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**IN-FIBER LIGHT-FLUOROPHORE INTERACTIONS
FOR SPECTROSCOPY AND PHOTOCHEMISTRY**

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Summary

Optical fibers have enabled the miniaturization of a host of optical devices and systems. The further development of photonic crystal fibers (PCFs) has broadened this miniaturization capability to fluidics. Exploiting the optofluidics offered by PCFs, in-fiber light-fluorophore interactions have been studied in this thesis for applications in fluorescence spectroscopy and the generation of photochemical reactions.

A compact optofluidic platform was first devised to facilitate these in-fiber studies. The simple application of a bend in the length of PCF created wavelength-specific core-cladding coupling that were observed as dips in the output spectrum. Variations in the refractive index of the PCF's air hole array were hence reflected as dip shifts. A performance study of the platform was next performed using this bending-induced sensitivity to refractive index changes.

Fluorospectroscopy was subsequently studied within bent lengths of PCFs, which were observed to possess heightened sensitivities in contrast to their non-bent counterparts. In order to account for light-fluorophore interactions within PCFs, a ray-tracing model was developed. Numerical results demonstrated good concurrence with experiments and were able to attribute the higher sensitivities to higher number of reflections in bent PCFs. Additionally, a ratiometric method of spectral analysis mitigated the effects of intensity fluctuations and enabled spectroscopy under a filterless regime.

Light-fluorophore interactions were further utilized in the generation of photochemical reactions. This enabled the novel demonstration of in-fiber surface functionalization through photochemical means. This method was simple and involved non-hazardous chemicals. The functionalization primarily entailed the photo-induced radicalization of a biotin-conjugated fluorophore. Intermediary products of the process further led to the radicalization of an amino acid residue in proteins adsorbed on the in-fiber surfaces. Consequently, radical-radical reactions would lead to bond formation and thus the immobilization of biotin. The subsequent introduction of fluorescent dye-

tagged streptavidin (a biotin-binding biomolecule) enabled quantification of these biomolecules' coverage density via means of in-fiber fluorospectroscopy.

The feasibility of creating bio-integrated photonic devices was next explored using the biomolecule-functionalized in-fiber surfaces. Liposomes (biomolecular constructs) loaded with fluorescent dyes were chosen for tethering within PCFs due to their likeness to dye-doped nanoparticles and ease of characterization by fluorospectroscopy. Investigation of these dye-loaded liposomes external of PCFs revealed an intriguing counterintuitive phenomenon. This was the observation of increasing fluorescence emission intensities upon photobleaching. A devised mathematical model accounted for this unique phenomenon, attributing it to a reduction in quencher concentration upon photobleaching. The original demonstration of in-fiber tethered liposomes was further substantiated by the observation of this phenomenon. Since the phenomenon only presented when fluorescent dye was present at high concentrations within liposomes, it indicated the maintenance of liposomal structural integrity post-surface attachment.

In sum, in-fiber light-fluorophore interactions were studied and exploited. This was facilitated by the developed optofluidic platform that provided infiltration-evacuation efficiency and repeatability. Ratiometry was demonstrated to enable fluorescence measurement capabilities comparable to other PCF-based techniques, but with an all-fiber configuration and just an LED source. The further devised ray-tracing model was generalized for all fluorospectroscopy using index-guiding PCFs. Next, the first biomolecular photo-immobilization and subsequent first successful tethering and probing of liposomes within PCFs were demonstrated. The first report of photobleaching-induced dequenching was also made, with its observation substantiating the intactness of the in-fiber tethered liposomes.

Future work would explore the application of the developed bio-integrated photonic device. This would comprise utilization of the current established understanding of light-fluorophore interactions for implanted waveguides as well as further investigate the potential of liposomes for use in photonics.

List of Abbreviations

15:0 PC	1,2-dipentadecanoyl-sn-glycero-3-phosphocholine
AttoSA	Atto 488-conjugated streptavidin
bLipo(CF)	Biotinylated-liposomes encapsulating carboxyfluorescein
B4F	Biotin-4-fluorescein
Biotin-DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl)
BSA	Bovine serum albumin
CF	Carboxyfluorescein
DIUF water	Distilled ultra-filtrated water
dcPCF	Defected core photonic crystal fiber
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
Fl	Fluorescein
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
LED	Light-emitting diode
Lipo(CF)	Liposomes encapsulating carboxyfluorescein
MOF	Microstructured optical fiber
pbB4F	Photobleached biotin-4-fluorescein
PBG	Photonic bandgap
PBS	Phosphate buffered saline
PCF	Photonic crystal fiber
PEG	Polyethylene glycol
RET	Resonance energy transfer
REV	Reverse-phase evaporation vesicles
SA	Streptavidin
scPCF	Solid core photonic crystal fiber
TIR	Total internal reflection
Tyr	Tyrosine residue
UV	Ultraviolet
WGM	Whispering gallery mode

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1 Introduction

1.1 Motivation and Objectives

The advent of fiber optic technology has not only revolutionized the telecommunications industry [1, 2] but also created an effective means of miniaturizing optics. This has enabled the development of fiber lasers and amplifiers [3] as well as led to a myriad of applications in other fields ranging from aerospace to medicine, encompassing techniques in sensing [4-7], imaging [7-10] and therapy [11-14]. This hype could be ascribed to the inherent advantages offered by optical fibers, specifically their (1) flexibility, compactness and durability; (2) electromagnetic interference resistance; and (3) ability to support broad bands of light [15-17].

The subsequent devise of photonic crystal fibers (PCFs) [18, 19], which are optical fibers possessing axially-running microstructured arrays, has opened further avenues for exploration. The unique means of optical guidance in PCFs has allowed for applications like high-power transmission [20, 21] and supercontinuum generation [22-24]; while the ease in incorporating doped rods into their microstructures has advanced fiber laser and amplifier technologies [25, 26]. Notably, air hole arrays in PCFs also serve the supplementary role as sample chambers or microfluidic channels. This property has not only facilitated the study of non-linear interactions of light with gases [27, 28] but also has been exploited in the field of optofluidics. This inherent ability in combining and miniaturizing fluidic and optical elements, has stimulated much research on in-fiber light-matter interactions for chemical and biochemical sensing as well as photochemical applications [29-35]. There have however been fewer reports on selectivity, which is of particular interest to studies on

interactions between biomolecules. The lackluster could primarily be attributed to the practical difficulties in immobilizing biomolecules within the microstructures of PCFs. This in turn stemmed from the absence of a commercial or even an efficient means of infiltrating and evacuating PCFs. In this regard, the following objectives would be pursued in this thesis:

1. Devise a platform capable of supporting optofluidic studies in PCFs.
2. Decipher the fundamentals of in-fiber light-matter interactions.
3. Demonstrate the in-fiber immobilization of biomolecules for selectivity.
4. Explore the further immobilization of biomolecular constructs within PCFs for potential applications in bio-integrated photonics.

1.2 Organization of Thesis

Chapter 1 begins with an overview on the domain of fiber optic and PCF technology that would highlight the motivation and indicate the objectives of this thesis. The organization of this thesis would then be listed.

Chapter 2 provides a background on the primary areas of focus – PCFs and fluorescence. The section on PCFs introduces its origin and focuses on their light guidance mechanisms and consequent in-fiber light-matter interactions. Subsequent discussion on the fundamentals of fluorescence would emphasize on the properties of absorption and emission, quantum yield, quenching and photobleaching. Current platforms used for light-fluorophore interactions would also be reviewed.

Chapter 3 presents the development and study of an optofluidic platform that would facilitate the study of light-matter interactions within PCFs. It then investigates the effects on bending in PCFs and further demonstrates the application in refractive index measurements.

Chapter 4 studies the use of bent PCFs in fluorescence spectroscopy. It details a ray-tracing model for the computation of light-fluorophore excitation and collection within PCFs and contrasts the calculated results to experimental data.

Chapter 5 investigates the feasibility of bioactive surface formation within PCFs via photochemical means. The successfully photo-immobilized biotin was verified through the further conjugation of streptavidin. The fluorescent dye-tag

on the streptavidin then enabled the quantification of the bioactive surface's coverage density using the earlier presented method of in-fiber fluorescence spectroscopy.

Chapter 6 explores the potential of combining biomolecular constructs with optics. It first studies the spectral behaviour of fluorescent dye-loaded liposomes under prolonged excitation and accounts for an observed counterintuitive phenomenon. The liposomes were next tethered within PCFs using the earlier discussed bioactive surface. Using in-fiber fluorescence spectroscopy the successful tethering of intact liposomes was verified.

Chapter 7 summarizes and draws conclusion from the preceding chapters. The contributions of this thesis would subsequently be highlighted. It also discusses future work on the optofluidic platform and potential bio-integrated photonics applications.

2

Literature Review

2.1 Photonic Crystal Fibers

2.1.1 A brief background

Optical fibers that possess micron-scaled two-dimensional features extending axially along their lengths are generally known as microstructured optical fibers (MOFs). The earliest MOF dates back to 1973, when Kaiser et al. [36, 37] reported a silica fiber comprising of a suspended-core supported by spoke-like membranes. Study of such fibers was however abandoned upon the introduction of modified chemical vapour deposition, which enabled the mass production of low loss optical fibers. It was not until 1991, when Prof Philip Russell had “a neat idea” [18, 38], that the notion truly advanced. This intriguing idea, involved the trapping of light within a hollow core encircled by a two-dimensional photonic bandgap (PBG) structure. This PBG structure comprised a periodic array of air holes – a form of photonic crystal. Following years of fashioning photonic crystal structures in optical fibers, the first silica-air photonic crystal fiber (PCF) was eventually realized in 1996 by Knight et al. [39, 40]. However, it did not possess the envisioned hollow core, instead it had a core of material consistent with the bulk silica background and operated on the principle of modified total internal reflection (TIR). This was primarily a result of technological limitations in fabrication that restricted the required air-filling fractions. The problem persisted until 1998 when the first PCF operating under PBG guidance still retained a solid core [41]. It was only in 1999, that the first hollow core PCF was devised, boasting a single-mode guidance within a vacuum core [42]. Following which, numerous other forms of PCFs and MOFs

as well as a myriad of applications have developed, seeing to its widespread study and employment [34, 43-46].

2.1.2 Types of PCF

Figure 2.1. Schematics of 4 basic PCF types – solid core, hollow core, all-solid and hybrid. Material composition of their microstructured arrays is denoted by a variation in colours – background silica in blue; high-index doped rods in dark blue; air holes in white. Standard microstructure parameters, pitch size (Λ) and hole diameter (d), are also indicated in the solid core PCF.

The host of PCF designs that have emerged over the years can generally be classified into four basic types [44] shown in Figure 2.1. The most prevalent types are the solid and hollow core PCFs, which comprise of periodically arranged microstructured air holes surrounding a silica (continuous with the background material) or air hole center respectively. All-solid PCFs, on the other hand, largely resemble solid core PCFs with the exception of the microstructured array, which consists of high-index doped rods instead of air holes. Lastly, as its name suggests, hybrid PCFs comprise a microstructured array of more than one material.

2.1.3 Fabrication

Figure 2.2. Schematics of a four-step stack-and-draw process. Approximate diameters of the structures during the process are also indicated to highlight the effects of drawing.

The fabrication of PCFs is less sophisticated in contrast to that of conventional optical fibers, as it often comprises only a single material – silica. In order to realize the assortment of intricate microstructure arrays, a variety of different techniques have been devised. The most frequented technique is the stack-and-draw process [39, 40] illustrated in Figure 2.2.

First, capillaries are drawn from larger tubes of silica by heating to $\sim 1950^\circ\text{C}$ and stretching in a fiber-drawing tower. These capillaries are next cut into shorter lengths before being stacked on a hexagonally shaped jig into the desired array arrangement. This versatile method of defining the array configuration enables a range of sophisticated assemblies to be made. Specifically, a host of combinations can be achieved by varying capillary sizes, shapes and material – with air holes attained through the exclusion of capillaries. Subsequently, the arranged stack is inserted into a hollow silica tube, where any remaining gaps between the hollow tube and stack are filled using additional silica tubes. The filled tube is then drawn into a preform within the fiber-drawing tower. Further drawing of the preform into fibers eventually produces the desired PCFs. Lastly, following quality control of the fiber diameter and microstructure a polymer coat is applied to provide mechanical enhancement to the produced PCFs.

2.1.4 Light-guidance mechanisms

In general, the mechanism of light guidance in PCFs is determined by the refractive index contrast between their core (n_{co}) and cladding (n_{cl}) regions. The

cladding region here refers to the microstructure array surrounding the core. When the core refractive index is higher than that of the cladding, light is guided via modified TIR. This is the case in solid core PCFs (or index-guiding PCFs) where the air hole array in the cladding possesses a lower refractive index than its silica core. On the other hand, when the inverse is present – a core refractive index lower than that of the cladding – light is guided through PBG guidance. This occurs in hollow core PCFs where the central air hole, constituting the core, possesses a lower refractive index in contrast to the air-silica air hole array in its cladding.

Figure 2.3. Light-guidance via TIR in a conventional optical fiber. Refractive index of the core and cladding are denoted by n_{co} and n_{cl} respectively. Blue solid arrows represent guided light, while blue graded triangles indicate evanescent fields. Components in white illustrate the conditions for guidance. α denotes the incident angle at the core-cladding interface. θ defines the propagation constant in $\beta = kn_{co} \cos\theta$, where k is the vacuum wave vector.

