

Assessment by Ames Test and Comet Assay of Toxicity Potential of Polymer used to Develop a Field Capable Rapid Detection Device to Analyze Environmental Samples

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ABSTRACT

Current methods of detecting food-borne pathogens take days to produce and confirm results. There is a need for a field-capable rapid-detection device, and such a device is being developed in a collaborative effort including FDA, MIT and other partners. In this study the materials developed for conductive and surface area character of the biosensor are assessed for potential cytotoxicity. The device is comprised of melt-spun polypropylene coupons which have been coated with Polymerized 3,4-Ethylenedioxythiophene and 3-Thiopheneethanol (P(EDOT-co-3TE)). The copolymer was deposited on the surface of the microfibers via oxidative Chemical Vapor Deposition (oCVD). The process creates a conductive conformal layer that is 100-300nm thick. Although there have been previous tests assessing cell viability after exposure of this copolymer, no long range effects were studied. The Ames Test and Comet Assay have been used in this study to examine the mutagenic and cytotoxic potential of the deposited copolymer. The assays conducted in this study provide evidence that the copolymer is non-toxic to humans.

Keywords: biosensor, cytotoxicity, P(EDOT-co-3TE)

1 INTRODUCTION

Employing nanomaterial may provide the potential to drastically decrease the time it takes to carry out and confirm food pathogen assays. Utilizing a rapid detection device could prevent outbreaks by confirming the presence of a pathogen prior to distribution or ingestion. Designing a real-time biosensor device to detect pathogens greatly decreases the chance of an outbreak, and if an outbreak still occurs, this device could confirm the cause of the outbreak. Since the benefits of such a device are clear, a determination of possible toxicity effects created from its use should be explored to prevent unintended consequences of its use.

Many foodborne pathogens are spread from surface contact, which makes environmental swabs an important part of Hazard Analysis Critical Control Plans (HACCP) at most food manufacturing companies. Over one thousand

environmental swabs are used each year at just one large facility of a major United States food processor. [1] There are many food companies in the US alone that could significantly benefit from a real-time device that would not only provide rapid, but highly sensitive results.

A prototype device has previously been generated that employed polypropylene microfibers coated with polypyrrole and functionalized with antibodies for *Escherichia coli* O157:H7.[2] The conductivity of the functionalized membrane was measured over time and could detect as few as 0-9 CFU per ml. [2]

Desirable characteristics of such a device for detection of bacteria include being durable with high surface area microfiber material. Larger surface area allows greater chance of attachment of biomolecules, which means this biosensor would have better sensitivity.[3-5] The microfibers employed in this biosensor are melt spun polypropylene and are coated on both sides with a very thin layer of conducting polymer, created from the monomers 3,4-ethylenedioxythiophene (EDOT) and 3-thiopheneethanol (3TE) via oxidative Chemical Vapor Deposition (oCVD). In this process, iron(III) chloride is heated in a vacuum to allow deposition on the fibers. This is followed by heating the monomer mixture in a vacuum allowing single molecules to be polymerized from the iron(III) chloride deposited earlier. [3;4]

After deposition, the samples were exposed to an acid rinse dopant exchange, in this study it was sulfuric acid rinse, then methanol, both of which are used to remove excess iron(III) chloride or monomers, which increases the conductivity and improves sensitivity.[3;4;6] Previous studies looked at cell viability of PEDOT which was doped using phosphate buffered saline or polystyrene sulfonate. The viability studies were carried out on PEDOT created by electrodeposition, not oCVD. None of these studies demonstrated significant change in viability. However, there are more subtle long range effects which need to be considered that do not overtly express as loss of viability but may have consequences via long-term exposure. [7-10]

By determining the toxicity potential of the chemicals in this study, it can be decided whether the polymerized copolymer coating is safe for use or whether another composition might be created that would have less risk.

There have been no published *in vitro* toxicity studies on the chemicals used for nano-layer oCVD deposition nor on the copolymer P(EDOT-co-3TE). A tenet of nanomaterials is that they often take on diverse characteristic(s) peculiar to the nano state. It is prudent therefore to examine toxicity potential of such materials. If current chemical composition of the coating was determined to be cytotoxic or environmentally hazardous, other options to reduce cytotoxic effect could be explored. Among possible options would be modifying the EDOT to 3TE ratio, using 3-Thiophene Acetic Acid and polypyrrole [5] or other conductive copolymers.

