Non-Covalent Functionalisation of Carbon Nanotube & Graphene Films

Introduction

In **?@sec-fabrication**, methods of fabricating carbon nanotube and graphene devices were discussed, and in **?@sec-pristine-characteristics**, it was demonstrated that these devices are highly sensitive to environmental changes in an aqueous environment. However, for specific sensing, the devices require biochemical functionalisation. The sensing signal is picked up by attached receptors, while the transistors act as transducers for the received signal. Receptors previously used with carbon nanotube and graphene devices include aptamers [@Khan2021; @Nguyen2021; @Shkodra2021; @Nekrasov2021; @Mishyn2022; @Cassie2023] and a range of proteins [@Lerner2014; @Ahn2020; @Tong2020; @Wang2020], including animal odorant receptors [@Goldsmith2011; @Lee2018; @Murugathas2019a; @Murugathas2020; @Moon2020; @Yoo2022]. A common approach to attaching receptors to the transducer involves the use of a linker molecule to tether the receptor to the transducer. Verifying that this linker molecule is bridging between the transducer and the receptor element is important for a complete understanding of the behaviour of these sensors. This verification involves providing evidence for effective attachment of linker molecule to the transducing device channel, then showing successful tethering of odorant receptors and other biomolecules to the attached linker molecule.

This chapter therefore takes some time exploring the following selection of available linker molecules for specific biosensing: 1-Pyrenebutanoic Acid N-hydroxysuccinimide Ester (PBASE), 1-Pyrenebutyric Acid (PBA), Pyrene-PEG-NTA (PPN) and Pyrene-PEG-Biotin (PPB). The mechanisms underlying functionalisation with each linker are described. A literature review and analysis techniques including Raman spectroscopy, fluorescence microscopy and electrical characterisation are used to understand the impact of various experimental parameters on the functionalisation process. Electrical characterisation of linker attachment to the transducer was also performed to act as a comparison tool when performing functionalisation with insect odorant receptors. Numerous obstacles to successful functionalisation are identified and discussed, including PBASE hydrolysis, linker coverage, non-specific attachment, photoresist contamination, channel hydrophobicity and the coffee-ring effect. Approaches to overcome these obstacles were identified, tested and the results characterised. This process provided assurance that successful attachment of linker molecule to the carbon nanotube network or graphene region could be achieved.

Attachment of 1-Pyrenebutanoic Acid N-Hydroxysuccinimide Ester

Pi-stacking

Pi-stacking or $\pi - \pi$ interaction is a specific type of non-covalent bonding which occurs due to dispersion forces between unsaturated polycyclic molecules [@Perez2015]. It has been argued

that this label is unhelpfully specific and a misrepresentation of what can be simply classed as a type of Van der Waals bonding [@Martinez2012; @Perez2015]. However, as the use of the term is widespread in the literature, it is also used here for the sake of clarity. A wide range of linker molecules with aromatic moieties, such as pyrene, have been used for modification of polycyclic carbon nanotubes and graphene via pi-stacking [@Hermanson2013-16; @Perez2015; @Zhou2019; @Mishyn2022]. Pyrene-based pi-stacking underlies all the functionalisation processes used in this thesis. Figure **??** demonstrates how a pyrene-based linker molecule can be used to attach a receptor element to a thin-film transducer. The linker element attaches to the biomolecule via covalent bonding with a nucleophilic functional group; linker attachment can occur via biomolecule aminos, carboxyls, hydroxyls, thiols/sulfhydryls, phenols, imidazoles and so on [@Fruh2011; @Dung2018].

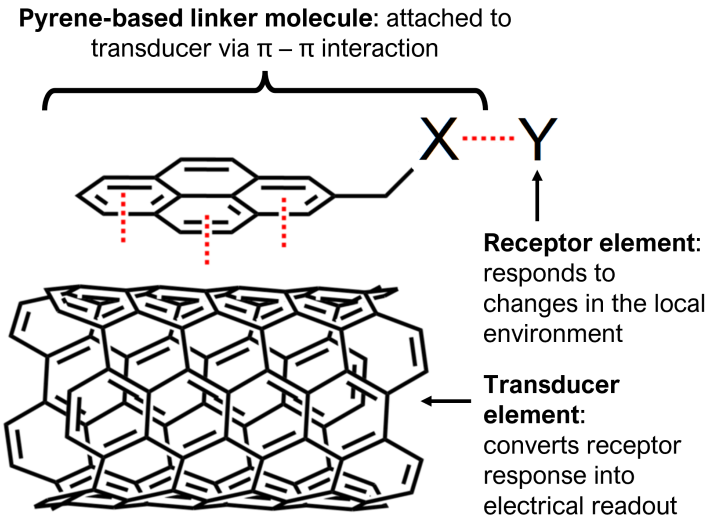

Figure 1: Attachment of pyrene-based linker molecule pyrene-X and receptor Y to a carbon nanotube, representing the transducer element of a field-effect transistor. Figure adapted from [@Carbonnanotube], used under the CC BY-SA 4.0 license.