In conventional optical fibers, light is confined in a high-index core by TIR at the core-cladding interface [47]. TIR occurs at an incident angle of α as illustrated in Figure 2.3, when α is greater than the critical angle α_{crit} defined by:

$$\alpha_{crit} = \sin^{-1}\left(\frac{n_{cl}}{n_{co}}\right) \quad (2.1)$$

However, the large index contrast between air ($n = 1.00$) and silica ($n = 1.46$) as well as the complex microstructures in PCFs makes its light guidance more difficult to determine. A means of identifying whether light is propagated or evanescent involves its parameterization in terms of an axial wave vector component, known as the propagation constant (β). At a particular wavelength

(λ) and thus a fixed vacuum wave constant ($k = 2\pi/\lambda$), the maximum value of β for a propagating wave is set by:

$$\beta = kn \quad (2.2)$$

Therefore, light propagates when $\beta < kn$ and is evanescent when $\beta > kn$, where n pertains to the region under consideration [18, 19, 42, 48]. This is further elucidated in Figure 2.3, where the parameterization is applied in a conventional optical fiber. Specifically, the ray has $\beta = kn_{co} \cos \theta$, where θ is the angle relative to the fiber axis. Since β in this case is smaller than kn_{co} , the ray is guided. In conventional optical fibers, TIR basically occurs when $kn_{cl} < \beta < kn_{co}$.

In PCFs, as β is solved numerically from Maxwell's equations, an effective refractive index (n_{eff}) can be obtained using Equation (2.2). In particular, a n_{eff} can be computed for their claddings that comprise of air hole arrays. Consequently, a solid core PCF would be able to guide light via TIR should its core have a higher n than the n_{eff} of its air hole array. In contrast, hollow core PCFs have β values lower than k (i.e. $n_{co} = 1.00$), allowing for light to propagate even within an air core. This however only occurs over particular spans of wavelengths (or transmission bands) known as band gaps and results from the collective scattering effect of the air hole array. These different modes of light guidance in PCFs further translates into largely different interactions between the guided light and the matter residing within their air holes.

2.1.5 In-fiber light-matter interactions

The interaction between light and fluids has been widely explored in the field of optofluidics. With the emergence of microfluidics, platforms for such interactions have been rapidly scaled down whilst still possessing enhanced function and performance. This has thus seen to a myriad of applications in sensing, analysis and micro-manipulation [49-53]. Notably, PCFs have been identified as such a platform, where they are highlighted to be inherently capable of combining fluidic and optical elements [29-35]. As mentioned earlier, the distinctive modes of light guidance result in different light-matter interactions within PCFs. These interactions further define their applicability.

Figure 2.4. Schematics of light-matter interactions within a (a) solid core and (b) hollow core PCF. Light is indicated in blue while the target matter for interaction is represented in red. Refractive indices of the PCFs' silica background and infiltrated media are denoted by n_{Si} and n_m , correspondingly.

The propagation of light within the air core in hollow core PCFs, allows for an almost complete overlap between the excitation light and the infiltrated sample (shown in Figure 2.4b). This unique mode of guidance within a low-index core has been rigorously exploited in enhancing the sensitivities and detection limits in absorbance [54-56] and fluorescence [56-58] spectroscopy. Specifically, more recent publications have reported limits of 1 pM [57] and 20 pM [58] using only tens of nanoliters in sample volumes.

Nevertheless, filling the air holes with samples would inevitably yield undesirable material-induced losses. This together with the restrictive transmission bands supported by hollow core PCFs have led to their more traditional applications in gas-phase sensing [54, 59-61]. On top of which, PBG guidance would also be altered due to the changes in the air holes' refractive index. Although this property has been utilized in refractive index sensing applications [62] where PBGs shift in accordance to the refractive index scaling law [63, 64], it also creates complications during analysis. This would present when the spectral effects of PBG shifts overlap with that of absorption and fluorescence, resulting in indecipherable spectral profile changes. Furthermore, for the PBG guidance to remain, infiltrated samples have to be of a lower refractive index than the silica background. The combined effects of these factors hence pose huge limitations the choice of samples usable in hollow core PCFs.

On the contrary, as light is guided via means of modified TIR in solid core PCFs, there fundamentally is no restriction imposed by narrow transmission bands. Moreover, since PBG effects do not guide light here, changing the air holes' refractive index generally does not result in spectral profile changes. Nevertheless, sensitivity to refractive index changes could still be induced within solid core PCFs. This could be achieved through the infiltration of samples with refractive indices higher than the silica background. When the air holes have a higher refractive index than the silica background, the mechanism of light guidance is transformed from TIR to PBG guidance. Consequently, varying the refractive index in this range ($>n_{\text{Si}}$) results in PBG shifts [65]. On the other hand, the inscription of Bragg [66, 67] and long period [68-70] gratings into the PCF's core has also generated sensitivities to low-index infiltrations.

Since light guidance in solid core PCFs occurs by modified TIR within its silica core, interaction with infiltrated samples only occurs via the evanescent field penetrating its surrounding air holes [71, 72] (shown in Figure 2.4a). Although the extent of overlap between the evanescent fields and samples is admittedly inferior to that in PBG guidance, evanescent interaction techniques boast unique advantages. These include: the earlier-discussed (1) broad band of transmission; (2) a controllable extent of penetration by altering the microstructure parameters [71]; and notably, (3) surface-specificity. The first two advantages have essentially highlighted the versatility of solid core PCFs and led to their broad applications. These include the absorption spectroscopy of liquids [73-75] and gases [76, 77], as well as fluorescence spectroscopy [58, 78]. Surface-specificity, on the other hand, has been advantageous for biosensing applications using evanescent wave techniques involving planar waveguides and conventional optical fibers [79, 80] as well as within MOFs [81].

2.2 Fluorescence

2.2.1 A brief background

In 1845, a remarkable phenomenon was observed and documented by Sir John Herschel in a solution of quinine sulphate [82, 83]. He described what he

witnessed as an “extremely vivid and beautiful celestial blue color” from what appeared to be a perfectly transparent and colourless solution. He further termed the observation as "dispersive reflection" as it only presented when viewed from certain angles in sunlight. However, the phenomenon could not be fully explained until several years later when Sir G. G. Stokes provided insight into the phenomenon in 1852 [84]. Using simple instruments, he highlighted several characteristics. Firstly, using blue-glass from a church window as a filter (<400 nm) for sunlight, he excited a solution of quinine with ultraviolet (UV) light. The exciting light was then filtered by a yellow-glass of wine (>400 nm), which allowed for the blue emission from quinine to be easily seen. Having absorbed light in the UV region, which was not visible to the human eye, explained why quinine was colourless. Today, it is known that quinine absorbs UV light and emits at 450 nm [85]. This shift in wavelength from excitation to emission is now known as Stokes' shift. In addition, Stokes gave an interesting description with regards to Herschel's observation of a blue glow appearing only at the surface by which light entered the quinine solution. He attributed the observation to a weakening of light after it passed the external most region of solution, which made it incapable of inducing the blue glow beyond the surface. This was what became known later as the inner filter effect.

The blue glow or emission was subsequently termed “fluorescence” by Stokes, after the mineral crystal fluorspar (or fluorite) that glows under UV light. Fluorescence-based methods have since been extensively used across the fields, ascribed to its ease of application, low cost, high sensitivity and non-toxicity (relative to radioactive tracers) [86].

2.2.2 Absorption and emission

Fluorescence is a form of luminescence that is emitted from electronically excited fluorescent substances or fluorophores. The excitation process typically involved the absorption of a photon by an orbital electron, which brings it an excited state. The subsequent return to a ground state resulted in the release of a photon that constituted to emission. [87]

Figure 2.5. A typical Jablonski diagram highlighting absorption and emission. Singlet ground as well as its first and second excited electronic states is represented by S_0 , S_1 and S_2 respectively. Triplet excited electronic state is denoted by T_1 . Their vibrational energy levels are further indicated by 0, 1, 2 etc. Absorbed photons are represented by $h\nu_A$, while photons emitted by fluorescence and phosphorescence are given by $h\nu_F$ and $h\nu_P$, correspondingly. Solid and dotted arrows correspond to transitions between state via photons and non-radiative means respectively.

The Jablonski diagram shown in Figure 2.5 further elucidates this absorption and emission process. Following the absorption of light, particularly a photon with energy $h\nu_{A,1}$ or $h\nu_{A,2}$, the fluorophore is excited from its singlet ground state (S_0) to the higher singlet electronic states of S_1 or S_2 respectively. This is rapidly followed by its rapid relaxation to the lowest vibrational energy level of S_1 through internal conversion. This non-radiative transition generally takes only 10^{-12} s or less and is faster than the typical fluorescence lifetime of 10^{-8} s. This disparity in rate thus results in an equilibrated excited state before emission ensues. The subsequent return to its S_0 state hence results in the fluorescence emission of a photon with energy $h\nu_{F,1}$ or $h\nu_{F,2}$.

From the S_1 state, a spin conversion to the first triplet state (T_1) could also occur via intersystem crossing. This is followed by the phosphorescence emission (the other form of luminescence) of a photon with energy $h\nu_P$, which has a much longer lifetime of 10^{-3} to 1 s. The mentioned triplet state refers to the three states

of which the fluorophore has a total electron spin angular momentum of 1. Similarly, the singlet state reflects the one state that the fluorophore has a total electron spin of zero, where its electron pairs have opposite spins [88]. This thesis, however, focuses on fluorescence-based emissions and this mention of the triplet state pertains only to subsequent discussions on photochemistry.

As illustrated in Figure 2.5, fluorescence emissions are of lower energies than that absorbed. This corresponded to shorter wavelengths for absorption and longer wavelengths for emissions, where this wavelength difference is known as Stokes' shift [84].

Figure 2.6. Peak normalized absorption and fluorescence emission spectra of 5(6)-Carboxyfluorescein in Tris buffer at pH 9 (data obtained from online database Fluorophores.org). The spectra are depicted in blue (left y-axis) and red (right y-axis) respectively.

The typical absorption and emission spectra for a fluorophore is illustrated in Figure 2.6 by data from 5(6)-Carboxyfluorescein in a Tris buffer at pH 9. Two characteristics are highlighted – (1) the earlier mentioned Stokes' shift and (2) the mirror image behaviour between absorption and emission. This symmetry between the spectra is attributed to the identical transitions that occur for absorption and emission. This was based upon the Frank-Condon principle and referred to vibrational energy levels within S_0 and S_1 states. Specifically a 0 to 2 transition from S_0 to S_1 is most probably followed by another 0 to 2 transition from S_1 to S_0 .

The efficiency of converting that absorbed to emission is described by an important characteristic of fluorescence, namely its quantum yield.

2.2.3 Quantum yield

The quantum yield of a fluorophore is simply the ratio between its number of absorbed and emitted photons. This parameter measures the efficiency of the fluorophore and is determined experimentally [89]. Mathematically, fluorescence quantum yield, Φ_{Fl} , can be expressed as:

$$\Phi_{\text{Fl}} = \frac{k_r}{k_r + k_{\text{nr}}} \quad (2.3)$$

Here, k_r and k_{nr} are the rate constants for radiative and non-radiative pathways respectively. In particular, these pathways refer to processes responsible for returning the fluorophore from S_1 to S_0 . Radiative pathways correspond to emission processes indicated in Figure 2.5. Non-radiative pathways, on the contrary, refer to processes that returned the fluorophore to the S_0 state without the release of photons. The sum of both pathways hence gives the total rate of the excited state's depopulation. It also corresponds to the total number of energy transition events and the total number of photon absorption events. On the other hand, k_r reflected only the photon emission events. The division of k_r by the sum of k_r and k_{nr} hence represents the ratio of absorbed photons that have successfully resulted in emitted photons. However, due to losses induced from processes such as internal conversion, Φ_{Fl} is always less than unity.

The introduction of an additional pathway that does not contribute to radiative component hence increases the dominance of the denominator in Equation (2.3). A pertinent process would be quenching.

2.2.4 Quenching

Fluorescence quenching can essentially refer to any process that causes a decrease in the intensity of fluorescence [90]. Quenching can result from a number of molecular interactions such collisional quenching, energy transfer, excited-state reactions and molecular rearrangements. This section would focus on the first two interactions, which are most relevant to subsequent discussions.

Collisional (or dynamic) quenching occurs when a quencher diffuses and comes into contact with a fluorophore during the latter's lifetime in the excited state. This causes the fluorophore to return to the ground state by non-radiative means, thus quenching it. This requirement for molecular contact hence allows molecular interactions to be deciphered, providing information to biochemical systems. The contributions from the quencher can be represented in the quantum yield equation as:

$$\Phi_{\text{Fl}} = \frac{k_r}{k_r + k_{\text{nr}} + k_q[\text{Q}]} \quad (2.4)$$

Here, k_q and $[\text{Q}]$ are the quencher's rate constant and concentration, correspondingly. This is more frequently expressed in the Stern-Volmer equation where the fluorescence intensities in the presence (I_{Fl}) and absence ($I_{\text{Fl},0}$) of a quencher are contrasted:

$$\begin{aligned} \frac{I_{\text{Fl},0}}{I_{\text{Fl}}} &= \frac{\Phi_{\text{Fl},0}}{\Phi} = \left(\frac{k_r}{k_r + k_{\text{nr}}} \right) \bigg/ \left(\frac{k_r}{k_r + k_{\text{nr}} + k_q[\text{Q}]} \right) \\ &= 1 + k_q \tau_0 [\text{Q}] \quad \left| \tau_0 = (k_r + k_{\text{nr}})^{-1} \right. \end{aligned} \quad (2.5)$$

Here, τ_0 is the lifetime of the fluorophore in the absence of a quencher, while its multiplication with k_q gives the Stern-Volmer quenching constant, K_{SV} . In the case of dynamic quenching, this is often represented by K_{D} .