The Ames Test determines mutagenic potential of each test chemical by exposing them to mutated strains of *Salmonella typhimurium* that need histidine to survive. *Salmonella* grows on minimal media and has a trace amount of histidine in a top agar. For metabolic activation, it is necessary to add pooled rat liver S9 fraction that is mixed with co-factors or a phosphate buffer to the top agar as well. [6;11-16]. Research on numerous known mutagens has demonstrated that they will have a higher count of colony forming units (CFU) than non mutagenic chemicals tested with a strain.[16] If the percentage of S9 fraction in the mix is too low, there is no significant difference in the negative and positive controls.[17] If the test chemical has the same heavy growth using various concentrations as the control mutagen, then the test chemical is considered to be mutagenic, and potentially carcinogenic

The strain used in this study was TA1535 but other strains that are known to be tested in this assay include TA1537, TA1538, and TA98. Each has a different type of mutation. When TA1535 undergoes a reverse mutation, a base-pair substitution has occurred. [11] By exposing the mutated strain to a known mutagen, there is a high amount of CFU since many reverse mutations occur. Without the mutagen, there are fewer colonies due to the randomness of a mutation. A test chemical is considered a mutagen when the mutagenic frequency is above 2.0. This number is determined by dividing the CFU of the test chemical by the mean of the CFU for the untreated sample. [11-17] By comparing the controls to the test chemicals, it can be determined whether they are potentially harmful.

The Comet Assay employs single cell gel electrophoresis. As a small amount of cells that are suspended in agarose gel become lysed, by one of two methods, the DNA unwinds, and then is subjected to gel electrophoresis. [18] Once these cells are stained with either SYBR Gold or SYBR Green, the comet-tail shape due to migration of small fragments of DNA from the cell damage becomes apparent in epifluorescence microscopy. The extent of DNA damage can then be scored. Alkaline reagents are used for lysis because it is suggested that this is the more sensitive treatment method allowing detection of single and double strand breaks. [18;19]The larger the comet tail, the more DNA damage has occurred. Little or no change in cells after test chemical exposure, indicates the chemical has a very low probability of toxicity.

Numerous articles have discussed the Comet Assay, over 7,400 on PubMed alone. This emphasizes its reliability and justifies its use. The cell lines tested in this study are FRhK, HEK 293t, HepG2, BHK-21, MDCK, Vero, and V9 cells. These cell types were chosen to provide diverse specie and tissue type examination. Testing different cell lines derived from a variety of tissue types aids in determining the potential for genotoxic affects of chemical exposure to different organs. [20]. A portion of the BHK-21 cell had copolymer electroporation to assure that the copolymer had been introduced internally to cells.

For comprehensive Comet Assay analysis, software has been developed to measure the comet tail and cell size and provide statistical analysis of such data collected.. However, other literature has shown that the toxicity can be statistically analyzed by viewing the slides using an epifluorescence microscope. This is done by counting the number of healthy looking cells and comparing them to the number of damaged ones. It is very clear when a cell is highly damaged, due to the large comet tail. [21-23] The high percentage of damaged cells indicates a great risk for toxicity. Viewing the comets without the software gives similar results and is a fraction of the cost.

After performing both toxicity assays, EDOT and 3TE show some potential of toxicity, but the deposited copolymer shows a much lower potential. Based on current results, P(EDOT-co-3TE) copolymer has been shown to be safe for use in environmental sampling analysis.

2 RESULTS

2.1 Comet Assay

In this study the Comet Assay had been performed with various cell lines: FRhK, BHK-21, BHK-21 electroporated with copolymer, MDCK, V79, HepG2, Vero, 293t. Analysis has been done by counting the number of damaged cells and comparing it to the number of healthy cells. By viewing the cells under an epifluorescence microscope, the approximate amount of damage is easily viewed , due to the width and length of the comet tail. (Fig. 1) Overall, it is clear that the P(EDOT-co-3TE) treated cells cause much less damage to the cells than the monomers

Sample Treatment	Total Number of Cells	% of unhealthy cells
Untreated Cells	207	28%
Hydrogen peroxide	223	100%
EDOT	206	95%
3TE	224	50%
Copolymer	230	27%

EDOT and 3TE (Table 1).

