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Detection of Non-nitro Compounds by Amplified Fluorescence Polymer (AFP): An Opportunity for Breath-Based Disease Diagnosis

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Amplified fluorescence polymers (AFP) are a set of unique polymers known for their ability to detect trace nitro explosives. The prior knowledge in the AFP field indicates that the functional group variation on the polymer backbone is responsible for the selectivity of an analyte. The mechanism of analyte detection is believed that only compounds with nitro functional groups are detected by AFP. Usually, AFP functional groups varied to detect nitro compounds and the non-nitro compound detection and the mechanism of the AFP were not completely understood. In this work, the AFP polymer was kept constant and studied with 136 analytes with different functional groups for analyzing few non-nitro compounds. Among the 136 compounds analyzed, about fourteen have been detected by AFP. It was observed that most of the fourteen compounds were non-nitro compounds. The mechanism proposed originally for nitro compounds and associated hypothesises the existence of a parking space on the polymer backbone. Present study suggested that the possibility of only nitro compounds interacting with AFP due to the three-dimensional shape of the analyte as the detrimental factor. The discovery of non-nitro compound detection by AFP opens up the use of AFP for gas-phase disease volatile organic compound detection. Future studies of functional group variation on the AFP backbone in relation to the analyte detection could provide insights into the relation of analyte detection by AFP and the parameters to optimize for obtaining the selectivity and specificity.

Keywords: Amplified fluorescent polymer, Volatile organic compounds, Gas phases detection, Quenching fluorescence.

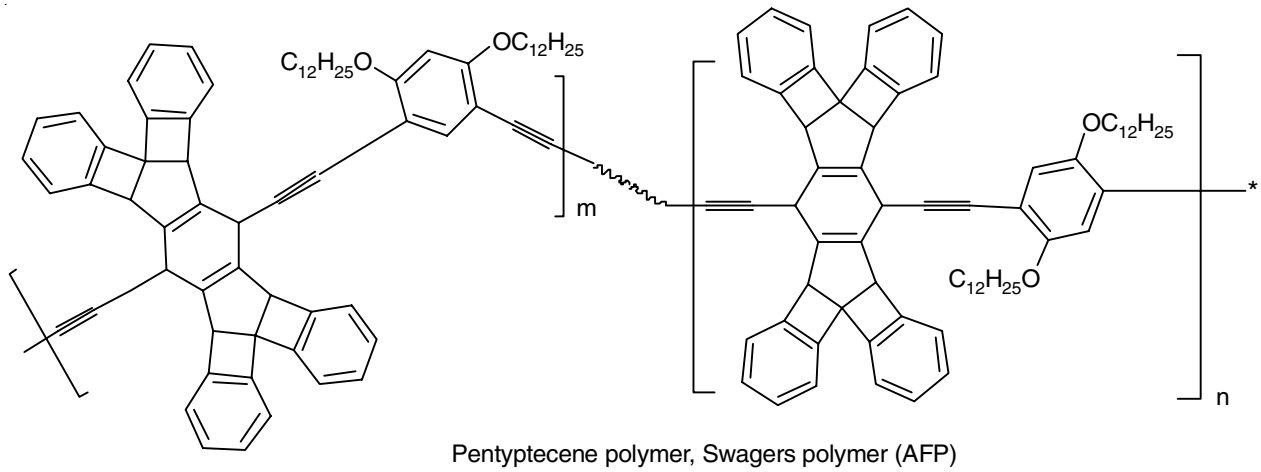
INTRODUCTION

Volatile metabolites detection from breath has become a new form of disease diagnosis [1-5]. The common approaches for the disease diagnosis are (i) detection of analyte/analytes using GC-MS signature [6-10], and (ii) pattern recognition using nanoarrays [6,11-13].

Another approach, which has received minimal attention on the disease diagnosis is the amplified fluorescence polymer (AFP) [14]. This method was proven to be incredibly useful for trace explosive detection of low vapour pressure molecules like TNT, RDX, *etc.* [15-18]. The amperometric [19-21] and resistive methods [22-24] of detection of analytes was far from perfect and GC-MS based portable devices have been minimally used in the field [10,14,25]. The amplified fluorescence polymer (AFP) is a conjugated fluorescence polymer, which elicits a block response to an analyte by turning off fluorescence from all the conjugated monomers [15,16,18,26].

