly decreased, thus increasing efficiency of the methodology. It proved successful: the sperm cells still fluoresced. Therefore, moving forward, the fluorescent dye protocol was used as follows: the samples were washed with 1,000µl of PBS and incubated at room temperature for five minutes; fifty microliters of the prepared Hoechst solution were added and incubated for fifteen minutes; finally, two more 1X PBS washes were performed with five-minute room-temperature incubations following each wash. Because of the abundance and overlapping of sperm cells in the neat semen samples, a 1:3 dilution was used. The sperm cells in the 1:3 dilution were also present in such abundance that overlapping would have made hand counting them difficult, if not impossible.

The next attempt was a 1:6 semen dilution. This ended up being the optimal dilution to quantify sperm cells because the number present in the 1:6 semen dilution was able to be accurately counted by hand. Subsequent samples were viewed using a fluorescence microscope; images were captured; and the fluorescent sperm cells were counted by hand. Fifty microliters of deionized water were pipetted onto each sample and then pipetted back off. This was done to simulate a “wash” step. The samples were again viewed using a fluorescence microscope; images were captured; and the fluorescent sperm cells remaining after the “wash” were counted by hand. This was done to determine the approximate percentage of sperm cells that endured the wash while on the ENM.

Simulated Sexual Assault Sample Preparation

Simulated sexual assault samples were prepared according to the preparation methods set forth by Vuichard et. al (2011). Buccal samples were prepared by rubbing swabs back and forth forcefully inside the cheek ten times. The buccal swabs dried at room temperature and were stored at room temperature until semen was deposited. A 1:50 dilution of semen from a commercial supplier was prepared using sterile water. Ten microliters of the prepared semen dilution were deposited onto each buccal swab. The swabs dried for two hours at room temperature and were stored at room temperature until extraction [14].

Differential Extraction/Pilot Protocol

Figure 4: Rendering of 2mL microcentrifuge tube with added ENM. Photo from https://bit.ly/37bhQbl. Drawing representative of ENM added by author.

To each 2mL microcentrifuge tube to be used in this study, a six-layered ENM was added. Each prepared sample swab tip was broken off into a prepared 2mL microcentrifuge tube. Known semen and epithelial samples served as positive controls to verify the reliability and consistency of the extraction process. A reagent blank served as a negative control to monitor contamination throughout the process from extraction to analysis. A modified version of the manufacturer's DNA IQ™ protocol was performed [42]. Two-hundred fifty microliters of lysis buffer were added to each tube and incubated at 50˚C for 45 minutes. Each swab was transferred into a spin basket, which was placed back into its original microcentrifuge tube. The tubes were centrifuged at maximum speed (13,200rpm) for 2 minutes. The spin baskets and swabs were discarded. While exercising caution to not disturb the sperm pellet, the supernatant was removed and placed into a new 2µl microcentrifuge tube. To the

remaining sperm pellet in the original microcentrifuge tube, 250µl of lysis buffer combined with 2.5µl of 1M DTT were added and incubated at 70˚C for 30 minutes. At this point, the remainder of the manufacturer's DNA IQ™ protocol was followed as written. Seven µl of resin were added to all tubes: those containing the supernatant and those containing the sperm fraction. The tubes were vortexed for 3 seconds. They were incubated at room temperature for 5 minutes. During the incubation, the tubes were vortexed for 3 seconds once every minute. Following the incubation, they were vortexed for 2 seconds before placing them in the magnetic stand. The solution was discarded without disturbing the resin. One hundred microliters of lysis buffer were added; the tubes were vortexed for 2 seconds, returned to the magnetic stand, and the lysis buffer removed. One hundred microliters of wash buffer were added; the tubes were vortexed for 2 seconds, returned to the magnetic stand, and the wash buffer removed. The wash step was repeated two more times for a total of three washes. All wash buffer was removed after the final wash. With the tubes still in the magnetic stand and the lids open, they were air-dried for five minutes. Fifty microliters of elution buffer were added; the tubes were vortexed for 2 seconds, incubated at 65˚C for 5 minutes, vortexed for 2 seconds, and returned to the magnetic stand. The DNA-containing solution was transferred to storage tubes.

Quantification

The samples were quantified using Applied Biosystems's Quantifiler™ Human DNA Quantification Kits PCR Amplification. A master mix was prepared containing 10.5µl of Quantifiler Human Primer Mix and 12.5µl of Quantifiler PCR Reaction Mix per reaction. Twenty-three microliters of the PCR mix were dispensed into each well of the reaction plate prior to adding 2µl of standard, sample, or control. The plate was sealed with an Optical Adhesive Cover, centrifuged to remove bubbles, then placed into a CFX96 Touch Real-Time

PCR Detection System. The following thermal cycler conditions were used: 50˚C for 2 minutes, 95˚C for 10 minutes, then 39 cycles of 95˚C for 15 seconds followed by 60˚C for 1 minute. The quantification data were viewed using CFX Maestro™ Software by Bio-Rad.