Comparing Attachment Methods

1-pyrenebutanoic acid N-hydroxysuccinimide ester (also known as 1-pyrenebutyric acid N-hydroxysuccinimide ester and 1-pyrenebutanoic acid succinimidyl ester, and by the acronyms PBASE, PBSE, PyBASE, PASE, PYSE, PSE, Pyr-NHS and PANHS) is a pyrene-based linker molecule commonly used for tethering biomolecules to the carbon rings of graphene and carbon nanotubes. A ball-and-stick model of the PBASE molecule is shown in Figure **??**. The pyrene moiety, highlighted blue in Figure **??**, non-covalently bonds to the carbon rings of the transducer. Previous modelling has shown that when PBASE attaches to graphene, it may take on one of two different locally stable conformations (one straight, one bent) [@Oishi2022]. The N-hydroxysuccinimide (NHS) ester group, found within the structure highlighted red in

Figure **??**, can undergo a nucleophilic substitution reaction with primary amines attached to biomolecules, tethering them with an amide or imide bond [@Chen2001; @Hermanson2013-16; @Hermanson2013-3; @Shkodra2021; @Mishyn2022].

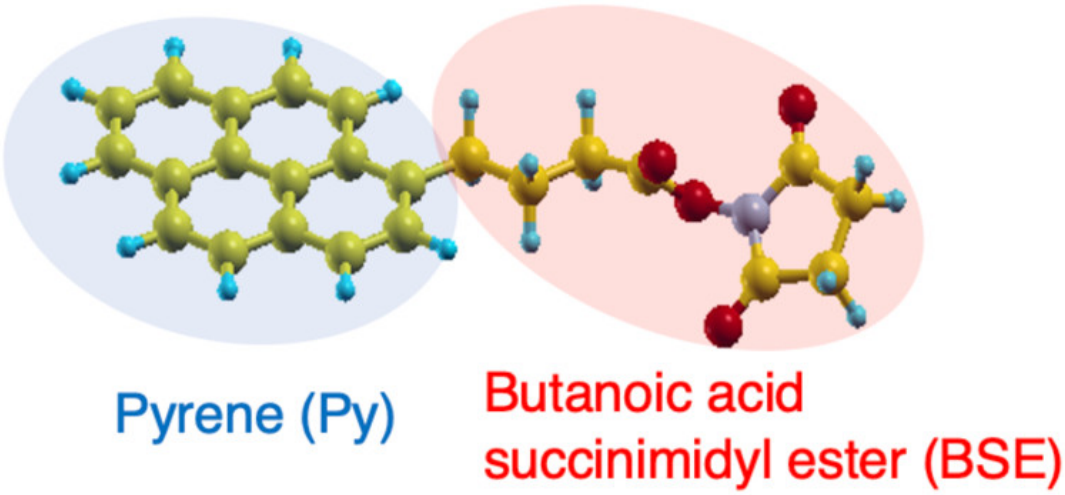

Figure 2: Structure of 1-pyrenebutanoic acid N-hydroxysuccinimide ester (PBASE) visualised in XCrySDen software [@Kokalj1999]. Blue corresponds to hydrogen, yellow to carbon, red to oxygen and grey to nitrogen. The NHS ester is the ring structure on the right hand side of the figure. Figure reproduced from [@Oishi2022], used under the CC BY-NC-ND 4.0 license.