The biomedical research applications of AFP are minimal even though the potential is immense [27-32]. The problems associated with AFP are the tunability and understanding of the mechanism of action. The nitro group sensitive polymers took few years for perfection before the actual field product "FIDO" was introduced in the market [33,34]. The electronic interaction of nitro groups with AFP polymer results in quenching of fluorescence, but all the AFP polymers do not interact in the same way with the nitro compound basis set. This enabled researchers to explore different substitutions on polymers as the TNT sensitive AFP (Fig. 1).

Though this polymer gives quenching upon exposure to almost all volatiles, slower recovery of fluorescence happens with compounds with nitro functionality. For every analyte or group of analytes, this interaction of AFP and its functionality variation is needed and considered to be a laborious process (Fig. 2). This could be the difference between trace explosive detection *vs.* breath VOCs. Breath VOC's concentration in air



Pentyptecene polymer, Swagers polymer (AFP)

Fig. 1. Pentyptecne polymer also called Swager's polymer and is representative of amplified fluorescent polymer (AFP)

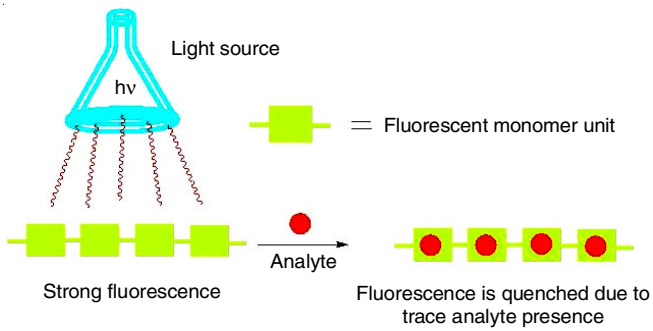


Fig. 2. Principle of amplified fluorescence polymer with trace analyte detection

is much higher than typical explosives present on surfaces. The breath-based volatile organic compounds (VOCs) are known to be present in the ppm range. In contrast, explosives in the air are of the order of part per billion (ppb) and part per trillion (ppt), which clearly implying that the challenge of detection is unique and the procedure of testing and developing reason why AFP ased biomedical breath-based VOC sensors are non-existent.

Haick *et al.* [1,35,36] have pioneered a breath-based VOC detection. His group has initially used GC-MS based lung cancer VOCs detection [37,38] and later moved on to pattern-based VOCs detection using nanoarrays [39-41]. Haick's sensors are being used for disease detection in many countries and the reported selectivity and specificity ranges of the same ranges from 70-80% with real patient samples. Developing countries like India, with a 1.35 billion population need solutions, which can enable large population screening with minimal infrastructure. Volatile organic compounds (VOCs) detection using AFP is a realistic possibility and could offer a complementary platform for nanoarrays. This study is further to improve our understanding of amplified fluorescence polymer (AFP) behaviour for the volatile organic compounds detection. The trace explosives are typically present in ppt/ppb level, whereas the VOCs are at ppm level and thus the mechanism of AFP understanding is critical. This publication discusses a new line of research wherein we have evidence for the detection of non-nitro VOC by amplified fluorescence polymers.

EXPERIMENTAL

The analytical grade solvents acetone and chloroform were purchased from E. Merck while the 13 types of volatile organic compounds (VOCs) were procured from Sigma-Aldrich. All chemicals, solvents & AFP procured are used as such without further purification. Swagger's polymer, also known as amplified fluorescence polymer (AFP) was synthesized as reported [30]. The Eppendorf tubes were procured from Tarsons, while the glass sensing elements were purchased from local glass blowers. Beagle-Z device is meant for research purpose only and obtained from Bigtec Labs, Bangalore, India.

Beagle-Z device and sensing element: The functional performance device construction, optics, electronics, validation housing, software, relay firmware, validation with gold standard, *etc.*, were performed according to the validation methods [42]. The Beagle-Z houses, a fluorescence setup comprising of optical block, collimating lens, a diode array, an appropriate wavelength LED excitation and emission filters, relay electronics, a glass sensing element, an air inlet orifice, air outlet, *etc.* and the excitation source and emission source were perpendicular to each other. The firmware translates optical signal to electric signal was monitored by the onboard software. The AFP detection methodology uses a reduction in signal due to an analyte and hence the initial signal was set about 75% of the maximum possible (4.8 V). The reduction in signal with respect to time upon analyte exposure was monitored and in the onboard chip, which can be transferred to a computer for the rate of decay analysis.