Amplification

The samples were amplified using Applied Biosystems's GlobalFiler™ PCR Amplification Kit. A reaction mix was prepared containing 7.5µl of Master Mix and 2.5µl of Primer Set per reaction. Ten microliters of the reaction mix were dispensed into each PCR tube prior to adding each sample or control. Based on collected quantification data (Table 3 in "Results" section), calculations were performed to normalize the samples and controls – to determine the amount needed in order to have a concentration of 1ng of DNA. The total reaction volume per tube was 25µl: 10µl of the reaction mix, the required amount of DNA extract calculated, and the remaining µl as ultrapure water. The tubes were vortexed and centrifuged prior to amplification then placed into a GeneAmp PCR System 9700. The thermal cycling conditions were as follows: a 95˚C hold step for 1 minute, 29 cycles of a 94˚C denaturation step for 10 seconds followed by a 59˚C annealing/extension step for 90 seconds, and a 60˚C final extension step for 10 minutes. The samples were held at 4˚C until electrophoresis.

Analysis

The amplified samples were run on an Applied Biosystems 3500 Genetic Analyzer. A reaction mix was prepared that contained 0.4µl of GeneScan™ 600 LIZ™ Size Standard v2.0 per reaction and 9.6 µl of Hi-Di™ Formamide per reaction. Into each well of a MicroAmp™ Optical 96-Well Reaction Plate, 10µl of the reaction mix were dispensed followed by 1µl of PCR product or allelic ladder. The plate was sealed with septa, vortexed briefly, then centrifuged. It was heated in a thermal cycler at 95˚C for 3 minutes followed immediately by resting on ice for

3 minutes. The plate was placed onto the autosampler and run on the Genetic Analyzer. The data were analyzed using GeneMapper *ID-X* Software.

Figure 5: Overview schematic of experiments performed in this collaborative pilot research study. The three experiments displayed in Figure 5 were performed independently of one another and in succession. First, ENMs were used as a filter in an attempt to physically separate sperm cells from epithelial cells based on the size difference of the two cell types. Next, a film was attempted to be designed to physically separate sperm cells from epithelial cells based on the size difference of the two cell types. Finally, sperm cells were attempted to be separated from epithelial cells by capturing them using an ENM within a microcentrifuge tube.

Results and Discussion

Pilot Protocol: Physical Separation Via ENM Filtration

Table 1 displays the smallest pore size, largest pore size, and average pore size of the pores measured for 6, 12, 24, 36, 48, and 60 layered ENMs that were electrospun onto acrylic molds. This process involved collecting a single layer of unidirectional electrospun nanofibers followed by collecting an additional single layer of unidirectional electrospun nanofibers perpendicular to the first. This process was repeated until the specified number of layers was collected. The purpose of collecting each layer perpendicular to the previous layer was to establish clearly delineated pores that could be measured. A group of pores representative of the pore size variation was measured for each ENM, the six and twelve layered ENMs in triplicate; the measurement results are tabulated in Table 1.

Table 1	**ENM Pore Sizes**		
# of ENM Layers	*Smallest (µm)*	*Largest (µm)*	*Average (µm)*
6	4.673	29.739	14.789
12	0.813	8.427	3.056
24	1.660	8.480	3.221
36	0.696	4.869	1.654
48	0.740	2.286	1.164
60	0.923	1.897	1.167

A consistent and reproducible 10µm pore size was the goal because it would theoretically be small enough to allow sperm cells to pass through the pores while keeping epithelial cells from doing so. As can be seen in Table 1, the pores tend to decrease in size as the number of ENM layers increases; however, they do not do so consistently. The pore sizes were found to be inconsistent within a single ENM and among different ENMs. It was not possible to selectively create pores of the 10µm goal size. This is apparent in Figure 6. As previously stated, as the

number of layers increased, the size of the pores established by the additional layers was expected to be precise, controllable, and repeatable. This experiment demonstrated that, despite the number of additional layers, this was not possible.