The non-covalent functionalisation of proteins onto a single-walled carbon nanotube using PBASE was first reported by Chen *et al.* in 2001 [@Chen2001]. Two successful methods for protein functionalisation and immobilisation were reported, with the only differences being the solvent used to dissolve the PBASE powder (dimethylformamide, methanol) and the final concentration of the resulting solutions (6 mM, 1 mM respectively). PBASE powder appears to dissolve poorly in methanol at higher concentrations, which might explain the use of different concentrations of PBASE with each solvent. An extensive comparison of methods used in the literature for PBASE functionalisation of carbon nanotube and graphene devices with aptamers and proteins is given in **?@tbl-pbase-functionalisation**. Several listed works directly cite Chen *et al.* when discussing functionalisation with PBASE [@Cella2010; @Ohno2010; @Zheng2016]. The other works listed do not explicitly reference Chen *et al.* in their methodology; however, the frequency of methods describing the use of 6 mM PBASE in dimethylformamide (DMF) and 1 mM PBASE in methanol indicate that other groups typically emulate the original process from Chen *et al.*

However, it is also apparent from **?@tbl-pbase-functionalisation** that there is a large degree of variation in the methods used for PBASE functionalisation. Various electrical characterisation, microscopy and spectroscopy techniques have been used to demonstrate successful functionalisation. Until recently, there has been little justification provided for the selection of

variables used in the functionalisation procedure (*e.g.* length of time submerged in solvent containing PBASE), despite the widespread use of this process in the literature [@Hinnemo2017; @Zhen2018; @Wang2020]. Furthermore, a detailed investigation of PBASE functionalisation process variables has only been undertaken for graphene-based devices [@Zhen2018; @Hao2020; @Wang2020; @Mishyn2022]. This is surprising, given that multiple sources make an explicit link between sensitivity of functionalised devices and the density of surface functionalisation with PBASE [@White2008; @Hermanson2013-3; @Chen2014].

Zhen *et al.* [@Zhen2018], Wang *et al.* [@Wang2020] and Mishyn *et al.* [@Mishyn2022] have all claimed that carefully tuning the surface concentration of PBASE is required to avoid multilayer coverage of the graphene surface, as this negatively impacts sensing. Mishyn *et al.* [@Mishyn2022] used cyclic voltammetry to demonstrate that less receptor attachment to the graphene surface occurs when multiple layers of PBASE are present. However, none of these groups have presented analyte sensing results from their functionalised graphene devices. In contrast, Hao *et al.* [@Hao2020] found that maximising the PBASE surface coverage of a channel resulted in more sensitive aptameric sensing. The inconsistency in these recent findings mean more work is needed to understand the PBASE functionalisation process and achieve optimal biosensor sensitivity.

It may also be the case that a specific functionalisation process is required for optimal sensitivity with the use of a specific type of receptor.

Once fastened to a bioreceptor via an amide or imide bond, the attachment to the linker molecule is not easily broken. However, prior to use in functionalisation processes, the NHS ester may react with any water present. This ester hydrolysis converts PBASE to its corresponding carboxylic acid, 1-pyrenebutyric acid (PBA), leaving it unavailable to react further with amine groups [@Hermanson2013-3; @Hermanson2013-5; @Mishyn2022]. If the amine group functionalisation is performed at close to neutral pH, within a $\sim$ 1 hour period, and with a high concentration of bioreceptor present, competing hydrolysis should not have a significantly adverse impact on the functionalisation process [@Hermanson2013-3]. If PBASE is exposed to water during storage, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) can be used to restore the NHS ester and enable the substitution reaction to take place (see Section **??**).

Examining 1-Pyrenebutanoic Acid N-Hydroxysuccinimide Ester Purity

I purchased PBASE from two suppliers, Sigma-Aldrich and Setareh Biotech. Sigma-Aldrich listed DMF and methanol as suitable solvents for dissolving PBASE, alongside chloroform and dimethyl sulfoxide (DMSO). Setareh Biotech indicated methanol can be used for dissolving PBASE. The two suppliers had conflicting information for suitable storage of PBASE, where Sigma recommended room temperature storage, while Setareh Biotech recommended storage of -5 to -30 °C alongside protection from light and moisture. Nuclear magnetic resonance (NMR) spectroscopy was used to verify the purity of PBASE from various suppliers. As water

can react with PBASE to form unwanted byproducts, it appears that protection from moisture is particularly important. A particular emphasis was placed on detecting water presence in the received samples, considering the long travel time of the PBASE with uncertain storage conditions.

