

APPLICATION OF SOLID-STATE NANOPORE TECHNOLOGY TO THE
TRANSLATIONAL ANALYSIS OF OXIDATIVE DAMAGE THROUGH QUANTIFICATION
OF 8-OXO-7, 8 DIHYDROGUANINE IN GENOMIC DNA

BY

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A Dissertation Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

In Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Cancer Biology

January, 2025

Winston-Salem, North Carolina

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ACKNOWLEDGMENTS

I extend my profound appreciation to my advisor, Dr. Adam R. Hall, whose unwavering guidance, patience, and extensive knowledge have been instrumental throughout my doctoral journey. Your belief in my abilities and your encouragement to pursue diverse opportunities have been invaluable.

My sincere thanks go to my esteemed committee members: Dr. Cristina M. Furdui, Dr. Ravi Nandan Singh, Dr. Peiqing Sun, and Dr. Joseph Robertson. Your expert insights and thoughtful recommendations have significantly enhanced the quality of this work. I am deeply grateful for your commitment to my project and the time you've invested in ensuring its quality.

I would like to thank my incredible lab mates who have made this journey both rewarding and enjoyable. To Ian, Komal, Dorothea, Mohamed, Suruchi, Haddie, Daria, Max, and Tyler: your camaraderie, support, and intellectual discussions have enriched my PhD journey. A special mention goes to Ian and Komal, whose unwavering support and friendship have truly made our lab feel like a second home.

I am also profoundly grateful to my family and husband for their constant support and encouragement. Their belief in me and valuable advice have been indispensable throughout this journey.

To my dear children, Amr and Yara Kawas, your arrival during my Ph.D. studies taught me the true value of sleep. Despite the challenges, you have been a source of joy and inspiration, making every moment worthwhile.

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LIST OF ABBREVIATIONS

4PL	4-Parameter Logistic
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
8-oxoGua	8-oxo-7,8-dihydroguanine
A	Adenine
AA	L-Ascorbic Acid
AP site	Abasic Site
APE1	AP endonuclease 1
BER	Base excision repair
BSA	Bovine Serum Albumin
C	Cytosine
DAO	D-amino Acid Oxidase
ddNTP	2',3'-Dideoxyadenosine-5'-triphosphate
DDR	DNA Damage Response
dG	2'-Deoxyguanine
ds-	Double Stranded
dsDNA	Double-stranded Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electromobility Shift Assay
Endo IV	Endonuclease IV
FAM-labeled	Fluorescently Labeled
FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine

FBS	Fetal Bovine Serum
Fpg	Formamidopyrimidine DNA Glycosylase
G	Guanine
gDNA	Genomic DNA
GST	Glutathione-S-Transferase-Tagged
HIM	Helium-Ion Milling
hMS	Polyhistidine-Tagged Monovalent Streptavidin
HNSCC	Head and Neck Squamous Cell Carcinoma
HPLC	High Performance Liquid Chromatography
HyPer	Hydrogen Peroxide Fluorescent Probe
IP	Immunoprecipitation
IR	Ionizing Radiation
I-V	Current-Voltage
LC-MS/MS	Liquid Chromatography-Mass spectrometry
LOQ	Limit of Quantification
MBP	Maltose-Binding Protein
miRNA	Micro-RNA
MS	Monovalent Streptavidin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOX	NADPH oxidase
nt	nucleotides
OGG1	8-oxoGuanine DNA Glycosylase 1
PARP1	poly (ADP-ribose) polymerase 1
PCR	Polymerase Chain Reaction

PI	Isoelectric Points
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription-Quantitative Polymerase Chain Reaction
SiNx	Silicon Nitride
SNR	Signal-to-Noise Ratio
ss-	Single Stranded
SS-nanopore	Solid-State Nanopore
T	Thymine
WT	Wild-Type

ABSTRACT

The quantification of 8-oxo-7,8-dihydroguanine (8-oxoGua) in genomic DNA (gDNA) is crucial for understanding its role in disease development and progression, particularly in cancer. However, current analytical techniques display a wide range of limitations that could be overcome by solid-state (SS)-nanopores, offering robustness, high sensitivity and convenience to the quantification of 8-oxoGua. We developed a selective SS-nanopore assay leveraged to quantify 8-oxoGua when coupled with tagging its specific location in the DNA with biotin. In this dissertation, we aimed at validating the utility of this assay in 8-oxoGua quantification in gDNA models from various sources.

Several key objectives were achieved in this work. In chapter II, we enhanced the performance of the SS-nanopore assay by incorporating a polyhistidine-tagged monovalent streptavidin (hMS) and optimizing measurement conditions. Modifying the buffer pH and salt concentration enabled significant improvements in the stability, sensitivity and selectivity of the assay evidenced by micro-RNA (miRNA) quantification in human plasma at low concentrations. The assay's feasibility and convenience were also enhanced evidenced by the observed broadening of device diameters feasible for the measurement.

In chapter III, we validated the SS-nanopore assay for quantifying 8-oxoGua in gDNA. We first achieved high efficiency and specificity in 8-oxoGua labeling reaching up to 97%. Additionally, we successfully quantified 8-oxoGua in gDNA exposed to Hydrogen Peroxide (H_2O_2), showing strong correlations with mass spectrometry, the golden standard.

Finally, we applied the optimized SS-nanopore assay to study 8-oxoGua formation and repair in a matched model of head and neck squamous cell carcinoma (HNSCC) cell lines

with varied radiosensitivity to establish its translational capability in chapter IV. Our approach was able to detect potential mechanistic differences between the radio-sensitive and resistant phenotypes.

Through this work, we established the SS-nanopore assay developed by our lab as a promising tool able to accurately quantify 8-oxoGua in gDNA. This method addresses the limitations of traditional techniques, providing a reliable and convenient tool. Our findings also contributed to our understanding of 8-oxoGua's role in cancer radio-resistance and opened new avenues for its use as a biomarker in cancer research and treatment response assessment.

CHAPTER I

This unpublished chapter was composed by Sara Abu Jalboush with editorial guidance from

Adam R. Hall

BACKGROUND

I.1 The SS-nanopore platform

SS-nanopores present a promising and innovative technology that is gaining recognition for its ability to detect and characterize biomolecules [1] with remarkable sensitivity, and cost-effectiveness. The fundamental principle underlying the SS-nanopores' function is resistive pulse sensing [2] wherein objects passing through a narrow aperture produces interruptions in pathway resistance (i.e. ionic current) that can be measured and interpreted to report on the nature of the object. This mechanism, analogous to that employed in Coulter counter technology for cell characterization [3], has been miniaturized successfully to enable sensing at a single-biomolecule level.

A typical SS-nanopore comprises a nanometer-scale aperture drilled in a thin silicon nitride (SiN_x) membrane, supported by a frame for easy handling. This membrane is mounted in a flow cell comprised of two isolated reservoirs of electrolyte solution, the *cis* and *trans* chambers, with the pore serving as the sole connection point (Figure I.1).

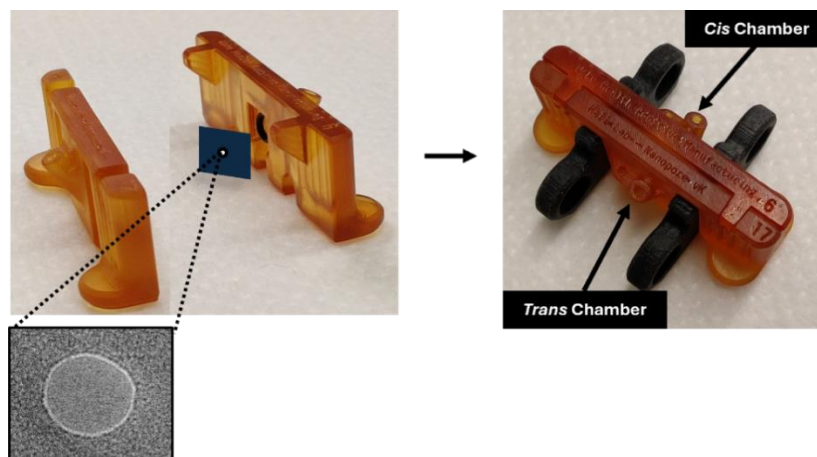


Figure 1.1: The SS-nanopore platform. A silicon chip supporting a SiNx membrane featuring a nanopore (illustrated in a TEM image) prior to mounting in a flow cell (left panel). The right panel demonstrates a fully mounted SS-nanopore in a flow cell containing two chambers of electrolyte solution; the cis and trans chambers, where the pore serves as the only connection point between them.

When a voltage is applied across the membrane, it generates a highly localized electric field within the pore, resulting in a baseline ionic current with a magnitude that is determined by both the electrolyte composition and pore dimensions [4]. Charged molecules introduced to one side of the pore will be subjected to electrophoretic forces propelling them to the opposite chamber. Throughout this process, each passing molecule will result in a transient displacement of the ions contributing to the baseline current, causing a reduction that rapidly returns to the baseline value upon completion of molecule translocation (Figure 1.2). These transient current blockades are referred to as translocation 'events'. These events provide valuable information through careful analysis of their properties, including amplitude, duration, and rate which can be used to

characterize the passing molecule, including cross-sectional area [5], molecular weight [6], and concentration [7].

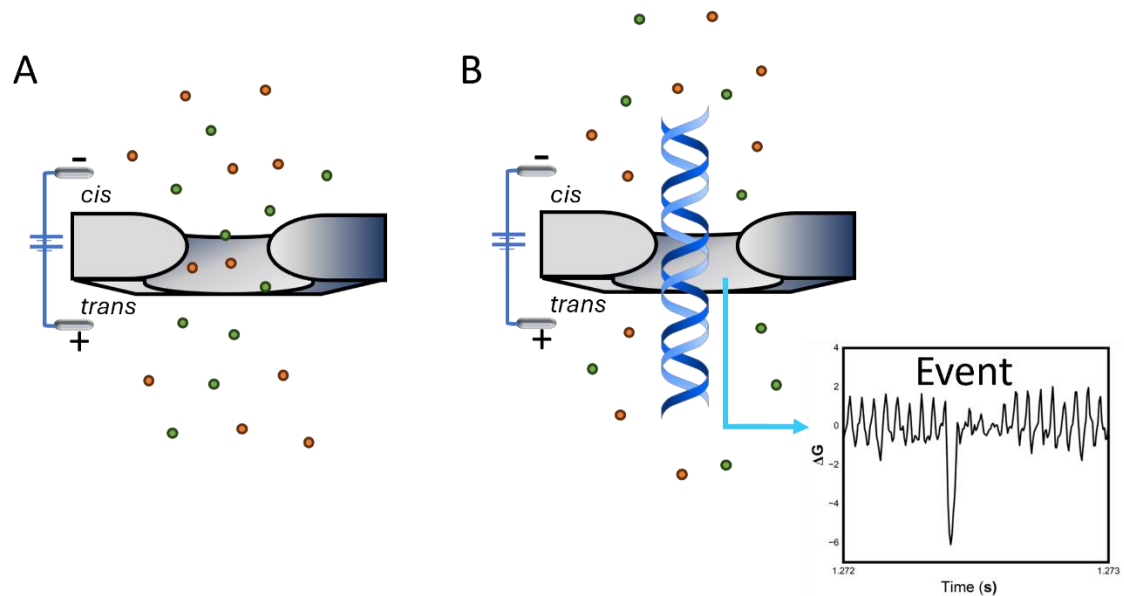


Figure I.2: Cross sectional view of a fully mounted SS-nanopore prior to and during biomolecule translocation. A. a schematic view of a fully mounted SS-nanopore conducting an ionic current (orange and green circles represent positive and negative ions diffusing across the nanopore) when a voltage is applied. B. Electrophoretic translocation of DNA across the pore leading to displacement of ions and a transient blockade of the ionic current that rapidly returns to baseline value when the translocation is completed. This transient blockade in conductance is known as an event.

Over the past decade, SS-nanopores have been employed in the detection, quantification, and characterization of single molecules including DNA [4, 8-13], RNA [14-18], protein [19-26], small molecules [11, 27, 28], and biomolecule complexes [5, 21, 29-31]. Also, they have been used for discrimination of DNA and protein conformations [32-34], epigenetic DNA modifications [35-39], various pathogens [40-44], as well as single nucleotides for DNA sequencing [45-47]. Nanopores, and especially SS-nanopores introduce several advantages in comparison to existing technologies including rapid detection, convenience, single-base level quantification, sequence preservation, minimal sample size requirements, elimination of prior amplification steps, reduced preprocessing, and enhanced versatility and robustness. However, the majority of these applications have been conducted in controlled, synthetic specimens that do not approximate the complexity of clinical samples. This limitation primarily stems from lack of intrinsic selectivity in conventional measurements because all translocating molecules can produce events. Consequently, distinguishing signals resulting from specific molecule types within a mixture proves challenging and necessitates rigorous downstream analyses.

I.2 Selective SS-nanopore assay

To improve the selectivity and resolution of SS-nanopore detection, multiple approaches have been implemented with limited success including (1) modifying the pore membrane material to lower the electrical noise and enhance the signal (reviewed in [48]), (2) slowing down the translocating molecule and using brute force analysis of event signatures [10, 49, 50], (3) surface modifications and membrane functionalization (reviewed in [51, 52]). Others have used DNA nanostructures [18] and probes coupled with viral protein p19 [17] to pre-select specific sequences of short nucleic acids for SS-nanopore detection. Alternatively, our lab developed a selective SS-nanopore assay designed to produce a signal from the target molecule only, allowing us to specifically probe one biomarker

present in a mixture of interfering molecules (See Figure 1 in chapter II). The assay is composed of two components: a short biotin-labeled double-stranded (ds-) nucleic acid, and a mutant variant of streptavidin protein which can only bind one biotin instead of four (monovalent streptavidin, or MS) [53]. When passed through a pore of an appropriate diameter individually, neither the nucleic acid nor the protein produces a signal due to their fast translocation that cannot be resolved by conventional electronics. However, when a biotin-streptavidin complex is formed, the nucleoprotein is large enough to interact with the walls of the pore slowing its translocation to a resolvable speed resulting in a detectable signal which allows for the quantification of the complex present in the measured sample [14, 39, 40, 54].

The sensitivity and selectivity of our assay depends critically on many experimental parameters that include nanopore diameter, DNA length, and applied voltage and we have explored each of these systematically to identify optimal conditions for assay performance [35, 36, 39]. The diameter of the nanopore is the most critical factor affecting the performance of this selective assay. The smallest viable diameter is approximately 7.5 nm, which matches the combined cross-section of a duplex nucleic acid (~2.2 nm) and the MS tetramer (~5.6 nm). Smaller pores require deformation of the complex to pass through, leading to signal instability and fouling. As the pore diameter increases, steric hindrance decreases, allowing more nucleoprotein complexes to translocate rapidly, which results in an exponential decrease in the event rate. However, under certain conditions, high selectivity can be achieved with SS-nanopores up to 16 nm in diameter as we will show in this dissertation. The length of the duplex nucleic acid construct is also important. The assay is effective across sizes from 23 bp to 250 bp, but two competing effects arise. Larger nucleic acids yield more resolvable nucleoprotein complexes, increasing the event rate, while smaller constructs reduce sensitivity. Conversely, larger constructs also allow

for easier resolution of nucleic acid fragments, which diminishes selectivity and impacts the assay's effectiveness. We will address the effect of DNA length in more detail in this dissertation.

I.3 SS-nanopores for the detection and quantification of nucleic acid biomarkers

Chemical modifications of DNA bases are commonly seen in gDNA due to their crucial roles in various cellular functions including regulation of stem cell differentiation, gene expression, RNA splicing, DNA replication, and chromatin organization (reviewed in detail in [55]). Alterations in their abundance and regulatory/repair mechanisms have been associated with the development and progression of a wide range of diseases such as metabolic disorders [56, 57], neurological disorders [58, 59], cardiovascular diseases [60], age related disorders [61] and cancer [62-64]. Their clinical significance as biomarkers is underscored by their high mutagenic potential, their ability to disrupt methylation patterns, ability to regulate gene expression, and to predict the magnitude of response to DNA damage after exposure to a damaging agent such as oxidation or alkylation.

Although these modifications hold significant clinical potential, their adoption as biomarkers has been hindered by the high cost and complexity of current analytical methods. Many existing techniques face technical challenges such as cross-reactivity, sample size limitations, and artifactual oxidation. Our selective SS-nanopore assay offers a potential solution to these issues, providing a cost-effective, rapid, and versatile tool for quantifying various single-base modifications. This is accomplished by introducing an affinity tag (e.g., biotin) at each modification site, preparing the tagged dsDNA for binding with MS, and enabling downstream quantification through SS-nanopores.

I.3.1 SS-nanopore Quantification of 5-hydroxymethylcytosine (5hmC)

To assess the utility of the selective SS-nanopore assay in the quantification of single base modifications, we applied the assay to quantify global levels of 5hmC in mammalian gDNA known to acquire elevated levels of the modified base as a model [35]. an established labeling approach was employed to incorporate an N3- β -glucosyl-biotin at 5hmC sites [65] after which labeled materials were subjected to SS-nanopore analysis and their concentration was determined. To validate these measurements, the results were compared to LC-MS/MS quantitation of the same samples. As 5hmC levels from two independent SS-nanopore devices yielded results in quantitative agreement with LC-MS/MS measurement. These findings verified the ability of the SS-nanopore assay to quantify 5hmC in gDNA revealing a significant correlation with conventional quantitative methods.

Additionally, the selective SS-nanopore assay was used to measure 5hmC content in human breast cancer tissues and cell lines [36]. SS-nanopore analysis enabled the quantification of the base, finding that the reduction in the global levels of 5hmC reported in literature [66] is associated with invasive but not early-stage cancer cells.

Those studies mark a significant step toward quantitative analysis of epigenetic modifications in human gDNA using solid-state nanopores, opening avenues for further research in utilizing this technology for biomarker quantification clinically.

I.3.2 SS-nanopore quantification of other DNA base modifications

The labeling technique described above is limited to 5hmC and a few other elements of the cytosine demethylation pathway, thus cannot be widely used for other DNA base lesions of interest such as 8-oxoGua. Therefore, our lab introduced a novel modular site-specific labeling approach that utilizes the stepwise enzymatic process of the base

excision repair pathway (BER) [39]. Briefly, a modified base is recognized and excised using a DNA glycosylase specific for that modification leaving an abasic (AP) site. Next, an AP endonuclease is used to cleave the phosphodiester bond 5' to the AP site and to process the exposed 3' phosphate, leaving a hydroxyl group that is amenable to the last step, which involves introducing a gap-filling polymerase that lacks the 3'–5' exonuclease activity and is offered with only one of the cognate biotin-dNTPs to incorporate it at the site of the modification (Figure I.3).

This approach was applied to distinctly label and detect four different DNA base modification via SS-nanopores, namely, uracil, 8-oxoGua, T:G mismatch, and 1,N6-Ethenoadenine [39]. Furthermore, adaptations were developed to distinguish elements of the cytosine demethylation pathway that were difficult to probe previously, including 5-methylcytosine (5mC), 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) [67].

The modular and enzymatic nature of this labeling strategy provides a versatile tool for the comprehensive analysis of various DNA base modifications in gDNA.

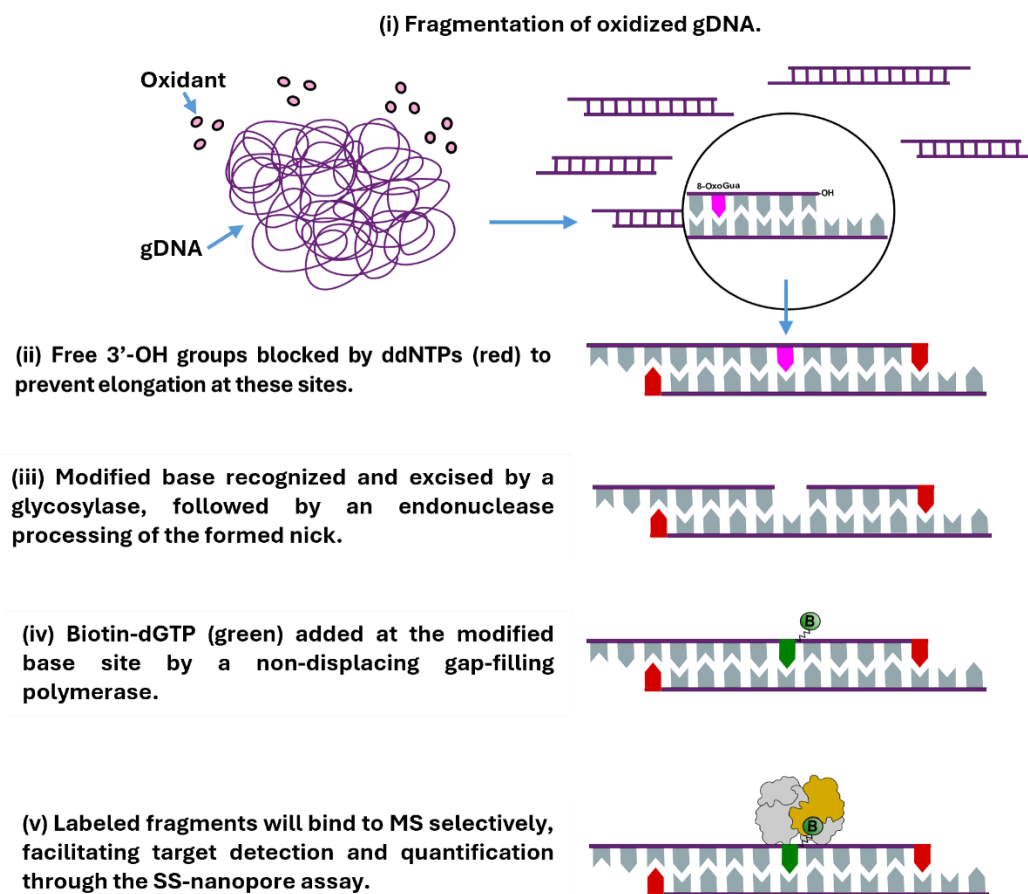


Figure I.3. Site-specific labeling of modified DNA bases. A Schematic representation of the site-specific labeling protocol starting with gDNA oxidation which induces modified base formation (pink), followed by (i) Fragmentation of oxidized DNA into segments at lengths feasible for the assay. Some fragments may contain modified bases. (ii) Blocking of free 3'-OH ends with ddNTPs (red), (iii) Recognition and excision of the modified base, backbone nick formation 3' to its site by a bifunctional glycosylase, GAP formation and processing of 3'-P ends into 3'-OH ready for elongation, and (iv) Filling the GAP site with Biotin-dGTPs (green) via a gap-filling non-displacing DNA polymerase. (v) Labeled fragments are bound to MS to facilitate their quantification through the SS-nanopore assay

I.4 Significance of 8-oxoGua

I.4.1 Reactive oxygen species (ROS)

ROS are highly reactive molecules derived from oxygen [68, 69]. At physiological levels, ROS have been found to play crucial roles in cell signaling, immune response, and in modulating redox homeostasis. However, excessive ROS production or impaired antioxidant defense mechanisms can lead to oxidative stress, causing damage to biomolecules and disrupting cellular functions and consequently contributing to the pathogenesis of various diseases including cancer [70].

ROS can be generated from endogenous (intracellular) and exogenous sources. Endogenous sources of ROS are intracellular enzymes and organelles that generate ROS as byproducts of normal cellular metabolism or as part of their physiological functions. The major endogenous sources include the Mitochondrial electron transport chain, NADPH oxidases (NOXs), and other enzymatic sources. On the other hand, exogenous sources of ROS are external factors or environmental stimuli that can induce ROS generation within cells or tissues. The major exogenous source of ROS generation is exposure to ionizing radiation (IR), such as X-rays, gamma rays, and particle radiation and UV radiation production. Other exogenous sources include environmental pollutants such as particulate matter, ozone, cigarette smoke, and certain chemicals (e.g., paraquat, quinones, nitroaromatics) [70, 71].

While some ROS possess limited chemical reactivity, others are considered highly reactive and strong oxidizing agents based on their redox potentials, lifetime, and their diffusion capabilities. For instance, $O_2^{\bullet-}$ is less reactive and has confined effects, while H_2O_2 is membrane diffusible and is readily converted to the more reactive $\bullet OH$ upon encounter with transition metals such as Fe (II) and Cu (I) by what is known as the Fenton

reaction (Harber-Weiss reaction). In turn, $\bullet\text{OH}$ has the ability to interact with almost all macromolecules found in living cells including the gDNA leading to the formation of various DNA base modifications [72].

I.4.2 8-oxoGua

Guanine (G) is the most readily oxidized base in the DNA due to its low redox potential [73]. Numerous studies have shown that 8-oxoGua is the most abundant product of guanine oxidation [72, 74]. 8-oxoGua arises from the attack of $\bullet\text{OH}$ on the C-8 position of the purine ring of G which gives rise to 8-hydroxy-7,8-dihydro-7-yl radical that is considered the precursor of both 8-oxoGua and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG). Subsequently, one-electron reaction in the presence of Oxygen (O_2) results in the formation of 8-oxoGua [74] (Figure I.4, A).