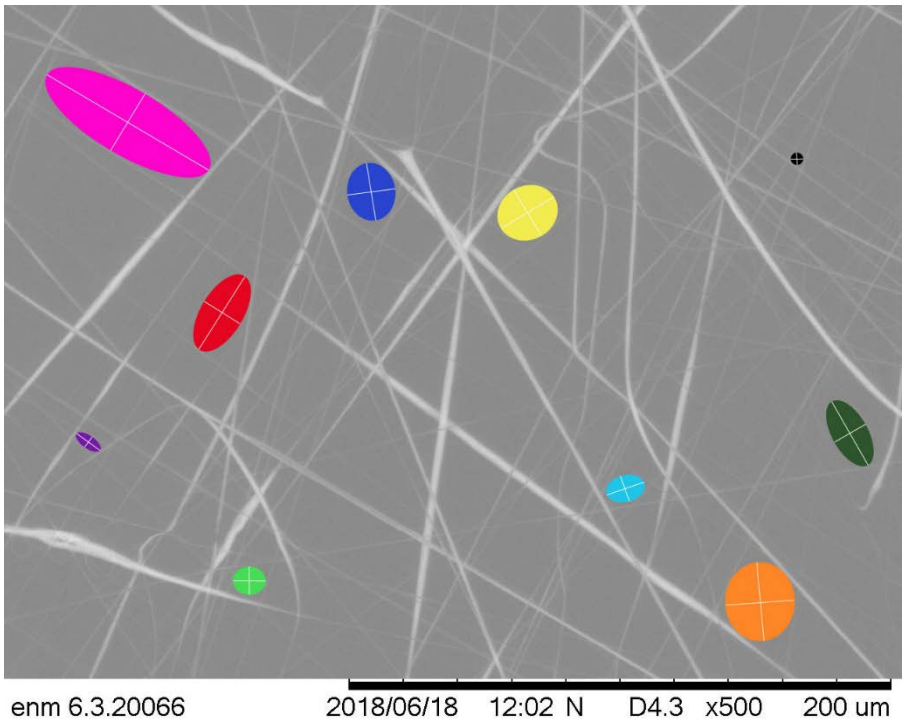

Figure 6: SEM image of a six-layer ENM. A six-layer ENM viewed under a scanning electron microscope with pores of different sizes delineated.

Although the ENMs proved to have neither consistent nor reproducible pores, attempts were made to determine their capturing capabilities. This experiment resulted in two conclusions. The first conclusion is that the current means of electrospinning is unable to yield consistent, reliable ENMs and because the pores are inherently inconsistent, the ability to capture is also inconsistent. This was determined to be true regardless of the number of layers within the ENM. The second conclusion is that the ENMs are not strong enough to withstand vortexing or centrifugation, both crucial steps in the analysis of sexual assault samples. Because the pores, and thus, the capturing capabilities of ENMs were found to be inconsistent and unreliable, and because they were not found to be strong enough to withstand vortexing or centrifugation, the

focus of this pilot study shifted away from using ENMs as a mechanism for physical separation of sperm cells from epithelial cells in sexual assault samples. In order for the idea to be plausible, advances must be made in the electrospinning process, namely using an automated system as opposed to manual spinning.

Pilot Protocol: Physical Separation via Polycaprolactone Film

The polycaprolactone films produced were solid: they did not have pores. A laser was used to make holes through the film. Two problems were encountered with the laser. The first is that it was unable to achieve a 10μm hole [which is greater than the size of a sperm head (approximately 5μm by 3μm) and smaller than a vaginal epithelial cell (12-60μm)][43]. The length of its focus lens is such that the smallest hole it can make is 150μm. The second is that the laser melted the polycaprolactone; thus, the holes drilled were larger than the specifications set on the laser because the film surrounding the laser-cut holes melted away. The result was that the holes were neither the desired size nor shape.

In an attempt to achieve consistent and reproducible pores for physical separation of sperm cells from epithelial cells, experimentation was performed on films composed of the same polymer as the ENMs (polycaprolactone). Because the laser used to drill the pores through the film was unable to achieve the desired pore size due to its specifications and due to the fact that the polycaprolactone melted when hit with the laser, the focus of this pilot study shifted away from polycaprolactone films as a mechanism for physical separation of sperm cells from epithelial cells in sexual assault samples.

Neither physical separation via ENM filtration nor physical separation via polycaprolactone film is attainable with the current engineering processes used by the Nano Biology Research Lab at the University of Central Oklahoma. The products required for the

success of this pilot protocol must be precise, controllable, and repeatable. The ENMs and films from the current engineering processes used by the Nano Biology Research Lab at the University of Central Oklahoma are not.

Sperm Capture via ENM within Microcentrifuge Tube

Six-layered ENMs were spun and used for all experimentation regarding sperm capture within microcentrifuge tubes. In previous research by the Nano Biology Research Lab at the University of Central Oklahoma, ENMs composed of 1, 4, and 6 layers were compared. The researchers concluded that four and six-layered ENMs are suitable for biological applications. They also determined that more cell adhesion was observed the higher the number of layers in an ENM. [44]. Therefore, six-layered ENMs were used in this research in order to allow for as much sperm adhesion as possible.

Quantification of Spermatozoa

Figure 7 displays neat semen dyed using the full protocol Click-iT™ EdU Cell Proliferation Kit for Imaging. The goal was to be able to quantify by hand the number of sperm cells present in a sample. Using the results in Figure 7, this would have been difficult, if not impossible, due to the abundance and overlapping of cells. Figure 8 displays a 1:3 semen dilution dyed using only the DNA staining portion of the Click-iT™ EdU Cell Proliferation Kit for Imaging. Because using the DNA staining portion of the protocol proved successful, it was used for all remaining experimentation. This increased efficiency and cost-effectiveness. Again, the sperm cells were present in such abundance that overlapping would have made hand counting them difficult, if not impossible. Figure 9 displays a 1:6 semen dilution. This ended up being the

dilution used to quantify sperm cells because the number present in it was able to be accurately counted by hand.