A complicating factor to the analysis of collisional quenching is static quenching. It results when the fluorophore and quencher combine into a non-fluorescent ground-state complex that upon absorbing light immediately returns to the ground state without any emission. This could simply be represented in the Stern-Volmer equation by replacing the quenching constant with the association constant, K_{S} , which is defined as:

$$K_{\text{S}} = \frac{[\text{Fl-Q}]}{[\text{Fl}][\text{Q}]} \quad (2.6)$$

Here, $[\text{Fl-Q}]$, $[\text{Fl}]$ and $[\text{Q}]$ are the concentrations of the fluorophore-quencher complex, the free fluorophore and quencher respectively.

The combination of dynamic and static quenching hence yields the second order equation:

$$\frac{I_{\text{Fl},0}}{I_{\text{Fl}}} = (1 + K_{\text{D}}[\text{Q}]) (1 + K_{\text{S}}[\text{Q}]) \quad (2.7)$$

On the other hand, although similarly resulting in a decrease in fluorescence intensity, resonance energy transfer (RET) is differentiated from quenching [91]. This is because unlike quenching there is no dissipation of the excited fluorophore's electronic energy as heat. Instead, energy is transferred from a donor to an acceptor that could be fluorescent or non-fluorescent, when the absorption spectrum of the former overlaps the emission spectrum of the latter. Furthermore, RET occurs across a space measuring tens of angstroms, with the distance at which it occurs at 50% efficiency being known as the Förster distance, R_0 . Hence, the process is also often referred to as Förster resonance energy transfer (FRET) [92, 93]. Similarly, RET contributes to the non-radiative pathways that return the excited fluorophore to its ground state, where its distance-dependent rate constant is given by:

$$k_{\text{RET}}(r) = \frac{1}{\tau_{\text{D}}} \left(\frac{R_0}{r} \right)^6 \quad (2.8)$$

Here, τ_{D} is the donor's lifetime without the presence of an acceptor and r is the donor-acceptor center-to-center distance. Although not specifically mentioned in literature, the relation to distance depicted in Equation (2.8) could also be further associated to concentration. Hence, this property could likewise be incorporated into the Stern-Volmer equation.

In the case of high concentration fluorophore solutions, the observation of quenching has been primarily attributed to the above-discussed processes. Additionally, as the quenchers in these solutions are the fluorophores themselves, the effect is commonly known as concentration quenching or self-quenching [94, 95].

2.2.5 Photobleaching

Apart from radiative and non-radiative pathways discussed above, fluorophores in the excited state may undergo chemical reactions that result in their degradation and a consequent loss in their ability to fluoresce [96]. This is a permanent photochemical modification of the fluorophore known as

photobleaching and is observed as a decrease in emission intensity over time. This decrease is often simplified into a negative exponential function given by [97]:

$$I_{\text{Fl}} = I_{\text{Fl},0} \exp(-k_{\text{pb}} t) \quad (2.9)$$

Here, I_{Fl} and $I_{\text{Fl},0}$ are the fluorescence emission intensities at times t and $t = 0$ respectively; k_{pb} is photobleaching rate constant.

Although the mechanism of photobleaching is still poorly understood, several proposed theories (summarized in [97]) point to the primary involvement of the excited triplet state. For simplicity, subsequent mention of the excited singlet and triplet states would be denoted by * – i.e. S^* and T^* respectively. Likewise, S and T would represent the ground states, correspondingly. Following excitation of the singlet ground state by the absorption of a photon:

The excited singlet state undergoes intersystem crossing to the long-lived excited triplet state:

The longer lifetime of the excited triplet state hence increases its interaction time with the environment, which includes: (1) other fluorophores in the excited triplet state or ground single state and (2) oxygen molecules. Reaction with other fluorophores generates radicals through an electron transfer process:

Here, R and X represent the semi -reduced and -oxidized radical forms of the fluorophore. The above two reactions compete respectively with the quenching processes:

Quenching of T^* also occurs via reactions with R and X without the consumption of the quenchers:

On the other hand, reaction with naturally occurring triplet oxygen ($^3\text{O}_2$), results in the quenching of T^* and the generation of reactive and long-lived singlet oxygen ($^1\text{O}_2$) [98]:

Alternatively, a slower reaction also occurs with the generation of fluorophore and hydroperoxyl ($\text{HO}_2^\bullet$) radicals:

The excited triplet state fluorophore is hence elucidated by the above reactions to produce products or are themselves highlight reactive, resulting in irreversible chemical changes on top of being unable to fluoresce. The dynamics of photobleaching have been exploited in the extraction of information about biological systems. This encompasses techniques like fluorescence recovery after photobleaching (FRAP) [99, 100], fluorescence loss in photobleaching (FLIP) [100, 101] and Förster resonance energy transfer (FRET) acceptor photobleaching [102, 103].

2.3 Light-Fluorophore Interactions

Figure 2.7. A simplified diagram for light-fluorophore interactions in a conventional cuvette-based configuration. Key components include: a light source, a sample holder and a detector. Excitation and emission optical elements are simplifications of optics involved in the respective paths.

The excitation of fluorophores and the subsequent collection of emission require three basic elements: a light source, a sample holder and a detector. The light source and detector are essentially dependent on the absorption and emission characteristics of the fluorophore. In a conventional configuration for light-fluorophore interactions, as illustrated in Figure 2.7, the sample holder is typically a cuvette. In such a configuration, light is directed into and out of the cuvette via a series of optical elements [104, 105]. This often also requires the cuvette to be loaded with more than a milliliter of sample. However, such configurations comprise inherently bulky free-space optics. Additionally, they entail volume requirements impractical for applications in biochemistry [106] and biomedical research [107] – samples often come in micro and nanoliters. These drawbacks have largely been addressed by advancements in optics miniaturization and microfluidics. Considering the focus of light-fluorophore interactions, this section would specifically cover elements that facilitate said interactions. With the overarching essential concept of miniaturization, only constructs capable of combining both excitation and emission optical elements would be discussed.

2.3.1 Integrated optics

The concept of integrated optics stems from that of integrated (electronic) circuits, where components of varied functionalities are combined into a single chip to carry out a more complex function. In comparison to conventional optical systems, integrated optics offers the compelling advantages of smaller space requirements, lower manufacturing costs and superior flexibility in design. [108]

Figure 2.8. Schematics of common integrated optical waveguide geometries – square channel, planar and ridge. Material composition is denoted by a variation in colours – the darker blue represents a higher refractive index material.

In integrated optics, the optical waveguide element serves as the fundamental building block. This is because it acts like the backbone of the system by guiding and delivering light to and from desired target(s). In such waveguides, light is guided by TIR within a region of higher refractive index (core) relative to the surrounding (cladding). This mechanism is identical to that in conventional optical fibers as detailed earlier in Section 2.1.4. These waveguides are typically fabricated from materials like silica (SiO_2) and silicon (Si). Higher refractive index cores in silica waveguides are subsequently achieved through doping with chemicals like germanium [109], phosphorous [110] and boron [111]. Alternatively, waveguides can also be grown on silica substrates from inherently higher refractive index materials like silicon nitride [112] and silicon oxynitride [113]. This is done through chemical vapour deposition techniques, and the deposited layer can be further shaped and patterned via means of photolithography. The common geometries of such waveguides are illustrated in Figure 2.8.

In geometries where the waveguide core is exposed, evanescent fields penetrate the core surface. When a liquid cladding layer is flown over the exposed core, the evanescent fields interact with the liquid's contents. [114] Hence, light-fluorophore interactions can be achieved. Planar waveguide configurations have thus been successfully used in fluorescence detection regimes for applications like protein [115] and nucleic acid [116] detection. In order to increase the amount of light-fluorophore interaction, the extent of penetration has to be increased. This has been accomplished in reverse-symmetry waveguide

geometries [117]. In such waveguides, the chosen substrate has a refractive index lower than the liquid cladding. This extends of the distribution of the propagating light in the direction of the liquid cladding, increasing the evanescent field's penetration depth from a few hundred nanometers to several microns. Apart from this, several other approaches have also been successful in increasing the extent of light-fluorophore interactions. This has been attained in liquid-liquid core waveguides [118] and liquid core anti-resonant reflecting optical waveguides (ARROWs) [119]. In the former, the sample liquid (core) is flown while being sandwiched by another liquid of a lower refractive index (cladding). Light is thus confined within the sample liquid by TIR. In the latter, light is confined within the lower refractive index liquid core by coherent interference effects created by the ARROWs. These approaches, however, comprise either elaborate microfluidics or require sophisticated fabrication processes.

2.3.2 Optical fibers

Optical fibers were originally devised for telecommunications, which implied minimal losses over extended distances of light propagation. In order to tap on the guided light for light-fluorophore interactions, various modifications have to be made. Specifically, this meant exposing the evanescent fields (illustrated by blue graded triangles in Figure 2.3) to the target sample.

Figure 2.9. Schematics of common optical fiber modifications required for exposing evanescent fields – de-cladded, tapered and side-polished. High refractive index cores are differentiated from their claddings by a darker shade of gray.

The most straightforward way of achieving this was by physically removing the optical fiber's cladding through means of chemical etching. Such de-cladded optical fibers have successfully been applied in fluorescence detection [120, 121]. However, the penetration of evanescent fields in such optical fibers is low. This has been addressed by bending the de-cladded section of the optical fiber into a U-shape [122]. Bending creates higher order modes and extends of the distribution of the propagating light outside the core, increasing the extent of interactions. This was demonstrated in absorption spectroscopy studies [123]. Apart from chemical etching, the optical fiber cladding has also been successfully removed by the mechanical method of polishing [124]. This typically involves embedding the optical fiber in a grooved block, followed by coarse and fine polishing processes. Application in absorption spectroscopy for pH detection has similarly been demonstrated [125]. The addition of a thin metallic coating has also been shown to improve light-fluorophore interactions through plasmonic enhancements [126]. Other than removing the cladding, the evanescent field of an optical fiber can also be exposed by reducing its cross-section. This is achieved in a taper where a heated section of a cladded or de-cladded optical fiber is stretched [127]. A reduced cross-section thus causes a larger distribution of the guided light to reside outside the optical fiber as evanescent fields. Tapered optical fibers have thus been reported to provide higher efficiencies than their de-cladded counterparts in absorption spectroscopy studies [128]. They have also been successfully used in fluorescence spectroscopy [129]. More recently, tapers close to or smaller than the wavelength of guided light have also been reported [130, 131]. Such dimensions create even stronger evanescent fields and exhibit other interesting properties.

Although the various optical fiber modifications have been discussed to be proficient in light-fluorophore interactions, sample introduction still necessitates flow or sample chambers. This requirement inevitably increases the size of the system and contradicts the miniaturization offered by optical fibers.

2.3.3 Fluid-filled optical fibers

On top of PCFs discussed in Section 2.1, liquid-core optical fibers [132] and other types of MOFs [133] have also been explored for in-fiber light-fluorophore interactions. In the former, a capillary (cladding) is filled with a liquid (core) within which light is guided by TIR. TIR was possible with the use of the low refractive index fluoropolymer, Teflon® AF ($n = 1.29 - 1.31$), as the cladding material. Such optical fibers are however less proficient in light guidance.

Figure 2.10. Schematics of common MOFs used in in-fiber interactions – Bragg fiber and suspended-core fiber. Material composition is denoted by a variation in colours – the darker blue represents a higher refractive index material. White spaces indicate air.

On the contrary, MOFs are specifically devised for efficient light guidance. In Bragg fibers, light is reflected off its inner walls and confined within its hollow core [134]. This relies on a periodic dielectric multilayer with alternating refractive indices, which utilizes coherent interference to reflect specific wavelength bands of light. Although Bragg fibers offer a similar extent of light-fluorophore interaction as hollow core PCFs, there has been little report on their use in absorption [135] and fluorescence [136] measurements. This is most likely due to their complex fabrication process, which comprises multiple steps and materials. In that regard, suspended-core fibers have been more popular [137]. This stemmed from its simple design and strong evanescent fields. The latter is a result of wavelength-scaled core diameters that caused much of the guided light to reside outside the core itself – very much like in tapered optical fibers. It also offers ease of filling due to its relatively larger air holes. These advantages have created proficiency in fluorescence spectroscopy [78] and facilitated subsequent applications in protein [138] and nucleic acid [139] detection.

3

An Optofluidic Platform

3.1 Introduction

This chapter would present the development and evaluation of an optofluidic platform for PCFs. The platform would provide a means of completely filling and emptying PCFs while optically aligning them to other fiber optic components. The platform would be evaluated in its optofluidic performance and repeatability. It would serve to enable subsequent studies on in-fiber light-fluorophore interactions that would be covered in the succeeding chapters of this thesis.

3.2 Background

Optofluidics is the combined manipulation of light and fluids, so as to utilize their interactions in applications such as sensing and analysis [49-53]. The integration of optical and fluidic components into a system thus constitutes an optofluidic device. This often consists of fluidic components that controls one or several fluid media and optical elements that manipulate light with regards to its generation, guidance and selectivity. In the recent decade, the advancement of optofluidics has been rapid and cross-disciplinary, with much of it driven by the maturation of microfluidics. Likewise, optical components have undergone extensive miniaturization.

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3.2.1 An overview on optofluidic devices

Optofluidic devices have largely been realized by microfluidic chip prototyping. Most of these implementations were achieved through the use of soft lithography [140, 141]. In soft lithography, polymeric materials are cast from a hard master stamp or mold, enabling the rapid patterning of microstructures. Commonly used low-cost materials are polydimethylsiloxane (PDMS) and polymethylmethacrylate (PMMA). The elasticity, optical quality and transparency of PDMS have further made it an attractive option in optofluidics [142]. On the other hand, processes such as photolithography [143] and laser micromachining [144] are direct and independent of the stamp or mold for consistency in quality, but are generally less cost-effective. More recently, ultrafast lasers have been demonstrated to be proficient in optofluidic fabrication [145, 146]. Their extremely high peak powers enable 3D localization of material modification even in transparent materials. This hence facilitates the direct writing of 3D microstructures in conventional optical materials like fused silica.