8-oxoGua is readily repaired by the BER in the cells. Although this defense mechanism is efficient, some adducts are not repaired. 8-oxoGua have been shown to possess high mutagenic potential if persisted in the DNA. Structural studies demonstrated that 8-oxoGua has the propensity to form a Hoogsteen base pair with Adenine (A) in a syn conformation (Figure I.4, B) converting G into thymine (T) at the site of the adduct if not repaired during DNA replication. This mutation is frequently observed in cancer cells, especially with impaired repair mechanisms [75]. Moreover, 8-oxoGua may be erroneously incorporated from the nucleotide pool by DNA polymerases opposite to cytosine (C) and even A, but to a lesser extent. Consequently, two distinct mutations may arise: misincorporation of 8-oxoGua opposite to A results in A-C transversion mutation, and misincorporation of 8-oxoGua opposite to C results in G-T transversion mutation [72].

development and progression of cancer [77, 78]. Numerous studies have shown increased levels of 8-oxoGua in the blood, urine and tumor tissues of advanced stage cancer patients compared to healthy controls. This has been related to high ROS generation coupled with low antioxidant activity in the tumor environment, or potential alterations in repair mechanisms, specifically the base excision repair pathway [64, 79-81]. Furthermore, 8-oxoGua is one of the most commonly observed DNA base adducts in tumor tissues and irradiated cells suggesting its potential role in risk assessment and response to therapy [82, 83]. For instance, in Head and neck cancer where radiation is used significantly in patients' treatment, 8-oxoGua could serve as an important biomarker for response to treatment. A study conducted by Roszkowski et al. collected both urine and blood samples from HNSCC patients 24 hours after radiotherapy and showed increased levels of the modified base but not the nucleoside in the urine of most patients, indicating potential DNA damage and repair caused by radiotherapy. However, a subset of patients showed a decrease in 8-oxoGua in urine and an increase of the nucleoside in DNA isolated from leukocytes, which may suggest impaired DNA repair in those patients [84]. In another study, Roszkowski et al. have shown that HNSCC patients undergoing radiotherapy displayed increased levels of 8-oxoGua in their urine and no concomitant change in 8-oxoGua in leukocytes after 24 hours of the first fraction of radical radiotherapy which was associated with better response to treatment [85]. These are the only studies to date demonstrating the potential predictive role of 8-oxoGua in radiotherapy response for HNSCC patients. Thus, more studies are warranted to better understand the formation and repair of 8-oxoGua and its association with radio-resistance which may exhibit significant clinical implications for those patients.

On another note, formation of DNA base lesions including 8-oxoGua is indicative of increased ROS production which is a known phenomenon of cancer cells. Tumor cells

commonly compensate by elevating repair pathways which renders the tumor resistant and more aggressive. Extensive research has been employed in the past decade to exploit elements of the DNA damage response (DDR) as therapeutic target either solely or in combination with conventional therapeutic modalities [86-88]. Inhibitors targeting elements of the BER pathway, which is involved in the repair of 8-oxGua, have been developed to overcome resistance or to enhance the activity of cytotoxic reagents. A great example of these targets showing tremendous success clinically are AP endonuclease 1 (APE1) and poly (ADP-ribose) polymerase 1 (PARP1) inhibitors. Hence, investigating the net abundance of 8-oxoGua in a particular tumor could provide an insight of the magnitude of oxidative damage exerted on that tumor and the status of its DNA damage repair pathways leading to identification of therapeutic targets.

I.5 Limitations of the current analytical techniques

The clinical utility of 8-oxoGua has been hampered in recent years due to limitations associated with the current detection and measurement techniques. Many high-throughput sequencing techniques have been developed to probe 8-oxoGua in gDNA. The results from these studies varied considerably due to biases arising from the dynamic nature and non-uniform distribution of 8-oxoGua at the studied promoters. Furthermore, those techniques typically require prior amplification steps which may lead to artifactual generation of oxidative lesions, and full-genome sequencing at high coverage which is very expensive [75, 89]. A global view of 8-oxoGua formation in the genome has been explored using antibody-based techniques such as enzyme-linked immunosorbent assay (ELISA), microarrays, or immunoprecipitation (IP). Such techniques suffer from cross reactivity, non-specificity, and low resolution [90, 91]. High performance liquid chromatography (HPLC) and mass spectrometry have been used to quantify the net abundance of 8-oxoGua in cells, tissue, and bodily fluids. Although this method is the most

sensitive and specific method, it remains expensive and labor intensive. It also requires multiple preprocessing steps which may cause artifactual oxidation of the DNA, lose sample yield and necessitates the use of a large sample size that might not be available in a clinical setting. Additionally, it is destructive to the DNA and hinders further sequence-related analyses [92, 93]. Therefore, there is a pressing need for convenient, reliable, and high-resolution technologies to quantify global levels of 8-oxoGua in the genome while preserving the sequence for further analyses.

I.6 Overall objectives

Given the potential clinical advantages of SS-nanopores and the importance of 8-oxoGua as a predictive biomarker in the context of radiotherapy, the objective of this study is to optimize our SS-nanopore assay to improve its sensitivity in the detection and quantification of 8-oxoGua in gDNA, validate its accuracy by comparing it to mass spectrometry, and demonstrate its translational capability. The proposed experiments in this study are strongly supported by previous data from our lab demonstrating that: 1) DNA base lesions including 8-oxoGua featured in synthetic dsDNA constructs were probed with high specificity and detected by SS-nanopores [39]; 2) SS-nanopore quantification of 5hmC as a model DNA base modification in mammalian tissues yields measurements in quantitative agreement with mass spectrometry [35, 36].

Although 8-oxoGua has been probed and detected successfully using our SS-nanopore assay, the resolution can be improved and the limits of detection are yet to be established to achieve physiologically relevant measurements, especially in a cellular system where 8-oxoGua is present in low abundance. Additionally, SS-nanopore quantification of 8-oxoGua has not been applied to a cellular system. Thus, we aim to address these open questions in this project.

Our central hypothesis states that our SS-nanopore assay will demonstrate sufficient sensitivity to quantify 8-oxoGua in nucleic acids from various sources with high sensitivity and specificity and will demonstrate translational value in the context of 8-oxoGua quantification in an HNSCC model of radio-resistance. In this work, we aimed at validating the selective SS-nanopore assay against mass spectrometry, determine its limits of quantification, and employing it to quantify 8-oxoGua in the gDNA of two different models. Additionally, we implemented the optimized and validated assay to probe difference in 8-oxoGua formation and repair in a matched model of radio-sensitive/resistant HNSCC cell lines.

Our long-term goal is to establish our SS-nanopore approach as a reliable, cost-effective, and convenient tool for quantifying 8-oxoGua not only in cells but in samples derived from human sources in order to be employed in clinical applications.

I.7 References

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CHAPTER II

IMPROVING THE PERFORMANCE OF SELECTIVE SOLID-STATE NANOPORE SENSING USING A POLYHISTIDINE-TAGGED MONOVALENT STREPTAVIDIN

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The following manuscript was published in *ACS Sensors*, volume 9/issue 3, pages 1602-1610, March 2024 and is reprinted with permission. The stylistic variations are due to the requirements of the journal.

Author contributions: S.A.J., I.D.W., K.S. contributed equally to this work. Specific contributions include: 1- conceptualization and research design: A.R.H., S.A.J., I.D.W., K.S.; 2- Buffer pH related studies: S.A.J.; 3- Salt concentration related studies: I.D.W.; 4- Pore diameter related studies: K.S., S.A.J., I.D.W.; 5- Monovalent streptavidin protein expression related studies: L.C.R., I.D.W., T.H., A.R.H.; 6- Drafting the manuscript: S.A.J., I.D.W., K.S.; 7- Manuscript revision and edits: A.R.H., S.A.J., I.D.W., K.S.; All authors have read and agreed to the published version of the manuscript.

II.1 Abstract

SS-nanopore sensing has gained tremendous attention in recent years but has been constrained by its intrinsic lack of selectivity. To address this, we have previously established a novel SS-nanopore assay that produces translocation signals only when a target biotinylated nucleic acid fragment binds to MS, a protein variant with a single high-affinity biotin-binding domain. While this approach has enabled selective quantification of diverse nucleic acid biomarkers, sensitivity enhancements are needed to improve the detection of low-abundance translational targets. Because the translocation dynamics that determine assay efficacy are largely governed by constituent charge characteristics, we here incorporate a polyhistidine-tagged MS (hMS) to alter component detectability. We investigate the effects of buffer pH, salt concentration, and SS-nanopore diameter on the performance with the alternate reagent and achieve significant improvements in measurement sensitivity and selectivity and expand the range of device dimensions viable for the assay. We use this improvement to detect as little as 1 nM of miRNA spiked into human plasma. Overall, our findings improve the potential for broader applications of SS-nanopores in quantitative analyses of molecular biomarkers.

II.2 Key Words

Solid-state nanopores, nucleic acids, monovalent streptavidin, amplification-free detection, selective detection, biomarkers

II.3 Introduction

SS-nanopores are an emerging technology for biological sensing at the single-molecule level that has garnered interest due to remarkable sensitivity, versatility, robustness, and cost-effectiveness^{1,2}. The device consists of a nanometer-scale aperture in a thin membrane that is positioned such that the pore is the only connection between two chambers of ionic solution. When a voltage is applied across the membrane, a strong electric field is produced in the pore and an ionic current can be detected and monitored. Charged molecules introduced to one side of the membrane are subjected to electrical forces that translocate them through the pore and to the opposite chamber. During this translocation process, passing molecules produce transient blockades (*i.e.*, events) in the ionic current that are typically used to report on aspects of the molecules themselves through event properties such as amplitude, duration, and rate. Over the past decade, the platform has been employed in the analysis of diverse (bio)molecules including DNA^{3–6}, RNA^{7–9}, proteins^{10–12}, biomolecular complexes^{13–16}, and small molecules^{17–19}.

Despite the utility of SS-nanopore detection, a major limitation has been its lack of intrinsic selectivity; all translocating molecules produce signals and thus must be discriminated based on often subtle differences in event characteristics. This has been especially challenging for molecular disease biomarkers that are typically found in heterogeneous biological fluids where molecules of similar sizes and physical properties may be present. To address this, multiple strategies have been implemented to improve event differentiation^{3,20–25} and to pre-select for target analytes^{10,26,27}. Alternatively, we have developed an approach that achieves selectivity through molecular conjugation and steric interactions with the pore^{7,28–30}. Our assay (**Figure II.1a**) is comprised of two elements: a short, biotin-labeled nucleic acid and a monovalent variant of the protein streptavidin that possesses only one active biotin-binding subunit^{31,32}. When passed through a pore of an

appropriate diameter individually, neither the nucleic acid nor the protein produces significant events due to their rapid threading that cannot be detected easily by conventional electronics. However, when the two bind to form a nucleoprotein complex, its larger size interacts with the walls of the pore during translocation, slowing it to a resolvable speed.

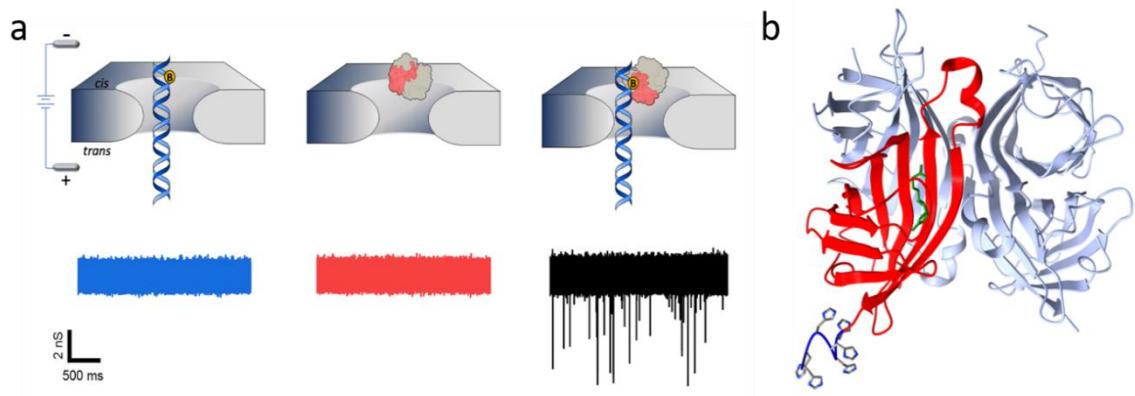


Figure II.2. The selective SS-nanopore assay concept. (a) Schematic (top) and example conductance traces (bottom) demonstrating the selective SS-nanopore assay. When translocated individually through a SS-nanopore, neither DNA (left) nor MS (center) yields significant events in the conductance trace (blue and red, respectively). When in complex (right) events are readily observed (black trace). (b) Structural representation of the polyhistidine tagged MS. The streptavidin tetramer contains three inactive protomers (grey) and one active protomer (red) capable of binding biotin (green). The C-terminal polyhistidine tag on the active monomer is shown as stick representation.

The sensitivity and selectivity of our assay depend critically on many experimental parameters that have been explored systematically in prior studies to identify optimal conditions for assay performance^{28,33,34}. One critical factor determining the dynamics of

translocation is the charge associated with both the molecular components and with the SS-nanopore itself. We recently investigated³⁰ the impact of modified salt conditions on our assay, thereby varying charge screening and influencing electrokinetics. While strong effects were observed, the systemic nature of buffer ionic concentration alone as an experimental variable limited our ability to modify charges independently; because all assay components are contacted by the buffer, it was not possible to vary the charge of any one component without affecting the others. Therefore, we reasoned that employing MS specifically with a less negative charge profile would alter translocation dynamics of the nucleoprotein complex, potentially making it more detectable by reducing electrokinetic transport through the pore while maintaining stericity.

Here, we investigate this by utilizing an MS on which the conventional glutamate tag³² has been replaced with a polyhistidine peptide tag (**Figure II.1b**). Polyhistidine comprises a bulky ionizable side chain moiety in the form of an imidazole ring that is partially protonated at neutral pH, decreasing its net charge by about 6.5 e⁻ per protein relative to glutamic acid. Using this new reagent, we explore key experimental conditions including buffer pH, buffer salt concentration, and SS-nanopore diameter to determine their impacts on assay performance, ultimately demonstrating a significant improvement in both sensitivity and selectivity. Finally, we used these improvements to demonstrate enhanced translational biosensing with the assay by detecting a synthetic miRNA spiked into human plasma.

II.4 Materials & Methods

II.4.1 Biomolecules

All synthetic DNAs and RNAs were purchased from Integrated DNA Technologies (Coralville, IA, USA). For assay optimization, monobiotinylated DNA oligo (60 nt) with the sequence ATC AAC TGT TTC AGC CAC TGC TTC GCA GGC TGA CGT A**I**C TGA CGT GGT GCC AGC GAC GGA (where **I** indicates biotinylated thymine) was used to form double stranded constructs via annealing with an unmodified complementary oligo. For human plasma measurements, a synthetic RNA oligo with the sequence UAC CCG UAA UCU UCA UAA UCC GAG was used, matching cel-miR-54, a 24 nt miRNA associated with *C. elegans* and not found in human specimens. A complement monobiotinylated DNA oligo probe (CTC GGA TTA **I**GA AGA TTA CGG GTA) was utilized as a probe to form RNA/DNA heteroduplex constructs.

Recombinant hMS was produced in-house as a heterogeneous tetrameric variant of streptavidin containing one wild-type (WT) monomer with high-affinity biotin binding capacity and three mutant (inactive) monomers^{31,32}. The genes encoding the streptavidin^{WT} and streptavidin^{N12A/S16D/S34A} proteins were each expressed as a fusion with the maltose-binding protein (MBP) in a pET28 vector. The sequences included the rhinovirus 3C protease recognition site between the MBP and streptavidin to allow for cleavage and removal of the MBP. The streptavidin^{WT} sequence included a C-terminal polyhistidine sequence. WT and mutant fusion monomers were expressed independently in BL21* *E. coli*. Each resulting fusion protein was then captured separately on an amylose resin column (New England Biolabs, Ipswich, MA, USA), washed, and eluted. The fusion proteins were then cleaved by glutathione-S-transferase-tagged (GST-) PreScission Protease (GenScript, Piscataway, NJ, USA), separating the streptavidin from MBP.

Following cleavage of the fusion protein, GST-PreScission Protease was removed by a glutathione resin column (New England Biolabs, Ipswich, MA, USA) to produce stocks of mutant and WT proteins. The WT and mutant streptavidin proteins were then mixed at a molar ratio of 1:4 and denatured in 8M guanidinium hydrochloride (pH 1.5) (Sigma-Aldrich, Saint Louis, MO, USA) at 50 °C for 1 hr. The denatured protein mixture was added dropwise into cold 1X PBS under constant stirring and then left stirring overnight to induce protein refolding. The MBP was then removed by passing the protein mixture over an amylose resin column a second time after which monovalent streptavidin tetramer was isolated and purified using a HisTrap HP nickel column (Cytiva, Marlborough, MA, USA). Finally, hMS was concentrated by spin column (Sartorius, Goettingen, Germany). hMS protein purity was confirmed by SDS-PAGE and its activity was verified by electromobility shift assay (**Figure II.2**).

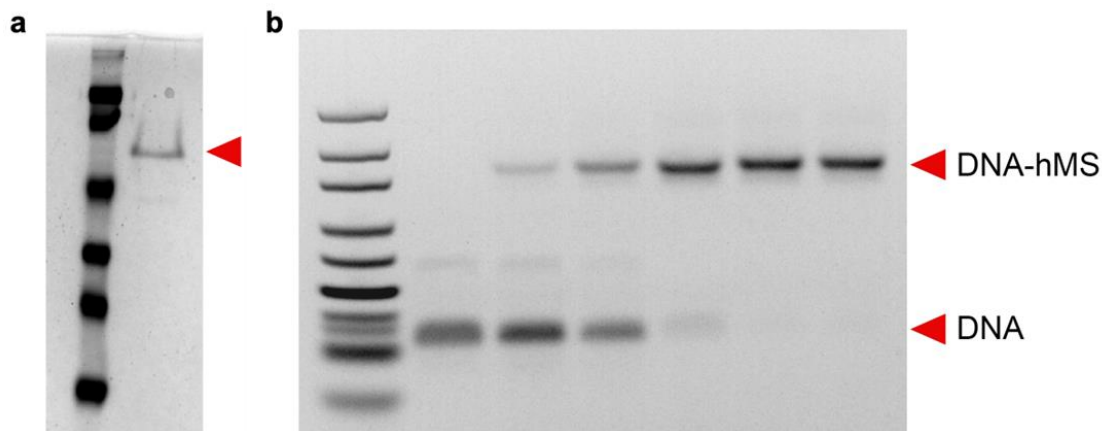


Figure II.2. Visualization of hMS binding to Biotinylated DNA. (a) SDS-PAGE analysis of a molecular weight ladder (lane 1) and hMS (lane 2; 54.3 kDa, red arrow), demonstrating the high purity of the protein produced in-house. From top to bottom, the ladder bands are 103, 77, 50, 34.3, 28.8, and 20.7 kDa. (b) Agarose gel analysis of an electromobility shift assay showing hMS titrated against biotinylated DNA at molar ratios (hMS:DNA) of (l-r) 0, 0.25, 0.5, 0.75, 1, and 2. A biotinylated 60 nt synthetic ssDNA oligo was used to form a nucleoprotein complex to determine the biotin-binding efficiency of the hMS.

II.4.2 SS-nanopore measurements

SS-nanopores were fabricated with a helium-ion milling (HIM) technique described elsewhere³⁵ in 20 nm SiNx membranes supported by a silicon chip (4.4 x 4.4 mm). Prior to measurements, each device was first exposed to 10 W air plasma (Harrick Plasma, Ithaca, NY, USA) for 2 min on each side and then mounted in a custom flow cell fabricated with a commercial 3D printer (Carbon, Redwood City, CA, USA). Both sides of the chip were then contacted with measurement buffer consisting of 10 mM tris-base, 1 mM EDTA, and NaCl concentration and pH as described in the text. Ag/AgCl electrodes introduced to each reservoir were used to apply voltage and measure ionic current using a commercial

patch-clamp amplifier (Axopatch 200B, Axon Instruments, San Jose, CA, USA). The diameter d_{pore} of a SS-nanopore device was determined from its open-pore current-voltage (I-V) response using the expression²⁶

$$I = V\sigma \left[\left(\frac{4L}{d_{pore}^2} \right) + \left(\frac{1}{d_{pore}} \right) \right]^{-1}$$

where I is current, V is applied voltage, σ is bulk conductivity of the solution, and L is the effective length of the pore (considered to be 1/3 total membrane thickness following previous reports²⁶).

All presented measurements were performed under a 300 mV applied bias. Translocation data were collected at 200 kHz with a 100 kHz Bessel filter and recorded using in-house data acquisition software (LabView, National Instruments, Austin, TX, USA). An additional 10 kHz low-pass filter was applied digitally during analysis. Transient reductions in measured SS-nanopore conductance were considered only if their durations were between 25 and 2500 μ s and their amplitudes were at least 4.5 standard deviations from baseline noise. Data were recorded in discrete 3.2 s segments from which the average rate was calculated. All measurements were performed on 100 nM DNA, 500 nM hMS, or a mixture of the two incubated for 30 min at room temperature to produce the DNA-hMS complex.

For pH optimization, measurements were performed on SS-nanopores with diameters between 7.3 and 10.1 nm. At least 4 separate nanopores were utilized for each condition. For salt concentration optimization, measurements were performed on SS-nanopores with diameters between 7.7 and 10.6 nm. Analysis at each condition was repeated using three different SS-nanopores except for 1.0 M ($n = 4$) and 1.25 M ($n = 5$) NaCl. For diameter dependence measurements, 14 independent SS-nanopores were employed with

diameters from 7.3 to 23.1 nm. All measurements were performed for at least 5 min except for pH 7.0 due to instability and clogging.

II.4.3 miRNA quantification

Whole blood acquired from a healthy human subject (Atrium Wake Forest Baptist protocol number IRB00039804) was immediately processed by centrifugation at 1,500 $\times g$ at 4 °C for 15 min. Separated plasma was stored at -80 °C prior to use. An miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germantown, MD, USA) was used to isolate small RNA (including miRNA) from 200 μ L of plasma. Synthetic cel-miR-54 miRNA (described above) was spiked into the plasma at concentrations of 1, 10, or 100 nM after the addition of the protein precipitate buffer (kit buffer 'RPP') to ensure the stability of the bare synthetic RNA. Purification was performed according to the manufacturer's instructions but with the final elution step conducted twice to maximize recovery³⁶. RNA isolate from an unspiked plasma aliquot was also acquired using the same procedure and used as a negative control. After isolation, biotinylated DNA complement probe (100 nM) was added to all isolates and heated to 95 °C for 10 min then cooled to 39 °C over 2 hrs to promote annealing to miRNA oligos. hMS was then added (500 nM) to bind to the biotinylated DNA and form the RNA/DNA heteroduplex-hMS complex where spike-in was present. Finally, the samples were measured with a single SS-nanopore for consistency (beginning diameter 9.4 nm, final diameter 10.5 nm), utilizing the experimental conditions optimized in this study.

II.5 Results and Discussion

Buffer pH can influence the translocation dynamics of biomolecules through SS-nanopores³⁷ by modifying the surface charges of all components in contact with the buffer or in extreme conditions by altering molecular structure³⁸. These changes can impact the speed and direction of translocation³⁹, the molecular capture rate, and the level of interaction between the pore surface and target molecules. Consequently, we initially sought to probe the effect of buffer pH on the performance of the assay incorporating hMS. For this purpose, we performed a series of SS-nanopore measurements across a pH range of 7.0 to 8.0, chosen to be above the isoelectric points (pI) of all assay components to ensure their net negative charge: the pI for both the SS-nanopore surface³⁹ and the double stranded DNA (dsDNA)⁴⁰ were taken to be ~5.0 from prior literature while the pI of the hMS was 6.0 as calculated⁴¹ using the pK values⁴² of all amino acids in its sequence, including the polyhistidine tag itself.

For each pH condition, analyses were conducted on at least four independent SS-nanopore devices and in standard 1 M NaCl. Measurements included DNA only (using 60 bp dsDNA with a single biotin as a standard; 100 nM), hMS only (500 nM), and finally an incubated mixture of the two constituents to form a DNA-hMS complex. Considering representative conductance traces for all investigated conditions (**Figure II.3a**), more translocation signals per unit time were observed at low pH. Quantitative analyses confirmed this, showing that the measured event rates for all three groups reduced as pH increased (**Figure II.3b**). There are several possible explanations for this observation. First, the progressively more negative net charge of the molecular constituents as pH is increased above the pI could have had the effect of reducing event durations due to higher electrophoretic driving force, making translocations more difficult to resolve and thereby reducing the apparent event rate. Indeed, like other proteins, the charge of streptavidin is

known to change significantly with pH^{39,43}; even a simple summation of all amino acid pKa values in hMS suggests a two-fold increase in net negative charge could be expected between pH 7.0 and 8.0. However, the charge of DNA has been shown to be nearly invariant across the same range^{44,45} and the observed rate for DNA alone in our measurements (**Figure II.3b**) decreased by almost the same amount as hMS (**Figure II.3b**) across the pH window. In addition, the charge of the nanopore surface itself has been shown experimentally to become more negative across this pH range as well^{39,46}. The resulting electroosmotic flow would slow translocation speeds, which could be expected to make them more detectable and increase measured event rate, counter to our observation. Consequently, we consider these electrodynamic effects to be unlikely explanations for the data.

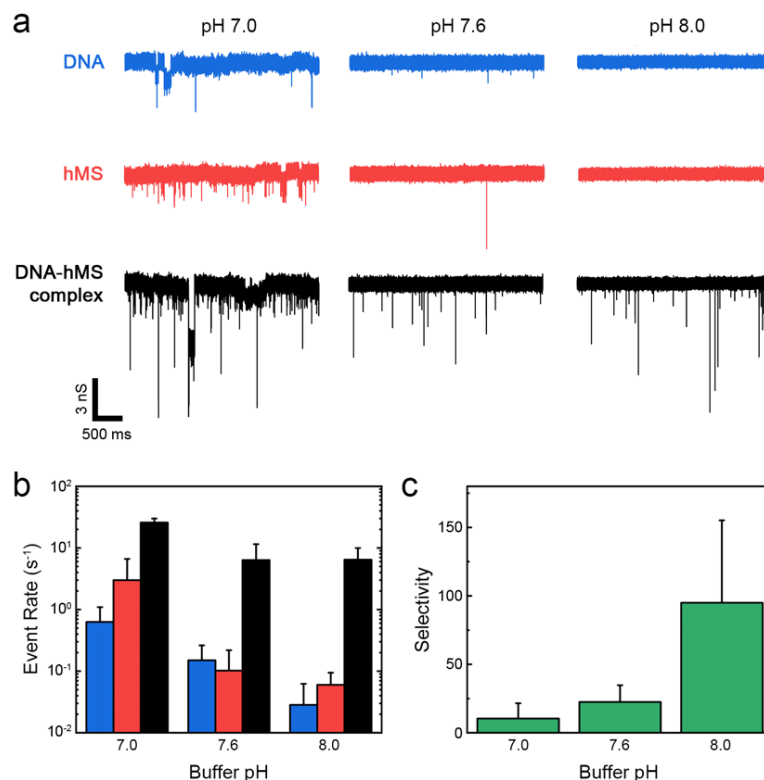


Figure II.3. Effect of buffer pH. (a) Representative conductance traces for DNA only (blue), and hMS only (red), and DNA-hMS complex (black) at (l-r) pH 7.0, 7.6, and 8.0. (b) Translocation event rates ($n \geq 3$) measured for DNA only (blue), and hMS only (red), and DNA-hMS complex (black) for all examined pH conditions. (c) Measured selectivity ($n \geq 3$) for all examined pH conditions. Error bars represent the standard deviation of the measurements.