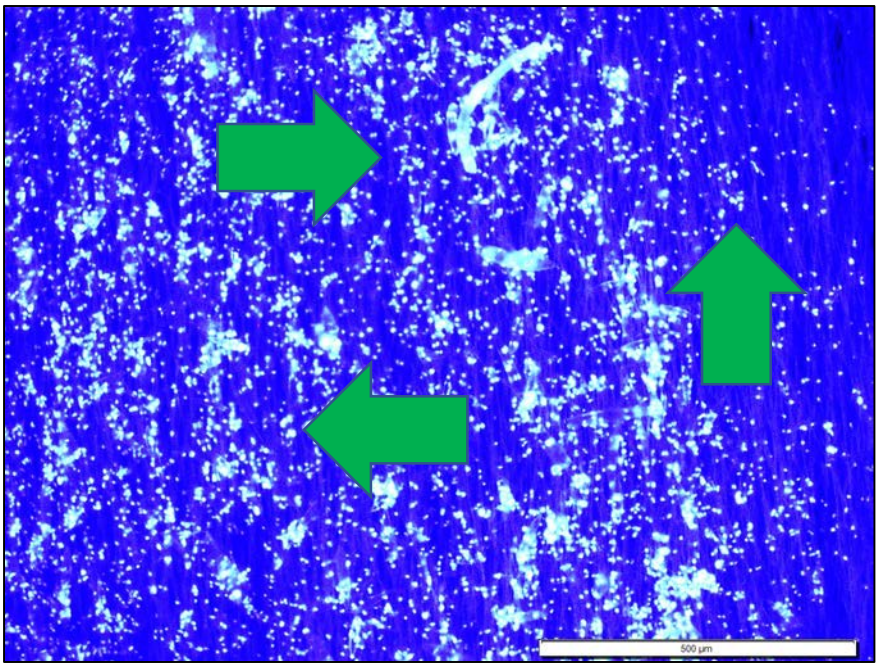

Figure 7: Image of neat semen. Fluorescence microscope image of neat semen dyed using the Click-iT™ EdU Cell Proliferation Kit for Imaging. Each bright spot or group of spots is a cell or group of cells. Due to the abundance and visible overlapping of the cells thus leading to the difficulty or impossibility that would have been involved with counting them, a 1:3 dilution was attempted next (Figure 8).

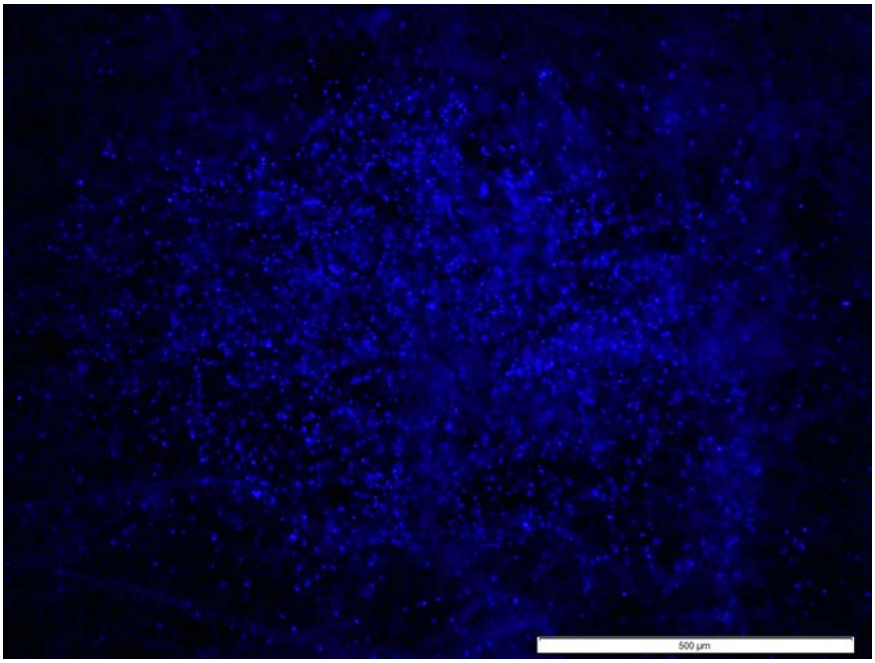

Figure 8: Image of a 1:3 semen dilution. Fluorescence microscope image of 1:3 semen dyed using only the DNA staining portion of the Click-iT™ EdU Cell Proliferation Kit for Imaging. Each bright spot or group of spots is a cell or group of cells. Due to the abundance and visible overlapping of the cells thus leading to the difficulty or impossibility that would have been involved with counting them, a 1:6 dilution was attempted next (Figure 9).