The microstructures that can be fabricated include microfluidic components like channels, valves, pumps and mixers [147]; as well as optical components like microcavities [148], gratings [149] and waveguides [150]. This presents a host of component combinations that allow for versatile and comprehensive microsystem design. The additional advantages of high sensitivity and throughput have consequently favoured the use of optofluidic devices in various sensing applications and measurements [51, 52]. This includes refractive index measurements as well as fluorescence and Raman spectroscopy. However, in order to interface such optofluidic devices with typical light sources and detectors, the additional incorporation of optical fibers is often necessitated [151-154]. This thus entails an additional step in the device's fabrication, with the implied hassle of tedious optical alignment. The inherent ability to support optofluidics has therefore positioned PCFs as a practical alternative to microfluidic chip-based techniques.

3.2.2 Optofluidic platforms for PCFs

Since its advent, numerous reviews have discussed the applications of PCFs [34, 43-46, 155]. Despite the common emphasis pertaining to the use of PCFs' air holes as sample chambers for light-matter interactions, capillary forces was still the frequently employed method in achieving filled lengths. Filling PCFs via capillary action, however, not only took extremely long durations but have also been reported to result in the formation of numerous microbubbles [56]. These microbubbles were further mentioned to scatter the guided light, adversely affecting the collected output. On top of which, as capillary action was fundamentally unable to evacuate filled lengths of PCFs, their reuse and application in real-time sensing and measurements were largely impeded. Hence, it becomes imperative that an effective means of infiltration and evacuation be devised. Ideally, this fluidic control should be concomitant with optical alignment, enabling more proficient studies of light-matter interactions within PCFs.

Although, there have been several reported instances of facilitated infiltration and evacuation utilized in coating processes within PCFs, their set-ups focused only on fluidic manipulation. This comprised the use of bulky and complex equipment such as high-pressure liquid chromatography (HPLC) [156] or vacuum [157] pumps. In order to interface these thin threads of PCFs in the former, HPLC tubing and connectors were used. In the latter however, no specified means of interconnection was mentioned.

The first reported platform that combined fluidic control with optical alignment was one that integrated of optical fiber components into a microfluidic chip [55]. This entailed the machined channels that served to align the optical fibers as well as provide fluidic access to the PCF component. The platform offered compactness and robustness, but involved a tedious fabrication and assembly process. Furthermore, having the PCF component embedded implied that the entire chip had to be discarded following each use. This integration with microfluidic chips was mimicked in a preliminary work [158]. However, it was realized to suffer from contamination issues on top of its one-time-use and elaborate fabrication and assembly.

Notably, the more recent publications by P. Russell's group [31, 34, 58] described a custom pressure cell. It enabled the fluidic control in PCFs via HPLC tubing and connectors and possessed optical windows that provided access to other external optics. Although offering an extremely versatile platform, where the PCF component could be easily replaced, the method of interfacing the platform to standard optics was bulky and component intensive. These were very much contradictory to the flexibility and compactness boasted by optical fibers. Despite mitigating the dead volume through the subsequent development of a microfluidic chip-based interface [33], the mentioned drawbacks still existed.

Therefore, an optofluidic platform possessing the traits of compactness, simplicity and versatility was to be devised. Further to which, a means of verifying the platform's performance through the incorporated PCF component had to be selected.

3.2.3 Refractive index measurements as a means of evaluating efficiency

Refractive index measurements have been widely employed in bio- and chemical-sensing applications for the determination of sample concentration or composition. Generally, refractive indices of samples increased with increasing concentrations [159]. In order to enable interactions between the guided light and the sample, fiber optics often required modifications. This included Bragg gratings [160, 161], long period gratings [162], tapering [163] and deposition of thin metal films [164, 165].

PCFs, on the contrary, possess the inherent ability to perform refractive index sensing without the need for sophisticated post-modifications. By simply infiltrating hollow core PCFs, a refractive index change could be made to their air holes. This affects the PBG guidance, altering the bands of light that are confined and become observed as wavelength shifts in their bandgaps [62]. The application of such PCFs however is limited to their respective supported wavelength bands, predetermined by the configuration of their air hole array.

In solid core PCFs, a similar wavelength-based change could be induced. This could be achieved by either (1) drastically increasing the refractive index of the

air holes or by (2) inducing a resonant coupling between the core and cladding modes. The former entailed and was limited to the detection of high refractive index infiltrations [65]. The latter, on the other hand, required a physical change in the refractive index profile of the air hole array. This could be achieved by a selective infiltration of the cladding region [166], but was difficult to realize experimentally. A simpler and more pragmatic approach was bending [167]. The bending-induced losses being highly dependent on the refractive index profile of the PCF, thus offered a means of refractive index sensing. Moreover, the wavelength-dependent sensing is insensitive to variable losses, provided an additional advantage over intensity-based measurements [17].

All in all, wavelength-dependent bending-inducing losses in solid core PCFs presented a pragmatic and straightforward approach to determining the efficiency of the devised optofluidic platform.

3.3 Scope

This chapter focuses on: (1) the development of an optofluidic platform for PCFs, which highlights the compactness and flexibility of optical fibers as well as facilitates the easy replacement of its PCF component; (2) an evaluation of the platform's fluidic performance by determining infiltration and evacuation times; (3) the conduct of refractive index measurements using a bent length of PCF within the devised platform and supporting observed trends through simulations; and (4) subjecting the platform to a measurement of various ethanol concentrations.

3.4 Methodology

3.4.1 Materials

Refractive index liquids were purchased from Cargille. Ethanol absolute was obtained from Merck Millipore. All other chemicals were of reagent grade. PCFs were manufactured by Yangtze Optical Fibre and Cable Company Ltd.

3.4.2 Optofluidic manipulator

Figure 3.1. Schematics (not drawn to scale) of the optofluidic manipulator -based platform. It comprised a 105 mm length of PCF bent at 12.5 mm and coupled to a light source (input) and a computer-linked spectrometer (output) at opposite ends. This was enabled by a series of FC fiber connectors, mating sleeves and multi-mode fibers (MMFs). Red dotted and black solid arrows indicate the sample flow and light propagation respectively. Inset: Enlarged diagram of optofluidic manipulator. Yellow circles denote “plugs”, which are airtight junctions formed between the jacketed edge of a fiber and the inner channel of a fiber connector.

As shown in Figure 3.1, the devised optofluidic manipulator interfaced optical fiber components to a negative pressure system. It comprised two primary parts joined together by a silicon sealant, namely (1) a modified 3-way control valve (BD Connecta Plus 3) and (2) a ceramic split mating sleeve for fiber connectors. The silicon formed airtight seals whilst still giving the mating sleeve a degree of flexibility for the subsequent insertion of (commercially available) FC fiber connectors. Optical fibers were then inserted into their

respective fiber connectors. All optical fibers had both ends stripped of 10 mm of their polymer jackets. The remainder of the jacket generally served to provide mechanical protection, particularly for the bent segment of the PCF. In addition, the jacketed-unjacketed interface further served to form a “plug” with the inner channel of the fiber connector, at the point where it narrows – also at 10 mm from its end. The “plugs” and silicon hence created a chamber within the mating sleeve that is only open to the 3-way control valve and the air holes of the inserted PCF. Infiltration and evacuation could hence be facilitated via the attachment of a syringe-pump at the Luer taper fitting of the 3-way valve. On top of which, the optical fiber inter-connections retained their native purpose of optical alignment, where coupling loss was measured to be at a maximum of 3 dB across these junctions.

Figure 3.2. Micrograph of a solid core PCF’s cross-section, with the dimensions: exterior diameter = 125 μm ; air hole diameter = 5.4 μm ; and pitch = 9.6 μm .

A solid core PCF (scPCF) (shown in Figure 3.2 and reported in [65]) was employed for this phase of experiments. Specifically, a 105 mm length of scPCF was used. This length consisted of a semi-circular section of bending radius, $R_{\text{bend}} = 12.5$ mm, flanked by straight sections for insertion into the fiber connectors. The fiber connectors and mating sleeves, being the larger and sturdier components, were used as anchoring points by a series of nuts and bolts. It is also good to highlight here that this method of securing the components provided practicality and flexibility, especially to the PCF component, allowing it to take up straight and bent configurations.

Prior to optical measurements, the platform's fluidic performance was first measured. Infiltration and evacuation durations were timed in the platform using water and refractive index liquids (Cargille Labs), representative of aqueous and viscous solutions respectively. This was performed for both the straight and bent configurations of the scPCF. A fully drawn 50 ml syringe was used as the negative pressure system, while verification of complete filling and emptying was done under a light microscope.

3.4.3 Refractive index measurements

The device was first studied with refractive index liquids (Cargille Labs) of the refractive indices, $n = 1.31, 1.32, 1.33, 1.34$ and 1.35 . This was performed in two different formats: (1) a calibration study where a fresh scPCF is used in the measurement of each refractive index liquid; (2) a study of hysteresis with continuous infiltration-evacuation cycle for increments of n from 1.31 to 1.33 to 1.35 (step-up) followed by decrements from 1.35 to 1.33 to 1.31 (step-down) with the same scPCF. Each format was conducted in triplicate. For these experiments, the input light source was a halogen white-light (Yokogawa, Ando AQ-4304B) and spectral acquisition was performed with a computer-linked spectrometer (Ocean Optics, Maya2000).

Simulations of an identically bent scPCF were performed with a finite difference beam propagation method using RSoft's BeamProp software. Specifically, a three-layer hexagonal array of air holes was simulated with the parameters: air hole diameter of $5.4 \mu\text{m}$; lattice spacing of $9.6 \mu\text{m}$; bending radius of $12500 \mu\text{m}$; fiber length of $39270 \mu\text{m}$; background index, $n = 1.4457$; and infiltrations of indices, $n = 1.31, 1.33$ and 1.35 . Numerical calculations of the effective mode refractive indices (n_{eff}) were performed over the wavelength range of 500 to 1100 nm . Results were then contrasted with that obtained experimentally.

Varying concentrations of ethanol solutions (in percentage by weight) were prepared with ethanol absolute and ultra-filtrated distilled water. This was subsequently infiltrated into the scPCF via the platform. Spectral acquisitions were made for the ethanol concentrations of 0% , 25% , 50% , 75% and 100% . Each concentration was measured in triplicate.

3.5 Results and Discussion

The fluidic performance of the platform was first evaluated. Following that, the observed bending-induced losses would be accounted for and a brief study on the bending radius would be discussed. The results from refractive index liquid calibrations would next be presented and contrasted with simulations. Lastly, would a discussion of results from ethanol concentration measurements.

3.5.1 Optofluidic performance

3.5.1.1 Fluidic performance

Table 3.1. Summary of durations required for infiltration and evacuation of water and oils for straight and bent PCF configurations via the platform.

PCF	Infiltration time		Evacuation time	
	Water	Oil	Water	Oil
Straight	20 s	25 min	120 s	35 min
Bent	30 s	30 min	125 s	40 min

The durations required for infiltration and evacuation are summarized above in Table 3.1. Results showed a good ten-fold improvement to infiltration when compared to filling via capillary action, and a notable achievement of complete evacuation – a process fundamentally impossible to capillary forces. In addition, due to the faster rates, fluidic manipulations were generally performed in the straight configuration. The alternation between configurations in subsequent experimentation was largely facilitated by the flexibility of the platform. Additionally, the fastest flow rate was calculated at 4.30 nl/s under the straight configuration for water. This was comparable to rates reported by P. Russell’s group’s custom pressure cell (14.0 nl/s) [31, 34, 58] and microfluidic chip-based interface (4.25 nl/s) [33].

3.5.1.2 Bending-induced optical loss

Figure 3.3. (a) Normalized intensity of output spectra collected from the optofluidic manipulator -based platform. The red downward-pointing arrow indicated the spectral dip. Corresponding (b) loss and (c) dispersion curves obtained from simulations are aligned accordingly. Black and blue plots correspond to the straight and bent ($R_{\text{bend}} = 12.5$ mm) PCF configurations respectively. Vertical dotted lines indicate the wavelengths at which core-cladding coupling occurred as predicted by simulations. Insets: Mode-field distributions (exterior of bend is towards the right) in the bent configuration at corresponding wavelengths.

Bending an empty length of scPCF within the device resulted in a notable dip in the output spectrum shown in Figure 3.3(a). This dip, residing at approximately 750 nm, reflected a wavelength-localized region of loss that closely concurred with the simulation results in Figure 3.3(b) and (c).

Effective mode refractive indices (n_{eff}) obtained from the simulations were used in the calculations of leakage loss (L) using the following equation:

$$L = \frac{0.2}{\ln(10)} k \text{Im}(n_{\text{eff}}) \quad (3.1)$$

Here, L is expressed in dB/cm, while k is the vacuum wave vector and $\text{Im}(n_{\text{eff}})$ corresponded to the imaginary component of n_{eff} . As shown in Figure 3.3(b),

the calculated peak loss resided at a wavelength corresponded well with that of the experimentally observed dip.

Dispersion curves in Figure 3.3(c), which are the real components of n_{eff} plotted against wavelength, further supported the observed dip. Specifically, under phase-matching conditions where the n_{eff} of the core ($n_{\text{eff,co}}$) and cladding ($n_{\text{eff,cl}}$) were the same, core-cladding coupling occurred [166, 167]. This condition occurred when the dispersion curves of the core intersected or met with that of the cladding. In particular, two prominent cladding modes were identified to intersect the core mode and were subsequently named Cladding A and B for easier reference. The earlier described peak loss was however only matched by the intersection of the Core and Cladding B curves. On the other hand, although the Cladding A and Core curves similarly intersected, the equivalent loss at this intersection was calculated to be smaller and more gradual. As a result, no corresponding dip was observed in the output spectrum.