Figure **??** compares the shapes of hydrogen NMR spectra of PBASE from each supplier when dissolved in deuterated DMSO, alongside a blank deuterated DMSO spectrum. Both PBASE samples possessed characteristic chemical shift features between $2.1 - 2.2$ ppm, $2.8 - 2.9$ ppm, and $3.4 - 3.5$ ppm. These chemical shifts roughly correspond to those seen in previous NMR spectra for PBASE [@NMR2]. The feature at 2.5 ppm represents the deuterated DMSO solvent, while the single peak between $3.3 - 3.4$ ppm represents the water present in the sample. By comparing the area of these peaks, a rough estimate of the amount of water originally present in the PBASE sample can be obtained.

The H_2O:DMSO ratio is 1:7 in the blank spectrum, but $\sim$ 1:3 in the provided samples, possibly indicating the introduction of water to the PBASE during production or storage. However, DMSO is strongly hygroscopic and slight differences in DMSO storage time, as well as differences in humidity during sample preparation, may have had a significant impact on this result [@Lebel1962]. Other impurities are also seen on both PBASE spectra, though their small size indicates they make up only a small percentage of each sample. Strack *et al.* [@Strack2013] recommend leaving frozen PBASE at room temperature for 15 minutes before exposing it to air to prevent condensation near the PBASE, as this can cause unnecessary H_2O contamination.

Electrical Characterisation

The transfer characteristics of the carbon nanotube or graphene transistor are often used to verify successful functionalisation and make a statement about the effects of chemical modification. However, this verification usually does not account for the effects of exposing the transistor channel to solvent. Figure **??** (a) and Figure **??** (b) show that by exposing a steam-deposited carbon nanotube network channel to solvents commonly used in PBASE functionalisation processes (**?@tbl-pbase-functionalisation**), such as methanol (MeOH) or dimethyl sulfoxide (DMSO), a significant negative shift in channel threshold voltage occurs even after thorough rinsing with deionised water. It appears that the carbon nanotubes have adsorped solvent which persists even after thoroughly rinsing the device. From the shape of the change in the transfer curve, it seems the residual polar solvent molecules capacitively gate the channel [@Artyukhin2006; @Heller2008]. Besteman *et al.* reported observing a similar effect from prolonged exposure of a single carbon nanotube to dimethylformamide (DMF) [@Besteman2003].

Capacitive gating results from dense coverage of adsorped molecules on the carbon nanotube surface which have a low permittivity relative to the surrounding electrolyte [@Heller2008]. The relative permittivity of MeOH and DMSO are $\sim$ 33 [@Mohsen-Nia2010] and $\sim$ 47 [@Hunger2010] respectively, which are both much lower than the relative permittivity of PBS,