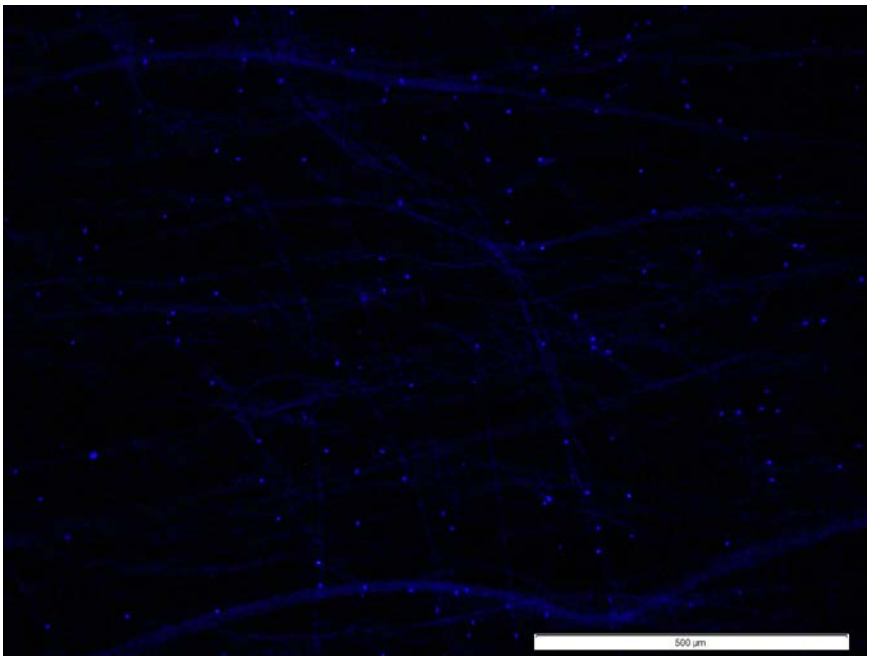

Figure 9: Image of a 1:6 semen dilution. Fluorescently dyed sperm cells from a 1:6 semen dilution viewed under a fluorescence microscope. Each bright spot is a cell. Due to the number of cells present, the cells present in this 1:6 dilution were able to be accurately quantified.

Because the number of sperm cells present in the 1:6 semen dilution was established as being able to be accurately counted by hand, four 1:6 semen dilution samples on ENMs were viewed using a fluorescence microscope; images were captured; and the fluorescent sperm cells were counted by hand. Fifty microliters of deionized water were pipetted onto each sample before being pipetted back off. This was done to simulate a "wash" step. The samples were again viewed using a fluorescence microscope; images were captured; and the fluorescent sperm cells remaining after the "wash" were counted by hand. This was done to determine the approximate percentage of sperm cells that endured the wash while on the ENM. Table 2 displays the hand-counted number of sperm cells present on the ENMs before and after the wash step.

Table 2	**Quantification of Spermatozoa (1:6 Semen Dilution)**	
Sample #	*# of Sperm Before*	*# of Sperm After*
1	187	72
2	122	69
3	124	35
4	174	79
Percent	42% Retention of Sperm Cells After a Simulated Wash	

The fact that differential extractions have been shown to be capable of losing ninety-four to ninety-eight percent of sperm DNA [14] means that they retain merely two to six percent of sperm DNA. On average, the ENMs tested retained forty-two percent of sperm cells after a simulated wash step. That is a potential seven to twenty-one-fold increase in sperm DNA retention. Implementing ENMs into differential extraction protocols has the potential to increase sperm DNA retention to aid in obtaining more complete, if not full, single-source DNA profiles from sperm cells in sexual assault samples.

Differential Extraction/ Pilot Protocol Quantitation, Amplification, & Analysis

Table 3 displays the quantification data obtained from the simulated sexual assault samples after they were differentially extracted via the pilot protocol.

Table 3	**Quantification of DNA in Simulated Sexual Assault Samples**	
Sample #	*Epithelial Fraction (ng/µl)*	*Sperm Fraction (ng/µl)*
SIM 3	5.255	3.782
SIM 4	17.880	6.296
SIM 5*	11.140	0.6765
SIM 6*	4.567	1.573
SIM 10	13.960	2.043
SIM 11	5.788	0.5269
SIM 12	29.16	1.584

*removed ENM prior to 2nd incubation for comparison of those with melted ENM VS. without

Although DNA was successfully extracted via the pilot protocol, then quantified, amplified, and analyzed, the electropherograms obtained were mixed profiles. Modifications were attempted but unsuccessful. Modifications include different incubation temperatures and lengths, different methods of removing the supernatant, different reagents used, and different amounts of reagents used. The electropherograms of the positive controls demonstrated that the processes were performed correctly because they were single source and matched the expected

profiles of the semen and epithelial cells respectively. The electropherograms of the negative controls demonstrated that contamination was not an issue.

Although complete, single-source DNA profiles from sperm cells in sexual assault samples were not obtained by implementing the use of ENMs into differential extraction protocols, positive outcomes were achieved from this study. It was discovered that ENMs have the capability of retaining sperm cells and that approximately 42% of sperm cells are retained by ENMs following a wash. Considering that differential extractions typically retain two to six percent of sperm cells, which are already the minor contributor, incorporating ENMs increases the recovery of sperm cells by seven to twenty-one times.