The mode-field distributions in Figure 3.3(b) further attested to the lower losses at the point of coupling between the Cladding A and Core. In particular, a less leaky pattern was depicted, where no field was observed to reside in the region outside the air hole array. This could also be intuitively understood by the closer proximity of the Cladding A mode to the physical core of the PCF, which led to better light confinement at the core region. These factors substantiated the focus on the dip at 750 nm, resulting from the coupling between Core and Cladding B modes.

In contrast, for the same PCF in the straight configuration, simulation results showed negligible losses and non-crossing dispersion curves, which corresponded to the absence of any dips in its output spectrum. On the other hand, it is also noteworthy that the output spectra for the straight and bent configurations closely resemble each other beyond 850 nm. This was inclusive of a dip located at 945 nm, which was thus able to serve as an ideal reference point for normalization as used in Figure 3.3(a).

Figure 3.4. (a) Schematic representation of the scPCF under study with an indicated x-axis. (b) Physical refractive index profile corresponding to the drawn axis in (a). Λ indicates the pitch of the air hole array. Black solid plot and blue dashed plot represent the refractive index profiles of the scPCF in the straight and bent configurations respectively. The latter was the calculated equivalent refractive index upon bending.

The bending-induced crossing of the dispersion curves could also be conceptually understood by the changes in the PCF's refractive index profile upon bending (n_{bend}). This bending-induced refractive index change was governed by the following conformal transformation-derived equation [168, 169]:

$$n_{\text{bend}} = n \left(1 + \frac{x}{R_{\text{bend}}} \right) \quad (3.2)$$

Here, n is the original spatial refractive index distribution of the PCF's cross-section; R_{bend} is the bending radius; and x is the horizontal location along the indicated axis in Figure 3.4(a). It should also be noted that x originated at the center of the PCF, while the positive x direction was towards the exterior of the bend. The second term represented the change in refractive index upon application of a strain, equivalent to x/R_{bend} . Applying a bend (i.e. $R_{\text{bend}} \neq \infty$) would therefore increase the refractive index towards the exterior of the bend as shown by the blue dashed plot in in Figure 3.4(b). This increment in n in the cladding region thus brought $n_{\text{eff,cl}}$ closer towards $n_{\text{eff,co}}$, allowing for phase-matching conditions to be achieved. It should be noted that the plot in Figure 3.4(b), corresponded to the physical refractive index of the structure and not the

n_{eff} that is related to light propagation in waveguide. Therefore, n_{core} was not observed to reach n_{airholes} in this case.

3.5.1.3 Varying the bending radius

Figure 3.5. Output spectra from various bending radius, R_{bend} = (a) 10.0 mm and (b) 12.5 mm. Black solid and blue dotted lines represent empty and water-filled scPCFs, correspondingly. Red downward-pointing triangles indicate the dips under study.

Prior to the experiment proper, a simple dip study was performed to determine the effects of different bending radii. This was done through comparison of output spectra between empty ($n_{\text{sample}} = 1.00$) and water-filled ($n_{\text{sample}} = 1.33$) PCFs for the different bending radius. Particular focus was made on the dips' shift and Q-factor as well as practicality factors. In general, as the bending radius decreased, the extent of shift per refractive index unit (RIU) or sensitivity increased from 120 nm/RIU to 190 nm/RIU. Q-factor was next calculated using $\lambda_0/\Delta\lambda$, where λ_0 is the dip wavelength and $\Delta\lambda$ is the full width half maximum of the dip taken at approximately mid-height. Calculations then revealed a Q-factor that decreased from 53.2 to 27.4 when bending radius decreased from 12.5 mm to 10.0 mm, which implied lowered resolution and higher deviations. Moreover, due to the sharp bending at $R_{\text{bend}} = 10.0$ mm, the collected spectra were generally of much lower intensities, implying a lower signal-to-noise ratio. On the other hand, at larger bending radii ($R_{\text{bend}} = 15.0$ mm) where collected spectra were of higher intensities, dips were difficult to discern.

The relatively decent sensitivity and Q-factor, coupled with its ease of handling and incorporation in commercially available optomechanical components hence substantiated the use of $R_{\text{bend}} = 12.5$ mm.

3.5.2 Refractive index measurements

3.5.2.1 Studies performed with refractive index liquids

Figure 3.6. (a) Normalized intensity of output spectra collected from the optofluidic manipulator -based platform for infiltrated refractive index liquids of $n = 1.31$ (in black); 1.33 (in blue); 1.35 (in grey). Corresponding (b) loss and (c) dispersion curves obtained from simulations are aligned accordingly. Vertical dotted lines indicate the wavelengths at which core-cladding coupling occur as predicted by the peak losses in (b).

In the calibration study, a fresh length of scPCF was used in each spectral acquisition. This eliminated any possible effects of cross-contamination between the different refractive index liquids. At least three pieces were used per refractive index to determine repeatability as well as account for fluctuations in the cut length. Similarly, a red shift in the studied dip was observed with the increasing refractive index of infiltrations as shown in Figure 3.6(a). These dips were observed to coincide well with peaks in the calculated losses in Figure 3.6(b). The reason for the red shift could however be attributed

to larger increase in $n_{\text{eff,cl}}$ as compared to $n_{\text{eff,co}}$ upon an increase in the n_{airholes} due to the infiltrated sample of n_{sample} . This was due to the evidently greatly influence n_{airholes} has on the former as the air holes physically comprise the majority of cladding region. Similarly, and was also reflected by the dispersion curves of Figure 3.6(c) where a more significant upward drift was observed for Cladding B curves in contrast to the Core curves. As a result, the point of intersection between the Core and Cladding B curves were shifted to longer wavelengths, ascribing to the observed red shift.

Figure 3.7. Dip and peak loss wavelengths for varying refractive indices of the infiltrated samples. Black squares represent results obtained from calibration; Blue upward-pointing and grey downward-pointing triangles correspond to results from the step-up and step-down study respectively; Red crosses represent peak losses calculated from simulation results. Linear fit equations for each are also included in the dotted box.

Next, to study the scPCF's reusability, a study of hysteresis was performed. Refractive index liquids were infiltrated one after the other, in increments (step-up) and subsequently in decrements (step-down) of n_{sample} . In between infiltrations of refractive index liquids, complete evacuation was achieved before subsequent infiltration. Input and output ports (mating sleeves) were also cleaned with alcohol wipes upon a simple disassembly of the platform. The entire cleaning and disassembly-reassembly process required no more than 5 min. This, together with the infiltration and evacuation durations listed in Table 3.1, amounted to approximately an hour per refractive index measurement.

Each set of data comprised six such infiltration-evacuation cycles performed on a single length of PCF.

As shown in Figure 3.7, the step-up and step-down results closely matched those obtained from the earlier calibration to within 1 nm. This showed the device's reliability in eliminating contamination effects, enabling clear differentiation between the different samples despite of the polarity of change in refractive index. The small deviation in sensitivities was also noteworthy, as shown to be within 7 nm/RIU that amounted to about 2% in variation, further bolstering the device's use in real time applications.

When compared with simulation results, experimental results demonstrated a sensitivity that was marginally higher by approximately 14%. This corresponded to each n_{sample} point differing by less than 3 nm. The difference could however be attributed to two factors, namely: (1) Minor length variations in the cut PCFs and itsunjacketed regions, both with an accuracy of ± 0.5 mm. This hence translated to deviations in bending radius, which has been shown earlier to affect the dip wavelengths. (2) Small discrepancies in the set-up's alignment due to repeated manual handling, could have also affected bending radius. Nevertheless, the differences in dip wavelength were within expectations, as the average standard deviation calculated from calibration results approximated to 3 nm. Moreover, since the simulation results resided within the experimental data deviations, it could be concluded that the device demonstrated good consistency.

3.5.2.2 Measurement of ethanol concentrations

Figure 3.8. Dip wavelengths for different infiltrated concentrations of ethanol. Black squares represent experimentally obtained results; Red crosses correspond to predicted results – calculated from calibration results and the known refractive indices of ethanol solutions.

Following calibration, the device was used in the measurement of ethanol solution concentrations between 0 and 100% by weight. The solutions spanned the refractive index range of 1.333 to 1.364 (based on online databases). As shown in Figure 3.8, experimentally obtained results closely matched the predicted dip wavelengths with differences of only up to 2.5 nm. The predicted dip wavelengths were calculated using the calibration curve ($y = 329x + 354$) and online available refractive index values of ethanol solutions. This exhibited the device's ability in determining non-calibration solutions of differing refractive indices. Nevertheless, it was also noted that a solution's refractive index does not necessarily increase consistently with its concentration, due to the fact that water and alcohol tend to segregate to form percolated networks [170]. This was reflected in the turning point at 75% concentration. As a result, the device was therefore only capable of identifying concentrations of unique refractive indices, which was below 50% concentration in the case of ethanol.

Ethanol was specifically chosen for its low latent heat of vaporization, implying that it would rapidly evaporate when exposed to the atmosphere. This would thus result in an undesired concentration decrease over time, making

time-dependent studies inaccurate. However, this effect could be mitigated via reduction of the exposed surfaces, which is a particular advantage of infiltrating the sample into PCFs. Specifically, the miniscule-sized air holes on either ends of the fiber produced a total area of $1650 \mu\text{m}^2$, which is about 1/1000 times that of the $1500000 \mu\text{m}^2$ surface found on an identical volume of liquid in a spherical droplet.

3.5.2.3 Discussion on platform optimization and limitations

As discussed in Section 3.5.1.3 an R_{bend} of 12.5 mm was selected based on its balanced performance and practicality. This decision stemmed from a superior Q-factor and signal-to-noise ratio (versus $R_{\text{bend}} = 10.0$ mm), as well as a better discernibility of dips (versus $R_{\text{bend}} = 15.0$ mm). Although higher sensitivities could be achieved with small bending radii, the returns were outweighed by the above-mentioned factors as well as the ease and efficiency of performing the experiments. Considering that the platform's functionality is principally dependent on the effects of bending, subsequent parameter optimizations were consequently centered upon $R_{\text{bend}} = 12.5$ mm.

The desired bend was achieved by forming a semi-circle at the middle of the length of PCF, resulting in a bent section of length equal to $R_{\text{bend}} \times \pi$. This is flanked on both ends by straight sections that are to be inserted into FC fiber connectors as illustrated in Figure 3.1. Consequently, for a fixed R_{bend} , the length of the fiber connectors essentially determines the total length of the PCF. Since the platform's operation is R_{bend} -dependent, these straight sections are understood to not contribute to its functionality. This was also noted experimentally while using a different brand of fiber connectors. However, a longer length of PCF does translate to a longer duration for infiltration and evacuation. Ideally, shorter fiber connectors would be preferred in the optofluidic platform, so long as optical alignment requirements are met.

Likewise, the longer durations of infiltration also resulted in slower response times. This further bolsters the preference for shorter fiber connectors. It was noted in experiments that spectral outputs were stable once the infiltration was performed for at least two-thirds the duration required for complete infiltration. However, complete infiltration was always performed in experiments so as to

ensure consistency of results. Additionally, although the durations reported in the spanned tens of minutes, it should be noted that this was a consequence of the refractive index liquids' high viscosity. In contrast, durations required by aqueous solutions were reported to be within several minutes, mere fractions of their high-viscosity counterparts. Should repeated use not be required (i.e. only infiltration), each measurement can be performed in under a minute.

The one-time use of each PCF also sees to the complete elimination of cross-contamination. Although cross-contamination has been demonstrated to be negligible by the insignificant hysteresis in Figure 3.7, it applies only to sample solutions that do not interact with the in-fiber surfaces. As would be demonstrated in Chapter 5, solutions such as proteins are inherently capable of adhering to in-fiber surfaces. This implies that it is impossible to achieve complete evacuation and hence the result of "contamination".

Lastly, although linear fit equations were obtained from the calibration in Figure 3.7, extrapolation beyond the calibrated refractive index range ($n = 1.31$ to 1.35) is unlikely to yield accurate results. This is due to the gradual flattening of the curve at higher n_{sample} . Therefore, the linear relationship between the dip wavelengths and n_{sample} is only valid within small refractive index ranges. Regardless, as aqueous solutions are the primary sample format in subsequent studies, their refractive indices are expected to not differ significantly from that of water ($n = 1.33$). Hence, the studied refractive index range is deemed to be sufficient.

3.6 Conclusion

The devised optofluidic platform was demonstrated to be proficient in the complete infiltration and evacuation of PCFs while concurrently enabling optical alignment to other fiber optic components. Its efficiency was experimentally validated through the conduct of refractive index measurements using bent lengths of solid core PCFs.

The platform comprised an optofluidic manipulator that aligned optical fibers and provided a connection to a negative pressure system. Its all-fiber nature made it compact and flexible. Optical losses across connections were measured to be within 3 dB, while filling rates were reported to be significantly faster than capillary forces. In addition, complete evacuation was notably achieved.

Bending was observed to induce wavelength-dependent losses in solid core PCFs, which presented as dips in the output spectrum. These losses were attributed to a core-cladding coupling condition at particular wavelengths. Coupling could be attained upon the bending-induced refractive index profile change in PCFs, allowing $n_{\text{eff,co}}$ to be matched by $n_{\text{eff,cl}}$. Physically varying the refractive index of the air holes thus induced changes to the refractive index profile and consequently shifted the wavelength of the losses. The R_{bend} of 12.5 mm was chosen based upon its sensitivity, Q-factor, output intensity and experimental handling. Simulation results were observed to support the experiments, with the wavelength location of calculated peak losses and intersection of core-cladding dispersion curves concurring well with the experimentally observed dips. This was similarly achieved in continuous sample infiltration-evacuation cycles with sequential increments and decrements in refractive indices, which reflected good repeatability and reusability. Lastly, the device's ability in refractive index measurements was verified through its successful determination of ethanol solutions at varied concentrations, up to 50% by weight.