The sensing element is a borosilicate glass tube comprising of 6 mm outer diameter and four inner diameters and about 4 cm in length. The exterior and interior of the borosilicate are finely polished for smoothness and for effective coating of the AFP. The AFP at 0.5 g/mL concentration is typically dissolved in chloroform or acetone and 20 µL of the polymer solutions were placed at one end of the borosilicate tube. The liquid was moved back and forth in the glass sensing elements three times to form a thin film in the inner wall of the sensing element. After the movement, a 2 mm Hg vacuum was applied for 2-3 s and the liquid contents of the tube were removed using the

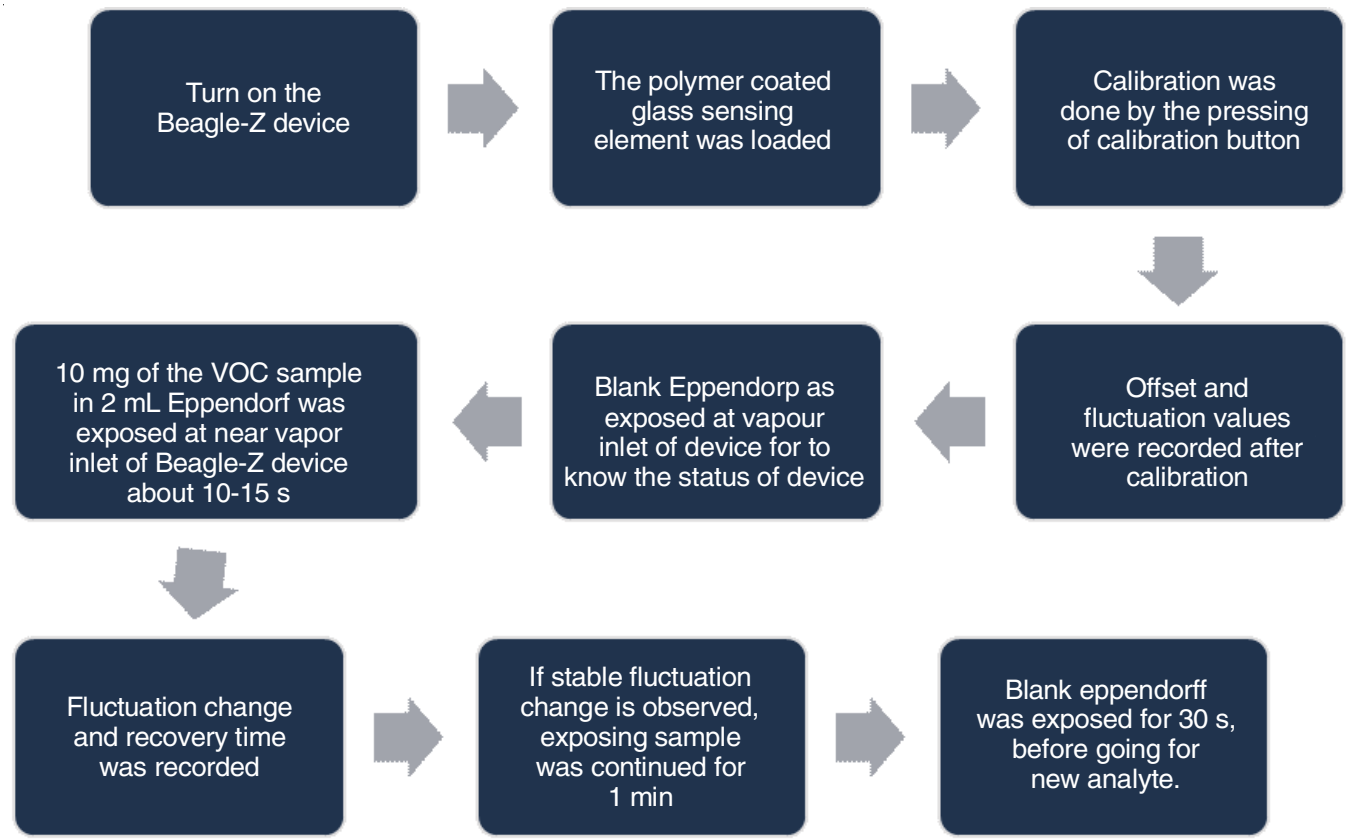


Fig. 3. Beagle-Z device and the replicable borosilicate glass tube. The device is reported in literature and variations of the same are used for trace explosive detection

vacuum pump. The sensing element was placed inside the beagle-Z device and the total fluorescence from the tube was observed through the offset voltage of the device and the total voltage corresponds to the total fluorescence signal generated from the AFP tube, the reduction in fluorescence signal due to analyte activity (Fig. 3). The AFP, known for its unique property of an analyte presence on the polymer backbone, results in a reduction of fluorescence from the whole polymer. As per this sensor, a fall in the total fluorescence can be correlated to the concentration of analyte.