Another positive outcome was the discovery that melting the ENMs during incubation does not inhibit PCR or any other aspect of downstream processing. The results of the samples in which the ENMs had melted were not affected. By melting the fibers, the need to pipette around them without clogging the pipette tip was removed. Melting the ENMs theoretically allows for the capture of all sperm cells that adhere to them. The initial incubation in the protocol is 50˚C. The melting point of polycaprolactone is 60˚C [45]. Therefore, the ENM in any given sample withstands the initial incubation while also retaining sperm cells. After the 50˚C incubation, the supernatant is removed, leaving the ENM with its attached sperm cells. At this point, a 70˚C incubation takes place. The ENM melts. Since the supernatant has already been removed, all that should remain are sperm cells, or more specifically, sperm cell DNA since the second incubation is used to lyse the sperm cells.

A third positive outcome was that the resulting protocol merges the typical differential extraction protocol with a solid-phase DNA extraction, a process which has been automated. The protocol developed and used in this research adds one incubation in addition to the already

existing incubation required by the solid-phase DNA extraction protocol. This presents the potential to automate the developed protocol.

Automating a protocol that separates epithelial from sperm cells within one system would be groundbreaking. The Qiagen QIAcube HID Differential Washing Station is capable of separating epithelial cells from sperm cells and lysing the sperm pellet for up to twelve samples in approximately an hour and a half [46]. Downstream processing via QIAGEN EZ1® and QIAamp® DNA Investigator® purifies the samples in approximately 20 minutes. It is important to note that two different instruments are required to accomplish both separation and purification. The DNA IQ™ Casework Pro Kit for Maxwell® 16, a solid-phase DNA extraction kit, on the other hand, can purify up to sixteen samples in approximately thirty minutes using a Maxwell® 16 [47]. The Differex™ System by Promega is a preprocessing separation of sperm from epithelial DNA that can be used manually prior to Casework Pro Kit purification method for the Maxwell® 16 Instrument. According to the manufacturer's protocol, the total separation time is approximately 2 hours, including a 90-minute Pro K digestion [48]. The DNA purification then takes approximately thirty minutes. Ultimately, the separation and purification can both be accomplished in as little as two and a half hours. The proposed experimental protocol includes back-to-back manual 45-minute and 30-minute incubations followed by the solid-phase purification process. Theoretically, by using an automated purification process such as the DNA IQ™ Casework Pro Kit for Maxwell® 16 that takes 30 minutes, the total time for both extraction and purification would be 1 hour and 45 minutes using one instrument. This total time is 45 minutes shorter than the manufacturer's protocol for sexual assault samples. These totals are summarized in Table 4.

Table 4	**Comparison of Automated Systems**				
System	*# of Instruments*	*# of Samples*	*Separation/ Extraction Time*	*Purification Time*	*Total Time*
Qiagen QIAcube HID Differential Washing Station combined with QIAGEN EZ1® and QIAamp® DNA Investigator®	2	12	1.5 hours	20 minutes	1.83 hours
Differex™ System combined with DNA IQ™ Casework Pro Kit for Maxwell® 16	1	16	2 hours	30 minutes	2.5 hours
Proposed experimental protocol combined with DNA IQ™ Casework Pro Kit for Maxwell® 16	1	16	1.25 hours	30 minutes	1.75 hours

Conclusion

To address the problem of untested SAKs, attempts were made at enhancing the current methodology for their analysis. The first attempt was physical separation of sperm cells from epithelial cells via ENM filtration. The second attempt was physical separation of sperm cells from epithelial cells via an ENM film. The final attempt was sperm capture via ENM within a microcentrifuge tube. The different approaches were assessed by means of experimental trial and error. Throughout each experiment, the overarching goal was to contribute to the development of an objective, more efficient method for the analysis and interpretation of sexual assault samples. This was to be accomplished using ENMs, and as a result, would give them relevance within the field of forensic science. Achieving this goal would assist in expediting the identification of perpetrators in the hundreds of thousands of sexual assaults that occur annually.

The attempt at physical separation via ENM filtration led to the realization that the pores, and thus, the capturing capabilities of ENMs are inconsistent, unreliable, and not strong enough to withstand vortexing or centrifugation. These discoveries eliminated ENMs as a viable filtration mechanism. The attempt at physical separation via a polycaprolactone film led to holes that were neither the size nor shape required to filter sperm cells from epithelial cells. This was due to the fact that the laser caused the polycaprolactone to melt beyond the set specifications of the laser, resulting in holes with inconsistently melted perimeters. This eliminated polycaprolactone films as a viable option for physical separation.

The attempt at sperm capture via ENM within a tube yielded the greatest success of the three pilot protocols. It was demonstrated that ENMs are capable of retaining sperm cells. A modified differential extraction was performed on simulated sexual assault samples using microcentrifuge tubes that had ENMs inside of them. The introduction of the ENM into the

process did not yield single-source DNA profiles from the sperm cells as was the hope. Instead, all resulting DNA profiles were mixed. Regardless, the discoveries that ENMs are capable of retaining sperm cells and that melting them during the extraction process does not inhibit downstream processing contributes to the field and opens the door for continued research. The potential remains to optimize ENMs in the analysis of sexual assault samples.