Table 1: Percentage of unhealthy FRhK cells post chemical treatment.

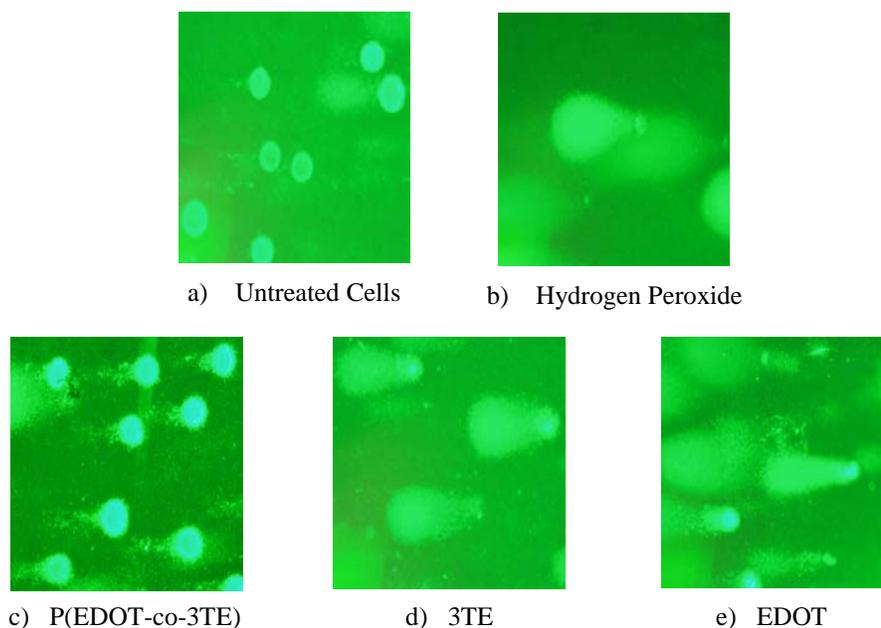


Figure 1: (a) FRhK cells, (b) after exposure to hydrogen peroxide, (e) EDOT, (d) 3TE, and (c) P(EDOT-co-3TE) stained with SYBR Green.

2.2 Ames Test

All treatments were done in triplicate. The chemicals with a mutagenic frequency above 1.6 or well below 1.0 are considered highly mutagenic. The positive control, sodium azide, was 2.6. The 3TE had the least amount of growth, which indicates it could actually be highly toxic since fewer colonies grew with this treatment. The copolymer had a very similar number of colony growth to the control, giving the mutagenic frequency of 0.92.

Chemical Added	Average CFU/plate	Mutagenic Frequency
Sodium azide	130	2.6
None (only <i>Salmonella</i>)	50	n/a
Copolymer from glass slide	46	0.92
EDOT	43	0.86
3TE	28	0.56
Mutagenic Frequency is measured by dividing the negative control CFUs (just <i>Salmonella</i>) with the other test chemicals and known mutagen.		

Table 2: The Mutagenic Frequency of Chemicals using the Ames Test with *Salmonella* TA1535.

3 DISCUSSION

In this study, the Ames test using strain TA1535 showed that the mutagenic frequency of the copolymer was close to 1.0, indicating there is little to no mutagenic potential. EDOT and 3TE have a lower mutagenic frequency than the copolymer, which shows strain TA1535 did not grow well after exposure to these chemicals. In the case of 3TE it is low enough that Ames concludes it may indicate toxicity potential.

The Comet Assay shows there is about the same percentage (27-28%) of damaged cells in the copolymer treatment as the untreated, which indicates there is no cytotoxic potential. The pictures in Figure 1 show that the P(EDOT-co-3TE) has a much smaller comet tail than the monomers 3TE and EDOT. Previous viability studies also suggest there is no toxicity, however only short-term viability tests were done. The Ames Test and Comet Assay provide evidence that the deposited copolymer is nontoxic to humans. These results indicate using the P(EDOT-co-3TE) coating would not be harmful to humans.

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