In conclusion, a compact and versatile optofluidic platform for PCFs was developed and evaluated via refractive index measurements. This platform would serve to facilitate subsequent studies on light-matter interactions within PCFs.

4

In-fiber Fluorospectroscopy

4.1 Introduction

This chapter would detail a numerical model for the computation of light-fluorophore interactions within PCFs. Through this model, the fundamentals of in-fiber light-fluorophore interactions would be understood. The validity of the model would be evaluated through comparison with experimental results – obtained via the optofluidic platform described in the earlier chapter. This would provide a basis for the effective use of PCFs in fluorospectroscopy. This is essential in subsequent chapters for the characterization of PCF contents.

4.2 Background

Light guidance via modified TIR in index-guiding PCFs, offer not only broadband guidance but also surface-specific light-matter interactions [39]. The extent of interaction being manipulable by variation of its microstructure [71], has allowed for their applications in absorption spectroscopy of liquids [73-75] and gases [76, 77], as well as fluorescence spectroscopy [58, 78].

In the above employment of PCFs, however, bulky and component-intensive set-ups were often required to interface them to conventional optics. Furthermore, fluorospectroscopy entailed the additional requirement of optical filters or laser sources, which served to eliminate or enable easy disregard of the remaining excitation respectively. All of which, were often costly and extremely space consuming, largely overshadowing the compactness and

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flexibility boasted by fiber optics. The following hence discusses the alternatives.

4.2.1 Micro-filters and filterless techniques

Several other approaches that have circumvented the need for traditional or physical optical filters have also been reported, these included the use of: (1) sophisticated micro-architectures; (2) highly precise time-resolved detectors; (3) cumbersome arrangements to separate excitation and fluorescence emission. These are detailed as follows.

4.2.1.1 Micro-architectures

Numerous approaches have been explored to achieve miniaturization of fluorescence spectroscopy and sensing, with a majority centered upon the use of similarly microscopic optical hardware [171], such as the following:

(a) Interference filters

Interference filters operate on the principles of distributed Bragg reflectors [172], which are essentially mirrors comprising alternating layers of materials with different refractive indices and sub-wavelength thicknesses. Different wavelengths of incident light thus experience either constructive or destructive interference, which translate into transmission or reflection respectively. The versatility and availability of various fabrication techniques have thus seen to their incorporation in a host of microfluidic and lab-on-a-chip devices [173-176] and even directly onto complementary metal oxide semiconductor (CMOS) imagers [177]. However, such filters are extremely sensitive to small variations in layer thickness, making their effectiveness largely dependent on fabrication precision. Furthermore, other factors, such as the extent of Stokes' shift of the fluorescent dye under probe as well as the incident angle's influence on the filtered wavelengths, pose additional practical challenges.

(b) Absorption filters

In contrast, absorption filters governed by the Beer-Lambert law, are significantly simpler in structure – comprising just a single layer. Their ability to filter, on the contrary, is derived from the wavelength-dependent absorption

of the material comprising the absorbing layer. Such filters have likewise been employed in numerous microfluidic devices, owing to its straightforward process of fabrication. Their materials' absorptive wavelengths ranged from ultraviolet-to-blue in semiconductors [178, 179] to visible bands in various dyes [176, 180, 181]. On the other hand, absorption filters possess limited tailorability, where an absorptive material only complements a specific fluorescent dye under probe. This results in difficulties when fabrication techniques have to be completely altered to accommodate the varying properties of the different absorbing material used.

(c) Spectrally selective detectors

Apart from light blocking in filtering techniques, a contemporary approach entails the discrimination of fluorescence and excitation via electronic means – semiconductor detectors. Such detectors operate primarily on the principles of absorption and the fact that shorter wavelengths are absorbed closer to the surface, while longer wavelengths penetrate deeper. The detected absorption at various depths thus corresponded to the different wavelengths present. This was initially realized in fixed stacks of layered structures [182] and subsequently in detectors with voltage-controllable collection depths [183-186]. Although able to provide simultaneous multi-wavelength detection, the fabrication of such detectors is significantly more sophisticated and expensive.

(d) Light-guiding elements

Similar to the spectral selection above, light-guiding elements provide a means of spatially separating the excitation source and fluorescence emission. The positioning of the detector hence determines which of the two is collected. This has been accomplished via the use of micro-lens arrays. Such arrays enable the direction of excitation light into the sample chamber and away from the detector upon its exit [187]. On the other hand, an alternate means of achieving spatial separation was through the use of diffraction gratings [188, 189]. The different wavelengths of the excitation and emission translated to differing diffraction angles, which could thus be collected at distinctive detector positions. Both techniques, however, entail fabrication complexities inherent to

micro-architectures, with the latter requiring a space-consuming platform to facilitate the various angles of collection.

4.2.1.2 Highly precise time-resolved detectors

Instead of physical elimination, time-resolved methods rely on a time-based separation of the excitation and fluorescence emission. Fluorescence lifetime measurements, in particular, quantify fluorescence by measuring its decay over time, following a pulsed excitation [190, 191]. However, most fluorophores possess extremely short lifetimes, spanning only several hundred picoseconds to tens of nanoseconds. Hence, highly specialized and complex instruments are required to modulate between excitation and emission collection as well as perform the measurement itself. Although more advanced techniques like time-correlated single photon counting [192] have been developed, equipment requirements have becoming similarly more elaborate.

4.2.1.3 Source and detector placement

Alternatively, there have also been methods that exploit the properties of ray optics in achieving a spatial form of separation between the excitation and emission. In general, this involves the utilization of geometry to prevent the excitation light from reaching the detector, enabled by the presence and absence of directionality in the excitation source and fluorescence emission respectively. This is demonstrated in configurations where the detector and source are positioned orthogonally or at an angle to each other [193, 194], which is sometimes also coupled with a TIR-based means of excitation [79, 195]. However, such systems are either bulky due to the nature of its components' arrangement or require complex fabrication techniques to achieve miniaturization.

The above-discussed methods demonstrated effectiveness through the miniaturization, circumvention or replacement of optical filtering techniques. Regardless, they also entailed a host of disadvantages centered upon complicated fabrication techniques as well as equipment that were costly and often bulky. These methods, were therefore not applicable to the optofluidic platform discussed in Chapter 3 and [196], as the addition and replacement of components would undo its all-fiber regime and low-cost. Hence, an alternative

approach, which was neither component-centric nor required a restructuring of the platform, had to be devised.

4.2.2 A mathematical approach

Inspiration was drawn from wavelength-ratiometry [197], which used an additional emitter as a reference to the fluorescence emission under investigation. Although the method discussed a rather different class of fluorescence spectroscopy, it highlighted the use of a mathematical approach coupled with the simplest possible instrumentation. These factors thus held potential in preserving the all-fiber nature of the platform and its low-cost. In brief, instead of comparing the fluorescence emission intensity to the reference emitter, the approach to be presented would contrast it with the remaining excitation. This would hence provide a dimensionless relationship between the emission and concentration of the fluorescent dye, which further mitigates the undesirable intensity fluctuations common to optical set-ups.

In order to accomplish this ratiometric approach, the in-fiber light-matter interactions had to first be modeled. An available method was detailed in [78], where excitation and fluorescence collection within a suspended-core fiber was studied. It comprised the solving of Maxwell's equations via Finite Element Methods in a commercial software (COMSOL) and focused on determining the influence to which in-fiber light-fluorophore overlap had on fluorescence sensing capabilities. This was however a computationally intensive method that required specialized software. On the other hand, a significantly less complex approach that uses simple geometry has also been widely used in the domain of fiber optics – namely, ray-tracing [198-202]. Amongst the reported approaches, [198] depicted a ray-tracing method that modeled the excitation and fluorescence collection at the surface of a conventional optical fiber's exposed core. This largely resembled the in-fiber configuration of a core surrounded by fluorescent dye-filled air holes. Since the region of light propagation within the PCF's core possessed dimensions relatively larger than the studied wavelengths [200, 203], this ray-tracing approach was deemed a suitable approximation for subsequent tailoring.

4.3 Scope

This chapter focuses on: (1) the development of a ray-tracing model that accounts for the propagation of individual rays and their consequent interactions with the ambient fluorescent dye; (2) tweaking of the model to accommodate the ratiometric interpretation of the relation between the remaining excitation and collected fluorescence emission; and (3) a detailed comparison of the model-derived calculations with results obtained experimentally. These were performed for both a defected core PCF (dcPCF) [74] and a solid core PCF (scPCF) [65] for varying concentrations of a common fluorescent dye – carboxyfluorescein.

4.4 Methodology

4.4.1 Materials

5(6)-Carboxyfluorescein (CF) was purchased from Sigma-Aldrich . 1M Tris buffer at pH 8.8 was obtained from 1st Base. All other chemicals were of reagent grade. PCFs were manufactured by Yangtze Optical Fibre and Cable Company Ltd.

4.4.2 In-fiber fluorescence spectroscopy of carboxyfluorescein

The excitation input of the earlier discussed platform was replaced with a 2.3 mW 490 nm fiber-coupled light-emitting diode (LED) (Thorlabs, M490F1) adjusted to 80% its maximum power. Spectral acquisition was performed with the same computer-linked spectrometer (Ocean Optics, Maya2000).

A 100 mM stock of CF was prepared by dissolving 0.564 g of CF powder in a mix of ultra-filtrated distilled water and 1.0 M sodium hydroxide with 10.5 ml and 4.5 ml, correspondingly. 105 mm lengths of PCFs were cut and infiltrated with varying concentrations of CF, between 10 μ M and 100 μ M – diluted from the earlier prepared stock with 321 mM Tris buffer.

Corresponding output spectral were acquired for each infiltration under both the straight and bent PCF configurations, where at least a triplicate was performed per concentration. For the purposes of calibration, a fresh length of PCF was used for each infiltration. The intensity ratio between $\lambda_{exc} = 490$ nm and $\lambda_{ems} = 510$ nm, $I(\lambda_{ems})/I(\lambda_{exc})$, was subsequently calculated.

4.4.3 Computation of in-fiber light-fluorophore interactions

4.4.3.1 Ray-tracing in bent PCF

A ray-tracing model was adapted from that of a conventional optical fiber. The model described fluorophore excitation and its subsequent fluorescence emission collection at the exposed surface of an optical fiber's core [198]. Its direct use, however, was primarily deterred by the structural non-conformity to the standard cylindrical structure of conventional optical fibers – a bent configuration and a defected core.

A ray-by-ray discretization method was hence employed. Each ray, defined by its initial spatial location and angle of launch, has its trajectory and incident angles traced via simple geometrical constraints. From the light-fluorophore interactions derived at each point of incidence and its number of occurrences, the spectral variations per concentration of CF, characterized by $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$, could thus be computed.

Figure 4.1. (a) Micrograph of dcPCF's microstructure with the region of interest (its core) demarcated by white dotted circles. (dcPCF dimensions: Air hole diameter = 6.2 μm ; central air hole diameter = 3.1 μm ; pitch = 6.6 μm) Corresponding (b) transverse and (c) longitudinal cross-section define the model's coordinate system (in black and green) and launch parameters (in blue). The indicated launched ray (in red) has the initial spatial location of $x\text{Pos}=y\text{Pos}=0$, tilt of 0° and launch angle of θ_{START} .

As illustrated in Figure 4.1b and c, each ray was determined by a combination of launch parameters, namely its (1) x- and y-position of launch ($x\text{Pos}$ and $y\text{Pos}$ respectively); (2) angle of launch (θ_{START}), on its plane of launch; (3) tilt (ψ) of its plane of launch. This was coupled with a simplification of the PCF's core into a cylinder with of diameter D_{core} . The aforementioned core was defined as the central region surrounded by the innermost ring of air holes as indicated by the outer white dotted circle in Figure 4.1a. In the case of a dcPCF, a hollow core of diameter $D_{\text{def-core}}$ was incorporated. On the other hand, $D_{\text{def-core}}$ was ignored in a scPCF.

A graded core refractive index (n_{co}) profile was adopted in order to incorporate the effect of bending. The bending-induced refractive index change derived from conformal transformation [168, 169] and used earlier in Chapter 3 was rewritten as follows:

$$n_{co,bend} = n_{co} \left(1 + \frac{x'}{R_{bend}}\right) \quad (4.1)$$

Here, n_{co} refers to the original core refractive index (i.e. unbent); x' (as indicated by the green axis in Figure 4.1b) was positive towards the exterior of the bend with a bending radius of R_{bend} . Since bending only deforms the PCF core in the x-direction, n_{co} varied only along the x-axis and remained constant in the y- and z- direction. Also, bending was assumed here to have a negligible effect on the medium refractive index (n_m), which was fluid in nature.

Discretization in the x-direction was defined by the layer thickness (t_{Lay}), which was equivalent to the division of D_{core} by the total number of layers (Lay_{Tot}). A corresponding $n_{co,bent}$ discretized distribution could hence be obtained. Applying the same discretization in the y-direction establishes the x-y grid illustrated in Figure 4.1b. This created a mesh density approximately equivalent to Lay_{Tot}^2 , with each x-y coordinate pair corresponding to a point of launch as indicated by the intersecting dotted lines.

Figure 4.2. Path of a typical ray (in red) traversing the entire 3-layer discretized core, generating the incident angles α_{Top} and α_{Btm} at the Layer 3-Top and Layer 1-Bottom (Btm) interfaces respectively, as well as a longitudinal displacement of L_z between the two incidences. Path of an intra-core reflected ray (in orange) is similarly indicated. (Note: Exterior of bend is towards the bottom, hence as calculated from Equation (4.1) $n_{co,1} > n_{co,2} > n_{co,3}$ and consequently $\theta_1 < \theta_2 < \theta_3$.)