As a second possibility, the baseline noise of the conductance could have increased with pH and made events harder to discern from the noise floor, resulting in a reduction in measured rate. However, we found that the baseline noise (standard deviation) of the baseline actually decreased with pH (**Figure II.4**), in agreement with recent work⁴⁷ that

showed the electrical noise in a SS-nanopores is greatest at the pI. Therefore, this is not considered an adequate rationalization for the observed results.

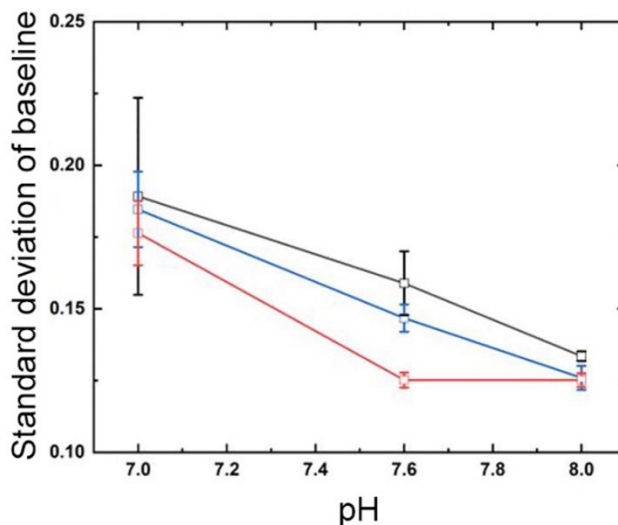


Figure II.4. Baseline noise during SS-nanopore measurements under different pH conditions. Standard deviation of the baseline during measurement of DNA only (blue), MS only (red), and DNA-hMS complex (black) as a function of buffer pH. All measurements were performed on a single nanopore at each pH condition. Mean $\pm$ standard deviation.

Instead, we suggest that the additional events recorded at low pH were caused by more frequent and longer-lasting stochastic collisions between the biomolecules and the silicon nitride surface, driven by weaker charge differentials at those conditions. We have shown previously⁴⁸ that apparent events can manifest from transient interactions with the sensing region near the SS-nanopore. These events tend to be low-amplitude due to their partial blockage of the pore and can occur even without molecular translocation. Examining the

measured conductance traces and event properties (**Figure II.5**) closely, we observed that the low-amplitude events with a broad range of durations found at pH 7.0 were less abundant as pH was increased. We also noted a difference in stability across conditions, with a low frequency variation in the baseline conductance at pH 7.0 that made measurements more difficult to perform, suggesting strong and unpredictable interactions. At higher pH where these interactions were less significant and non-translocative events were reduced, very few signals for either the DNA or the hMS alone were observed even while the nucleoprotein complex was still detected readily. This effect could be quantified through assay selectivity, defined as the ratio of the nucleoprotein complex event rate to the combined rates of the independent DNA and hMS constituents³⁰. We found that selectivity increased significantly with pH (**Figure II.3c**), reaching >100 at pH 8.0 – more than twice the value that had been achieved with prior MS under similar conditions³⁰. Consequently, pH 8.0 was identified as optimal for subsequent measurements.

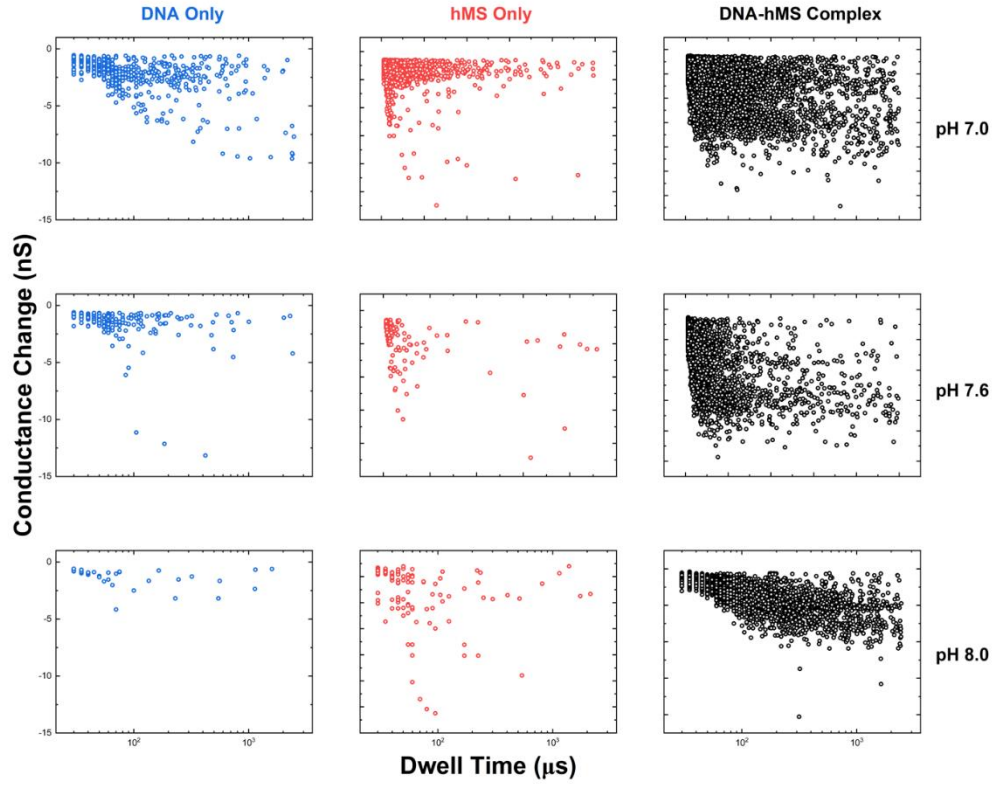


Figure II.5. Conductance change vs. dwell time scatter plots of all measured events under different pH conditions. Conductance change (ΔG) vs dwell time scatter plots for all measured DNA only (blue), hMS only (red), and DNA-hMS complex (black) events at each pH condition. At pH 7.0 (l-r) $n = 740, 3122, 18976$, at pH 7.6 $n = 243, 199, 12443$, and at pH 8.0 $n = 44, 130, 10880$

Having established a buffer pH condition to achieve enhanced assay selectivity, we next sought to optimize measurement sensitivity by varying salt concentration. Ionic strength is known to influence both event properties and analyte translocation dynamics in SS-nanopore measurements^{23,49,50}. Because the source of selectivity for our approach in

particular is the relative detectability of the different molecular components, salt conditions have also been shown³⁰ to impact its efficacy. High ionic strength has competing effects in the assay, making events easier to resolve by increasing event amplitude and duration⁴⁹ and decreasing baseline noise^{51,52}, but also decreasing their frequency by a reducing electrophoretic driving force via efficient charge screening. Because these effects are influenced by molecular charge, we expected that our incorporation of hMS could impact assay performance. To investigate this, we conducted another series of measurements on DNA alone, hMS alone, and DNA-hMS complex at the same molecular concentrations as above and at a pH of 8.0 but across a broad range of NaCl from 0.4 to 1.25 M.

We first observed that both DNA and hMS alone maintained a low event rate at or near zero with no significant variation across the entire NaCl range (**Figure II.6a**). Especially for the lower concentrations, this matched expectations; since events for both constituents were essentially undetectable at 1 M NaCl (*c.f.*, **Figure II.6a**), reducing the salt concentration further would only make their detection more challenging due to reduced signal-to-noise ratio (SNR) even if the total number of translocating molecules increased. However, the rate measured for the DNA-hMS complex varied considerably across the same span (**Figure II.6a**). The highest concentration investigated (1.25 M NaCl) yielded a relatively low event rate. Here, most or all translocating nucleoprotein complexes were detectable, but relatively few translocations occurred due to efficient counterion screening. As salt concentration was reduced, we observed a concomitant increase in the measured rate. We interpreted this behavior to result from an increasing capture rate, induced by weakened charge screening of the nucleoprotein complex due to reduced counterion availability. This appeared to be the dominant effect even though SNR and event duration were both expected to decline over the same range, thus making low-amplitude and fast events harder to resolve. For example, event amplitudes were found to decrease

progressively with decreasing salt concentration (**Figure II.6b, top**; **Figure II.7**), confirming the SNR reduction. For event duration, while similar analyses seemed initially to show an unexpected increase in dwell time at low salt conditions where the DNA-hMS complex was efficiently detected (**Figure II.6b, bottom**), the observed shifts actually resulted from fast events that became increasingly indistinguishable from the noise at low SNR conditions. We postulated that salt-dependent differences in DNA-hMS complex orientation may have been a main factor in translocation event temporal detectability.

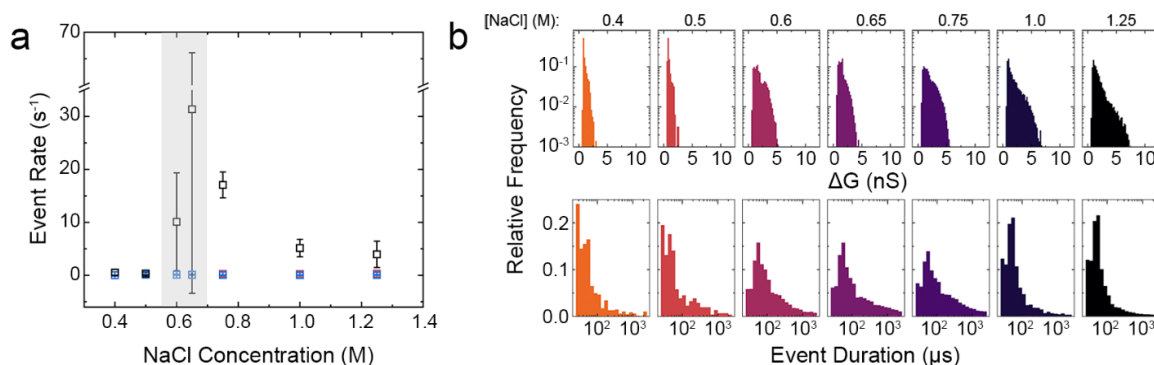


Figure II.6. Effect of salt concentration. (a) Translocation event rate ($n \geq 3$) for DNA only (blue), hMS only (red), and DNA-hMS complex (black) as a function of buffer NaCl concentration. The shaded region indicates a transitional region where measured rates were highly variable. Error bars represent the standard deviation of measurements taken on at least three independent SS-nanopore devices. (b) Conductance change (ΔG , top) and event duration (bottom) histograms for measured DNA-hMS events across all NaCl (l-r: $n = 711, 313, 8724, 15391, 17383, 7344$, and 7332).

Upon reducing NaCl concentration to 0.65 and then 0.6 M, we found a transitional state (**Figure II.6a, shaded region**) in which measured event rates were found to be highly variable between replicates. This behavior was attributed to a significant portion of events being barely detectable above the noise and consequently yielding rates that depended

on small differences between independent SS-nanopores. Additionally, conductance traces measured at these conditions were unstable (**Figure II.8**), possibly suggesting increased interactions between the DNA-hMS complex and the pore walls due to weak counterion screening. These interactions may have impacted event detection randomly, contributing to the rate variability. At low salt concentrations of 0.5 and 0.4 M, DNA-hMS event rates were very low and were not distinguishable from those of the DNA or hMS alone due to the low SNR that prevented any events from being resolved.

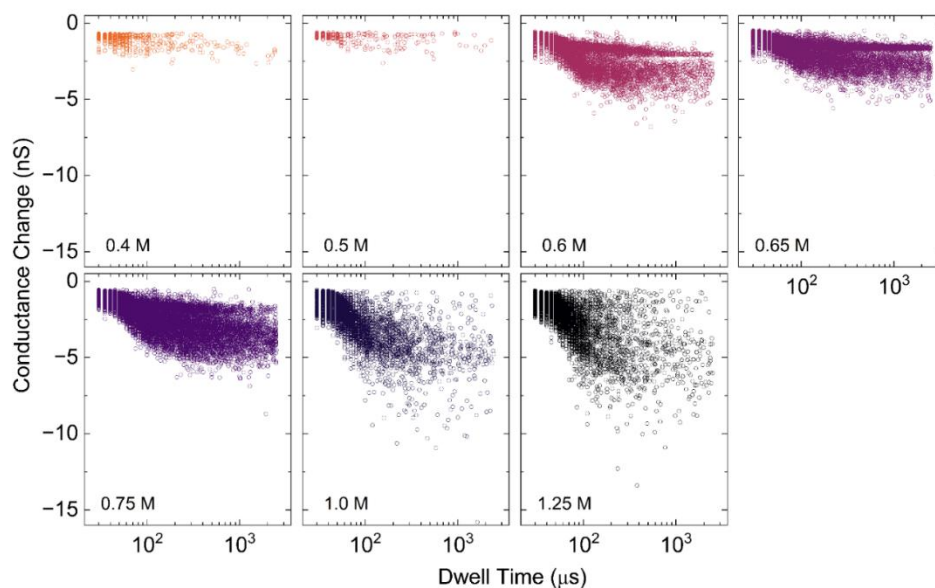


Figure II.7. Conductance change vs. dwell time scatter plots of all measured events under different salt conditions. Conductance change (ΔG) vs dwell time scatter plots for all measured DNA-hMS complex events at NaCl concentrations indicated in the lower left corner of each panel. From lowest to highest concentration, $n = 711, 313, 8724, 15391, 17383, 7344, \text{ and } 7332$.

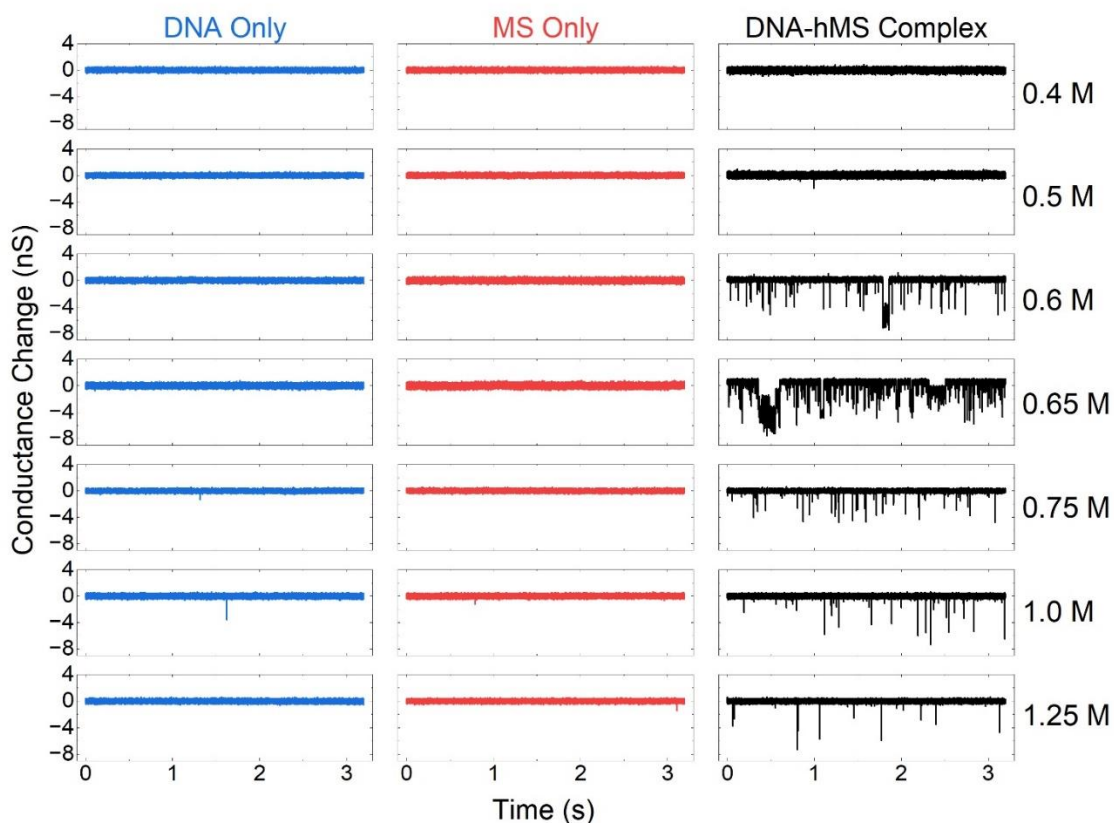


Figure II.8. Representative conductance traces of all assay components under different salt conditions. Representative conductance traces for DNA only (100 nM) (blue), MS only (500 nM) (red), and DNA-hMS complex (100 nM) (black) for all investigated NaCl concentrations listed on the right.

We note that our results with hMS had several key similarities with past measurements using an MS with higher net charge³⁰. For example, prior data also indicated the emergence of detectable nucleoprotein events at approximately 0.6 M NaCl because this detection threshold is driven by the noise of the measurement apparatus rather than molecular properties like charge. Additionally, event rate at the high salt limit measured here ($4.0 \pm 2.5 \text{ s}^{-1}$) is close to the prior study ($4.6 \pm 0.6 \text{ s}^{-1}$), demonstrating that the net

charges of the constructs at counterion saturation are comparable. However, the rate maximization observed here at intermediate salt concentrations was unique to the DNA-hMS complex. Whereas the prior construct was saturated with counterions at all NaCl concentration above approximately 0.6 M, the reduced negative charge associated with the polyhistidine tag here afforded our present measurements a greater window of detectability where partial screening enabled the capture rate enhancement to be resolved. As a result, 0.75 M NaCl yielded an approximately three-fold higher sensitivity than reported previously³⁰ and considerably higher selectivity than past results at the same salt concentration using glutamate-tagged MS³⁰. We note that while the selectivity observed here (~55) is somewhat less than reported above for 1 M NaCl (~100, see **Figure II.3c**), this is a consequence of a very slight increase in constituent rate and is still robust.

Because steric interactions drive the discriminatory detection in our assay, SS-nanopore diameter is another critical factor affecting selectivity³⁴. Pore diameters that closely match the size of the nucleoprotein complex generally increase the probability of interactions, thereby yielding the highest possible event rates in contrast to larger diameter pores that permit more rapid translocations to go undetected. Indeed, small differences in diameter can partially account for the variability that has been observed between measurements and can in principle be corrected for through a quantitative understanding of its dependence. While pore diameter dependence has been measured for our assay previously³⁴, its impact on the improved performance realized with hMS must be determined explicitly. To achieve this, we finally probed DNA, hMS, and DNA-hMS complex using a series of 14 independent SS-nanopores across the diameter range of 7.3 to 23.1 nm. Molecular concentrations were identical to those above and optimized buffer conditions of pH 8.0 and 0.75 M NaCl were used for all measurements.

Considering DNA-hMS complex event rates and summed hMS and DNA event rates separately (**Figure II.9b**), we confirmed both that events associated with the constituents remained consistently low and that nucleoprotein events decreased drastically with increasing pore diameter (**Figure II.9a; II.9b**). The reduction was well-described by a semi-log fit (linear on a semi-log scale, **Figure II.9b, black line**), similar to past results³⁴. For the smallest SS-nanopore studied here (7.3 nm), we observed significant transient clogging of the device compared to larger diameter pores (**Figure II.10**), demonstrating that steric hindrance was too strong at that condition to enable smooth translocations. From this, we concluded that 7.5 nm was the minimum diameter possible for assay implementation, correlating with the dimensions of the nucleoprotein complex and with our prior findings⁷. As in prior studies, fewer resolvable translocations were observed for large diameter SS-nanopores because of limited interaction with the pore walls. However, the enhanced sensitivity achieved here with optimized conditions still afforded a wide dynamic range for the assay; the DNA-hMS complex yielded an event rate that was more than one order of magnitude higher than the mean rate of the summed constituents up to approximately 16 nm, relieving prior constraints³⁴ that limited the assay to pores below 10 nm. This ultimately reduces the variability of results obtained with SS-nanopores of similar dimensions and facilitates easier fabrication of devices viable for the application.

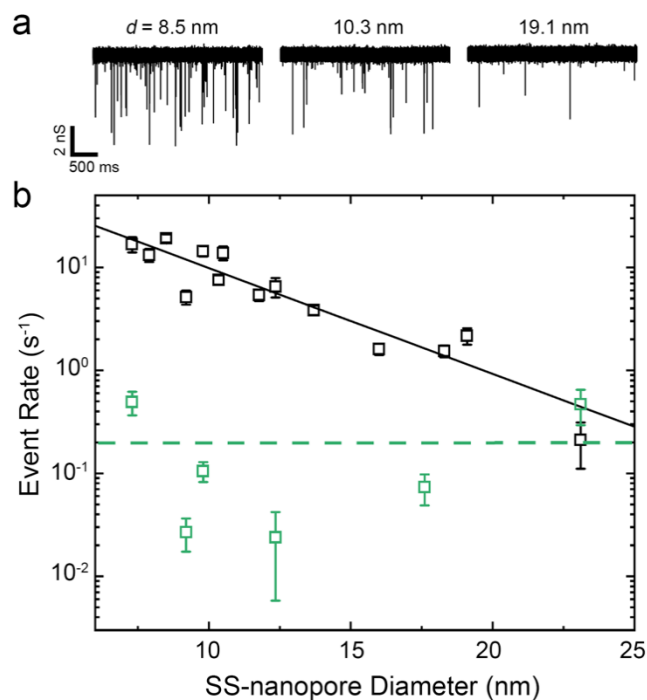


Figure II.9. Effect of the SS-nanopore diameter. (a) Example conductance traces for DNA-hMS measured with SS-nanopores of diameter 8.5 (left), 10.3 (center), and 19.1 nm (right). (b) Event rate as a function of SS-nanopore diameter for the DNA-hMS complex (black) and the summation of the DNA and hMS independently (green). Error bars represent the standard deviation of the measurements. The black line is a semi-log fit to the DNA-hMS data. The dashed green line is the mean value of the summed constituent event rates.

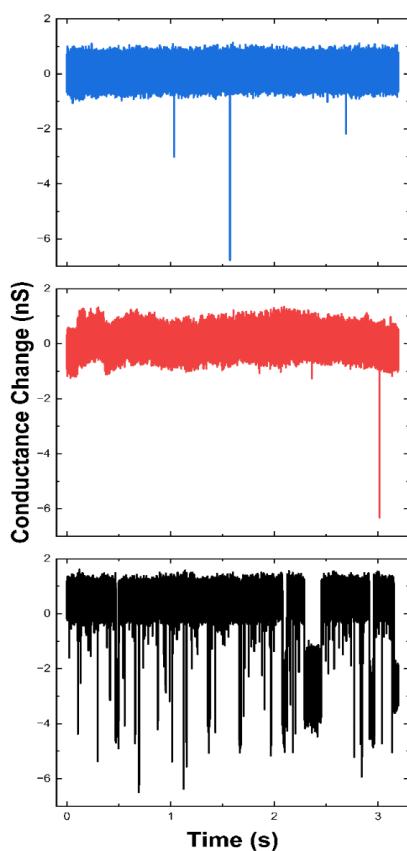


Figure II.10. Representative conductance traces of all assay components at 7.3 SS-nanopore diameter. Representative conductance traces for DNA only (100 nM, blue), hMS only (500 nM, red), and DNA-hMS complex (black) using a 7.3 nm SS-nanopore. The small diameter caused significant instability during translocations of the nucleoprotein complex in particular.

Having optimized measurement conditions for improved sensitivity and selectivity with the hMS reagent, we finally sought to leverage these improvements to enhance biosensing with the SS-nanopore assay. To do this, we investigated the quantitative measurement of miRNA, an analysis that typically requires specialized methods of reverse transcription quantitative polymerase chain reaction (RT-qPCR)^{53–55} to perform due to the small size (20–25 nt) of the target. We have previously⁷ demonstrated that specific miRNA sequences can be detected using an adaptation of our selective assay in which a biotinylated single-strand (ss-) DNA probe and MS are introduced to solution and translocation events are resolved only if the probe hybridizes with its complementary target; discrimination arises from the observation that ssDNA-MS does not produce events in contrast to duplex

nucleoprotein complexes, as described above. In our past work, we established this approach with miRNA in clean buffer with no interfering molecules. Here, we challenge our assay by discriminating a miRNA spiked into human plasma containing a mixture of native, off-target nucleic acids.

For our measurements, four aliquots of human plasma were prepared (**Figure II.11a**): one kept native and three spiked with a synthetic 24 nt miRNA (see *Materials & Methods*) at concentrations of 1, 10, and 100 nM. Small RNAs (including miRNAs) were then isolated from the samples independently and a biotinylated ssDNA probe with a sequence complementary to the miRNA spike-in was added to each at 100 nM concentration to enable the formation of RNA/DNA heteroduplexes. Finally, hMS was added to the aliquots as above (500 nM) to form complexes with all biotinylated constructs and the samples were analyzed by SS-nanopore using optimal conditions (0.75 M NaCl, pH 8.0, 9.4 nm beginning pore diameter). The event rate for the native, unspiked sample (**Figure II.11b, grey**) was low but significant at $1.2 \pm 0.3 \text{ s}^{-1}$. This reflected the contribution of unhybridized probe-hMS complexes as well as translocations of off-target miRNAs and other larger RNAs from the plasma. Because all of these components were present in all conditions and defined a consistent background signal, the event rate from plasma alone (no probe or hMS) had no direct impact on discriminatory power and was not measured here. Subsequent identical measurements of the spiked samples produced changes in event rate (**Figure II.11b, green; Figure II.12**), yielding values of $5.5 \pm 0.7 \text{ s}^{-1}$ (100 nM), $2.3 \pm 0.4 \text{ s}^{-1}$ (10 nM), and $1.6 \pm 0.4 \text{ s}^{-1}$ (1 nM). The observed concentration-dependence not only demonstrated the capacity of the hMS-optimized assay to detect a sequence-specific miRNA biomarker in complex biofluid but also suggests its ability to quantify it across a broad range of concentrations. In prior *in vitro* measurements⁷ using an identical measurement procedure but with a conventional glutamate-tagged MS, we resolved only

down to 10 nM miRNA even with no background molecules. Critically, event rates for all concentrations investigated here were significantly greater than the unspiked control down to at least 1 nM, signifying that the hMS reagent and optimized measurement conditions yielded significant improvements in our assay performance and highlighting its translational potential.