Future Research

Nanosieves have been demonstrated to survive at temperatures of up to 900 ˚C. This allows for the use of nanosieves in environments in which polymeric membranes melt [40]. The polymeric polycaprolactone films used in this pilot study melted when a laser was used to drill holes through them. Future research could be performed on membranes that can withstand the heat of a laser. Ten micrometer diameter pores could be drilled through those membranes, forming nanosieves, at which point the remainder of the pilot protocol attempted within this study could be performed to determine whether or not nanosieves are able to physically separate sperm cells from epithelial cells in sexual assault samples.

An immediate way to improve the functionality of the ENM within the microcentrifuge tube used in the pilot protocol is to determine a way to adhere the ENMs to the insides of the tubes. The current pilot protocol setup requires pipetting finesse around the free-floating ENMs, which an automated system would be unable to do. By adhering them, however, an automated system would be able to perform as usual without interfering with the ENM. It would also be worth performing the proposed manual protocol again using a microcentrifuge tube with an adhered ENM to determine whether greater separation of sperm from epithelial cells could be achieved. It is possible that by remaining in a fixed location, less carryover could be achieved by eliminating the ENM from floating around in solution. After achieving successful results, statistical analysis should be applied to determine the reliability of the process. Analysis should also be conducted to determine cost function of the production of microcentrifuge tubes with adhered ENMs.

To improve functionality of ENMs in general and within forensic molecular biology, an automated electrospinning system capable of producing consistent, uniform ENMs needs to be

developed. When this is accomplished, the concept of using ENMs as a physical filtration device can be revisited. Furthermore, when forensic DNA analysis reaches the point that it can use antibodies to distinguish between the DNA of more than one individual, those antibodies should theoretically be able to be incorporated into the ENMs. This would result in a separation device capable of distinguishing between multiple characteristics, which would make ENMs even more valuable than as a size-based filtration mechanism.

References

1. Network, R.A.I.N. *Statistics*. [cited 2020; Available from: https://www.rainn.org/statistics.

2. *End the Backlog*. [cited 2018; Available from: http://www.endthebacklog.org/.

3. Nelson, M., *Making sense of DNA backlogs, 2010: Myths vs. reality*. 2011, DIANE Publishing.

4. Morgan, R.E.K., Grace. *Criminal Victimization, 2016*. 2017; Available from: https://www.bjs.gov/content/pub/pdf/cv16.pdf.

5. Ritter, N., *The Road Ahead: Unanalyzed Evidence in Sexual Assault Cases*. 2011, National Institute of Justice. p. 1-21.

6. Strom, K.J. and M.J. Hickman, *Unanalyzed evidence in law-enforcement agencies: A national examination of forensic processing in police departments.* Criminology & Public Policy, 2010. **9**(2): p. 381-404.

7. Campbell, R., et al., *The national problem of untested sexual assault kits (SAKs): Scope, causes, and future directions for research, policy, and practice.* Trauma, Violence, & Abuse, 2017. **18**(4): p. 363-376.

8. Gill, P., A.J. Jeffreys, and D.J. Werrett, *Forensic application of DNA 'fingerprints'.* Nature, 1985. **318**(6046): p. 577-9.

9. Klein, S.B. and M.R. Buoncristiani, *Evaluating the efficacy of DNA differential extraction methods for sexual assault evidence.* Forensic science international: genetics, 2017. **29**: p. 109-117.

10. Garvin, A.M., et al., *Isolating DNA from sexual assault cases: a comparison of standard methods with a nuclease-based approach.* Investigative genetics, 2012. **3**(1): p. 25.

11. Balhorn, R., *A model for the structure of chromatin in mammalian sperm.* The Journal of cell biology, 1982. **93**(2): p. 298-305.

12. Butler, J.M., *Advanced topics in forensic DNA typing: methodology*. 2011: Academic Press.

13. Wiegand, P., M. Schürenkamp, and U. Schütte, *DNA extraction from mixtures of body fluid using mild preferential lysis.* International journal of legal medicine, 1992. **104**(6): p. 359-360.

14. Vuichard, S., et al., *Differential DNA extraction of challenging simulated sexual-assault samples: a Swiss collaborative study.* Investigative genetics, 2011. **2**(1): p. 11.

15. Timken, M.D., S.B. Klein, and M.R. Buoncristiani, *Improving the efficacy of the standard DNA differential extraction method for sexual assault evidence.* Forensic science international: genetics, 2018. **34**: p. 170-177.

16. Brevnov, M.G., et al., *Developmental validation of the PrepFiler™ forensic DNA extraction kit for extraction of genomic DNA from biological samples.* Journal of forensic sciences, 2009. **54**(3): p. 599-607.

17. Psifidi, A., et al., *Comparison of eleven methods for genomic DNA extraction suitable for large-scale whole-genome genotyping and long-term DNA banking using blood samples.* PLoS One, 2015. **10**(1): p. e0115960.