Each launched ray traversed the discretized PCF core as shown in Figure 4.2, where a simple 3-layer discretization was depicted. At each layer-layer interface, the ray underwent either refraction or reflection as illustrated at the Layer 1-Layer 2 interface by the red and orange arrows, correspondingly. In the case of refraction (experienced by the typical ray), the ray bent away from the

normal as it propagated in the positive x-direction as $n_{\text{co,bent}}$ decreased. Each refracted angle (θ_r) was calculated from the refractive indices of the ray's entry (n_i) and exit (n_r) as well as angle of incidence (θ_i) using Snell's law as follows:

$$\theta_r = \sin^{-1} \left(\frac{n_i}{n_r} \sin \theta_i \right) \quad (4.2)$$

On the other hand, the unique scenario of intra-core TIR occurred when the calculated θ_r became imaginary as $\frac{n_i}{n_r} \sin \theta_i > 1$. This was a combined effect of a higher n_i and θ_i , consequent of a bending-increased n_{co} and a θ_{START} that tended towards 90° respectively. Should refraction persist until the core-medium interface, TIR would too occur at an incident angle (α) of α_{Top} , referencing the top core-medium interface. Likewise, TIR at the bottom core-medium interface resulted in an incident angle of α_{Btm} . Note that for cases involving intra-core TIR, only the latter bottom core-medium incident occurred. The ray-tracing model entailed the computation of three main components: (1) α_{Top} and α_{Btm} ; (2) n_{co} of the layer where TIR occurred was recorded; and (3) the number of reflections, N_{Refl} – calculated from the longitudinal distance between TIR events (L_z) and the total PCF length (L_{PCF}). These are summarized in Table 4.1 below.

Table 4.1. Summary of computed outputs for launched rays in Figure 4.2.

Output	Typical Top-Btm reflection	Intra-core reflection
α_{Top}	$\alpha_{\text{Top}} = \theta_3 = \sin^{-1} \left(\frac{n_{\text{co},2}}{n_{\text{co},3}} \sin \theta_2 \right)$	NA
α_{Btm}	$\alpha_{\text{Btm}} = \theta_{\text{START}} = \theta_1 = \sin^{-1} \left(\frac{n_{\text{co},2}}{n_{\text{co},1}} \sin \theta_2 \right)$	$\alpha_{\text{Btm}} = \theta_{\text{START}}$
$n_{\text{co,Top}}$	$n_{\text{co},3}$	NA
$n_{\text{co,Btm}}$	$n_{\text{co},1}$	$n_{\text{co},1}$
N_{Refl}	$N_{\text{Refl}} = \left\lceil \frac{L_{\text{PCF}}}{L_z} \right\rceil + 1$	$N_{\text{Refl}} = \left\lceil \frac{\left\lceil \frac{L_{\text{PCF}}}{L_z} \right\rceil + 1}{2} \right\rceil$

Utilizing these fundamentals, the same outputs were computed from a 3D form of the above-discussed 2D regime.

Figure 4.3. Path of ray (in red) undergoing (a, c) refraction and (b, d) reflection in a 3D domain launched at a tilt of ψ . Plane-of-launch (for (a, c)) or incidence (for (b, d)) and Plane-of-refraction is highlighted in blue and yellow, correspondingly. (c) and (d) are alternate views (90° counter-clockwise rotation about x-axis) of (a) and (b) respectively.

Each discretized layer was transformed into a cuboidal element as illustrated in Figure 4.3. The rays were then launched with the additional parameter of tilt (ψ). $\psi \neq 0$ corresponded to a ray launched within its plane-of-launch, which has been rotated clockwise (from the x-axis) about its pivot along the z-axis. From this, a plane-of-refraction – orthogonal to the layer-layer interface – was derived in order to enable the subsequent computation of refracted angles as the ray traversed the layers. This entailed the derivation for the symbols (A to E) indicated in Figure 4.3a and c, listed as follows:

$$A = \frac{t_{\text{Lay}}}{\cos \psi} \quad (4.3)$$

$$B = L_{z,1} = \frac{t_{\text{Lay}}}{\cos \psi} \tan \theta_{\text{START}} = t_{\text{Lay}} \tan \theta_1 \cos \xi \quad (4.4)$$

$$C = t_{\text{Lay}} \tan \psi \quad (4.5)$$

$$D = \xi = \tan^{-1} \left(\frac{C}{B} \right) = \tan^{-1} \left(\frac{\sin \psi}{\tan \theta_{\text{START}}} \right) \quad (4.6)$$

$$E = \frac{t_{\text{Lay}} \tan \psi}{\sin \xi} = t_{\text{Lay}} \tan \theta_1 \quad (4.7)$$

From which, the refracted angle (θ_2) and consequent longitudinal displacement ($L_{z,2}$), as indicated in Figure 4.3a and c, could be attained:

$$\theta_2 = \sin^{-1} \left(\frac{n_1}{n_2} \sin \left(\tan^{-1} \left(\frac{\tan \psi}{\sin \xi} \right) \right) \right) \quad (4.8)$$

$$L_{z,2} = t_{\text{Lay}} \tan \theta_2 \cos \xi \quad (4.9)$$

Like in the 2D regime, the ray would similarly experience TIR. In contrast to refraction, TIR occurred in the plane-of-incidence, which was defined in a similar manner to the plane-of-launch as shown in Figure 4.3b and d. Its list of symbols (F to I) are likewise derived as follows:

$$F = L_{z,2} = t_{\text{Lay}} \tan \theta_2 \cos \xi \quad (4.10)$$

$$G = t_{\text{Lay}} \tan \theta_2 \sin \xi \quad (4.11)$$

$$H = \psi_2 = \tan^{-1} \left(\frac{G}{t_{\text{Lay}}} \right) = \tan^{-1} \left(\tan \theta_2 \sin \xi \right) \quad (4.12)$$

$$I = \frac{t_{\text{Lay}}}{\cos \psi_2} \quad (4.13)$$

From which, the reflected angle or α (represented by θ_3' in Figure 4.3b and d) was obtained:

$$\theta_3' = \tan^{-1} \left(\frac{F}{I} \right) = \tan^{-1} \left(\tan \theta_2 \cos \xi \cos \psi_2 \right) \quad (4.14)$$

It should be noted here that due to the method of discretization, reflection could only occur at the cuboid edge, which sufficed as an approximation of the PCF core's cylindrical surface under dense enough meshes. Following the establishment of the ray-trace, a translation to experimental observations had to be performed.

4.4.3.2 Combination with ratiometry

Figure 4.4. Output spectrum of Tris buffer (solid black line) and 30 μ M CF (blue dotted line) loaded dcPCF normalized to $I_0(\lambda_{exc})$ and $I(\lambda_{exc})$ respectively.

The typical output spectrum of fluorescent dye-loaded PCF is depicted by the blue dotted plot in Figure 4.4. Here, fluorescence emission presented as a shoulder on the right flank of the spectrum, essentially an overlap with the remaining excitation that constitutes the bulk of the spectrum – stemming from the filterless nature of the spectroscopy regime. $I(\lambda)$ hence represented a superposition of the two spectral distributions: (1) remaining excitation, $I_{rem}(\lambda)$ and (2) fluorescence emission, $I_{Fl}(\lambda)$. In order to simplify the computation, representative wavelengths were selected for each, specifically $\lambda_{exc} = 490$ nm and $\lambda_{ems} = 510$ nm that corresponded to the peak absorption and emission wavelengths respectively. To further mitigate the common but undesirable effects of intensity fluctuations, typical of optical set-ups, a dimensionless ratiometric relationship was derived from the corresponding intensities at λ_{exc} and λ_{ems} :

$$\frac{I(\lambda_{\text{ems}})}{I(\lambda_{\text{exc}})} = \frac{I_{\text{Fl}}(\lambda_{\text{ems}}) + I_{\text{rem}}(\lambda_{\text{ems}})}{I_{\text{Fl}}(\lambda_{\text{exc}}) + I_{\text{rem}}(\lambda_{\text{exc}})} \quad (4.15)$$

Rewriting $I_{\text{rem}}(\lambda)$ into the subtraction of the absorbed intensity, $I_{\text{abs}}(\lambda)$, from the input intensity, $I_0(\lambda)$, gave:

$$\frac{I(\lambda_{\text{ems}})}{I(\lambda_{\text{exc}})} = \frac{I_{\text{Fl}}(\lambda_{\text{ems}}) + I_0(\lambda_{\text{ems}}) - I_{\text{abs}}(\lambda_{\text{ems}})}{I_{\text{Fl}}(\lambda_{\text{exc}}) + I_0(\lambda_{\text{exc}}) - I_{\text{abs}}(\lambda_{\text{exc}})} \quad (4.16)$$

Introducing a fraction of absorption, $f_{\text{abs}} = \frac{I_{\text{abs}}(\lambda)}{I_0(\lambda)}$, and a further overall normalization to $I_0(\lambda_{\text{exc}})$ led to:

$$\frac{I(\lambda_{\text{ems}})}{I(\lambda_{\text{exc}})} = \frac{I_{\text{Fl, norm}}(\lambda_{\text{ems}}) + \frac{I_0(\lambda_{\text{ems}})}{I_0(\lambda_{\text{exc}})}(1 - f_{\text{abs, ems}})}{I_{\text{Fl, norm}}(\lambda_{\text{exc}}) + (1 - f_{\text{abs, exc}})} \quad (4.17)$$

The $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ ratio in Equation (4.17) would subsequently be calculated for each launched ray. This resulted in a four-dimensional matrix consisting of xPos by θ_{START} by yPos by ψ , representing all combinations of launch conditions. Averaging consequently produced a $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ per concentration of fluorescent dye. Prior to which, $I_{\text{Fl, norm}}$ and f_{abs} – depictees of light-fluorophore interactions at the core-medium interface – had to first be computed from each point of reflection.

$I_{\text{Fl, norm}}$ was derived from the sum of emissions generated and collected from each ray at each point of reflection, with each described by:

$$S_{\text{ray}}(\lambda) = c\varepsilon(\lambda)\Phi_{\text{Fl}} f_{\text{Fl}, \lambda} \frac{I_0(\lambda)}{I_0(\lambda_{\text{exc}})} \cos \alpha \int_0^\infty F_{\text{abs}} P_{\text{ems}} d\delta \quad (4.18)$$

The above represented the collected emission resulting from the excitation at each λ by a normalized excitation intensity of $I_0(\lambda)/I_0(\lambda_{\text{exc}})$. c , ε and Φ_{Fl} denoted the concentration, extinction coefficient and quantum yield of the fluorescent dye, correspondingly. $f_{\text{Fl}, \lambda}$ is the fraction of emission at λ_{ems} or λ_{exc} . The final terms, F_{abs} and P_{ems} are the factor of absorption and the probability of emission collection respectively. These two terms were integrated over the perpendicular

distance from the core-medium interface, δ , extended infinitely into the medium.

F_{abs} , a dimensionless factor indicating the extent of power absorbed (P_{abs}) with respect to the normal of the incident power flux ($I_0(\lambda) \cos \alpha$), was defined by:

$$\begin{aligned} F_{\text{abs}} &= \frac{P_{\text{abs}}}{c\epsilon(\lambda)I_0(\lambda)\cos\alpha} \\ &= 2\cos\alpha \left(\frac{n_{\text{rel}}}{n_{\text{rel}}^2 - 1} \right) \left(1 + \frac{2n_{\text{rel}}^2 \sin^2 \alpha - 1}{(n_{\text{rel}}^2 + 1)\sin^2 \alpha - 1} \right) \exp\left(-\frac{\delta}{d_p}\right) \end{aligned} \quad (4.19)$$

Here, $n_{\text{rel}} = \frac{n_{\text{co,bent}}}{n_m}$, represented the relative refractive index between the core and medium, where the former corresponds to the $n_{\text{co,bent}}$ at the point of reflection calculated from Equation (4.1). Also, d_p was the penetration depth:

$$d_p = \frac{\lambda}{4\pi n_m \sqrt{n_{\text{rel}}^2 \sin^2 \alpha - 1}} \quad (4.20)$$

It should be highlighted that in all calculations of n_{rel} , these assumptions were made: (1) n_m was a constant equivalent to that of water, due to the negligible influence of low solute (fluorescent dye) concentration on refractive index; (2) n_{rel} for λ_{ems} or λ_{exc} was the same, due to negligible dispersion differences between wavelengths in the studied band.

P_{ems} , on the other hand, determined the likelihood of a dipole's (fluorescent dye molecule) radiation being collected and propagated in the positive z-direction:

$$P_{\text{ems}} = \frac{\int_{\alpha_{\text{crit}}}^{\frac{\pi}{2}} \cos^{-1}\left(\frac{\sin \alpha_{\text{crit}}}{\sin \alpha}\right) \frac{dP_2}{d\Omega} \sin \alpha d\alpha}{\pi \left(\int_0^{\alpha_{\text{crit}}} \frac{dP_1}{d\Omega} \sin \alpha d\alpha + \int_{\alpha_{\text{crit}}}^{\frac{\pi}{2}} \frac{dP_2}{d\Omega} \sin \alpha d\alpha + \int_{\frac{\pi}{2}}^{\pi} \frac{dP_3}{d\Omega} \sin \alpha d\alpha \right)} \quad (4.21)$$

Where $dP_{\#}/d\Omega$ was the dipole radiation near the dielectric (core-medium) interface per unit solid angle, Ω , averaged over all dipole orientations. The subscript “#” corresponded to the different ranges of α between 0 and π .