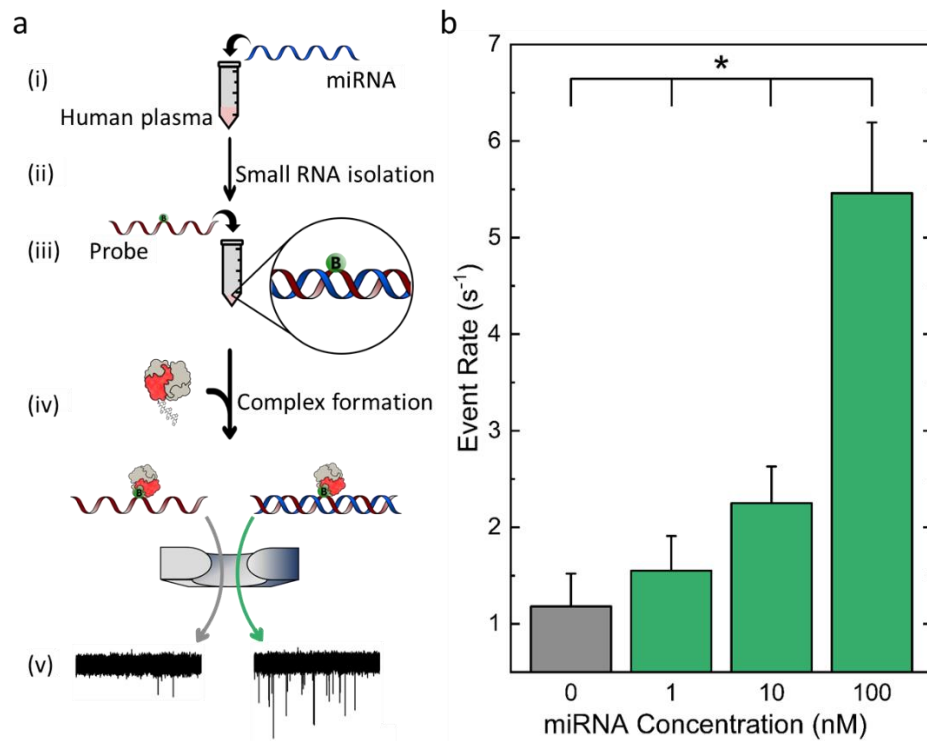


Figure II.11. miRNA quantification via the SS-nanopore assay. (a) Schematic of workflow for miRNA spike-in and analysis, showing (i) spike-in of miRNA (blue) in human plasma, (ii) isolation of small RNAs (including miRNA), (iii) addition of biotinylated ssDNA complementary probe (red) for heteroduplex formation, (iv) addition of hMS to form nucleoprotein complexes, and (v) SS-nanopore analysis. Off-target and background RNAs not shown for clarity. (b) Nanopore event rates measured across a range of miRNA

spike-in concentrations. Error bars represent the standard deviation of the segmented measurements (see Materials & Methods for details; segment counts were (l-r) 108, 138, 164, and 121). All concentrations differed significantly ($p < 0.001$) as determined by ANOVA and Tukey's HSD post-hoc analysis.

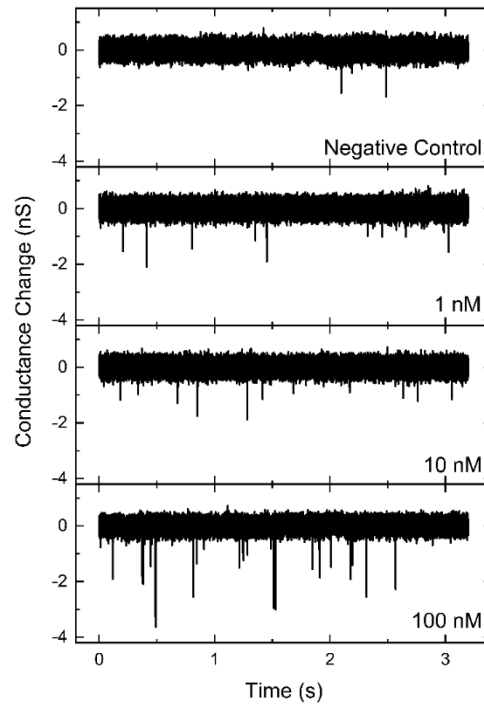


Figure II.12. Representative conductance traces of all assay components at different miRNA spike-in concentrations. Representative conductance traces for miRNA spike-in concentration listed in the bottom right corner.

II.6 Conclusions

In total, we have examined here the performance of a selective SS-nanopore assay after integrating hMS, a monovalent streptavidin featuring a polyhistidine tag that yields a lower net negative charge than previous versions of the protein. Increasing the pH of the buffer decreased noise levels in the measurement and resulted in an increased number of true events as well as a minimization of non-translocative signals. Although this decreased the overall measured event rate, it enhanced the selectivity of the assay markedly. Subsequent optimization of salt concentration in the measurement buffer enhanced the detection of low amplitude events and significantly enhanced the sensitivity of our assay while maintaining high selectivity. Assay performance was evaluated across a range of SS-nanopore diameters, demonstrating a broad dynamic range of approximately 7.5 to 16 nm. Finally, we used the optimized conditions to enhance miRNA assessment in human plasma, resolving concentrations as little as 1 nM above baseline without amplification, or an order of magnitude better than achieved previously⁷. Together, these findings establish the capacity of the hMS reagent to improve the selective SS-nanopore assay significantly, strengthening its value for the quantitative analyses of molecular biomarkers that include miRNAs, conserved pathogen sequences, and DNA epigenetic modifications and base lesions. Ultimately, our findings enhance the translational value of the platform.

II.7 Supporting Information

Additional experimental analysis and results including SDS-PAGE and EMSA gel images for hMS verification, SS-nanopore baseline noise analyses, translocation event characteristics across pH and NaCl conditions, and representative conductance traces.

II.8 Acknowledgements

This project was supported by NIH awards R21CA193067, R33CA246448, and P41EB020594 (ARH). SS-nanopore fabrication was performed at the Rutgers University Laboratory for Surface Modification.

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CHAPTER III

Manuscript in preparation for publication

OPTIMIZATION OF 8-OXO-7, 8-DIHYDROGUANINE SITE-SPECIFIC LABELING AND VALIDATION OF THE SS-NANOPORE ASSAY AS A TOOL TO QUANTIFY 8-OXOGUA IN ISOLATED AND CELLULAR GDNA

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III.1 Introduction

Chemical modifications of DNA bases are commonly seen in gDNA due to their crucial roles in various cellular functions including regulation of stem cell differentiation, gene expression, RNA splicing, DNA replication, and chromatin organization (reviewed in detail in [1]). Alterations in the abundance and regulatory/repair mechanisms of these chemically modified bases have been associated with the development and progression of a wide range of diseases such as metabolic disorders [2, 3], neurological disorders [4, 5], cardiovascular diseases [6], age related disorders [7] and cancer [8-10]. 8-oxoGua is one of the most abundant and well-studied DNA base modifications in cancer [11, 12]. It comprises high mutagenic potential leading to a G-T transversion mutation at the site of the adduct if not repaired during DNA replication [13]. This mutation is frequently observed in cancer cells, especially with impaired repair mechanisms [14]. In addition to point mutations, 8-oxoGua have been shown by recent studies to regulate gene expression through epigenetic modifications that affects regulatory elements in promoters, methylation of CpG islands, and distribution of histone modifications which may contribute to cancer development and progression [14]. Additionally, 8-oxoGua has been considered as a biomarker of oxidative DNA damage for decades. It has been shown to be one of the most observed DNA base adducts upon exposure to IR and certain Xenobiotics [11, 15]. In addition, Increased levels of 8-oxoGua and its repair machineries have been linked to advanced stages of various cancers and shown promise as predictive biomarkers in cancer patients, especially patients undergoing radiotherapy, which further underscores its clinical significance in risk assessment, response to therapy, and identification of therapeutic targets [10, 16].

Despite the clinical significance of 8-oxoGua, its adoption as a biomarker has been hampered by the expense and complexity of the current analytical methods. Typically, 8-oxoGua is quantified by conventional technologies like mass spectrometry and liquid chromatography and mass spectrometry (LC-MS/MS) and immunological methods like the ELISA, both of which are associated with technical challenges (cross reactivity, sample size, artifactual oxidation...etc.), requirement of rigorous training of personnel and special expertise, and requirement of sophisticated, high-cost instrumentation not readily available in clinical settings. Our selective SS-nanopore assay may overcome some of these challenges and provide an alternative cost-efficient tool for rapid, convenient, and versatile quantification of various single base modifications including 8-oxoGua. This is achieved by introducing an affinity tag (i.e., biotin) specifically at each modification site to prepare the tagged dsDNA containing the modification for binding with hMS and downstream quantification through SS-nanopores [17]. The selective SS-nanopore assay is explained in detail in chapter II. Additionally, a schematic representation of its principle and components is illustrated in Figure 1A.

Previous work in our lab employed the selective SS-nanopore assay to quantify global levels of 5hmC in mammalian gDNA as a model DNA base modification [18, 19]. An established technique was used to install N3- β -glucosyl-biotin in place of 5hmC bases in gDNA extracted from brain tissues of a murine model. This DNA was then subjected to SS-nanopore measurement and yielded results that agreed quantitatively with mass spectrometry which is the golden standard method [18]. However, that approach is intrinsically limited to 5hmC. To further expand the applications of the assay for a wider range of targets, our lab developed a modular enzymatic labeling assay that allows for the selective labeling of a wide range of DNA base modifications including 8-oxoGua [20]. The assay utilizes components of the BER pathway to exchange the modified base with

a biotin tag. Simply, a DNA glycosylase excises its designated base modification from the DNA leaving an AP site. If it is a bifunctional glycosylase as in the case of 8-oxoGua repairing glycosylases, it will result in an incision in the DNA backbone leaving a single strand nick. The phosphodiester bond 5' to the nick and the exposed 3' phosphate group are then processed by an AP endonuclease to form a hydroxyl group ready for elongation. Lastly, a gap-filling DNA polymerase is used to incorporate one biotin-conjugated nucleotide at the site where the modified base was removed. The assay has been shown to be highly adaptable and specific to the targeted modification when employed on synthetic short dsDNA constructs [20]. However, it hasn't been applied to gDNA where 8-oxoGua is present in low abundance. Here, we aimed at utilizing this novel SS-nanopore approach to quantify 8-oxoGua in cellular gDNA with high sensitivity and validate the accuracy of our measurements by comparing to mass spectrometry which is the golden standard method for 8-oxoGua quantification. We hypothesize that the high sensitivity of our assay using hMS at optimal buffer conditions will enable us to quantify low levels of 8-oxoGua in gDNA from various origins without requiring a large sample size or amplification and will yield rapid, convenient quantitation that is comparable to mass spectrometry.

III.2 Materials and Methods

III.2.1 Biomolecules

All synthetic oligonucleotides and primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). For 24, and 60 bp standard curves, DNA oligonucleotides of 24, and 60 nucleotides (nt) in length featuring a single biotin residue (Table 1) were used to form dsDNA constructs by annealing the biotinylated oligo with its

respective nonbiotinylated complementary sequence in a 1:1 mixture in 1X TE buffer at pH 8. For 150, and 200 bp standard curves, biotinylated 150, and 200 bp dsDNA constructs were formed through polymerase chain reaction (PCR) using synthetic Lambda DNA as a template (New England BioLabs, Ipswich, MA, USA). The biotin moiety was introduced to PCR products by a common reverse primer featuring a biotin-dT (see Table 1 for primer sequences). The length of all the synthesized dsDNA constructs was confirmed by running an aliquot on a 3% agarose gel prepared in 1X TBE buffer with GelRed nucleic acid stain (Phenix Research Products, Candler, NC, USA) and visualizing using a Gel Doc™ system (BioRad, Hercules, CA, USA). For 8-oxoGua site-specific labeling protocol, fluorescently (FAM-) labeled DNA oligonucleotide (40 nt) featuring a single 8-oxoGua in the 29th position (Table 1) was used. To form dsDNA constructs, the aforementioned oligonucleotide was annealed with a complementary 40 nt, or 46 nt sequence (Table 1) representing blunt, and sticky end dsDNA fragments, respectively, at an equimolar ratio in DNase, RNase free deionized water by incubating the mixture at 75°C for 10 minutes then cooling to room temperature overnight. The formed dsDNA constructs were used to assess labeling efficiency during protocol optimization via visualization on denaturing polyacrylamide gel, and alongside gDNA experimental samples via agarose gel analysis. All SS-nanopore assay analyses were carried out using a recombinant hMS protein produced in house with high yield and purity as shown in CHAPTER II.

Table III.1. A list of oligonucleotides used in the study

Oligonucleotide	Sequence
Biotinylated oligonucleotide-24	5'-CTC GGA TTA TGA AGA TTA CGG GTA-3'
Biotinylated oligonucleotide-60	5'-ATC AAC TGT TTC AGC CAC TGC TTC GCA GGC TGA CGT ATC TGA CGT GGT GCC AGC GAC GGA-3'
Forward primer-150	5'-AAC AAC TGT TTC AGC CAC TGC TTC-3'

Forward primer-200	5'-AAA TCC CGT CTC TCA GGA GG-3'
Biotinylated reverse primer	5'-CAG TTG AGG ATC CCC ATA A/iBiodT/G CGG CTG TTT TCT G-3'
FAM-oxoG oligonucleotide-40	5'-/56-FAM/TCA CGA CTA GTG TTA ACA TGT CGA CCT C/i8oxodG/A GAA TGA GAA T-3'
Complementary oligonucleotide-40-blunt end	5'-ATT CTC ATT CTC GAG GTC GAC ATG TTA ACA CTA GTC GTG A-3'
Complementary oligonucleotide-40-sticky end	5'-TTT TTT ATT CTC ATT CTC GAG GTC GAC ATG TTA ACA CTA GTC GTG A-3'

III.2.2 Site-specific labeling of 8-oxoGua

8-oxoGua labeling was performed on 1.5 µg FAM-labeled 40 bp dsDNA constructs containing a single 8-oxoGua moiety or 1 µg of fragmented gDNA. To block free 3'-OH blunt ends, DNA was incubated with U of Terminal transferase enzyme (NEB #M0315) in a 50 µl reaction containing 5 µl of 10x Terminal transferase buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate pH 7.9 @ 25°C), 0.25 mM CoCl₂, and 0.2 mM 2',3'-Dideoxyadenosine-5'-triphosphate (ddATP) for 30 minutes at 37°C. To block free 3'-OH sticky ends (5'-overhangs generated from enzymatic or mechanical fragmentation of gDNA), DNA was incubated with 2 U of Bsu DNA Polymerase, Large Fragment (NEB, #M0330) in 50 µl reaction containing 5 µl NEBuffer 2 (NEB #B7002; 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9@25°C), 0.2 mM of each of the ddNTP for 30 minutes at 37°C. Samples were then purified using QIAGEN nucleotide removal kit (QIAGEN#) and resuspended in 30 µl DNase, RNase free deionized water. To excise 8-oxoGua, DNA was incubated with 32 U of formamidopyrimidine DNA glycosylase (Fpg) (NEB #M0240) and 40 U of Endonuclease IV (Endo IV) (NEB #M0304) in one 50 µl reaction containing 5 µl NEBuffer 2, and 5 µl 10X bovine serum albumin (BSA) for 1 hour at 37°C. Without purification, 1.25 U of Bsu DNA Polymerase, Large

Fragment, and 16.6 μM Biotin-11-dGTP (AAT Bioquest #17015) were added to the reaction and incubated for 10 minutes at 37°C to replace 8-oxoGua with a biotinylated dGTP. Then, samples were purified using QIAGEN nucleotide removal kit (QIAGEN# 28306) and resuspended in 30 μl DNase, RNase free deionized water. Non-specific labeling was performed similarly with the exception of the Fpg/Endo IV step were in this case the enzymes were replaced with deionized water. 5 μl aliquots were preserved at each step during the labeling of the FAM-labeled 40 bp dsDNA constructs for gel analyses. For denaturing polyacrylamide gel analysis, aliquots were quantified using photospectroscopy (NanoDrop, Fisher Scientific, Waltham, MA, USA), then 0.2 μM of the sample was mixed with gel Loading Buffer II (Denaturing PAGE) (Fisher Scientific # AM8546G) in a final volume of 10 μl . Then samples were heated for 5 minutes at 95°C and loaded on a 23% polyacrylamide gel containing 7 M urea in 1xTBE buffer and ran on constant current of 2-3 mA for at least 4 hours then visualized on a gel documentation system. For electromobility shift assay analysis (EMSA), about 0.3 μM of the labeled samples were incubated with/without hMS at a 1:10 ratio in 1X TE buffer pH 8 for 30 minutes in a final volume of 10 μl . Then, samples were loaded on a 3% agarose gel stained with GelRed nucleic acid stain in 1X TBE buffer and ran at constant voltage of 180 V for 30 minutes. Gels were visualized using a Gel Doc™ system (BioRad, Hercules, CA, USA). Quantitative assessment of the images was carried out via Gels analysis tool in ImageJ software.

III.2.3 Lambda DNA oxidation by H_2O_2 /AA

About 10 μg of synthetic Lambda DNA (NEB #3011) was oxidized using 10 mM H_2O_2 and 100 μM L-Ascorbic Acid (AA) (SIGMA # A92902) in 150 mM phosphate buffer (pH 6.8) in a final volume of 100 μl for 0, 2, and 4 hours at 37°C. To stop the reaction, all samples

were immediately purified using gDNA clean and concentrator-10 kit (Zymo Research #D4010) and resuspended in 30 µl of DNase, RNase free deionized water.

Induction of 8-oxoGua formation in MCF-7 cell line by external H₂O₂:

The human breast cancer MCF-7 cell line transduced with roGFP (kindly provided by the Dr. Marcello Bonini group at Feinberg School of Medicine, North Western University) was used as a model cell line for 8-oxoGua induction. Cells were regularly cultured and passaged in MEM media (Gibco #11095080) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% Penicillin-Streptomycin (10,000 U/mL) (Gibco # 15140122) at 37°C and 5% CO₂. For induce 8-oxoGua formation, about 3.5x10⁶ cells were seeded in 100 mm cell culture treated dishes and adhered overnight. Cells were washed twice with 1X PBS and incubated with H₂O₂ at concentrations ranging from 2-20 mM in 1X PBS for 1 hour at 37°C and 5% CO₂. Cells incubated with 1X PBS without H₂O₂ were regarded as controls. All conditions were done in triplicates. After incubation, cells were washed twice with 1X PBS and harvested using 0.25% Trypsin-EDTA solution and pellets were prepared for gDNA extraction. Extraction of gDNA was performed using the DNeasy blood and tissue kit (QIAGEN #69504) and resuspended in 100 µl deionized water.

III.2.4 MTT assay

Cell viability after exposure to H₂O₂ was evaluated using the CyQUANT MTT cell viability assay (Invitrogen #13154). Briefly, 19.7x10³ cells/well were seeded in a 96-well plate and adhered for 24 hours. H₂O₂ was administered to the cells in 1X PBS at concentrations ranging from 2-50 mM for 1 hour. Cells incubated with 1X PBS without H₂O₂ were regarded as controls. All treatment conditions were done in triplicates. Media containing the H₂O₂ was then aspirated, and cells were washed with 100 µl 1X PBS. Then, the MTT (3-(4,5-

dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) solution was added to the culture according to the manufacturer instructions. Cell viability was calculated by the formula: (A treatment/A control) *100% where A is the absorbance recorded at 540 nm.

III.2.5 Live cell imaging and image analysis

Live cell imaging of H₂O₂ buildup in the nucleus was carried out using a stable MCF-7 cell line transduced with the NLS-Orp1-roGFP2 expression vector encoding a nuclear localization tagged protein (Described elsewhere in [48]). Cells were seeded at 70% confluency in an ibidi μ -8-well chamber slide cover slip glass bottom and adhered overnight. A Zeiss LSM 710 scanning confocal microscope with Zeiss Zen microscopy software was used for imaging. First, dishes were placed in a sealed, 37°C humidified chamber supplemented with 5% CO₂ for the duration of the experiment. Images were acquired using a Plan-Neofluar 40x/1.3-NA oil-immersion objective every 2 minutes for 10 cycles. The first 2 images were acquired prior to treatment, while the rest of the images were acquired 5 minutes post treatment with 0.5 mM DTT or H₂O₂ at concentrations ranging from 10-500 μ M for a total duration of 20 minutes per well/field. To collect images, excitation of Orp1-roGFP2 was carried out via a 405 nm and 488 nm lasers followed by sequential emission at 509-531 nm. The generated images were analyzed by Fiji imageJ (1.53t) software using standard procedure [49]. Briefly, images were converted to 32-bit format followed by separating the channels corresponding to different wavelengths, namely, the 405 nm and 488 nm. Using the default threshold algorithm of imageJ, only the nuclear area of the cells was selected for subsequent analyses. Then, a ratio image is created by dividing the images corresponding to each channel (405 nm/488 nm) and visualized using a color lookup table (e.g. fire). Quantitative assessment of the ratio image was conducted using the default “measure” tool in imageJ for the entire threshold area to calculate a ratiometric value corresponding to each time point in the series.

III.2.6 gDNA fragmentation for downstream SS-nanopore analyses

Fragmentation of gDNA was carried out by two different approaches: enzymatic or mechanical. For enzymatic fragmentation, the NEBNext dsDNA Fragmentase enzyme-based reagent (NEB #0348) was used. Approximately, 1 µg of gDNA is digested in a 30 µl reaction containing 3 µl of 10X NEBNext dsDNA Fragmentase Reaction Buffer v2 (20 mM Tris-HCl, 15 mM MgCl₂, 50 mM NaCl, 0.1 mg/ml BSA, 0.15% Triton® X-100, pH 7.5 at 25°C), 3 µl of 10X BSA, 10 mM MgCl₂, and 4 µl Fragmentase enzyme for 30 minutes at 37°C. The reaction is stopped using 0.5 M EDTA and the samples are then purified using the QIAquick nucleotide removal kit (QIAGEN #28306). For mechanical fragmentation, focused ultrasonication was performed using ME220 focused ultrasonicator (Covaris #500506). Approximately, 5 µg of gDNA was suspended in 8 microTUBE-50 AFA Fiber H Slit Strip V2 (Covaris #520240) at a final volume of 55 µl. The instrument was operated at 50 W peak incident power, 30% Duty factor, 1000 cycles per burst for a total of 280 s (28 iterations) to yield DNA fragments of 150 bp in length. To determine the mean length of fragments, each sample was run on a 3% agarose gel. Quantitative assessment of gray values in the smear generated by each fragmented DNA sample was obtained using imageJ tools [51]. A predefined dsDNA ladder (Fisher Scientific #10597012) was used to obtain an exponential equation to calculate the lengths associated with each migration distance on the gel. By plotting the calculated lengths (x-axis) and gray values (y-axis), we could evaluate the fragments lengths in each sample by a lognormal fit. The peak value of the fit determined the fragment length in the sample.

III.2.7 Sample preparation and SS-nanopore measurements

Approximately, 0.5-1 μg of gDNA was digested and labeled as described above. To prepare the sample for the selective SS-nanopore assay measurement, 1-10 μl of each labeled sample was incubated with hMS at 1:10 molar ratio in 1X TE buffer (pH 8) for 30 minutes at room temperature. DNA only samples and non-specific labeling samples were prepared similarly at the same time as controls. DNA only samples were incubated with 1X TE pH 8.0 in the place of hMS. Then NaCl was added to the reaction at a final concentration of 0.75 M. 10 μl aliquot of the sample was used for SS-nanopore measurement. All measurements were carried out using HIM SS-nanopores in 20 nm thick SiNx membrane. Prior to measurements, each device was exposed to 10 W air plasma (Harrick Plasma, Ithaca, NY, USA) for 2 min on each side and then mounted in a custom flow cell fabricated with a commercial 3D printer (Carbon, Redwood City, CA, USA). Both sides of the chip were then contacted with measurement buffer consisting of 1X TE buffer pH 8 and 0.75 M NaCl. Ag/AgCl electrodes introduced to each reservoir were used to apply voltage and measure ionic current using a commercial patch-clamp amplifier (Axopatch 200B, Axon Instruments, San Jose, CA, USA). All measurements were performed under a 300 mV applied bias. Translocation data were collected at 200 kHz with a 100 kHz Bessel filter and recorded using in-house data acquisition software (LabView, National Instruments, Austin, TX, USA). An additional 10 kHz low-pass filter was applied digitally during analysis. Transient reductions in measured SS-nanopore conductance were considered only if their durations were between 25 and 2500 μs and their amplitudes were at least 4.5 standard deviations from baseline noise. Data were recorded in discrete 3.2 s segments from which the average rate was calculated.

III.2.8 gDNA digestion for downstream mass spectrometry analyses

To prepare gDNA samples for mass spectrometry analysis, at least 2 µg of gDNA was resuspended in DNase, RNase free water at a final volume of 30 µl and boiled at 95°C for 15 minutes prior to enzymatic digestion. Denatured samples were placed on ice immediately to prevent reannealing and 10 U of Nuclease P1 enzyme (NEB #M0660) per 0.5 µg of gDNA was added and samples were incubated at 42°C overnight. Then, 0.002 U of phosphodiesterase I enzyme (SIGMA #P3243) per 0.5 µg of gDNA was added in a 60 µl reaction containing 100 µM Ammonium bicarbonate (NH_4HCO_3) and samples were incubated for 2 hours at 37°C. Then, 0.5 U of Alkaline phosphatase (SIGMA #11097075001) per 0.5 µg of gDNA was added and samples were incubated for 2 hours at 37°C. the completeness of digestion was confirmed by agarose gel. All samples were then resuspended in 1 ml DNase, RNase free water and filtered through a 0.22 µm syringe filter (Sartorius 0.2 µm syringe filters (Diameter 4.0 mm) catalog #: 17821K).