VOC testing with Beagle Z and qualitative interpretation of false-positive & true positive: An analyte VOC of 10 mg was placed in 1.5 mL Eppendorf tube. Based on the vapour pressure of VOC and Eppendorf tube volume, the amount of VOC in head space of VOC was calculated. The exposure time is always fixed at 3 s and if the response is not observed in the stipulated time the VOC is classified as non-respondent VOC. If a particular VOC upon exposure results a dip in fluorescence, then recovery time and pattern of recovery to the base line is monitored. The time taken for the 90% recovery of signal is used as a basis for determining, if a particular VOC can be classified as true analyte or interferant (Fig. 4). As a nature, most of the solvents and commonly occurring alkaloids, *etc*. display a false positive (interferant) response with a characteristic of exposure of analyte. Wherever there is an interaction between analyte and AFP which results in delay in baseline is qualified as detected VOC. The "delay" defined here can be varied and by our experience, we set it 60 s, which

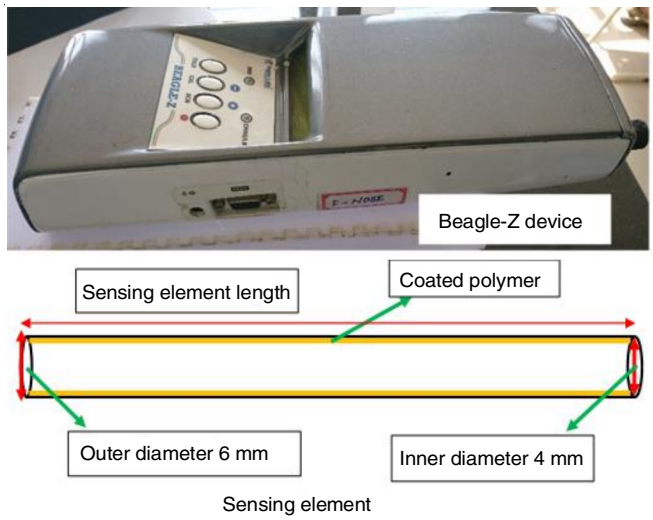


Fig. 4. Schematic of experimental procedure for detection of VOC using Beagle-Z device

is 10 times higher than false positive and there by avoids any doubt in mind about the classified analyte as false positive or true positive.

RESULTS AND DISCUSSION

Detection of nitro compounds: AFP is a unique class of polymers with commercial products based on this technology for trace nitro compounds detection. Swagger & Yang [43] has reported these polymers for TNT and another nitro compounds

detection and the mechanism is non-radiative quenching of fluorescence of AFP by nitro compounds. The non-availability of detection technologies for nitro compounds has made this a huge hit and the mechanism seems to have been well understood. Researchers observed that even though mostly AFP respond to organic nitro compounds vapours, only the series which had performed and given selective response. Incidentally, similar series were discovered for detection of RDX, PETN, *etc.*, based on AFP technology [16,17,44], the understanding of structural interaction between volatile nitro vapour and the AFP is still not very clear because of the trace nature of the analytes. The AFP based non-nitro compound detection has been a black box with marginal information available for a systematic study. The information available on AFP occasionally pointed out the same and most of the volatiles does cause a response, but the response recovers to baseline immediately after exposure. Hence, the AFP theory suggests that specific analytes like nitro derivatives cause a dip in the fluorescence of AFP and the signal has slow recovery. The general understanding is that the non-specific/non-nitro analytes may give a response but instantaneous recovery as shown in Fig. 5. Prospective, a trace explosive detection device can be used for VOC detection without any modification.