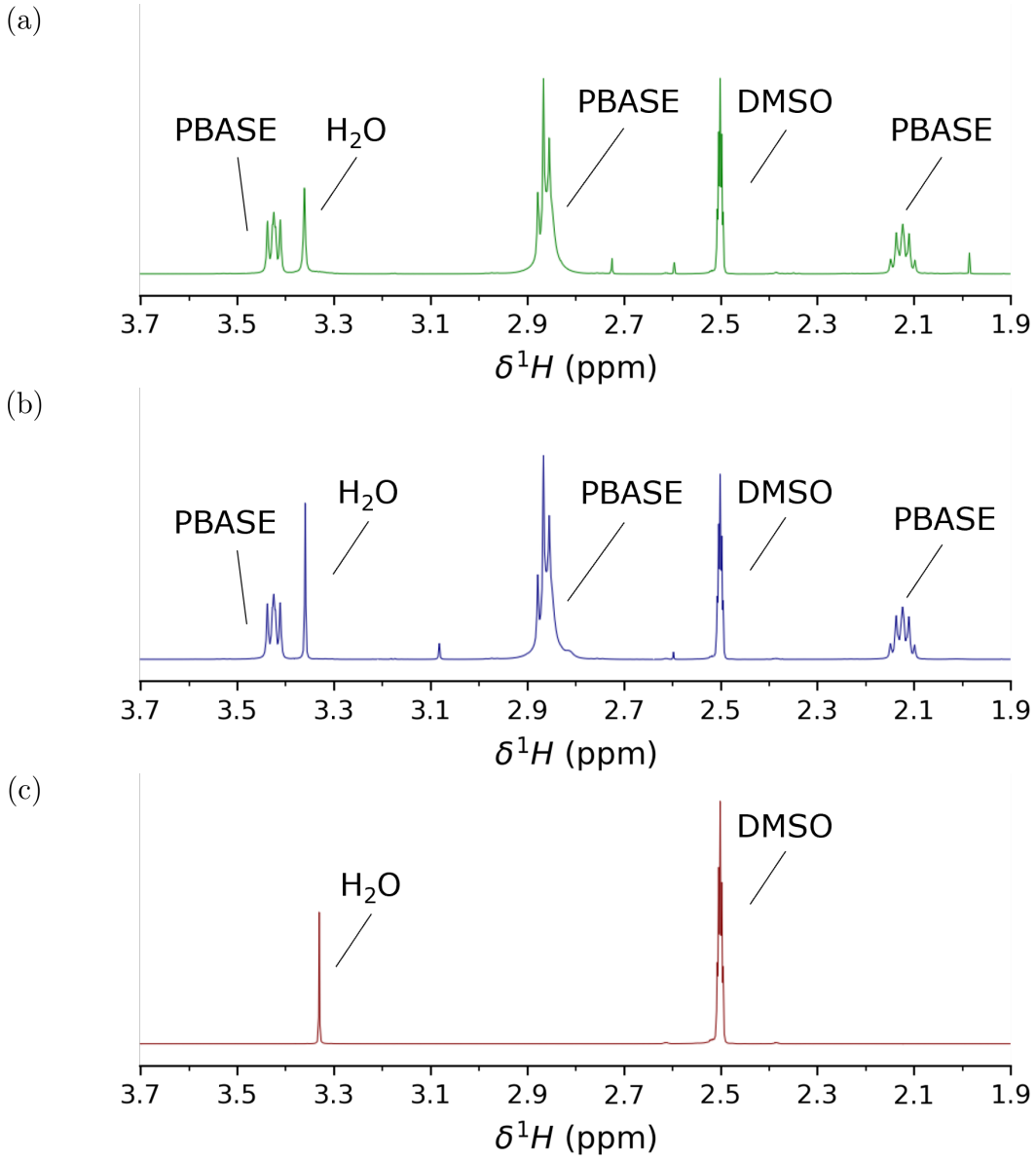

Figure 3: ^{1}H Nuclear Magnetic Resonance (NMR) spectra in the alkyl region. Spectra were taken using DMSO-d_6 as the NMR solvent. (a) and (b) show NMR spectrum for commercially purchased PBASE, from Sigma-Aldrich and Setareh Biotech respectively, while (c) shows the blank spectrum taken with only DMSO-d_6 present. Spectra were taken by Jennie Ramirez-Garcia, School of Chemical and Physical Sciences, Te Herenga Waka — Victoria University of Wellington. Unlabelled peaks correspond to sample impurities.