III.2.9 Liquid chromatography and mass spectrometry analysis

A Shimadzu Nexera UHPLC system (Shimadzu, Kyoto, Japan), consisting of a DGU-405 degassing unit, LC-40D binary pumps, SIL-40C autosampler, and CTO-40C column oven was coupled to an AB SCIEX 7500 triple quadrupole mass spectrometer (SciEx, Framingham, MA) was utilized for this study. Analytes were separated on an Agilent reverse-phase EclipsePlus C18 (100 x 2.1 mm, 1.8 µm) column using gradient elution consisting of 0.1% formic acid in water (A) and 100% methanol (B) at a flow rate of 0.4 mL/min. A 5 µL injection was loaded, separated, and eluted from the column using the following gradient: 5% B hold for 1 min, 30% B at 5 min, 90% B at 7 min, hold 90% for 2 min, 5% B at 9.1 min, re-equilibrate at 5% B for 2.4 min. The total run time was 11.5 minutes. The column temperature was maintained at 40 °C and the autosampler was

maintained at 15 °C. The mass spectrometer, AB SCIEX 7500 triple quadrupole (SciEx, Framingham, MA) was run in positive mode utilizing nitrogen gas for ion generation under the following conditions: Ion source gas 1 and 2 were set at 30 and 70 psi respectively, the curtain gas was set at 40 psi and the CAD gas was set to 12. The source temperature was set to 400 °C. Ion spray voltage was set to 4000 V. Compound specific parameters were optimized and summarized in Table 2.

Table III.2. Compound specific parameters used in mass spectrometry analyses

Compound ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (ms)	EP (V)	CE (V)	CXP (V)	Q0D (V)
2-deoxyguanosine-d5	273	157.021	100	10	10	33	10
2-mono-guanosine	268	152.03	100	10	39	49	10
2-mono-guanosine	268	135.01	100	10	52	40	10
8-deoxyguanosine	284.35	168.39	100	10	19	13	10

III.2.10 Statistical Analysis

All statistical analyses were performed using Jamovi software 2.3.28. All measurements were carried out in triplicates unless otherwise indicated in the text. Independent sample t-test was used to determine significant statistical differences between different conditions measured using the SS-nanopore or mass spectrometry and between SS-nanopore and mass spectrometry measurements. All measurements undergone normality testing by Shapiro-Wilks statistical test and homogeneity testing using Leven's test. Welch's t-test was used if Leven's test resulted in a p-value<0.05, and Mann Whitney U test was used if Shapiro Wilks test resulted in a p-value<0.05. The difference between groups was deemed significant if it resulted in a p-value<0.05. Specific tests, p-values, and sample sizes were identified in the respective figures.

III.3 Results and Discussion

III.3.1 Effect of dsDNA length on the sensitivity and selectivity of the SS-nanopore assay

In chapter II, we showed how the buffer conditions in terms of pH and salt concentration as well as the diameter of the SS-nanopore device itself affect the sensitivity and selectivity of our measurements using the selective SS-nanopore assay [21, 22]. Previous reports have shown that the configuration and length of dsDNA significantly affect its translocation dynamics through SS-nanopores [23-25]. Previous studies from our lab demonstrated that measurement of dsDNA constructs with various lengths using the selective SS-nanopore assay, even as small as 50 bp difference in the length, alter the event rates collected for each length at a wide range of concentrations [19]. Other studies have shown that length dependence is affected by measurement conditions such as the type of salt used and its concentration [23]. Therefore, rigorous assessment of the length of DNA prior to SS-nanopore measurement using this assay under the optimized conditions determined in chapter II is crucial. Notably, the length range of DNA suitable for the SS-nanopore assay is below 250 bp to maintain selectivity. This decreases the probability of configurations others than the linear form as these lengths are near the persistence length of DNA [26]. However, short dsDNA interaction with hMS may vary the conformation of the complex entering the SS-nanopore depending on the length and location of the biotin moiety on the DNA, hence changing the translocation dynamics of these molecules at different lengths [27]. Here we sought to investigate the effect of dsDNA length on the detection and quantification of the molecule via our selective SS-nanopore assay. To quantify length dependence, monobiotinylated dsDNA constructs (24, 60, 150, and 200 bp) within the functional range of lengths for our assay were selected and a series of SS-nanopore measurements were performed. Nucleoprotein (DNA/hMS) complexes were prepared for

each construct length individually at a range of concentrations ranging from 1-100 nM and both DNA only (100 nM) and hMS only (500 nM) were measured as controls. All measurements were conducted with at least three independent SS-nanopore devices in optimized measurement conditions (described in chapter II, [21]). Our results demonstrated that the measured event rate increases markedly as the DNA length increases, reaching up to 20-fold increase when comparing the 24 bp and 200 bp constructs event rates at 100 nM; measured event rate for the 24 bp monobiotinylated constructs at 100 nM is 0.7 ± 0.78 , and the measured event rate for 200 bp monobiotinylated construct at 100 nM is 20.97 ± 5.55 (Figure III.1 A). This agreed with previous observations from our lab showing that shorter lengths of DNA exhibit fast translocation that cannot be resolved by our system and that translates to loss of sensitivity. In contrast, longer DNA have been shown to produce events on its own which translates to loss of selectivity [17, 19, 28].

In Figure III.1 A, we illustrated a linear increase in event rate as the concentration of the analyte increases. A summary of the linear fits for the concentration-dependent curves at each DNA length is displayed in Table III.3.

Table III.3: Statistical description of the linear fits of the concentration-dependent curves at each DNA length							
Concentration	Average Event Rate (s ⁻¹)	Standard Deviation	Slope	Standard Error of Slope	R-Square	F statistic*	p-value
Biotin-24 bp			0.00696	0.00037	0.99132	342.44	0.0003
100 nM	0.70067	0.77942					
50 nM	0.15533	0.03691					
10 nM	0.0968	0.02342					
1 nM	0.1195	0.15377					
Biotin-60 bp			0.1066	0.00197	0.99762	2932.13	<0.0001
100 nM	10.85	7.70687					
50 nM	4.63	4.1508					
10 nM	0.43933	0.15405					
1 nM	0.0616	0.01521					
0.75 nM	0.10077	0.03103					
0.5 nM	0.07778	0.03116					
0.25 nM	0.07573	0.04241					
0.1 nM	0.05218	0.02089					
Biotin-150			0.12833	0.00033	0.99998	147356.43	<0.0001
100 nM	12.8225	5.75535					
50 nM	6.455	3.54011					
10 nM	1.081	0.39993					
1 nM	0.15533	0.03853					
Biotin-200			0.20351	0.0067	0.99247	922.04	<0.0001
100 nM	20.975	5.5524					
50 nM	7.655	2.73303					
10 nM	1.608	0.537					
1 nM	0.18957	0.05593					
*Used as a measure of the goodness of fit (ANOVA was performed)							

This increase is correlated with the increase in DNA length as evidenced by the increase in the slope of the curves with higher DNA length (Table III.3; Figure III.1 B).

Our goal is to quantify target nucleic acids derived from gDNA. Given the linear relationship between analyte concentration and event rate, our concentration-dependent measurements can be used as calibration curves to measure a target nucleic acid concentration from a gDNA sample. The appropriate calibration curve is selected depending on the length of DNA fragments in the sample. Given that fragmented gDNA samples are commonly presented as a heterogeneous mixture of various DNA lengths [18, 19] centered around a mean that can be determined experimentally (see section III.3.3), arbitrary calibration curves corresponding to any intermediate length can be inferred by interpolating the slopes of length-dependent calibrations (Figure III.1 B). This notion is supported by previous work showing that the measured event rate varies as a

function of analyte concentration in a linear relationship and only the slope of the curve is changed regularly with construct length [19, 29].

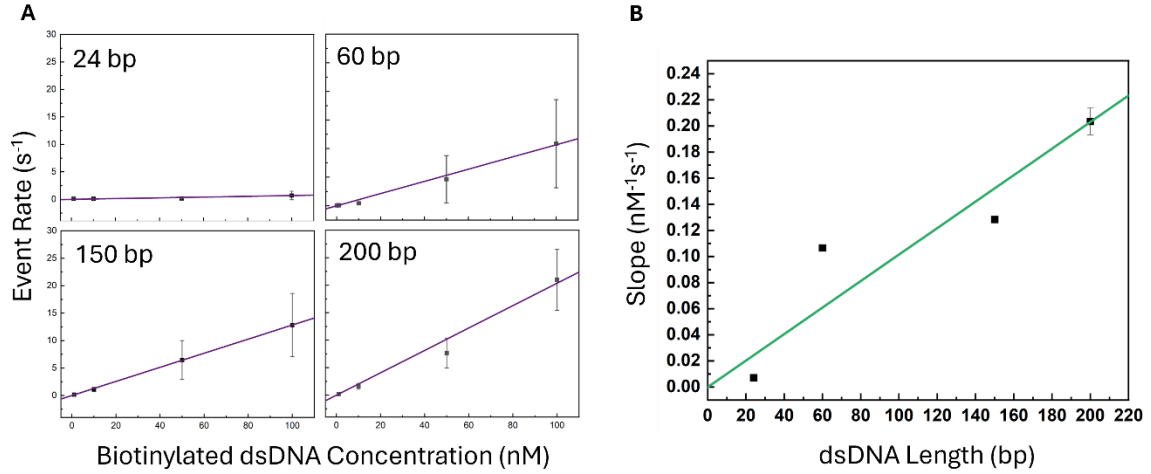


Figure III.1 Concentration and length dependence of the selective SS-nanopore assay measured event rates. A. Measured event rates (300mV) for Biotin-dsDNA/hMS complex as a function of Biotin-dsDNA concentration at various lengths (24, 60, 150, 200 bp). Purple lines are linear fits to each dataset. Error bars represent the standard deviation of measurements carried out on at least three separate SS-nanopore devices. B. Slopes of the linear fits in A as a function of Biotin-dsDNA length. Green line is a linear fit to the data (Slope: $0.00102 \pm 1.66233E-5$; R-square: 0.9992; F statistic: 3729.12; p-value: <0.0001). Error bars represent the standard error of the slope.

III.3.2 SS-nanopore assay limits of quantification deducted from calibration curves

Figure III.1 A illustrates that the event rates corresponding to each length at concentrations below 10 nM decreases significantly, hence reducing the detectability of the analyte at low concentrations (Figure III.1 A). To evaluate the quantification limit (LOQ) of the assay at optimal conditions, we conducted a series of SS-nanopore measurements using synthetic monobiotinylated 60 bp dsDNA construct at concentrations ranging between 0.001-1 nM. Measurements included DNA/hMS complexes, at the designated concentrations and appropriate controls (DNA only and hMS only). The measurements were carried out following standard optimized conditions as described in chapter II. With the enhanced performance of the SS-nanopore assay in the presence of the polyhistidine-tagged monovalent streptavidin in optimized conditions, we observed an increase in event rates collected at concentrations below 1 nM as well as a 10-fold increase in the slope of the Bio60 calibration curve relative to previous reports ($0.013 \pm 9.9\text{E-}4$ previously to 0.107 ± 0.0017) [19, 30] (Figure III.2). Our results indicated that the signal from DNA/hMS complexes remains above the DNA- only control signal down to 10 pM. This concentration is almost two orders of magnitude below what we could measure previously (Figure III.2, inset). However, evidence has shown that the precision in the measurement of response values decreases at low concentrations [31]. Therefore, we calculated the LOQ which we defined as the concentration at which the event rate is 10 standard deviations above the lowest DNA control measurement at 100 nM which was measured to be 0.0135 ± 0.0073 => $(10 * 0.0073) / 0.107$ [32]. In this way, we determined our LOQ to be approximately 0.682 nM of 60bp dsDNA fragments containing biotin which corresponds to 5.7 pM of 8-oxoGua after dividing by the length.

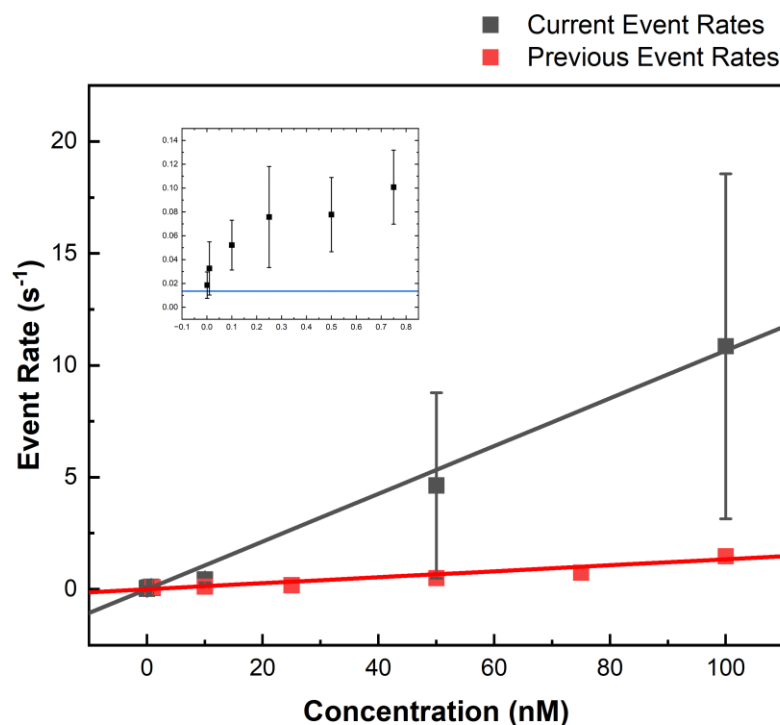


Figure III.2: Estimation of the LOQ using the optimized conditions for the SS-nanopore assay. Measured event rates of dsDNA-MS complexes at a wide range of concentrations (0.001 – 100 nM) using previously used MS and measurement conditions (red) compared to current measurement conditions optimized for hMS (black). Inset represents a magnified image of event rate values measured at low concentrations (0.001-0.75 nM). Blue line represents the lowest event rate measured at 100 nM biotin-60 bp Only without hMS. Error bars represent the standard deviation of event rates measured on at least 3 independent SS-nanopore devices for measurements with hMS, and 1 SS-nanopore device for measurements with the previously used MS.

III.3.3 Improving the site-specific labeling efficiency for downstream measurement of 8-oxoGua using the selective SS-nanopore assay

Based on the results above, the SS-nanopore assay can be leveraged to quantify a target modified nucleotide presented in the DNA at low abundance such as 8-oxoGua [33]. The modified base must be replaced/labeled with biotin to enable its quantification by the SS-nanopore assay. Site-specific labeling of 8-oxoGua followed an approach developed by Wang et al., [20] with slight modifications to enhance the labeling efficiency which was reported to be 83% using this method. An overview of the site-specific labeling assay is summarized in Figure I.3. First, Oxidized DNA is processed using enzymatic or mechanical fragmentation to yield dsDNA fragments at appropriate lengths for quantification via the SS-nanopores assay (24-250 bp). All free 3'-OH ends are blocked by ddNTPs followed by recognition and excision of 8-oxoGua. Excision of 8-oxoGua is carried out by a glycosylase that specifically recognizes 8-oxoGua. The glycosylase used here was Fpg which is the prokaryotic homolog of the human 8-oxoGuauanine DNA glycosylase 1 (hOGG1) responsible for the repair of 8-oxoGua. Fpg was used here due to limited availability of the recombinant hOGG1 enzyme and due to its high catalytic activity and affinity to the substrate [34-36]. Fpg acts as a bifunctional glycosylase; its N-glycosylase activity recognizes and cleaves 8-oxoGua generating an AP-site, and its AP-lyase activity cleaves the backbone 5' to the AP-site leaving a single nucleotide gap with a 5' and 3' phosphate termini. Next, Endo IV is used to catalyze the removal of the 3' phosphate at the nick site through its 3'-diesterase activity. Next, large fragment of Bsu DNA polymerase (*Bacillus subtilis* DNA polymerase I) is used for the addition of biotinylated-dGTPs at excised 8-oxoGua sites. The large fragment of this enzyme lacks the 3'-5' exonuclease activity which is particularly important for proper labeling with the biotinylated nucleotides. Similar to Fpg, Bsu DNA polymerase was used here in the place

of T4-polymerase (exo-) used previously due to its high catalytic activity and affinity to its substrate [37, 38].

To identify the optimal working conditions and evaluate the efficiency of the labeling, custom FAM-labeled dsDNA constructs (40 bp) featuring a single 8-oxoGua were used which allowed for defined 8-oxoGua concentration in all reactions. Additionally, fluorescent labeling of the oligos will enable us to visualize every step of the labeling procedure at a single nucleotide resolution level using a sequencing/denaturing acrylamide gel. Conditions for the function of each step in the labeling protocol were optimized individually. For 8-oxoGua recognition step, a pre-determined concentration of 8-oxoGua containing dsDNA constructs were treated with Fpg and incubated at 37°C for 1 hour. Then, denaturing acrylamide gel was used to assess 8-oxoGua excision efficiency. Excision of 8-oxoGua is visualized on the gel as a shift in the DNA fragment size from 40 nt to 28 nt as shown in Figure III.3 A, Lane 3. Notably, visualization of DNA constructs on a denaturing gel dictates the denaturation of the dsDNA into single stranded oligonucleotides. Thus, all denaturing gel images indicate the fluorescently labeled strand in the oligonucleotide containing 8-oxoGua at the 29th position. Next, the Bsu DNA polymerase function was optimized. We aimed at achieving a labeling efficiency that exceeds 90% in this step. This step was optimized by treatment of 8-oxoGua-dsDNA constructs with a wide range of enzyme concentrations and incubation at 37°C for 10 minutes post treatment with Fpg/EndoIV in a “one pot” reaction then visualizing using a denaturing gel. Efficient labeling (incorporation of a single biotinylated dGTP) is shown on the gel as a shift in the DNA fragment size from 28 nt to 29 nt as shown in Figure III.3 A, lane 4. At this point, labeling efficiency was calculated for each lane using image analysis software (ImageJ). Labeling efficiency is evaluated by measuring the area under the curve of the distribution of mean gray values of each DNA band in the 4th lane of the gel image. Our results show

that the labeling efficiency increases as the concentration of Bsu DNA polymerase increases reaching up to 97% (Figure III.3 A, lane 4). The optimization of treatment duration at each step ensured the achievement of maximum enzyme activity while maintaining minimum time to avoid artifactual formation of oxidized bases due to prolonged exposure to transition metals in the solution. Next, binding efficacy of the biotin-labeled molecules to hMS was evaluated using EMSA on agarose gel using a similar image analysis approach as with the denaturing gel (Figure III.3 B). Our results illustrated that the majority of the labeled DNA (about 85%) was bound to hMS at a molar ratio of 1:10 DNA:hMS in 30 min which resulted in the translocation shift observed in the +hMS lane on the gel in figure III.3, B.

An additional optimization step was carried out to ensure the labeling of sticky ends (5'-overhangs) which could be produced by fragmentation of gDNA (explained in more details in the next section). For this purpose, we designed a 46 nt ssDNA oligonucleotide complementary to the FAM-labeled 40 nt DNA oligonucleotide containing 8-oxoGua used above (Table III.1). Annealing of the two oligonucleotides produces a dsDNA construct containing a polydT 5'-overhang and featuring a single 8-oxoGua (fluorescently labeled to enable visualization on denaturing gel). Incorporation of ddNTPs which block the free 3'-OH ends at 5'overhangs to prevent erroneous biotin addition at these sites was carried out by Bsu DNA polymerase in this case which exhibits a 5'→3' polymerase activity using a template as well as single dA tailing at blunt ends. The rest of the labeling steps were carried out as described above. Successful addition of a single ddNTP is visualized on the gel as a shift of the DNA band from 40 nt to 41 nt as shown in Figure III.3 C, Lane 2. Our results show that the presence of 5'-overhangs did not affect the labeling efficiency which was calculated to be 94% (Figure III.3 C, lane 4).

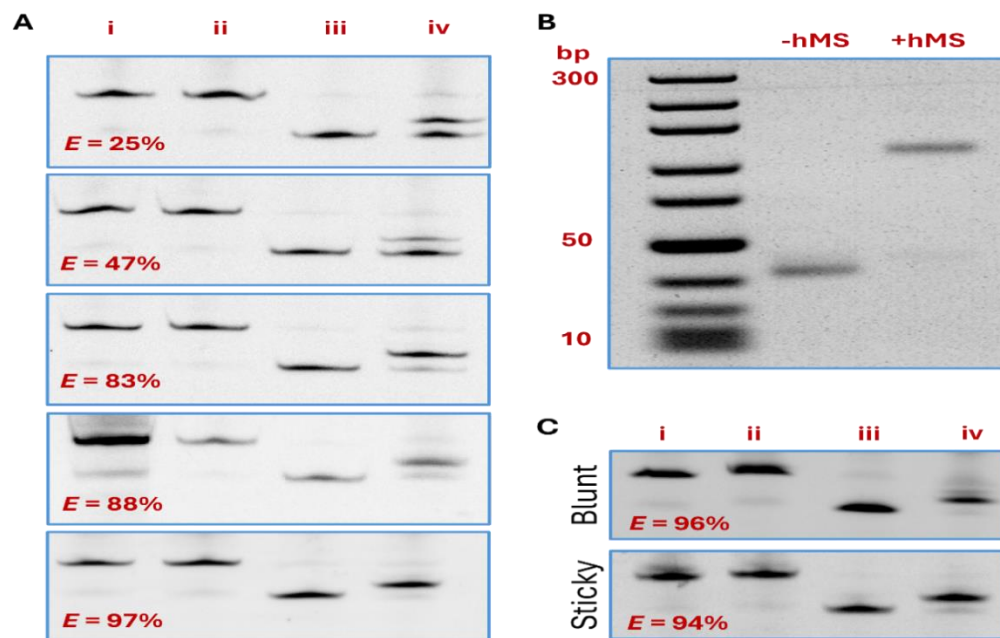


Figure III.3 Optimization of the site-specific labeling approach. A. Representative denaturing gel images obtained from the labeling of synthetic FAM-labeled, 40 bp dsDNA constructs featuring a single central 8-oxoGua. The steps are as follows: i. represents the full length denatured single stranded ss-constructs prior to labeling, ii. represents the ss-constructs after the addition of ddNTPs, iii. represents the same ss-constructs following the GAP formation by the glycosylase/endonuclease which is demonstrated by a band shift downwards, and iv. represents the ss-constructs after the addition of Biotin-dGTP at the GAP site which is demonstrated by a band shift upwards due to the presence of biotin. Lane 3 in these gels demonstrate the efficacy of 8-oxoGua excision by Fpg. Lane 4 demonstrates the increase in the site-specific labeling of 8-oxoGua as function of Bsu DNA polymerase dose (t-b: lowest to highest concentration). B. Representative agarose gel image demonstrating an EMSA; slows the DNA migration upon binding with hMS of the labeled materials in (B) (40 bp) with and without binding to hMS (lane 2, and 1 respectively). C. Denaturing gel images labeled as in (A) demonstrating the no difference in the labeling of blunt (top) and sticky ends (bottom).

III.3.4 SS-nanopore quantification of 8-oxoGua in isolated gDNA after exposure to H₂O₂

In previous sections, we have established optimal labeling efficiency for 8-oxoGua in dsDNA and optimized the SS-nanopore assay measurement conditions to enable the detection and quantification of low abundance target molecules. Here, we sought to quantify 8-oxoGua in gDNA after induction with an oxidant using the improved conditions of the labeling and SS-nanopore measurement. For this purpose, Lambda phage gDNA was used. This gDNA model is highly pure DNA with minimal baseline oxidation which allowed us to study 8-oxoGua formation in well-controlled conditions in terms of concentration and duration of exposure to the oxidant in the absence of other interfering cellular components. We used a mixture of H₂O₂ and AA to induce 8-oxoGua formation in native/unoxidized DNA [39]. 10 µg of Lambda DNA was treated with 10 mM H₂O₂ and 0.1 mM AA for 2, and 4 hours. Untreated samples were used as controls. To achieve functional lengths for downstream measurement with the SS-nanopore assay, gDNA was first fragmented into double stranded segments enzymatically using dsDNA Fragmentase enzyme. However, we found that enzymatic fragmentation yielded wide distributions of fragment lengths and was inconsistent. For example, simultaneous digestion of 3 Lambda DNA samples resulted in completely different distributions of gray values with widths ranging from $w=107-158$ and peak values ranging from 210-350 (Figure III.4 A). Wide distributions may be problematic in SS-nanopore measurements as it prevents concise assessment of length in the sample, hence affecting both the measurement itself (different lengths yield different event rates), and the downstream calculation of 8-oxoGua concentration. Another challenge we encountered were samples with multiple peaks when the gray values were fitted which made it difficult to determine which value to use in downstream calculations (Figure III.4 B). For cellular gDNA which we will discuss in the next section, enzymatic fragmentation yields incomplete digestion (DNA left in the wells

that has not migrated into the gel) which we attributed to the complexity of chromatin structures in those samples (Figure III.4 C). We thought this might introduce biases if 8-oxoGua was formed within these undigested areas of the genome.