Analysis of VOCs: Three nitro compounds *i.e.* 5-nitroquinoline, 1,3-dinitro-4-chlorobenzene and 4-nitrophenol, are detected by AFP and the recovery in the response is over 3 min conforming that the VOC response is 'true positive'. Two ammonium compounds *viz.* ceric ammonium nitrate and trimethyl phenyl ammonium tribromide, are present in the basis set and both of them are detected. It is assumed that quaternary ammonium salts are considered to be inert VOC and surprisingly detection can be attributed to the electron-deficient nature of the ammonium ions. Two naphthalene compounds were detected, but this is not across the trend as few naphthalene derivatives are undetected. The naphthalene itself did not cause the characteristic VOC true response and suggested that the structural motif is probably the reason. The phenyl group is the most preferred with diverse functional groups. The nature of the functional group could not be ascertained uniformly, but in general, the amino and phenolic groups are preferred. This is reflected in the detection of *o*-phenyl phenol, *p*-phenyl diamine and 4-tertbutyl catechol. Two nitro groups comprising phenyl compounds, namely 1,3-dinitro-4-chlorobenzene and 2-bramo-4-nitroacetophenone, are detected by AFP. In the metal containing compound list, tin(II)chloride dihydrate and ceric ammonium nitrate are detected and both are considered to be good reducing agents and have the ability to form an interaction with a double bond. The list of selected compounds contains four halogen containing compounds but among the screened VOC, only a few were detected clearly suggesting the presence of halogen is not a parameter, but the structural fit is the contributor. It is important to state observation of 3-iodo propionic acid, which recovered in the signal indicating that it is a false positive VOC and not true positive. In the segment, aliphatic compounds hydroxylamine hydrochloride and *N*-bromo succinamide were detected and this result needs further study. It is easy to ascertain that amine and bromo functionalities have a role in the detection. The inability of other molecules on the 136-odd basis to have similar responses is ratification of that functional groups, in general, have no role to play in analyte detection. The iodo functionality, which is historically known to be a quencher of fluorescence, is also not responsive for AFP.

Detection of non-nitro functional group compounds: About 136 chemicals covering functional groups like alcohol, ketone, iodo, bromo, nitro, acid, amide, *etc.* were selected for the study (Table-1). The screening is done at two levels *viz.* qualitative research and a semi-quantitative study. In the qualitative study, an analyte was exposed to the AFP tube in the Beagle-Z device and the compounds, which produced a characteristic true positive response are identified. Then in semi-quantitative screening, a set molar concentration of analyte vapour is used and a characteristic response was followed by maintaining recovery of signal post exposure. The time needed for recovery is chosen as a result and a typical nitro derivative takes anywhere between a few minutes to few tens of minutes to recover the fluorescence signal after quenching and this was used as the basis for identifying analytes that respond to AFP. When an analyte is exposed to AFP, a decrease in fluorescence