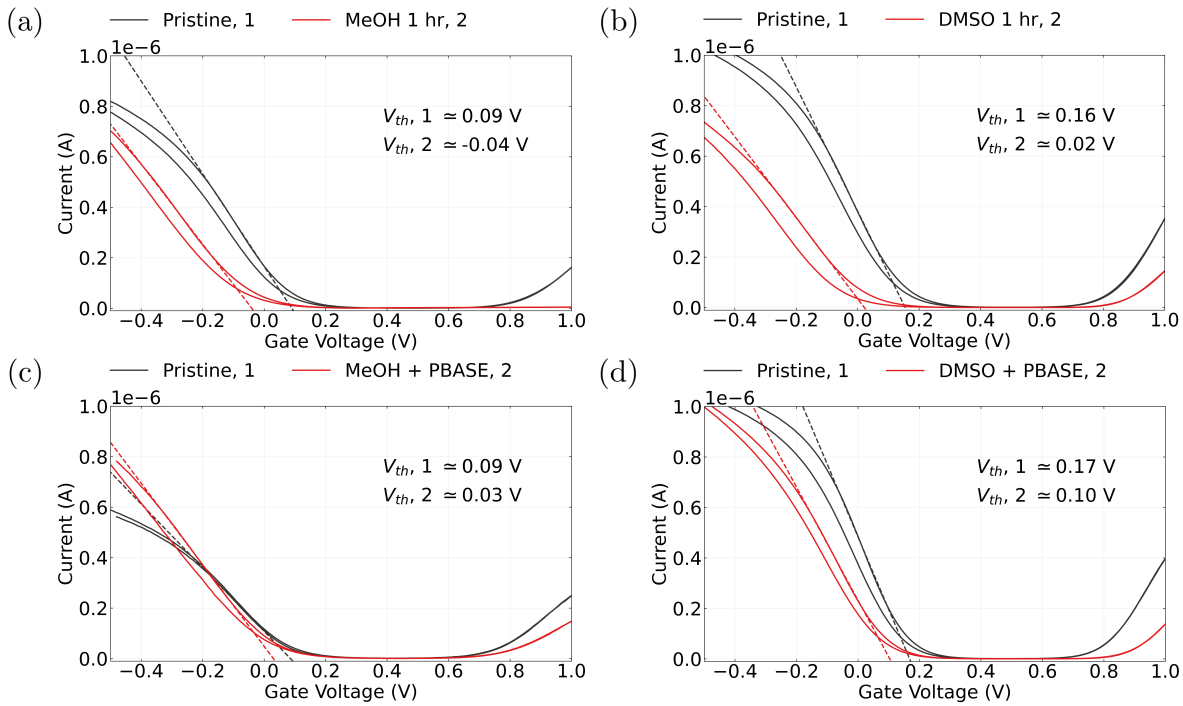

Figure 4: The transfer characteristics of carbon nanotube transistors (V_{ds} = 100 mV) before and after being submerged in MeOH (a) or DMSO (b) for one hour and subsequently rinsed with deionised water. The change in characteristics of similar transistor channels after being submerged in these same solvents containing 1 mM PBASE for one hour and then rinsed are shown in (c) and (d) respectively. The threshold voltage for the forward sweep of each transfer characteristic curve is also shown.

~ 80 [@Shkodra2021]. From Figure **??** (a) and Figure **??** (b), the threshold shift values found resulting from exposure to each solvent, taking the average of forward and reverse sweep values from a single device, were $\Delta V = -0.15 \pm 0.02$ V and $\Delta V = -0.15 \pm 0.01$ V for MeOH and DMSO respectively. The average threshold shift value for a second device exposed to MeOH was $\Delta V = -0.16 \pm 0.02$ V, indicating that this threshold shift result is reproducible.

Using the same characterisation process as in this work, Murugathas *et al.* [@Murugathas2019a] showed that the attachment of PBASE to a solvent-deposited carbon nanotube network had little effect on channel threshold voltage, implying the presence of PBASE had not significantly influenced channel gating. Here, an average threshold voltage shift of -0.06 ± 0.04 V is seen after PBASE functionalisation in MeOH and -0.06 ± 0.01 V after PBASE functionalisation in DMSO. These threshold voltage shifts are small compared to the shift values from solvent exposure. It is possible that the attachment of PBASE prevents solvent adsorption, and has a small negative gating effect on the channel. Alternatively, while the solvent negatively gates the channel, resulting in a threshold shift of -0.15 V, the PBASE may be counteracting this by positively gating the channel, resulting in a threshold shift of $+0.09$ V. Murugathas *et al.* also observed a slight increase in channel conductance after PBASE functionalisation [@Murugathas2019a]. Figure **??** also shows a slight increase in channel conductance post-functionalisation in both Figure **??** (c) and Figure **??** (d) relative to the solvent-only case in Figure **??** (a) and Figure **??** (b). This result implies that the presence of PBASE molecules increases channel mobility and therefore conductance [@Heller2008].

The absorption of organic solvent by the carbon nanotube network has unknown but potentially negative implications for biosensor functionalisation. Use of organic solvents in functionalisation can also attack the encapsulation layer of devices, promoting gate current leakage. In light of these issues, recent work has begun to explore alternative aqueous-based methods for functionalisation of biosensors [@Khan2021]. The discussion here also illustrates the importance of considering each substance used when characterising a device to verify if functionalisation has worked. The qualitative presence of a change in characteristics (or lack of one) over the full process is not sufficient to make conclusive remarks regarding successful functionalisation. A full set of control measurements are required for an understanding of electronic changes occurring during the functionalisation process, in the manner of Besteman *et al.* [@Besteman2003].

Attachment of 1-Pyrenebutyric Acid

Comparing Attachment Methods

Another linker molecule that can be used to attach receptor molecules to a carbon nanotube or graphene channel is 1-pyrenebutyric acid (PBA or PyBA). The pyrene group in PBA also undergoes pi-stacking with the channel surface. PBA can be reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC or EDAC) to form an *O*-acylisourea intermediate, which then reacts with an amine group on a biomolecule to form an amide or imide bond [@Sehgal1994; @Hermanson2013-4]. The water solubility of EDC means that,