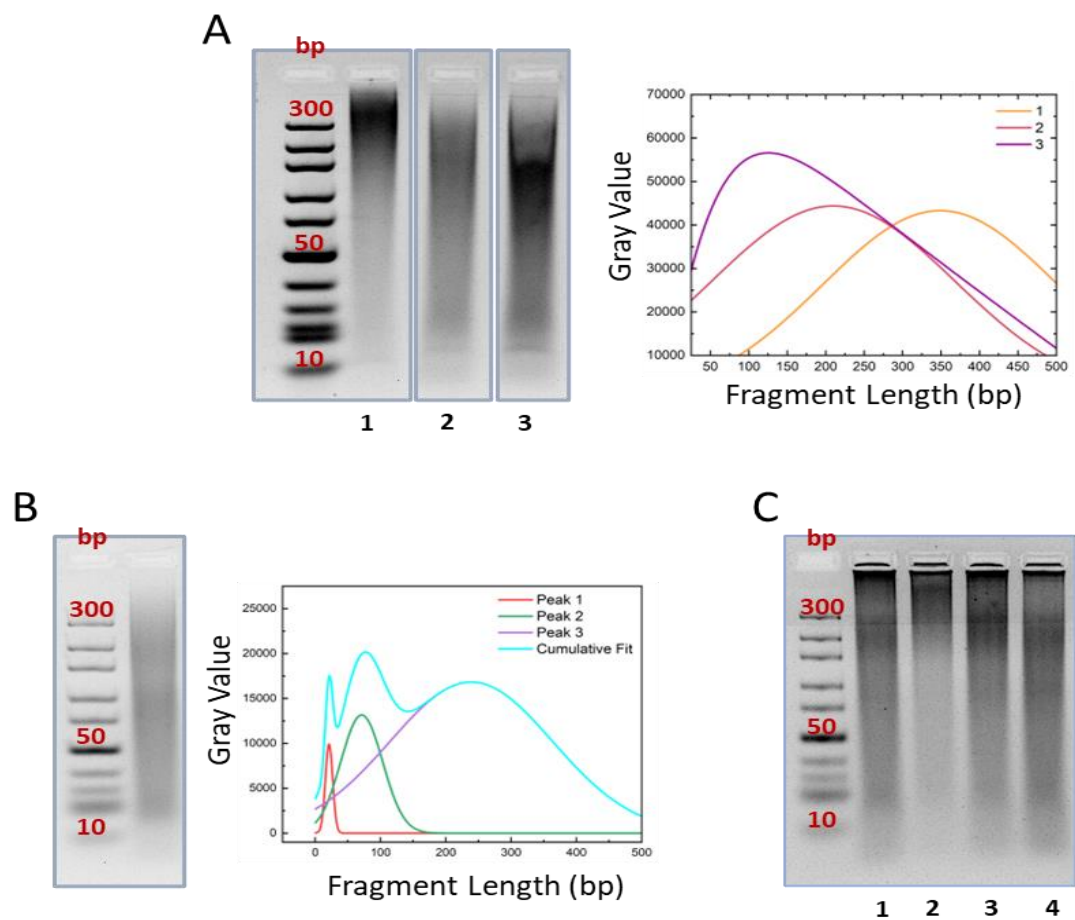


Figure III.4: Limitations of enzymatic fragmentation of gDNA. A. Representative agarose gel image demonstrating the fragmentation of the same Lambda DNA sample 3 times simultaneously using the exact same conditions (left panel). Right panel is quantitative assessment of the gray value intensities of the image in (A) demonstrating the average fragment length (peaks) for each sample and illustrating inconsistency of this approach. B. Representative agarose gel image demonstrating the fragmentation of one Lambda DNA samples that yielded populations of fragments at different locations on the gel (left panel). Right panel is quantitative assessment of the gray value intensities of the image in (B) demonstrating that the average fragment length couldn't be determined for that sample due to the presence of multiple peaks. C. Representative agarose gel image demonstrating the simultaneous fragmentation of MCF-7 gDNA samples using the exact same conditions and demonstrating incomplete digestion of DNA in these samples.

As an alternative to enzymatic fragmentation, we next investigated mechanical fragmentation using ultrasonic treatment. We found that this method yielded much more consistent fragments (peaks at 91.2, 86.5, and 89.5 bp for the untreated, 2h, and 4h exposure times respectively), and a narrower size distribution (Figure III.5) that would result in more precision during fragment length evaluation. All fits resulted in R-square values above 0.9.

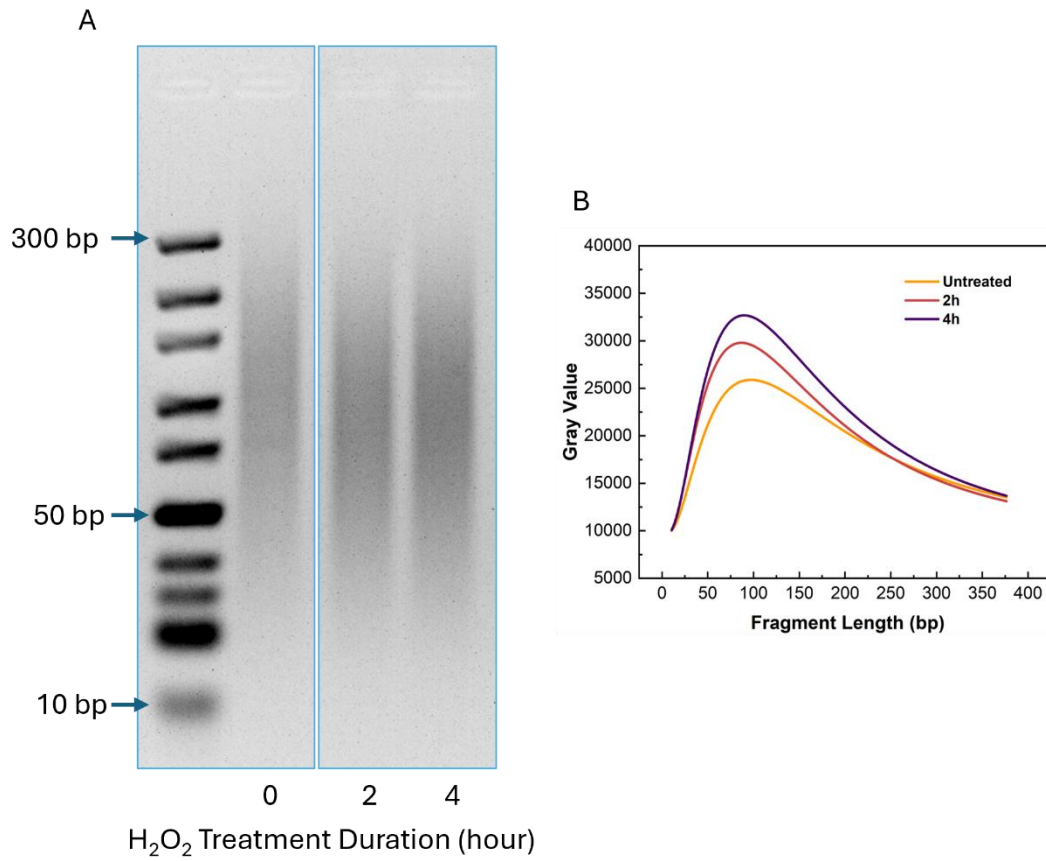


Figure III.5: Lambda DNA fragmentation by ultrasonication. A. Representative agarose gel image demonstrating the fragmentation of Lambda DNA samples treated with 10mM H₂O₂ for 0, 2, and 4 hours respectively. B. Quantitative assessment demonstrating lognormal fitting of the gray value intensities of all lanes in the image in (A) that was used to determine the fragment length for each sample from the peak value in each graph.

Next, to probe the formation of 8-oxoGua, oxidized and fragmented samples were then subjected to site-specific labeling of 8-oxoGua. After labeling, the samples will contain three different fragment populations including unlabeled, non-specifically labeled, and specifically labeled fragment populations (Figure III.6, A). The unlabeled population will comprise the majority of the fragments and will represent the background hMS-unbound DNA. This is under the assumption that 8-oxoGua comprises low abundance in gDNA even after exposure to an oxidant [33, 39, 40]. Non-specific labeling was defined as the population of fragments that incorporated the biotinylated dGTP at non-specific sites and were able to bind hMS regardless of 8-oxoGua excision with Fpg. The frequency of this populations can be deduced from ESMA analysis post binding to hMS for short dsDNA constructs featuring 8-oxoGua that were labeled alongside the gDNA samples. Figure III.6 B, demonstrates that the non-specifically labeled population comprises a low frequency that does not exceed 2.5% while 85% of the population that has been specifically labeled was bound to hMS and determined the labeling efficiency for further calculation of 8-oxoGua concentration.

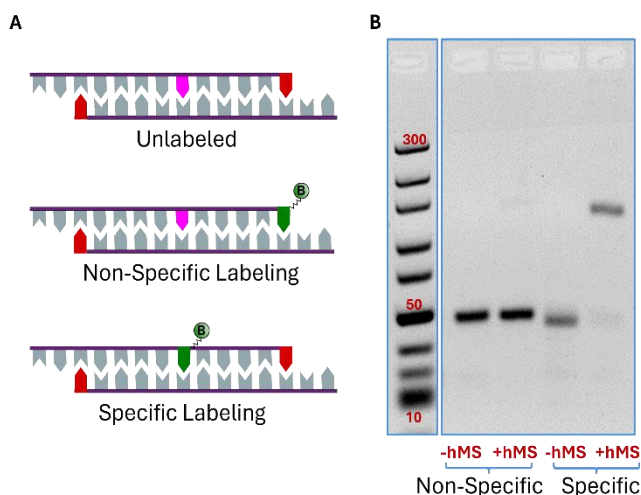


Figure III.6 Site-specific labeling of Lambda DNA samples treated with H_2O_2 and fragmented using ultrasonication. A. Schematic illustrations of the various fragment states present in the H_2O_2 -treated Lambda DNA samples post fragmentation and site-specific labeling. B. represents an EMSA image demonstrating the behavior of the non-specifically labeled (lane 1, and 2) and specifically labeled (Lanes 3, and 4) fragments after binding with hMS.

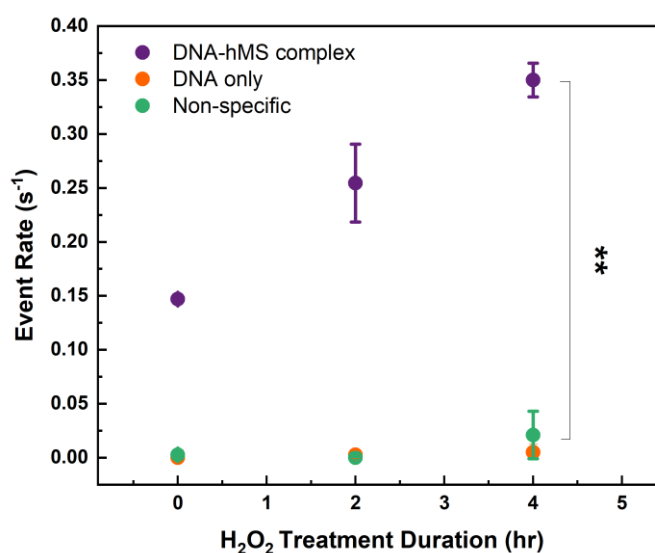


Figure III.7 Event rates of labeled Lambda DNA samples after site-specific labeling of 8-oxoGua. SS-nanopore measurements as a function of event rates of the labeled samples without hMS (orange), non-specifically labeled with hMS (green), and specifically labeled with hMS (purple) after treatment with 10 mM H_2O_2 for 1, 2, and 4 hours. Error bars represent the standard deviation of event rates collected from at least 2 independent SS-nanopore measurements for each condition. Asterisks represent p -values < 0.001 at pairwise comparison between slopes of the linear fits for each set of data.

Next, all samples including the hMS unbound DNA, non-specific labeling, and specific labeling populations were subjected to SS-nanopore measurements to collect event rates associated with 8-oxoGua formation at each dose of H₂O₂ treatment (Figure III.7). Our results demonstrate a dose-dependent increase in the measured event rate for the specifically labeled fragments which correlates with increased frequency of biotinylated fragments that in turn is associated with increased incidence of 8-oxoGua. The selectivity of the assay was maintained across all treatment conditions evidenced by the negligible event rate of unbound DNA samples and the non-specific labeling. The concentration of the non-specifically labeled population of fragments at each condition was considered for downstream 8-oxoGua concentration calculation.

The concentration of 8-oxoGua was calculated as the ratio of 8-oxoGua per 10⁴ 2'-deoxyguanine (dG) taking into account the labeling efficiency (E), the mean length of fragments in the sample (L), the GC-content (α) of the lambda DNA sequence (NC_001416.1 with mutations: 37589 C->T, 45352 G->A, 37742 C->T and 43082 G->A) which is calculated to be 25% using the expression:

$$\text{8-oxoGua}/10^4 \text{ dG Ratio} = \left(\frac{C_s - C_{ns}}{E * (C_i * \alpha) * 2L} \right) * 10000$$

Where C_s is 8-oxoGua concentration, C_{ns} is concentration of non-specifically labeled materials, C_i is the initial concentration of DNA in the sample. C_s and C_{ns} were calculated using the expression:

$$C = \frac{ER}{m}$$

Where ER is the measured event rate, and m is the slope of the linear fit of the calibration curve inferred specifically for each sample. The 8-oxoGua/dG ratio calculation yielded a

ratio of 14.9 ± 0.13 for the untreated samples, 20.9 ± 2.9 for 2-hour treatment, and 23.9 ± 0.31 for 4-hour treatment which demonstrates a statistically significant increase in 8-oxoGua levels upon H_2O_2 treatment for 4 hours (p-value=0.0007) (Table III.4; Figure III.8). The ratios obtained from the mass spectrometry analyses of the same samples yielded ratios of 36.5 ± 8.4 , 50.9 ± 3.8 , and 62.3 ± 7.6 for untreated, 2-hour, and 4-hour treatments respectively and displayed a significant dose-dependent increase in 8-oxoGua levels with increasing treatment time (p-value=0.02) (Table III.5; Figure III.8). According to this data, the SS-nanopore assay seems to underestimate 8-oxoGua levels. It is plausible that the SS-nanopore underestimates the absolute values of 8-oxoGua at high lesion density because fragments with multiple lesions only get counted once. At low density, like in physiological gDNA, the value should be more accurate between the two methods as demonstrated in the next chapter. Although numerically, the values obtained were different, time dependence was statistically identical between mass spectrometry and SS-nanopore measurements. For example, a 1.6-fold increase in the SS-nanopore dataset have been observed after 4 hours treatment, while a 1.5-fold increase in the mass spec dataset after 4 hours (p-value>0.05 between the two methods) (Figure III.8). Since we don't know at what 8-oxoGua level does this effect start, we instead focus on relative lesion density across conditions in cellular DNA measurements.

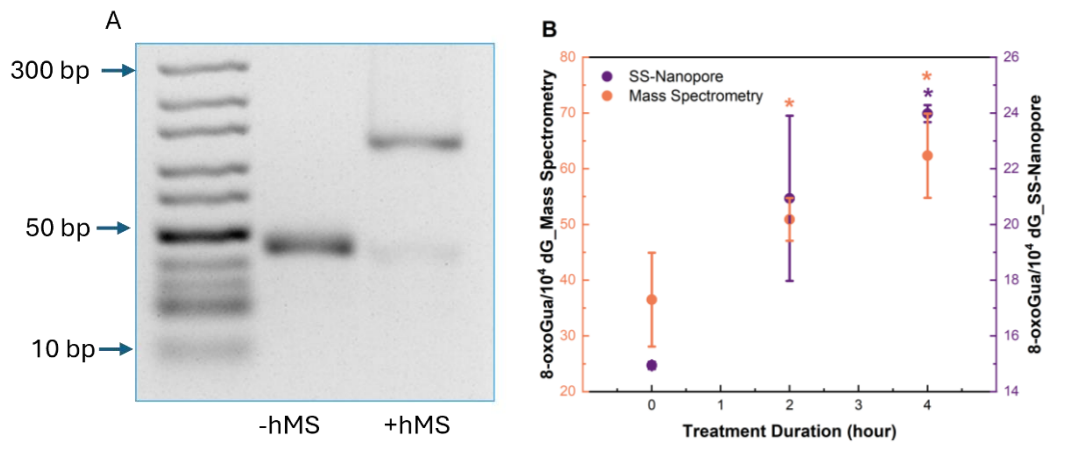


Figure III.8. Quantification of 8-oxoGua in Lambda DNA after H₂O₂ treatment using two methods; SS-nanopores (purple) and mass spectrometry (orange). A. EMSA for 40bp dsDNA DNA constructs labeled alongside the lambda DNA samples demonstrating binding with hMS successfully and labeling efficiency of 84%. B. 8-oxoGua/10⁴ dG ratio in DNA samples treated with H₂O₂ for a designated time after mass spectrometry measurement (left Y-axis) and SS-nanopore measurements (right Y-axis). Circles represent the mean ratio $\pm$ standard deviation of measurements carried out on at least 2 independent SS-nanopore devices and 3 mass spectrometry replicates. Asterisks represent p-values <0.05 (orange asterisks for mass spectrometry and purple asterisks for SS-nanopore measurement)

Table III.4: Pairwise comparisons of 8-oxoGua levels obtained at different time points after H₂O₂ treatment and quantified by SS-nanopore			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
Untreated-2 hour	-2.86	1	0.214
Untreated-4 hour	-38.4	1.38	0.005
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

Table III.5: Pairwise comparisons of 8-oxoGua levels obtained at different time points after H₂O₂ treatment and quantified by mass spectrometry			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
Untreated-2 hour	-2.7	4	0.027
Untreated-4 hour	-3.48	3	0.02
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

III.3.5 SS-nanopore quantification of 8-oxoGua in cellular gDNA of the MCF-7 cell line after exposure to H₂O₂

To further validate the capability of the SS-nanopore assay to quantify 8-oxoGua with high sensitivity and accuracy in a physiological system, we sought to quantify 8-oxoGua in the breast cancer cell line, MCF-7. This cell line is known to accumulate 8-oxoGua after exposure to H₂O₂ despite the presence of sufficient levels of hOGG1 in their nuclei [46, 47] which provide us with a good target. Cellular models are complex and allow for studying 8-oxoGua formation in the presence of variables within a cellular system such as chromatin structures, antioxidants, and repair machineries.

To ensure that the external H₂O₂ treatment results in sufficient ROS buildup in the nucleus, we first transduced MCF-7 cells with nuclear Orp1-roGFP constructs [48, 49]. Orp1-roGFP is a ratiometric redox-sensitive fluorescent probe that responds rapidly and specifically to H₂O₂ and can be localized to the nucleus [49]. Figure III.9 A, illustrates confocal microscopy images of the nuclei of MCF-7 cells prior to and 5 minutes after treatment with H₂O₂ at small concentrations (50-500 μ M). We found that the fluorescent levels of roGFP increases gradually with increasing H₂O₂ concentration up to 500 μ M where the signal exhibits a plateau shape or a slight decrease in the signal which can be explained by overoxidation of the probe (Figure III.9 B).

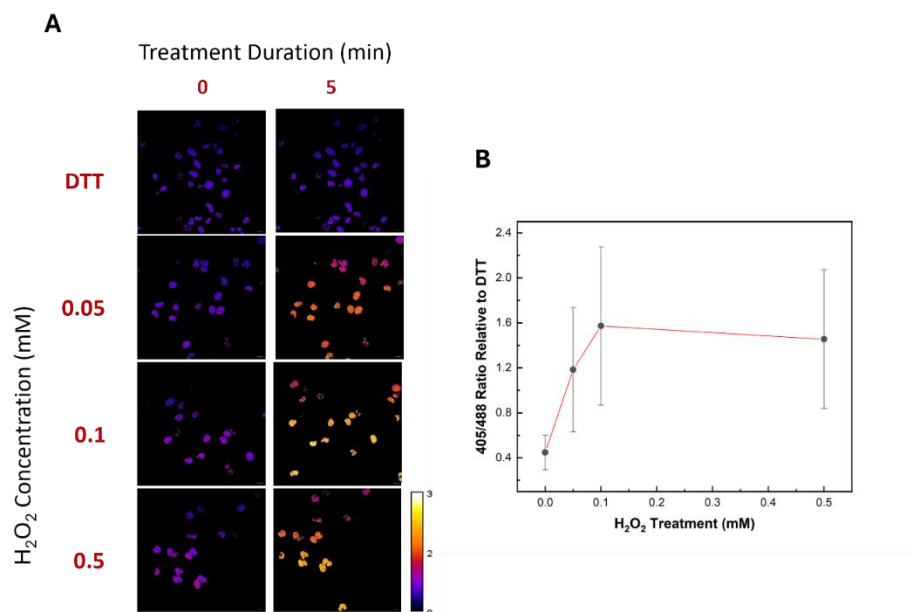


Figure III.9 Assessment of ROS buildup in MCF-7 cell nuclei via HyPer-DAO. A. Representative confocal images demonstrating the nuclei of MCF-7 cells transfected with the H₂O₂ sensor, roGFP. The images show an increase in the sensor's fluorescence as the H₂O₂ concentration increases until it reaches a plateau at 0.5 mM. B. quantitative assessment of fluorescence intensities from the images in (A).

Live cell imaging for 20 minutes in the presence of H₂O₂ at the aforementioned concentrations resulted in a drastic increase in the signal during the first 5 minutes which is followed by a constant nonchanging signal over the course of the next 20 minutes (Figure III.10 A). while the absolute H₂O₂ concentrations accumulated in the nuclei is not possible using these probes, we quantified 8-oxoGua in these cells at the aforementioned H₂O₂ concentrations over the course of 20 minutes to yield more information about 8-oxoGua formation as a result of ROS buildup in the nuclei. Our results demonstrated that

ROS buildup at low H_2O_2 concentrations (up to 500 μM) were not sufficient to induce the formation of 8-oxoGua in the treated cells relative to the untreated control (p -value= 0.7, 0.17, and 0.09 for cells treated with 0.05, 0.1, and 0.5 mM H_2O_2 , respectively, compared to the untreated controls). This could be due to the short treatment duration or the presence of phenol red in the treatment media that resulted in the neutralization of H_2O_2 (Figure III.10 B).

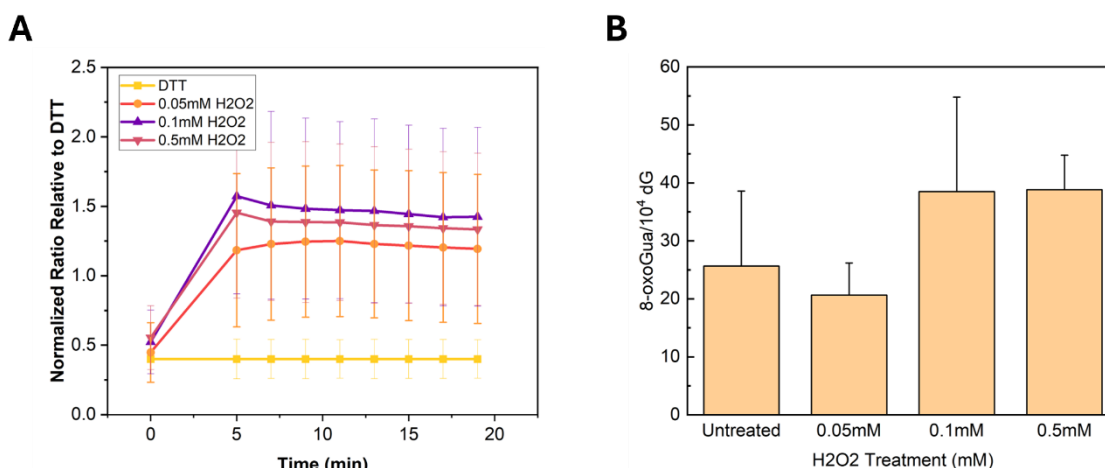


Figure III.10 Time-course quantification of roGFP ratio changes in MCF-7 cells upon addition of different concentrations of H_2O_2 . **A.** 405 nm/488 nm ratios from live cell imaging of MCF-7 cells nuclei transduced with roGFP over the course of 20 minutes at a wide range of H_2O_2 concentrations (0.05 – 0.5 mM). **B.** Mass spectrometry quantification of 8-oxoGua levels in gDNA isolated from MCF-7 cells treated with the designated H_2O_2 concentration.

To induce the formation of 8-oxoGua in the gDNA of MCF-7 cells, we treated the cells with external H_2O_2 at concentrations that do not result in a widespread cell death. An MTT assay was conducted to determine the appropriate treatment concentrations below the EC50 (Figure III.11 A). To determine the EC50 value, a 4-parameter logistic (4PL) regression fitting was applied after converting all H_2O_2 concentration values to a

logarithmic scale. For the fitting, the bottom asymptote was fixed at 0, and the top asymptote was fixed at 100 and EC50 was calculated using the following equation:

$$y = A1 + \frac{A2 - A1}{1 + 10^{(\log x_0 - x)p}}$$

Where A1 is bottom asymptote, A2 is the top asymptote, $\log x_0$ is the center, and p is the slope of the curve. The EC50 was determined from the fit to be approximately 22 mM of H_2O_2 in 1X PBS for 1 hour (Figure III.11 B). Thus, for SS-nanopore analysis, cells were treated with H_2O_2 concentrations ranging from 2-20 mM in 1X PBS for 1 hour after which 8-oxoGua was quantified.

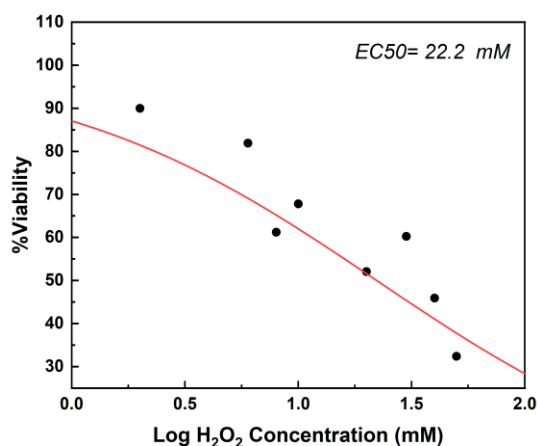


Figure III.11 MTT Cell viability of MCF-7 cells after exposure to H_2O_2 . Cell viability of MCF-7 cells after exposure to H_2O_2 at doses ranging from 2-50 mM for 1 hour. Closed circles represent the mean $\pm$ standard deviation of 3 measurements at each condition. A 4PL fit (red line) after converting H_2O_2 concentration values to a logarithmic scale was performed. EC50 was determined from this fit to be 22.2 mM.

For SS-nanopore measurements, gDNA was isolated immediately after treatment with each designated dose of H_2O_2 . Fragmentation of MCF-7 gDNA was conducted mechanically using ultrasonication as it yields more consistent fragmentation (peaks at 95, 116, 116, 120, 113 bp for the untreated, 2 mM, 6 mM, 10 mM, and 20 mM respectively) (Figure III.12 A). The labeling efficiency and frequency of non-specific labeling were determined via EMSA analysis of 8-oxoGua containing dsDNA constructs labeled alongside the gDNA samples (Figure III.12 B).