18. Hoff-Olsen, P., et al., *Extraction of DNA from decomposed human tissue: an evaluation of five extraction methods for short tandem repeat typing.* Forensic science international, 1999. **105**(3): p. 171-183.

19. Biosystems, A., *PrepFiler Express™ and PrepFiler Express BTA™ Forensic DNA Extraction Kits* 2010.

20. Jobling, M.A. and P. Gill, *Encoded evidence: DNA in forensic analysis.* Nature Reviews Genetics, 2004. **5**(10): p. 739.

21. Scientific, T. *Automation: Platform for Productivity*. [cited 2018; Available from: https://www.thermofisher.com/us/en/home/industrial/human-identification/human-identification-automation.html.

22. Horsman, K.M., et al., *Separation of sperm and epithelial cells in a microfabricated device: potential application to forensic analysis of sexual assault evidence.* Analytical chemistry, 2005. **77**(3): p. 742-749.

23. Greenspoon, S.A., et al., *Application of the BioMek® 2000 laboratory automation workstation and the DNA IQ™ system to the extraction of forensic casework samples.* Journal of Forensic Science, 2003. **49**(1): p. 1-11.

24. Chen, J., et al., *A physical method for separating spermatozoa from epithelial cells in sexual assault evidence.* Journal of Forensic Science, 1998. **43**(1): p. 114-118.

25. Ramakrishna, S., et al., *Electrospun nanofibers: solving global issues.* Materials today, 2006. **9**(3): p. 40-50.

26. Gibson, P., H. Schreuder-Gibson, and D. Rivin, *Transport Properties of Porous Membranes Based on Electrospun Nanofibers.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2001: p. 469-481.

27. Garg, K. and G.L. Bowlin, *Electrospinning jets and nanofibrous structures.* Biomicrofluidics, 2011. **5**(1): p. 013403.

28. Xue, J., et al., *Electrospun nanofibers: new concepts, materials, and applications.* Accounts of chemical research, 2017. **50**(8): p. 1976-1987.

29. Xue, J., et al., *Electrospun Nanofibers: New Concepts, Materials, and Applications.* Accounts of Chemical Research, 2017: p. 1976-1987.

30. Lim, C.T., *Nanofiber technology: current status and emerging developments.* Progress in Polymer Science, 2017. **70**: p. 1-17.

31. Khandaker, M., et al., *Microgroove and Collagen-poly (ε-caprolactone) Nanofiber Mesh Coating Improves the Mechanical Stability and Osseointegration of Titanium Implants.* Nanomaterials, 2017. **7**(6): p. 145.

32. Gibson, P., H. Schreuder-Gibson, and D. Rivin, *Transport properties of porous membranes based on electrospun nanofibers.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2001. **187**: p. 469-481.

33. Gopal, R., et al., *Electrospun nanofibrous filtration membrane.* Journal of Membrane Science, 2006. **281**(1-2): p. 581-586.

34. Bhat, G., *Polymeric nanofibers: recent technology advancements stimulating their growth.* Journal of Textile Science & Engineering, 2015. **5**(1): p. 1.

35. Kim, B.-S. and I.-S. Kim, *Recent nanofiber technologies.* Polymer Reviews, 2011. **51**(3): p. 235-238.

36. Casallas, L.H.C., *Classification of Squamous Cell Cervical Cytology*. 2012, Universidad Nacional de Colombia: Bogota D.C. Colombia.

37. Yoshimatsu, K., et al., *Selective molecular adsorption using electrospun nanofiber affinity membranes.* Biosensors and bioelectronics, 2008. **23**(7): p. 1208-1215.

38. Abràmoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ.* Biophotonics international, 2004. **11**(7): p. 36-42.

39. Khandaker, M., Y. Li, and T. Morris, *Micro and nano MgO particles for the improvement of fracture toughness of bone–cement interfaces.* Journal of Biomechanics, 2013. **46**(5): p. 1035-1039.

40. Tong, H.D., et al., *Silicon nitride nanosieve membrane.* Nano letters, 2004. **4**(2): p. 283-287.

41. Scientific, T.F., *DAPI Nucleic Acid Stain*. 2006. p. 5.

42. Corporation, P., *DNA IQ™ System-Database Protocol*. 2016: Madison, WI. p. 14.

43. Camargo Casallas, L.H., *Classification of squamous cell cervical cytology*. Universidad Nacional de Colombia.

44. Sultana, F., Riahinezhad, S., Vaughan, M., and Khandaker, M. , *Effect of architecture on the cell functions of an electrospun fiber membrane*. University of Central Oklahoma. p. 5.

45. Sarasam, A. and S.V. Madihally, *Characterization of chitosan–polycaprolactone blends for tissue engineering applications.* Biomaterials, 2005. **26**(27): p. 5500-5508.

46. QIAGEN®, *QIAcube® User Manual*. 2018. p. 164.

47. Corporation, P., *DNA IQ™ Casework Pro Kit for Maxwell® 16*. 2016: Madison, WI. p. 17.

48. Corporation, P., *Differex™ System--For Use With the Differex™ Magnet*. 2017: Madison, WI. p. 11.