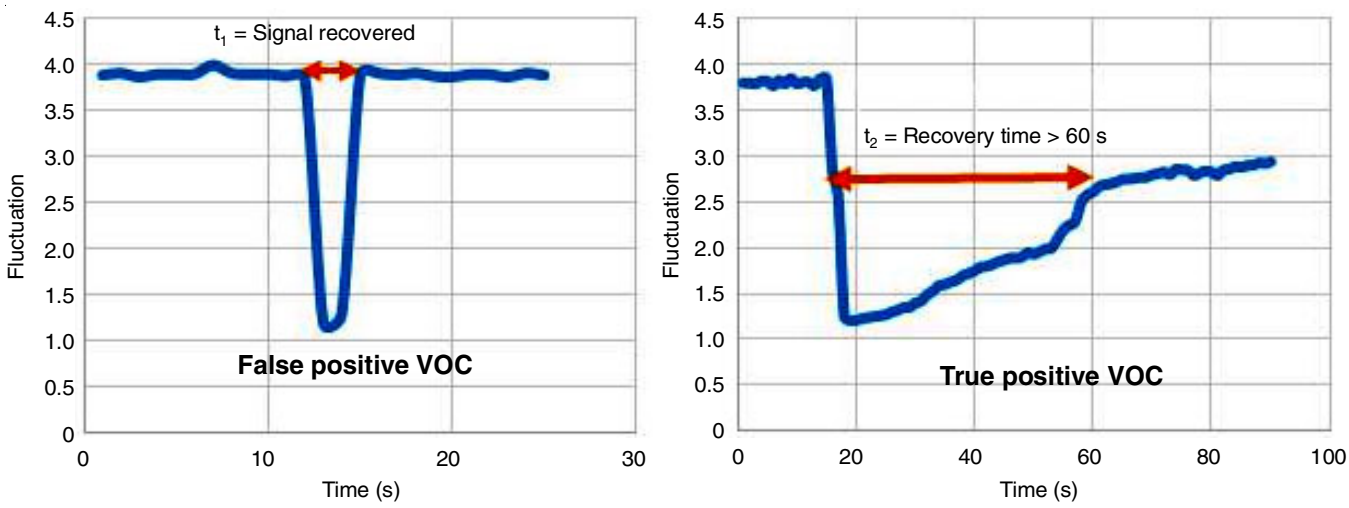


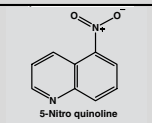
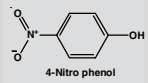
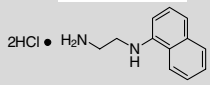
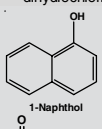
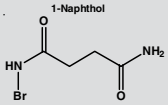
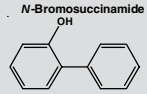
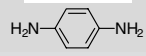
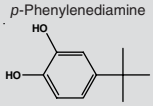
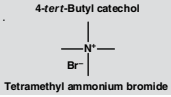
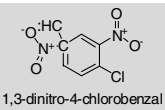
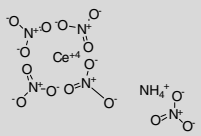
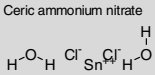
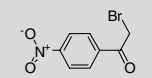
Fig. 5. Schematic of the false positive *vs.* true positive

TABLE-1

Functional group	Organic (aromatic/aliphatic) or inorganic	
Alcohol (-O.H.)	Aromatic alcohols	Aliphatic
	1. 4-*tert*-Butylcatechol	1. 1-Octadecanol
	2. 1-Naphthalene methanol	
	3. 4-Nitrophenol	
	4. 1-Naphthaol	
	5. *o*-Phenyl phenol	
	6. Inositol	
	7. Thymol	
	8. Bromophenol blue	
Ketone (-C=O)	1. Peptone	
	2. Mycological peptone	
	3. Anthraquinone	
	4. Benzophenone	
	5. 5-Amino-2,3-dihydro-1,4-phthalazinedione	
	6. 2-Bromo-4'-nitroacetophenone	
Iodo (I)	1. Iodobenzene	
	2. 1,4-Diiodobenzene	
Chloro (Cl)	Organic chlorides	Inorganic chlorides
	1. Thiamine hydrochloride	1. Cobalt(II) chloride
	2. 1,3-*bis*(2,4,6-Trimethylphenyl)imidazolium chloride	2. Tin(II)chloride dihydrate
	3. 1,3-Dinitro-4-chlorobenzene	
	4. Tetraethylammonium chloride	
	5. 4-(Phenylazo)benzoyl chloride	
	6. L-Cysteine hydrochloride, monohydrate	
	7. Cysteamine hydrochloride	
	8. Glucosamine hydrochloride	
	9. *p*-Toluene sulphonyl chloride	
Bromo (Br)	Organic bromides	Inorganic bromides
	1. Hexadecyltrimethylammonium bromide	1. Copper bromide
	2. Tetramethylammonium bromide	2. Potassium bromide
Nitro ($-NO_2$)/NO/ Nitrate (NO_3)	Organic NO_2 functional compounds	Inorganic (NO, $NO_{3,}$)
	1. 3-Nitro-1,8-naphthalic anhydride	1. Ammonium nitrate
	2. 5-Nitro quinoline	2. Ceric ammonium nitrate
	3. 4-Nitrophenol	3. Sodium nitroprusside
	4. Nitro blue tetrazolium chloride	
	5. 1,3-Dinitro-4-chlorobenzene	
	6. 5,5-Dithiobis (2-nitrobenzoic acid)	
	7. 3,5-Dinitrosalicylic acid	
	8. 2-Bromo-4'-nitroacetophenone	
Amine ($-NH_2$)	Aromatic amines	Aliphatic amines
	1. *N*-(1-Naphthyl)ethylenediamine	1. Hydroxylamine
	2. *N*-Phenyl-2-naphthylamine	2. 11-Amino decanoic acid
	3. *p*-Phenylenediamine	3. Tris (2-aminoethyl) amine
	4. *N*-Pheny-1-naphthylamine	
	5. 2-Amino anthracene	
	6. 5-Amino-2,3-dihydro-1,4-phthalazinedine	
	7. 3,3-Diaminobenzidine	
Amide/Sulfonamide ($R-CONH_2$/$RSOONH_2$)	Aromatic/aliphatic amides	Sulfonamide
	1. N-Hydroxy succinimide	1. Sulfanilamide
	2. N-Bromosuccinamide	
	3. Acetamide	
	4. Acrylamide	
	5. 5-Acetylsalicylamide	
Acid (-COOH)	1. 2,4-Dichloro phenyl acetic acid	1. 3-Idopropanoic acid
	2. Phosphomolybdic acid hydrate	2. Phosphomolybdicacid hydrate
	3. (+/-) α-Lipoicacid	3. DL-Isocitric acid
	4. 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate	4. 4-Aminobutyric acid
	5. Sulphanilic acid	5. Adipic acid
	6. 2,4-Dichloro phenyl acetic acid	6. Caproic acid
	7. 4-Aminobenzoic acid	7. L-Ascorbic acid
	8. 2,3-Dihydroxybenzoic acid	8. 6-Aminocaproic acid
	9. 2,5-Dibromobenzoic acid	9. Citric acid monohydrate
	10. 1-Diazo-2-naphthol-4-sulphonic acid	10. Diethylenetriaminepentaacetic acid
	11. 5,5-Dithiobis(2-nitrobenzoic acid)	11. 11-Amino decanoic acid
	12. *trans*-3-Indoleacrylic acid	
	13. 4-Hydroxy benzoic acid	
	14. 3,5-Dinitrosalicylic acid	
	15. 2-Bromophenylacetic acid	
Ester (-COOR)	1. 4-Methyl-2-pyridine boronic acid *N*-phenyldiethanolamine ester	