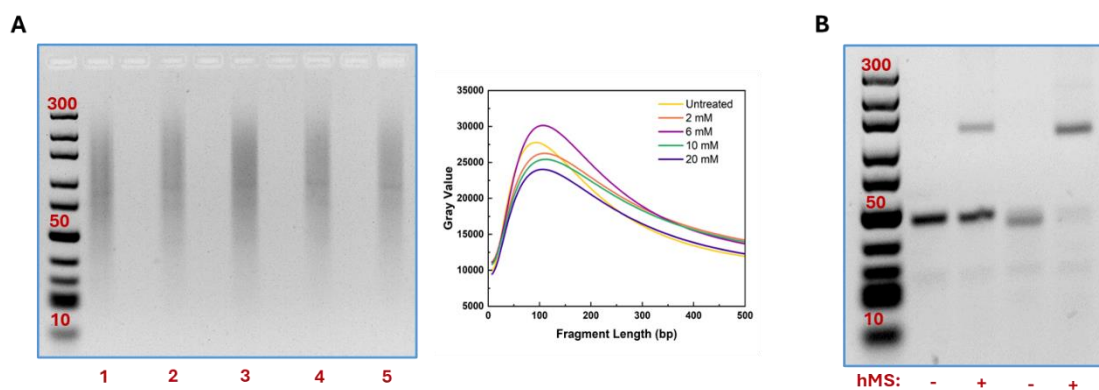


Figure III.12 Fragmentation and 8-oxoGua labeling efficiency assessment of the MCF-7 gDNA after exposure to different concentrations of H_2O_2 . A. Representative agarose gel image demonstrating the fragmentation with ultrasonication of isolated DNA from MCF-7 cells after treatment with various H_2O_2 doses ranging from 2-20mM for 1hr (left panel). Right panel is quantitative assessment of the gray value intensities of the image in (B) demonstrating the fragment length of each treatment condition which is determined as the peak of the lognormal fit. B. EMSA agarose gel image demonstrating the behavior of the non-specifically labeled (lane 1, and 2) and specifically labeled (Lanes

3, and 4) fragments after binding with hMS for synthetic dsDNA constructs labeled alongside the MCF-7 gDNA samples.

Labeled samples were then subjected to SS-nanopore measurement following standard protocols. Briefly, about 1 μg of each fragmented sample was labeled with biotin at 8-oxoGua sites, another 1 μg of the fragmented samples was labeled skipping the fpg excision step to probe for non-specific labeling (see section III.3.3). aliquots from each sample were used to form DNA/hMS complexes in a 40 μl reaction with an estimated final total DNA concentration of 10 nM. 10 μl was used to perform the SS-nanopore measurements. Each sample was measured on 3 independent SS-nanopore devices. For each condition, the non-specifically labeled samples as well as DNA only samples were measured and served as our controls. 8-oxoGua concentrations measured from DNA only samples were negligible which ensures selectivity is maintained while the concentration of the non-specifically labeled at each condition was considered for downstream 8-oxoGua ratio calculation. 8-oxoGua/ 10^4 dG ratios in samples from each condition was calculated from the measured event rates as described in section III.3.4 (8-oxoGua/ 10^4 dG Ratio equation). For these measurements, α was 0.2, L was measured from the gels shown in Figure III.12 A for each sample, and E was determined to be 0.85 (Figure III.12 B). SS-nanopore measurements yielded ratios of 50.14 ± 15.4 for untreated samples, 43.47 ± 2.5 for 2 mM H_2O_2 treatment, 61.5 ± 10.2 for 6 mM H_2O_2 treatment, 67.19 ± 19.3 for 10 mM H_2O_2 treatment, and 71.83 ± 22.5 for 20 mM H_2O_2 treatment which demonstrates an increase in 8-oxoGua levels upon H_2O_2 treatment but not to a statistically significant level (Table III.7; Figure III.13 A). These results were compared to mass spectrometry analyses of the same samples yielding 19.9 ± 9.2 , 11.9 ± 1.8 , 20.3 ± 0.8 , 29.3 ± 5.9 , and 38.7 ± 10.8 for untreated, 2, 6, 10, 20 mM respectively which displayed a significant increase in 8-oxoGua levels at 20 mM H_2O_2 ($p\text{-value} = 0.04$) (Table III.8; Figure III.13 B).

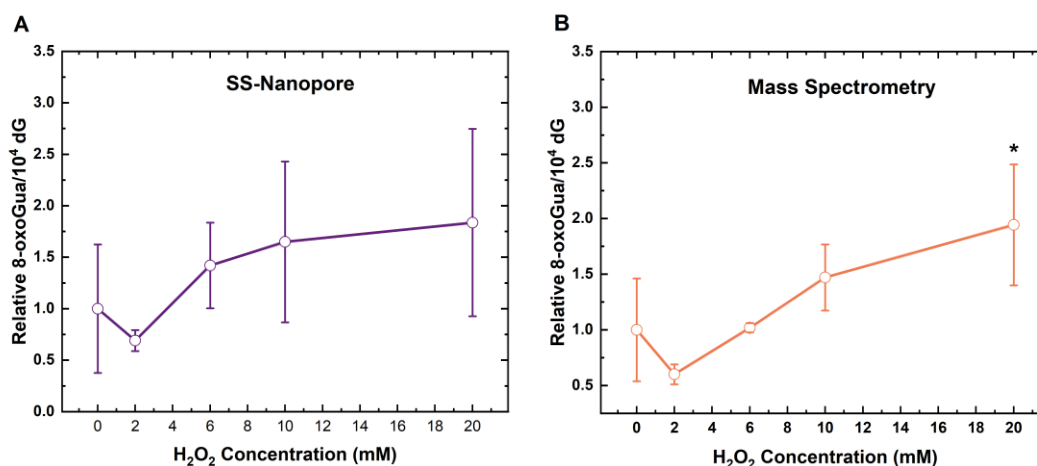


Figure III.13 Quantification of 8-oxoGua in MCF-7 gDNA after H₂O₂ treatment using two methods; SS-nanopores (purple) and mass spectrometry (coral). A. Relative 8-oxoGua/10⁴ dG ratio in DNA samples treated with H₂O₂ for 1 hour measured via SS-nanopore. Open circles represent the mean ratio $\pm$ standard deviation of measurements carried out on at least 3 independent SS-nanopore devices. B. Relative 8-oxoGua/10⁴ dG ratio in DNA samples treated with H₂O₂ for 1 hour measured via mass spectrometry. Open circles represent the mean ratio $\pm$ standard deviation of at least 3 independent measurements. * represent p-values <0.05 at pairwise comparison of each condition with the untreated control.

Table III.6: Pairwise comparisons of 8-oxoGua levels obtained at different H₂O₂ concentrations and quantified by SS-nanopores			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
Untreated-2 mM	0.849	4	0.444
Untreated-6 mM	-0.968	4	0.388
Untreated-10 mM	-1.18	5	0.293
Untreated-20 mM	-1.36	5	0.233
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

Table III.7: Pairwise comparisons of 8-oxoGua levels obtained at different H₂O₂ concentrations and quantified by mass spectrometry			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
Untreated-2 mM	1.47	4	0.892
Untreated-6 mM	-0.0693	4	0.474
Untreated-10 mM	-1.48	4	0.106
Untreated-20 mM	-2.29	4	0.042
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

Similar trends were observed in both the mass spectrometry and SS-nanopore analyses. A linear increase in 8-oxoGua levels have been observed in both methods as the concentration of H₂O₂ increased reaching a 1.9-fold increase for mass spectrometry measurement and a 1.8-fold increase in SS-nanopore measurements compared to their respective untreated samples (p -value>0.05). All relative 8-oxoGua concentrations yielded from the SS-nanopore analyses showed no statistical difference from the mass spectrometry analyses in pairwise comparison between the two methods (p -value=0.156, 0.086, 0.348, and 0.427 for the 2mM, 6mM, 10mM, and 20mM measurements respectively)

Interestingly, 8-oxoGua formation was not induced by H_2O_2 up to 2 mM but instead reduced by 0.6-fold. This reduction was detected using both approaches. This could be explained by an induction of repair mechanisms in the cells triggered by the exposure to H_2O_2 which can handle low levels of exposure but become less efficient at higher concentrations [46, 47, 50]. Higher H_2O_2 concentrations resulted in a dose-dependent increase in 8-oxoGua levels in the genome. The observed increase in 8-oxoGua levels did not exceed 3-folds using both measurement techniques which is consistent with previous reports using electrochemical detection and mass spectrometry [33]. These results further establish the utility of the SS-nanopore assay as a tool to quantify 8-oxoGua from various sources.

III.4 Conclusion

In this study, our main objective was to expand the utilization of the SS-nanopore assay and validate its ability to quantify 8-oxoGua which is considered a significant biomarker in a wide range of basic science and clinical applications. The main goals were to induce the formation of 8-oxoGua using H_2O_2 in two different gDNA models and use the SS-nanopore assay to quantify it. Our results showed that we were able to specifically probe 8-oxoGua in dsDNA of various origins with high efficiency and conduct highly sensitive and accurate quantification of 8-oxoGua in gDNA of both models using our SS-nanopore assay. Additionally, our platform was sensitive enough to quantify 8-oxoGua concentrations down to 0.6 nM. Our measurements were validated by mass spectrometry which is the current golden standard for 8-oxoGua quantification.

Furthermore, our results showed that the SS-nanopore assay measurements are length dependent which highlight the importance of rigorous assessment of DNA length during the quantification process as it plays a key role in downstream concentration calculations

of the target molecule. One of the challenges we encountered in this regard was in enzymatic digestion of gDNA to functional length suitable for the assay. Enzymatic digestion was inconsistent and resulted in wide distribution of DNA lengths within the sample which made it difficult to choose the appropriate calibration curve for concentration assessment and introduced outliers. This challenge was overcome by using ultrasonication which was more consistent and resulted in a more uniform digestion.

Additionally, concentration dependent calibration curves that were developed for this study enabled us to determine the LOQ of our measurement. Under optimized conditions, we were able to quantify target molecules down to 0.6 nM in concentration. These results were very promising as our target comprises low abundance in both native and oxidized gDNA. Nonetheless, we have anticipated lower LOQ based on results from the literature showing remarkable sensitivity of the nanopore platform. This could be attributed to the high standard deviation in our measurements. Device-device variation is considered a limitation for synthetic nanopores due to fabrication artifacts. Using internal standards may aid in reducing these variations. These aren't currently applied due to stability issues and efforts to increase the stability of the pores over long measurements are ongoing.

Finally, this study validated our SS-nanopore approach as a reliable tool for the quantification of 8-oxoGua in gDNA and set the groundwork to further expand its utilization as an alternative tool by providing several advantages in terms of convenience, time consumption, sample size requirement, sequence preservation and cost in translational applications (Table III.8). In the next chapter, we aim at determining the translational capability of our SS-nanopore assay by measuring changes in 8-oxoGua levels in HNSCC cell models that vary in their radiosensitivity.

Table III.8 Comparative assessment of the SS-Nanopore Technology and Mass Spectrometry for 8-oxoGua quantification focusing on cost, time, sensitivity, and reproducibility		
	Mass Spectrometry	SS-Nanopore
Cost (Includes materials cost only)	\$9 per sample	\$2 per sample
Time	4.5 hours per sample Preprocessing: 22.5 hours (3-step enzymatic digestion and filtration)	~5-10 minutes per sample Preprocessing: 4.5 hours (fragmentation, 3-step enzymatic labeling, and hMS binding)
Sample Size	2-10 µg	0.5-1 µg
Sequence Preservation	No	Yes
Reproducibility	• Technical replicates • Biological replicates • Appropriate controls • Internal standard • Concentration-dependent standard curve	• Technical replicates • Biological replicates • Appropriate controls • Concentration-dependent standard curve
Sensitivity (LOQ)	~0.3 pM	~6 pM

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CHAPTER IV

Manuscript in preparation for publication.

IDENTIFICATION OF DIFFERENCES IN 8-OXOGUA FORMATION AND REPAIR IN A MATCHED MODEL OF HEAD AND NECK CANCER CELL LINES WITH VARIED RADIOSENSITIVITY

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IV.1 Introduction

HNSCC accounts for 3% of all cancers with an estimated 58,450 diagnosed cases and 12,230 deaths in the United States in 2024. The incidence of HNSCC have been shown to increase by 1-2.3% annually [1]. HNSCC commonly involves multiple critical anatomical structures which renders it difficult to treat [2]. Most HNSCC patients receive radiation therapy at every stage of presentation either as primary treatment or as an adjuvant therapy [3, 4] which is commonly associated with adverse effects and decreased quality of life. Despite advances in radiotherapy approaches and enhanced treatment efficacy, a significant number of patients relapse within 6 months following radiation. The reason being a resistant population of tumor cells that repopulate the irradiated areas [4]. Currently, only one biomarker, namely HPV-status, has been utilized clinically to personalize treatment, and predict response to radiotherapy [5, 6], with limitations due to concerns of potential undertreatment of HPV-positive patients. In the past decade, tumor hypoxia has been researched extensively in relation to radio-resistance. However, due to the complex molecular mechanisms by which hypoxia modulates tumor resistance, not a single biomarker of hypoxia has been shown to predict resistance consistently in a clinical setting [6, 7]. Furthermore, some DNA damage response markers have been implicated in treatment failure and increased radio-resistance such as Ku80 and mutated P53 [8-10]. The lack of biomarkers stems from a lack of understanding of the biological processes surrounding oxidation. A deeper understanding of the molecular mechanisms of radio-resistance is crucial to improve the treatment of HNSCC patients. Recently, Bansal et al., developed a matched model of HNSCC radio-resistance using the parental radio-sensitive cell line SCC-61. The radio-resistant cell line rSCC-61 resulted from exposing SCC-61 cells to fractionated radiation for eight cycles after which rSCC-61 cells were randomly selected from surviving colonies grown in soft agar [11].

The radio-resistant rSCC-61 cells have been shown to acquire altered glucose and lipid metabolism relative to SCC-61 [12]. Additionally, the radio-resistant phenotype has been shown to be associated with increased mitochondrial NADPH production and exhibit enhanced ROS scavenging properties [13, 14]. A significant increase in global DNA methylation levels was also observed in rSCC-61 [15]. DDR is a key contributor to radiation resistance. A previous report comparing ROS levels and DNA damage in SCC-61 and rSCC-61 demonstrated that rSCC-61 comprise lower ROS levels, increased antioxidant activity, and increased DNA damage repair evidenced by reduced γ H2AX foci and high PARP1 expression [11]. Proteomic data from the same study revealed a number of significantly regulated pathways in rSCC-61 compared to SCC-61 which included the BER. Given that BER is responsible for the repair of DNA base modifications including 8-oxoGua [16], we wanted to further investigate how these two cell lines handle indirect oxidative DNA damage as a function of global 8-oxoGua levels. In this chapter, we will use the methods optimized and validated in the past two chapters to quantify 8-oxoGua in SCC-61 and rSCC-61 via our SS-nanopore assay and identify variations in its formation and repair after exposure to H_2O_2 to induce oxidative stress in these cell lines.

IV.2 Materials and Methods

IV.2.1 MTT assay for cell viability

Cell viability after exposure to H_2O_2 was evaluated using the CyQUANT MTT cell viability assay (Invitrogen #13154) as described in chapter III with slight modifications. Briefly, 30×10^3 cells/well were seeded in a 96-well plate and adhered for 24 hours. H_2O_2 was administered to the cells in 1X PBS at concentrations ranging from 0.1-20 mM for 1 hour. Cells incubated with 1X PBS without H_2O_2 were regarded as controls. All treatment conditions were done in triplicates. Media containing the H_2O_2 was then aspirated, and

cells were washed with 100 μ l 1X PBS. Then, the MTT solution was added to the culture according to the manufacturer instructions. Cell viability was calculated by the formula: (A treatment/A control) *100% where A is the absorbance recorded at 540 nm.

IV.2.2 HyPer-DAO transduction

To monitor H₂O₂ buildup in the nuclei of SCC-61 and rSCC-61 cell lines, cells were transduced with the NLS-HyPer-DAO fusion protein expression vector. Preparation of the lentiviral particles for transduction was described in [26]. To transduce SCC-61 and rSCC-61 cells, 5x10⁶ cells were seeded in 100 mm cell culture treated dishes and adhered overnight. Prior to transduction, cell suspension was supplemented with 10 μ g/ml polybrene for 30 minutes to enhance transduction efficiency. Then, 1 mM sodium butyrate as well as 1 ml of lentiviral particles were added to the culture media. Cells were treated with the viral particles for 48 hours after which transduction efficiency was assessed by monitoring GFP signal by fluorescent microscopy. Then, transduced cells were selected by incubation with 1.5 μ g/ml puromycin for 24 hours.

IV.2.3 Live cell imaging

Transduced SCC-61 and rSCC-61 were seeded on (Ibidi #80827) and adhered overnight to prepare for confocal microscopy. Live cell imaging was carried out using Olympus Evident FV4000 confocal microscope. Live imaging parameters were described in chapter III. Imaging was conducted for cells treated with 4 mM H₂O₂ for 20 minutes. DTT and D-Ala were used as a negative control and a positive control, respectively. Images were analyzed using Fiji imageJ software as described in chapter III.

IV.2.4 8-oxoGua Formation in SCC-61 and rSCC-61

SCC-61 and rSCC-61 head and neck cancer cells were regularly cultured and passaged in DMEM/F12 (1:1) media (Gibco 11320033) supplemented with 10% FBS (Invitrogen) and 1% Penicillin-Streptomycin (10,000 U/mL) (Gibco # 15140122) at 37°C and 5% CO₂. To induce 8-oxoGua formation, 10-12x10⁶ cells were seeded in 150 mm cell culture treated dishes and adhered overnight. Cells from both populations were then washed twice with 1X PBS and incubated with 4 mM H₂O₂ in serum-free, phenol red-free media (Gibco #31053028) for 2, 5, 15, and 30 minutes at 37°C and 5% CO₂. Cells incubated with DMEM media without H₂O₂ were regarded as controls. After incubation, cells were washed twice with 1X PBS and harvested using 0.25% Trypsin-EDTA solution and pellets were prepared for gDNA extraction. Extraction of gDNA was performed using the DNeasy blood and tissue kit (QIAGEN #69504) and resuspended in 100 µl deionized water. gDNA was then processed for SS-nanopore, and mass spectrometry analyses as described in chapter III.

IV.2.5 8-oxoGua Repair in SCC-61 and rSCC-61

SCC-61 and rSCC-61 head and neck cancer cells were regularly cultured and passaged in DMEM/F12 (1:1) media supplemented with 10% FBS and 1% Penicillin-Streptomycin (10,000 U/mL) at 37°C and 5% CO₂. 2x10⁶ cells were seeded in 100 mm cell culture treated dishes and adhered overnight. Treatments were conducted during the exponential growth phase of the cells. Cells from both populations were then washed with 1X PBS and treated with 4 mM H₂O₂ in serum-free, phenol red-free media for 30 minutes at 37°C and 5% CO₂ after which the oxidant was removed, and cells were washed thoroughly with 1X PBS and resuspended in DMEM/F12 (1:1) media for 6, and 24 hours. Cells were then harvested using 0.25% Trypsin-EDTA solution and pellets were prepared for gDNA extraction. Extraction of gDNA was performed using the DNeasy blood and tissue kit

(QIAGEN #69504) and resuspended in 50 µl deionized water. gDNA was then processed for SS-nanopore analysis as described in chapter III.

IV.3 Results and Discussion

IV.3.1 8-oxoGua formation and repair in a matched model of HNSCC cell lines, SCC-61 and rSCC-61

To induce 8-oxoGua formation in SCC-61 and rSCC-61, experiments were carried out where H₂O₂ was used as an oxidant alternative to radiation. H₂O₂ possess limited chemical reactivity. However, it has been shown to readily convert to the more reactive •OH upon encounter with transition metals such as Fe (II) and Cu (I) by what is known as the Fenton reaction (Harber-Weiss reaction). In turn, •OH has the ability to interact with DNA and form a wide range of oxidized bases including 8-oxoGua [17]. Note that this approach isolates chemical oxidation alone in contrast to radiation which can induce multiple forms of direct damage as well. Additionally, there is evidence that the identity of the individual oxidation products produced by hydroxyl radicals are the same regardless of the source of the radical, and the repair kinetics of those lesions are similar as well [19].

The concentration of H₂O₂ used for the treatments was determined based on results from an MTT viability assay as shown in Figure IV.1. To determine the EC₅₀ values for each cell population, a 4PL regression fitting was applied to each curve after converting all H₂O₂ concentration values to a logarithmic scale. For the fitting, the bottom asymptote was fixed at 0, and the top asymptote was fixed at 100 and EC₅₀ was calculated using the following equation:

$$y = A1 + \frac{A2 - A1}{1 + 10^{(\log x_0 - x)p}}$$

Where A1 is bottom asymptote, A2 is the top asymptote, $\log x_0$ is the center, and p is the slope of the curve. EC50 values were calculated for SCC-61 (8.5 ± 0.74 mM) and rSCC-61 (10.6 ± 1.9 mM). Cells were then treated with 4mM to ensure viabilities close to 80% in both cell populations during the course of the treatment while increasing the probability that 8-oxoGua will be formed in these cells.

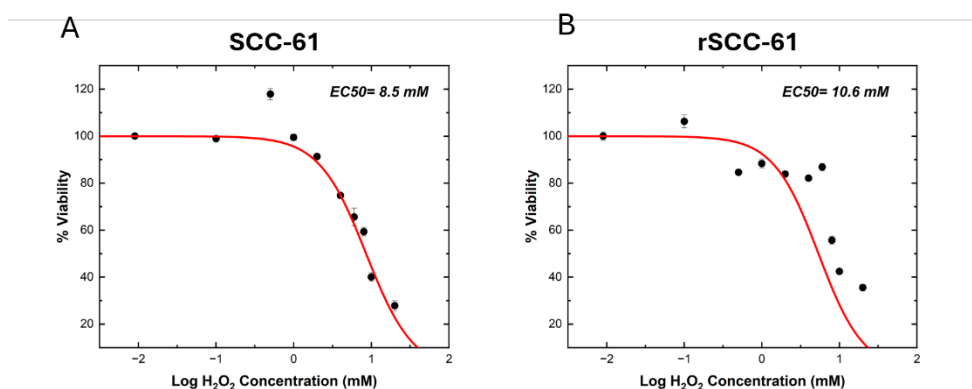


Figure IV.1: Cell viability upon exposure to H_2O_2 . A. MTT cell viability of SCC-61 cells after exposure to H_2O_2 at doses ranging from 0.1-20 mM for 1hr. EC50 (8.5 mM) was determined by applying a 4PL fit on each graph after converting H_2O_2 concentration values to a logarithmic scale. B. MTT cell viability of rSCC-61 cells after exposure to H_2O_2 at doses ranging from 0.1-20 mM for 1hr. EC50 (10.6 mM) was determined by applying a 4PL fit on each graph after converting H_2O_2 concentration values to a logarithmic scale.

To assess changes in 8-oxoGua levels upon exposure to H_2O_2 , we first conducted an experiment in which cells were treated with 4 mM H_2O_2 for 30 minutes after which the oxidant was removed and 8-oxoGua levels were measured after 6, and 24 hours. Cells at each time point were harvested and gDNA was extracted immediately after treatment. gDNA was then fragmented using ultrasonication and 8-oxoGua was labeled with biotin in preparation for quantification through the SS-nanopore assay. All Samples were subjected to SS-nanopore analyses as described in chapter III (See Materials and Methods). Briefly, at least 0.5 μg of DNA from treated cells was processed for each condition, and aliquots were used to form nucleoprotein complexes with hMS. Each sample was measured on a single SS-nanopore device for 7 minutes at least where approximately 100 blocks of 3.5 s were collected for each sample. Event rates were collected from each sample and 8-oxoGua/ 10^4 dG ratios were calculated.

Figure IV.2, A illustrates results of the SS-nanopore quantification of 8-oxoGua levels produced over time in the gDNA of SCC-61/rSCC-61 (Figure IV.2, A). Our results demonstrate that baseline levels of 8-oxoGua are not statistically different between the two cell lines despite differences in basal ROS levels (p -value = 0.605). Although a previous study has shown that rSCC-61 accumulate lower levels of ROS compared to SCC-61 [11], 8-oxoGua is highly dynamic in nature, especially, during the exponential phase of cellular growth and division where DNA might be exposed to various oxidants from the environment [20] and we only measure a ‘snapshot of time’ in the growth cycle of those cells.

With regard to 8-oxoGua levels after 30 minutes of H_2O_2 treatment, we did not observe any changes in 8-oxoGua levels in both cell lines compared to the untreated control (Table IV.1). After the removal of the oxidant, 8-oxoGua levels remained similar to the untreated cells in both cell lines up to 24 hours (Table IV.1; Figure IV.2, A) except an increase in 8-

oxoGua levels that was observed at 6 hours in rSCC-61 cells which might be related to normal cellular metabolism.

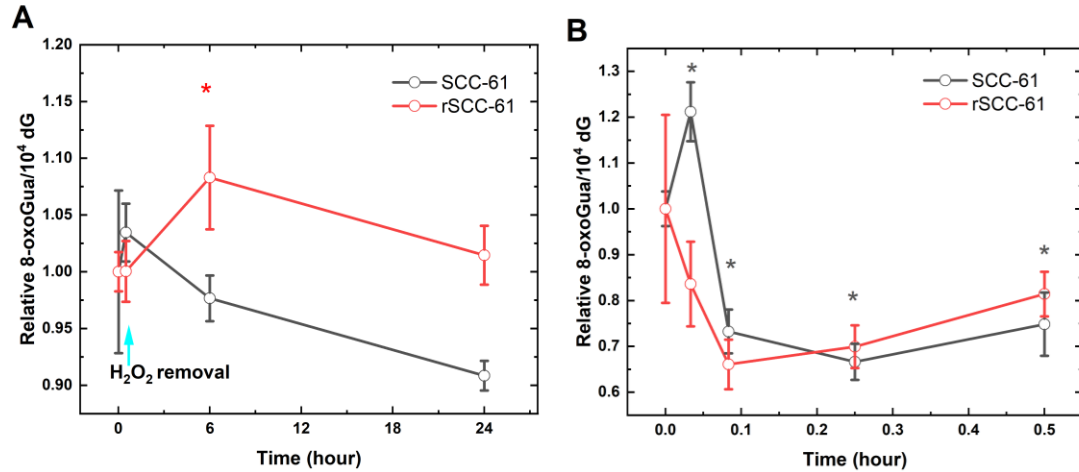


Figure IV.2: Quantification of 8-oxoGua in SCC-61 and rSCC-61 upon exposure to H₂O₂ via the SS-nanopore assay. A. illustrates SS-nanopore quantification of relative 8-oxoGua levels in SCC-61 and rSCC-61 upon exposure to 4mM H₂O₂ for 30 minutes after which the oxidant was removed (blue arrow), and cells were left to recover for 24 hours. B. illustrates SS-nanopore quantification of relative 8-oxoGua levels in SCC-61 and rSCC-61 upon exposure to 4mM H₂O₂ for designated durations ranging from 2-30 minutes. Filled squares represent the mean ratio $\pm$ standard deviation calculated from measurements conducted on a single SS-nanopore device for each time point. Gray and red asterisks represent p -values < 0.05 for SCC-61 and rSCC-61, respectively.