signal (quenching), should be associated with at least a minute's delay in recovery to baseline signal and is considered as a true positive response. Among the 136-odd analytes tested, about 14 analytes have given a true positive response and some of them are nitro compounds but the majority are non-nitro compounds. Table-2 shows the 14 selected compounds which give true positive responses with AFP. The significant time delay in recovery is predicted from nitro compounds, but some non-nitro compounds have caused similar time delay. This has never been noted in the literature. The AFP domain patent also vaguely mentions the possibility of detecting drug metabolites, narcotics, *etc.* Even in that case, AFP needs to be structurally tuned to detect the chosen analyte. The structural diversity offers a possibility of quantitative structure-activity relationship (QSAR) type models of medicinal chemistry, but the relation of functional group substitution on fluorescent polymer *versus* analyte functional groups needs study with multiple AFP, with the same polymer backbone and different substitutions.

TABLE-2
14 SELECTED VOC COMPOUNDS THAT GIVE TRUE-POSITIVE RESPONSES WITH AFP

Name of analyte VOC and structure	m.w. (g/mol)	Offset (Vol) (n = 3)	Fluorescence (Vol) Base signal (n = 3)	Quenching (Vol) (n = 3)
 5-Nitro quinoline	174.16	1.20	3.98 ± 0.01	2.84 ± 0.12
 4-Nitro phenol	139.11	1.30	3.97 ± 0.02	2.74 ± 0.17
 N-(1-naphthyl)ethylenediamine dihydrochloride	259.17	1.20	4.02 ± 0.03	2.77 ± 0.11
 1-Naphthol	144.17	1.10	3.99 ± 0.10	2.63 ± 0.31
 *N*-Bromosuccinamide	177.98	0.97	4.03 ± 0.03	3.27 ± 0.21
 *o*-Phenyl phenol	170.21	1.32	3.97 ± 0.02	3.07 ± 0.19
 *p*-Phenylenediamine	108.14	1.37	3.97 ± 0.32	2.99 ± 0.11
 4-*tert*-Butyl catechol	166.21	1.32	4.03 ± 0.03	3.32 ± 0.17
 Tetramethyl ammonium bromide	154.06	1.39	3.96 ± 0.04	2.87 ± 0.12
 1,3-dinitro-4-chlorobenzal	215.57	1.42	3.99 ± 002	2.93 ± 0.22
H_2N-OH Hydroxylamine	33.03	1.11	4.00 ± 0.02	2.79 ± 0.17
 Ceric ammonium nitrate	548.26	1.07	3.97 ± 0.12	2.98 ± 012
 Tin(II)chloride dihydrate	225.63	1.21	4.02 ± 0.03	3.17 ± 0.17
 2-Bromo-4'-nitroacetophenone	244.04	1.32	3.98 ± 0.02	3.21 ± 0.22

Possibility of the existence of parking space for analytes on the AFP backbone: The AFP relies on the principle of quenching due to non-radiative transfer caused due to overlap of the energy level of the analyte with polymer and the said analyte can quench the fluorescence of the polymer. From a sensitivity perspective, this offers additional 100-fold sensitivity but leaves ambiguity on the results obtained in present study. The traditional belief is that interference of analyte's LUMO with HOMO of polymer can explain quenching, but not recovery time is taken. In case of Swager's AFP, it was hypothesized that the longer recovery time is attributed to the interaction between nitro group energy levels with AFP's energy levels. If this was indeed a standalone reason, this research is the observation of non-nitro compound interaction is unexplained. Even if the traditional quenching of functional group (chloro, bromo, *etc.*) containing molecules is ignored, few amines, alcohol, acids, aliphatic and single ring aromatics have quenched the fluorescence of AFP and had slower recovery post exposure, a contrary to traditional understanding. Table-1 shows compounds with functional groups like bromo, chloro, iodo, naphthalene ring, biphenyl, *etc.* which have been traditionally known to quench and have not altered AFP's fluorescence. These observations bring to the conclusion that electronic interactions may not be the sole reason for quenching in AFP. The results suggest that the functional group might be playing a minor role, if at all any and it is due to more structural and other three dimensions interaction. The three-dimensional structure of these molecules could be fitting in the "parking space" of polymer and the extent of fit may be the reason for quenching. The appropriateness of 3D structures determines the quality of fits like lock and key and could be leading to a delay in recovery of the signal. The hydrophobic interactions between polymer and analyte and polymer and functional group, *etc.*, are post-binding parameters. If the appropriate fit of analyte on the AFP does not happen and then these parameters have no bearing on the extent of quenching and recovery.

Mechanism: Discussion of a position on the amplified fluorescence polymers (AFP) backbone for analyte was first proposed by Swager *et al.* [15,45]. A series of conjugated polymers under study and found that only few polymers had an ability to bind to TNT and have a maximum retention time of few minutes. It is also reported that the side chains in AFP were introduced to improve solubility and enhance thin film-forming ability. The polyacetylene backbone had the ability for TNT and other nitro derivatives but lacked sensitivity for RDX. Swager *et al.* [31,46] reported that having silicon in the AFP backbone encourages specific interaction with RDX, which is conceptually different from TNT, DNT, *etc.*, based on the ring structure. These studies suggested that the NO_2 group is not solely responsible for interaction and quenching whereas RDX is not detected by TNT sensitive AFP. At the same time, even the ring system in the analyte is also not solely responsible for analyte detection either because and then, we would have had almost the same affinity for all AFP from all nitroaromatics. Rochat & Swager [46] did also point that parking space for analyte detection is a must. Metabolically, human breath contains compounds which are hydrocarbons (linear or branched), oxygenated compounds and aromatics. If the diagnosis of a health condition using AFP should be a reality, understanding the interaction of these small molecules with AFP is important. Then using a single AFP, one could identify a significant amount of non-nitro compounds that interact, quench fluorescence, have slower recovery (longer in gas phases). Out of 130-odd compounds tested, only 14 respond to AFP suggests the specificity and selectivity. The HUMO, LUMO overlap seems to be not restricted by the presence of aromatic ring and significant variation in functional group in the detected compounds suggests that it is not functional group specific. The three-dimensional volume of the detected 14 molecules suggested that the structure, size based and fit of the molecule on the polymer is probably the single most factor. This research paves way for developing volatile organic compounds (VOCs) specific AFP by viewing the sensitivity originated from three-dimensional size, shape and volume of the VOCs. The concentrations at which this study operated is close to the actual breath VOCs concentration and not at the level of trace explosives. Hence, the literature correlation is non-existent and the higher concentration of VOCs could have led to this behaviour and the associated mechanism.

Choice of polymers and volatile organic compounds (VOCs): The polymer used in the study is amplified fluorescence polymers (AFP) and generally used in handheld sensor, which is capable of detecting single femtogram masses of vapour-phase nitroaromatic explosives also known as FIDO. The VOCs used for study as a basis set comprised of 130 volatiles, of which 14 are detected by the AFP. The VOCs studied and detected by AFP are given in Table-2.

Conclusion

It is observed that amplified fluorescence polymers (AFP), which is known for its ability to detect nitro compounds, also detects non-nitro compounds. Among the non-nitro compounds detected, apart from known quenchers like bromo, chloro, iodo, aromatics, there were few aliphatic compounds as well. This non-traditional quencher functional group interaction with AFP opens a new way to look at exciting state-ground state interactions of fluorescence polymers and analytes and definitely has far-reaching implications on the use of AFP for disease-specific VOC detection from breath.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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