Table IV.1: Pairwise comparisons of 8-oxoGua levels obtained at different time points after H₂O₂ treatment and quantified by SS-nanopores			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
SCC-61			
Untreated-30 min	-0.786	4	0.476
Untreated-360 min	0.544	4	0.616
Untreated-1440 min	2.18	2.13	0.153
rSCC-61			
Untreated-30 min	-0.0165	4	0.988
Untreated-360 min	-2.95	4	0.042
Untreated-1440 min	-0.808	4	0.464
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

Based on our prior measurements on MCF7 cells in Chapter III, we expected to observe a general accumulation of 8-oxoGua upon H₂O₂ exposure. One possible factor in our results could have been cell death; when more cells die, the surviving cells could be biased towards a less damaged phenotype. However, our selection of H₂O₂ concentration based on IC₅₀ should minimize this risk. A more probable explanation for this surprising observation is kinetics. Because we have no a priori knowledge of the timeframes of ROS buildup, 8-oxoGua formation, or repair processes, we hypothesized that the total experimental time and measurement frequency could cause us to miss changes in 8-oxoGua content. In response, we exposed the cells to H₂O₂ at a shorter period of time ranging from 2-30 minutes to capture the formation of 8-oxoGua. Cells were harvested at each time point and gDNA was extracted then processed for downstream SS-nanopore measurement. All samples were measured consecutively on the same SS-nanopore device and analyzed as described above for the 24 hour experiments.

Interestingly, 8-oxoGua buildup was absent in rSCC-61 cells and no significant change in 8-oxoGua levels have been observed in response to H₂O₂ treatment (Figure IV.2, B; Table IV.2). On the other hand, we captured a slight increase in 8-oxoGua level in SCC-61 that rapidly decreased after the 2-minute time point (Figure IV.2, B; Table IV.2). Those results were confirmed by mass spectrometry analyses of the same samples. Consistent with the SS-nanopore results, no change in 8-oxoGua levels have been observed in rSCC-61 in response to H₂O₂ treatment. On the other hand, similar trends of an initial increase in 8-oxoGua level at 2 minutes that decreases during the course of the treatment have been observed in SCC-61 (Figure IV.3; Table IV.3).

Table IV.2: Pairwise comparisons of 8-oxoGua levels obtained at different time points after H₂O₂ treatment and quantified by SS-nanopores			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
SCC-61			
Untreated-2 min	-4.61	4	0.01
Untreated-5 min	7.74	4	0.001
Untreated-15 min	10.6	4	< .001
Untreated-30 min	5.89	4	0.004
rSCC-61			
Untreated-2 min	0.829	4	0.454
Untreated-5 min	2.15	4	0.098
Untreated-15 min	1.82	4	0.143
Untreated-30 min	0.885	4	0.426
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

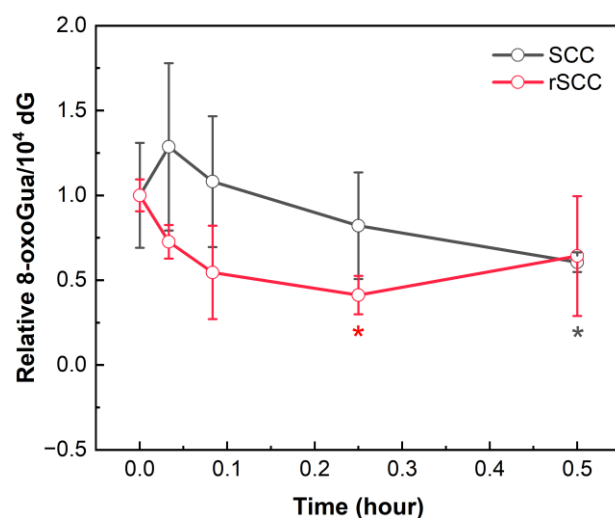


Figure IV.3: Mass spectrometry quantification of 8-oxoGua in SCC-61 and rSCC-61 upon exposure to H_2O_2 . Mass spectrometry quantification of relative 8-oxoGua levels in SCC-61 and rSCC-61 upon exposure to 4mM H_2O_2 for designated durations ranging from 2-30 minutes. Filled squares represent the mean $\pm$ standard deviation of at least 2 measurements. Gray and red asterisks represent p -values < 0.05 for SCC-61 and rSCC-61, respectively.

Table IV.3: Pairwise comparisons of 8-oxoGua levels obtained at different time points after H_2O_2 treatment and quantified by mass spectrometry			
Comparison	Independent samples t-test*		
	<i>t</i>	<i>df</i>	<i>p-value</i>
SCC-61			
Untreated-2 min	-1.01	7	0.348
Untreated-5 min	-0.328	6	0.754
Untreated-15 min	6	--	0.413
Untreated-30 min	2.51	6	0.046
rSCC-61			
Untreated-2 min	-0.628	4.19	0.562
Untreated-5 min	1	--	0.057
Untreated-15 min	7.96	6	< .001
Untreated-30 min	1.41	1.07	0.382
*Student t-test was used unless normality or equal variance assumptions were violated. In this case, Mann-Whitney or Welch's t-tests were used respectively.			

A plausible interpretation is that only the SCC-61 experiences a rise in 8-oxoGua whereas the rSCC-61 does not. A second interpretation would be that 8-oxoGua does in fact increase in the rSCC-61 as well as SCC-61 but that the rise is briefer and/or less pronounced than SCC-61 and may have happened earlier beyond the temporal window we have investigated. This could be the case for SCC-61 as well. Since the change is moderate, it is unclear if we are seeing the tail of the change or that this is the true peak. While this can be investigated further by developing methods by which to expose and extract gDNA at shorter time scales, within the limits of the present study, this data suggested a difference in the behaviors of the two cell lines.

Generally, the observed changes in 8-oxoGua levels were modest in both cell lines which might indicate a rapid and efficient repair response to regulate 8-oxoGua (Figure IV.2, B; Figure IV.3). In support of this explanation, studies have shown that OGG1, the enzyme responsible for the recognition and excision of 8-oxoGua in eukaryotic cells [21], binds 8-oxoGua containing DNA at high affinity, and the rate of binding increases exponentially within 2 minutes [22]. Studies have shown that OGG1 activity increases significantly within 15 minutes of H₂O₂ exposure [23]. Furthermore, the recognition of 8-oxoGua by OGG1 is extremely rapid. A study by Blainey et al., demonstrated that OGG1 scans the DNA for target bases at a rate of 10 Kb per second [24, 25]. Hence, it is plausible that H₂O₂ treatment triggered the expression of OGG1 which resulted in the rapid decrease in 8-oxoGua levels in both cell lines.

Another plausible explanation for the modest effects of the treatment is a lower than expected concentration of H₂O₂ in the nucleus. Because these cells are more sensitive (EC₅₀) to H₂O₂ than MCF7, we used a lower concentration to treat them, but it is also possible that the cells themselves prevent the transfer of external ROS into the nucleus due to their hyper-reductive nature. Previous studies have shown that SCC-61 and rSCC-

61 exhibit a highly reductive environment where antioxidants are highly expressed, and ROS is efficiently scavenged with the latter cell line showing more reducing properties [11]. This may explain why we couldn't observe any 8-oxoGua buildup through prolonged exposure to H_2O_2 as it was rapidly neutralized, most likely before it even reached the nucleus. To explore this, we collected some preliminary data from SCC-61 and rSCC-61 cells transduced with the nuclear H_2O_2 sensor HyPer coupled with D-amino acid oxidase enzyme (DAO) (HyPer-DAO). This way we could compare the two cell lines directly by intrinsic ROS formation within the nuclei. the results from these experiments suggested that external H_2O_2 treatment may not result in an increase in ROS buildup within the nuclei of both cell lines. Notably, intranuclear production of H_2O_2 by DAO using D-Ala as a substrate resulted in H_2O_2 accumulation in both cell lines with rSCC-61 appearing to be more resistant to ROS buildup even in the presence of high D-Ala concentration (Figure IV.4). It is worth mentioning that the 405 nm laser signal was weak during this imaging process, hence the reduction signal is not well depicted by this data. It is warranted to replicate this experiment using a better microscope to track the changes in H_2O_2 buildup in the nucleus. Also, improvements in the number of cells per well/field, selection criteria, and statistical analyses should be taken into consideration to gain conclusive results.

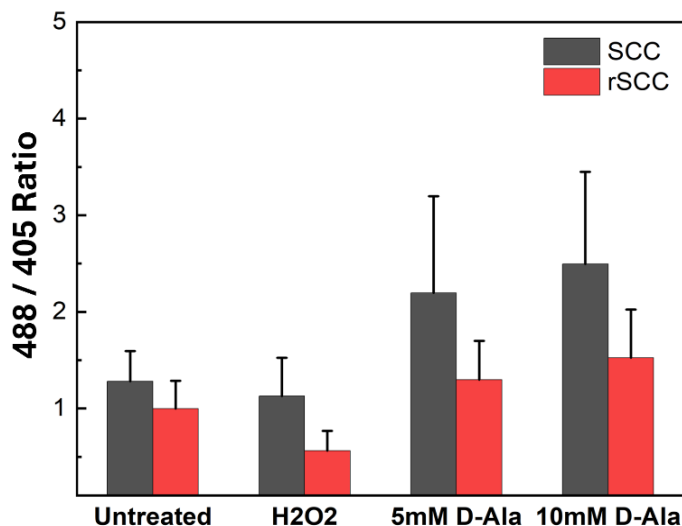


Figure IV.4: Quantitative analysis of confocal microscope images of SCC/rSCC-61 nuclei transduced with HyPer-DAO. Bar graph demonstrates HyPer-DAO fluorescence ratio (488/405 nm) in SCC-61 (gray) and rSCC-61 (red) nuclei measured in at least 3 cells for each condition. Error bars are the standard deviation of the mean ratio calculated for each image.

Another possible improvement would be bypassing the neutralization of H₂O₂ in the cytoplasm by intrinsic production of ROS directly in the nucleus by IR. Because ROS is a component of IR exposure, we can relate our findings directly to radiation effect. We performed a pilot study to quantify 8-oxoGua in SCC-61 and rSCC-61 cells post exposure to IR. Here, cells were exposed to 2 Gys of IR after which they were left to recover for 24 hours. 8-oxoGua was quantified using mass spectrometry at 0.25, 1, 3, 6, 12, and 24 hours post IR exposure. Obviously, IR exerts more damage to the cells, hence differences between the two cell lines were more pronounced. Similar to H₂O₂ treatment, 8-oxoGua accumulation appeared earlier and repair started rapidly in rSCC-61 while SCC-61 continued to accumulate 8-oxoGua for 3 hours after which a decline in 8-oxoGua level

was observed (Figure IV.5, A). at 6 hours, 8-oxoGua levels declined and were restored to baseline level by 24 hours (Figure IV.5, A). On the other hand, 8-oxoGua kept declining in SCC-61 cells up to 24 hours.

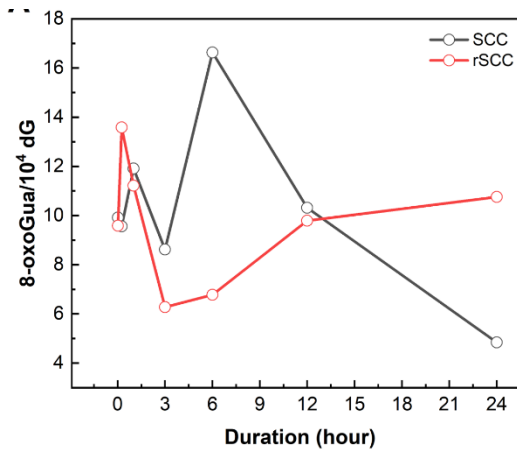


Figure IV.5: Mass spectrometry quantification of 8-oxoGua in SCC-61 and rSCC-61 cells after exposure to IR. Quantitative analysis via mass spectrometry of 8-oxoGua levels in SCC-61 and rSCC-61 cells 15 minutes, 1, 3, 6, 12, and 24 hours after exposure to 2 Gys of radiation.

IV.4 Conclusion

Radio-resistance remain a major challenge in the treatment of HNSCC patients. An in vitro preclinical model of matched HNSCC cell lines that vary in their radiosensitivity have been developed by Bansal et al. to gain a deeper understanding of the mechanisms of resistance in HNSCC. Differences in the redox state of these two cell lines have been identified and correlated with the radio-resistant phenotype. To further investigate alterations associated with response to oxidative stress in this model, we sought to monitor 8-oxoGua abundance in these cells under oxidative stress.

In this chapter, we utilized the validated SS-nanopore approach described in chapter III to quantify 8-oxoGua in HNSCC cells: the radio-sensitive SCC-61, and the radio-resistant rSCC-61. The translational capability of our approach was further validated in this chapter evidenced by our ability to capture the patterns of change in 8-oxoGua levels yielding results similar to mass spectrometry. An important aspect of using the SS-nanopore approach is convenience. It allowed us to quantify 8-oxoGua rapidly with high sensitivity. This approach required less time and sample size and is cost effective as well.

With respect to our aim regarding the identification of differences in SCC/rSCC-61 in their cellular response to oxidative stress, we obtained valuable insight on 8-oxoGua induction and repair upon oxidation with H_2O_2 and IR in the studied model. The resistant phenotype was found to combat the formation 8-oxoGua upon acute exposure to an oxidant and was found to be associated with an earlier response to repair/ decrease 8-oxoGua levels. Furthermore, we demonstrated that acute H_2O_2 exposure elicited subtle amount of stress where despite the small delay in the repair response in SCC-61, it was able to catch up with rSCC-61 and 8-oxoGua levels were significantly decreased within 15 minutes in both cell lines. Another intriguing observation was the reversal of repair regardless of H_2O_2

presence when 8-oxoGua were decreased significantly. This may suggest regulatory roles of 8-oxoGua in these cells where they need to be kept at a certain balance. Follow up experiments with chronic exposure to H_2O_2 is warranted to further confirm and better understand these findings. On the other hand, differences between the two cell lines were more obvious upon exposure to radiation. IR is known to impose higher levels of damage on the cells which are difficult to tackle. Nevertheless, we could identify similar trends to the H_2O_2 treatment where the resistant phenotype was associated with resilience and earlier repair of the damaged base.

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CHAPTER V

This unpublished chapter was composed by Sara Abu Jalboush with editorial guidance from Adam R. Hall

V.1 OVERALL DISCUSSION AND FUTURE DIRECTIONS

The work in this dissertation endeavored to emphasize and validate the utility of a novel SS-nanopore assay in the quantification of 8-oxoGua in isolated and cellular gDNA. 8-oxoGua is a significant biomarker of oxidative stress that has been implicated in the pathogenesis of cancer and in resistance to conventional cancer therapeutics such as IR. In depth study of 8-oxoGua in tumor cells by the current analytical techniques can be difficult due to artifacts, sample size requirements, high cost, and other challenges. The work presented here improved upon an established site-specific labeling technique coupled with downstream SS-nanopore approach to enable specific probing and highly sensitive quantification of 8-oxoGua in various cellular systems presenting low abundance of the lesion. This approach proved its reliability by yielding results that aligns with the golden standard method for 8-oxoGua quantification, mass spectrometry. It is also known to be cost-efficient overcoming by that many limitations of the available methods.

SS-nanopores have been gaining a lot of interest in the past years due to their remarkable sensing abilities at a single molecule level. However, its intrinsic lack of selectivity hindered its utility for translational applications. In response, our lab developed a selective SS-nanopore assay designed for selective detection of a target molecule in a heterogeneous mixture of non-specific materials. Adaptations to this assay have been employed to enable the quantification of DNA base modifications including 8-oxoGua. This is achieved by labeling their specific locations with a biotin moiety. Consequently, only tagged molecules can bind to MS which in turn allows for the selective quantification of these targets via SS-

nanopores. However, the low abundance of these lesions and their highly dynamic nature in the gDNA necessitates improved detection sensitivity, enhanced labeling efficiency followed by validation in a biological system.

In chapter II, we introduced hMS, comprised of lower net negative charge than previous versions of the proteins to the assay in order to enhance its performance for the detection of low abundance molecular biomarkers such as miRNA. We identified optimal conditions for the translocation of DNA-hMS complexes in terms of buffer pH and salt concentration that increases the sensitivity while maintaining selectivity of the assay. Our findings demonstrated that increasing the pH of the measurement buffer reduced the noise of the measurement, increased its stability, and decreased the incidence of non-translocative events. While increasing the pH decreased our detected signal, optimizing salt concentration restored the sensitivity by increasing the measured event rates while maintaining high level of selectivity. As a consequence of these optimizations, we were able to broaden the range of pore diameters feasible for the assay compared to previously reported [1]. Signal detection that is an order of magnitude higher than the controls was achieved across pore diameters from 7.5-16 nm which improved the convenience of nanopore analyses significantly. To further assess the applicability of the assay under these new conditions, we used the assay to quantify miRNA isolated from human plasma, resolving concentrations an order of magnitude better than achieved previously [2].

Another low abundance biomarker of interest in the genome of eukaryotic cells that has been difficult to probe is 8-oxoGua. In chapter III, we utilized the selective SS-nanopore assay in its improved performance to quantify 8-oxoGua in isolated and cellular gDNA. Unlike synthetic dsDNA constructs of defined lengths, gDNA may produce a signal on its own when translocating through a SS-nanopore due to its increased length, hence, fragmentation of gDNA to feasible lengths is required. Fragmentation usually results in a

pool of fragments with different lengths, and DNA length is known to affect the translocation dynamics via our SS-nanopore assay. Therefore, we first attempted to assess the effect of length on our analyses. Our results showed that measured event rates are length-dependent which necessitates rigorous assessment of DNA fragment lengths within the sample prior to the measurement. Enzymatic fragmentation used for shearing the isolated gDNA yielded inconsistent and wide distribution of fragment lengths which increased the variation in the measurements conducted across different SS-nanopore devices. That is because shorter DNA translocate without detection while longer DNA introduces clogging and instability. To illuminate these biases, we used ultrasonication to shear the DNA in subsequent analyses which proved to be consistent and yielded more accurate assessment of DNA length. Next, we optimized an established approach to tag the specific location of 8-oxoGua in the genome with biotin by introducing two enzymes with high catalytic activity and affinity to the substrate, namely, Fpg and Bsu DNA polymerase to the assay. Using these enzymes, we achieved at least 10% improvement in the labeling efficiency of 8-oxoGua relative to previous reports [3]. Taking all the aforementioned optimizations into account, we quantified global levels of 8-oxoGua in two gDNA models, the commercially available Lambda DNA and gDNA isolated from MCF-7 breast cancer cell line exposed to H₂O₂

as the source of oxidation. The validity of our measurements was confirmed using mass spectrometry which represent the golden standard method for 8-oxoGua quantification. Our approach yielded results that are in quantitative agreement with mass spectrometry analyses indicating its success and accuracy. These findings were promising as SS-nanopores may provide an alternative tool for 8-oxoGua detection and quantification that overcomes many of the limitations associated with the current available methods.

In chapter IV, we validated the translational capability of our SS-nanopore approach via implementing an analysis to investigate changes in 8-oxoGua levels under oxidative stress conditions in a matched model of HNSCC cell lines that vary in their radiosensitivity. Practically, SS-nanopore analyses were convenient, less time consuming, and required low sample sizes. The ample increase in the sensitivity of the assay and labeling of 8-oxoGua enabled us to quantify molar ratios below 10 8-oxoGua bases per 10^4 undamaged deoxyguanosine in this model which is remarkably sensitive [4]. It is common that 8-oxoGua levels measured by mass spectrometry to be reported as the ratio of 8-oxoGua per 10^5 undamaged deoxyguanosine. Ratios reported in the literature vary considerably ranging from 0.5-32 8-oxoGua/ 10^5 dG in tumor tissues of various origins (Reviewed in [4] and [6]). Furthermore, these reported ratios are context dependent. The lowest value measured by the SS-nanopore approach in this study was 8.4 8-oxoGua/ 10^5 dG which lies within the range of the reported 8-oxoGua values measured by mass spectrometry in the literature. Based on the calculated LOQ, the SS-nanopore approach may be able to measure ratios down to 3.3 8-oxoGua/ 10^5 dG for a sample that has a fragment length of 60 bp, and an initial concentration of 1 μ g. This value may vary depending on variables like fragment length and initial concentration. Given the wide variety of the reported 8-oxoGua levels in tumor tissues, further improvements in sensitivity and resolution are warranted. This could be achieved by using asymmetric salt concentrations [5] and thicker membranes for example. Limits of quantification and reduction in device-device variations could also be improved by the use of internal standards and standardization of fabrication techniques.

In addition to practical advantages, the work in this chapter provided evidence of potential mechanistic differences between radio-sensitive and radio-resistant HNSCC cell lines in their handling of 8-oxoGua adduct formation and repair upon exposure to oxidants such

as H₂O₂ and IR. This work laid the groundwork for SS-nanopore analyses to further elucidate differences in 8-oxoGua conveniently under a wide range of oxidative conditions such as chronic exposure to oxidants or therapeutics in this model and in other cancers in order to decipher the correlation between 8-oxoGua metabolism and therapeutic resistance as a future long-term goal.

V.2 References

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Curriculum Vitae

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EDUCATION

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TEACHING

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2013-2014	Jordan University of Science and Technology, Irbid, Jordan Biotechnology and Genetic Engineering Faculty Molecular Biology Lab, Teaching Assistant 15-20 Fourth-year undergraduate students from Biotechnology & Genetics and Forensic Sciences Faculties, 16 two-hour sessions per semester, Total Contact Hours: 32 hour per semester
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SCIENTIFIC AND SCHOLARLY ACTIVITIES

Research Impact:

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Poster and Presentations:

Abu Jalboush, S., Hall, A. R., (2023, March 31). Quantification of 8-Oxo-7,8-dihydroguanosine (8-OxoG) Using SS-Nanopores [Poster presentation]. Wake Forest Research Day, Wake Forest School of Medicine, Winston-Salem, NC, USA.

Abu Jalboush, S., Sethi K., Hall, A. R., (2024, March 01). Selective Enzymatic Labeling and Rapid Quantification of Chemically Induced 8-oxoGua in Isolated Genomic DNA via SS-Nanopores [Poster presentation]. Wake Forest Research Day, Wake Forest School of Medicine, Winston-Salem, NC, USA.

Abu Jalboush, S., Hall, A. R., (2024, March 25). Quantification of 8-Oxo-7,8-Dihydroguanine in Genomic and Cellular DNA Using Solid-State Nanopore Sensing [Oral presentation]. CRBM-MIG seminar series. Wake Forest Research Day, Wake Forest School of Medicine, Winston-Salem, NC, USA.

Peer Reviewed Manuscripts:

Abu Jalboush, S., Wadsworth, I. D., Sethi, K., Rogers, L. C., Hollis, T., & Hall, A. R. (2024). Improving the performance of selective solid-state nanopore sensing using a polyhistidine-tagged monovalent streptavidin. *ACS sensors*, 9(3), 1602-1610.

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Bodoor, K., Abu-Sheikha, A., Matalka, I., Alzou'bi, H., Batiha, O., Abu-Awad, A., ... & Albatayneh, K. (2018). Immunohistochemical analysis of heat shock proteins in triple negative breast cancer: HSP60 expression is a marker of poor prognosis. *Eur. J. Gynaecol. Oncol*, 39(6), 2018.

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Bodoor, K., Batiha, O., Abu-Awad, A., Al-Sarihin, K., Ziad, H., Jarun, Y., Abu-sheikha, A., **Abu Jalboush, S.,** Alibrahim, K. S. (2016). Identification of a novel WFS1 homozygous nonsense mutation in Jordanian children with Wolfram syndrome. *Meta Gene*, 9, 219-224.

Bodoor, K., **Jalboush, S. A.,** Matalka, I., Abu-Sheikha, A., Al Waqfi, R., Ebwaini, H., ... & Haddad, Y. (2016). Heat Shock Protein Association with Clinico-Pathological Characteristics of Gastric Cancer in Jordan: HSP70 is Predictive of Poor Prognosis. *Asian Pacific Journal of Cancer Prevention*, 17(8), 3927-3935.

Manuscripts Submitted for Review:

Wadsworth, I. D., Sethi, K., **Abu Jalboush, S.,** Rogers, L. C., Hollis, T., & Hall, A. R. (2024). Scalable production of monovalent streptavidin with high yield and purity. [Submitted for Review]

Ongoing Research:

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the Solid-State Nanopore Assay as a Tool to Quantify 8-oxo-7,8 Dihydroguanine in Isolated and Cellular Genomic DNA. [In Preparation]

Abu Jalboush, S., Sethi, K., Shukla, K., Underwood, E., Cox, A. O., Forshaw, T., Macnamara, L., Smitherman, P., Giauque, C., Furdui, C. M., Hall, A. R., Identification of Differences in 8-oxo-7,8 Dihydroguanine Formation and Repair in a Matched Model of Head and Neck Cancer Cell Lines with Varied Radiosensitivity. [In Preparation]

Book Chapters:

Hall AR, **Jalboush SA**, Wadsworth ID, Amin Elaguech M, Sethi K, Quantitative detection of cancer nucleic acid biomarkers with a selective solid-state nanopore assay. In: Rasooly A, Ossandon M, Prickril B eds. Emerging Technologies for Cancer Detection and Diagnosis. Boca Raton, FL: CRC Press; 2024. [In press]

Laboratory Skills:

- Nucleic acid extraction
- Gel electrophoresis
- Cell culture
- Cell transfection/transduction
- Stem cell renewal assays
- Cell viability assays
- PCR, and qPCR
- Exosomes and miRNA
- Drug combination assays
- Protein expression and purification
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