Specifically, for 1: $0 \leq \alpha \leq \alpha_{\text{Crit}}$, 2: $\alpha_{\text{Crit}} \leq \alpha \leq \pi/2$ and 3: $\pi/2 \leq \alpha \leq \pi$, where

$\alpha_{\text{Crit}} = \sin^{-1}\left(\frac{1}{n_{\text{rel}}}\right)$, each corresponding $dP_{\#}/d\Omega$ was given by:

$$\frac{dP_1}{d\Omega} = 4n_{\text{rel}}^3 \cos^2 \alpha \left(\frac{1}{\left(\sqrt{1 - n_{\text{rel}}^2 \sin^2 \alpha} + n_{\text{rel}} \cos \alpha \right)^2} \right) \left(\frac{1}{\left(n_{\text{rel}} \sqrt{1 - n_{\text{rel}}^2 \sin^2 \alpha} + \cos \alpha \right)^2} \right) \cdot \text{constant} \quad (4.22)$$

$$\frac{dP_2}{d\Omega} = \frac{4n_{\text{rel}}^3 \cos^2 \alpha}{n_{\text{rel}}^2 - 1} \left(1 + \frac{2n_{\text{rel}}^2 \sin^2 \alpha - 1}{(n_{\text{rel}}^2 + 1) \sin^2 \alpha - 1} \right) \exp\left(-\frac{\delta}{d_p}\right) \cdot \text{constant} \quad (4.23)$$

$$\frac{dP_3}{d\Omega} = \left(2 + r_{\perp}^2 + r_{\parallel}^2 + 2(r_{\perp} - r_{\parallel} \cos 2\alpha) \cos(2n_m \frac{2\pi}{\lambda} \delta \cos \alpha) \right) \cdot \text{constant} \quad (4.24)$$

Where the Fresnel coefficients are $r_{\perp} = \frac{\cos \alpha + \sqrt{n_{\text{rel}}^2 - \sin^2 \alpha}}{\cos \alpha - \sqrt{n_{\text{rel}}^2 - \sin^2 \alpha}}$ and

$r_{\parallel} = \frac{n_{\text{rel}}^2 \cos \alpha + \sqrt{n_{\text{rel}}^2 - \sin^2 \alpha}}{n_{\text{rel}}^2 \cos \alpha - \sqrt{n_{\text{rel}}^2 - \sin^2 \alpha}}$, and “constant” could eventually be ignored in Equation (4.21).

From F_{abs} , a more applicable form was next derived for use in Equation (4.17). Adopting relationships described in [204], an equation describing f_{abs} was acquired:

$$\begin{aligned} f_{\text{abs}} &= \frac{\int_0^{\infty} P_{\text{abs}} d\delta}{I_0(\lambda)} = \frac{\int_0^{\infty} F_{\text{abs}} c\varepsilon(\lambda) I_0(\lambda) \cos \alpha d\delta}{I_0(\lambda)} \\ &= c\varepsilon(\lambda) \cos \alpha \int_0^{\infty} F_{\text{abs}} d\delta \end{aligned} \quad (4.25)$$

Similarly, Equation (4.17) encompassed a summed form of S_{ray} , specifically $I_{\text{Fl,norm}}$. Further to which, as a fraction of I_0 is absorbed after each reflection, $I_{\text{Fl,norm}}$ was not a mere multiplication of S_{ray} by its total number of occurrence, given by N_{Refl} . Instead, it could only be expressed in the summations of successive absorptions given by a geometric progression. Each launched ray could undergo either the Top-Btm or intra-core reflection regime as depicted in Figure 4.2. Separate summative descriptors were thus derived, with top-and-bottom reflections ($\Sigma S_{\text{ray,TopBtm}}$) indicating the former and bottom-only

reflections ($\Sigma S_{\text{ray,BtmONLY}}$) depicting the latter. The derivation is detailed as follows.

Figure 4.5. Schematics of launched rays (in blue) (a) reflected off both top and bottom core-medium interfaces and (b) only off the bottom interface in intra-core reflections. Collected fluorescence emission (S_{ray}) for each point of reflection was also indicated in bluish green. Corresponding initial and remaining input intensities (I_0) were similarly indicated.

From the examples illustrated in Figure 4.5, a series of $I_0^{\#}$ and corresponding $S_{\text{ray}}^{\#}$ were expressed in terms of f_{abs} , with its progression indexed by “#”. f_{abs} was further differentiated into their respective Top ($f_{\text{abs,Top}}$) and Btm ($f_{\text{abs,Btm}}$) occurrences. These are summarized in the following tables.

Table 4.2. I_0 and S_{ray} for the Top-Btm reflection regime illustrated in Figure 4.5a.

I_0	S_{ray}
$I_0^0 = I_0^0$	$S_{\text{ray}}^0 = S_{\text{ray,Top}}$
$I_0^1 = I_0^0(1 - f_{\text{abs,Top}})$	$S_{\text{ray}}^1 = S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}})$
$I_0^2 = I_0^1(1 - f_{\text{abs,Btm}}) = I_0^0(1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}})$	$S_{\text{ray}}^2 = S_{\text{ray,Top}}(1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}})$
$I_0^3 = I_0^2(1 - f_{\text{abs,Top}}) = I_0^0(1 - f_{\text{abs,Top}})^2(1 - f_{\text{abs,Btm}})$	$S_{\text{ray}}^3 = S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}})^2(1 - f_{\text{abs,Btm}})$
$I_0^4 = I_0^3(1 - f_{\text{abs,Btm}}) = I_0^0(1 - f_{\text{abs,Top}})^2(1 - f_{\text{abs,Btm}})^2$	$S_{\text{ray}}^4 = S_{\text{ray,Top}}(1 - f_{\text{abs,Top}})^2(1 - f_{\text{abs,Btm}})^2$
$\vdots$	$\vdots$

Table 4.3. I_0 and S_{ray} for the intra-core reflection regime illustrated in Figure 4.5b.

I_0	S_{ray}
$I_0^0 = I_0^0$	$S_{\text{ray}}^0 = S_{\text{ray,Btm}}$
$I_0^1 = I_0^0(1 - f_{\text{abs,Btm}})$	$S_{\text{ray}}^1 = S_{\text{ray,Btm}}(1 - f_{\text{abs,Btm}})$
$I_0^2 = I_0^1(1 - f_{\text{abs,Btm}})^2$	$S_{\text{ray}}^2 = S_{\text{ray,Btm}}(1 - f_{\text{abs,Btm}})^2$
$\vdots$	$\vdots$

The subsequent summation of S_{ray} values, with consideration of the corresponding N_{Refl} at the Top ($N_{\text{Refl,Top}}$) and Btm ($N_{\text{Refl,Btm}}$) interfaces, gave:

$$\begin{aligned}
 \sum S_{\text{ray,TopBtm}} &= S_{\text{ray,Top}} + S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}}) \\
 &\quad + S_{\text{ray,Top}}(1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \\
 &\quad + S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}})^2(1 - f_{\text{abs,Btm}}) \\
 &\quad + S_{\text{ray,Top}}(1 - f_{\text{abs,Top}})^2(1 - f_{\text{abs,Btm}})^2 \\
 &\quad + S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}})^3(1 - f_{\text{abs,Btm}})^2 \\
 &\quad \vdots \\
 &\quad + S_{\text{ray,Top}} \left((1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \right)^{N_{\text{Refl,Top}} - 1} \\
 &\quad + S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}}) \left((1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \right)^{N_{\text{Refl,Btm}} - 1} \\
 &= S_{\text{ray,Top}} \frac{1 - \left((1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \right)^{N_{\text{Refl,Top}}}}{1 - \left((1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \right)} \\
 &\quad + S_{\text{ray,Btm}}(1 - f_{\text{abs,Top}}) \frac{1 - \left((1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \right)^{N_{\text{Refl,Btm}}}}{1 - \left((1 - f_{\text{abs,Top}})(1 - f_{\text{abs,Btm}}) \right)} \tag{4.26}
 \end{aligned}$$

$$\begin{aligned}
 \sum S_{\text{ray,BtmONLY}} &= S_{\text{ray,Btm}} + S_{\text{ray,Btm}}(1 - f_{\text{abs,Btm}}) + S_{\text{ray,Btm}}(1 - f_{\text{abs,Btm}})^2 + \dots \\
 &\quad + S_{\text{ray,Btm}}(1 - f_{\text{abs,Btm}})^{N_{\text{Refl,Btm}} - 1} \\
 &= S_{\text{ray,Btm}} \frac{1 - (1 - f_{\text{abs,Btm}})^{N_{\text{Refl,Btm}}}}{1 - (1 - f_{\text{abs,Btm}})} \tag{4.27}
 \end{aligned}$$

The further summation of Equation (4.26) and (4.27) for all λ hence gave the required $I_{\text{Fl,norm}}$ per launched ray:

$$I_{\text{Fl,norm}} = \sum_{\lambda} \left(\sum S_{\text{ray,TopBtm}} + \sum S_{\text{ray,BtmONLY}} \right) \tag{4.28}$$

Likewise, I_{rem} at λ_{ems} and λ_{exc} could be expressed in a similar manner:

$$1 - f_{\text{abs,ems}} = \left(1 - f_{\text{abs,Top}}(\lambda_{\text{ems}})\right)^{N_{\text{Refl,Top}}} \left(1 - f_{\text{abs,Btm}}(\lambda_{\text{ems}})\right)^{N_{\text{Refl,Btm}}} \quad (4.29)$$

$$1 - f_{\text{abs,exc}} = \left(1 - f_{\text{abs,Top}}(\lambda_{\text{exc}})\right)^{N_{\text{Refl,Top}}} \left(1 - f_{\text{abs,Btm}}(\lambda_{\text{exc}})\right)^{N_{\text{Refl,Btm}}} \quad (4.30)$$

Note that Equations (4.18) to (4.30) became invalid should the launched ray experience refraction at the core-medium interface. Failure in propagating through the entire L_{PCF} , resulted in no remaining excitation being collected at the output end (i.e. $I_{\text{rem}} = 0$). This reduced the $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ ratio in Equation (4.17) to a simple ratio between the $f_{\text{Fl},\lambda}$ at λ_{ems} and λ_{exc} :

$$\frac{I(\lambda_{\text{ems}})}{I(\lambda_{\text{exc}})} = \frac{I_{\text{Fl,norm}}(\lambda_{\text{ems}})}{I_{\text{Fl,norm}}(\lambda_{\text{exc}})} = \frac{f_{\text{Fl,lems}}}{f_{\text{Fl,lexc}}} \quad (4.31)$$

4.4.3.3 Definitions for algorithm

The above discussed were converted into an algorithm in MATLAB and employed in the computation of $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for a range of CF concentrations. The employed definitions and parameters are summarized in Table 4.4 below.

Table 4.4. Definitions and parameters used in ray-trace algorithm for the computation of $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$.

Type	Values
PCF definitions	$D_{\text{core}} = 10.0 \text{ } \mu\text{m}$; $D_{\text{def-core}} = 3.1 \text{ } \mu\text{m}$ $R_{\text{bend}} = 12.5 \text{ mm}$; $L_{\text{PCF}} = R_{\text{bend}} \times \pi$ $n_{\text{co}} = 1.4629$ (fused silica at $\lambda = 490 \text{ nm}$) [205]
Fluorescent dye definitions	$c = [\text{CF}] = 0 - 100 \mu\text{M}$ (at $10 \mu\text{M}$ intervals) $\lambda_{\text{exc}} = 490 \text{ nm}$; $\lambda_{\text{ems}} = 510 \text{ nm}$ $\varepsilon(\lambda)$ was obtained experimentally $\Phi_{\text{Fl}} = 0.5$ (arbitrarily determined) $f_{\text{Fl},\lambda_{\text{ems}}} = 0.0189$ (CF in pH9 Tris, fluorophores.org) $f_{\text{Fl},\lambda_{\text{exc}}} = 0.00249$ (CF in pH9 Tris, fluorophores.org) $n_{\text{m}} = 1.3373$ ($20.0 \text{ } ^\circ\text{C}$ Water at $\lambda = 490 \text{ nm}$) [206] $I_0(\lambda)/I_0(\lambda_{\text{exc}})$ was obtained from Tris buffer-filled PCF
Ray parameters	$\lambda = 400 - 600 \text{ nm}$ $\text{Lay}_{\text{Tot}} = 101$; $t_{\text{Lay}} = D_{\text{core}}/\text{Lay}_{\text{Tot}}$

$$\begin{aligned}
xPos &= 0 - D_{\text{core}} \text{ (at intervals of } t_{\text{Lay}}) \\
yPos &= 0, t_{\text{Lay}}/2 - (D_{\text{core}}/2 - t_{\text{Lay}}) \text{ (at intervals of } t_{\text{Lay}})^* \\
\theta_{\text{Crit}} &= \sin^{-1}(n_m/n_{\text{co}}) = 66.1^\circ \\
\theta_{\text{START}} &= \theta_{\text{Crit}} - (\theta_{\text{Crit}} + 0.09^\circ) \text{ (at intervals of } 0.01^\circ), \\
&\quad (\theta_{\text{Crit}} + 0.1^\circ) - (\theta_{\text{Crit}} + 0.9^\circ) \text{ (at intervals of } 0.1^\circ), \\
&\quad (\theta_{\text{Crit}} + 1.0^\circ) - 88.0^\circ \text{ (at intervals of } 1.0^\circ), \\
&\quad 88.0^\circ - 89.9^\circ \text{ (at intervals of } 0.1^\circ) \\
\psi &= 0^\circ - 170^\circ \text{ (at intervals of } 10^\circ)^*
\end{aligned}$$

** It should be noted that yPos and ψ were only calculated for half the true span due to symmetry about the xPos axis.*

Considering the varied intervals for the ray parameters listed in Table 4.4, a corresponding assortment of weightage factors were consequently applied. This ensured that $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for each launched ray was normalized to every other ray, regardless of the dissimilarity of its intervals. Generally, this entailed the multiplication of computed $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ values by the weightage factor(s) corresponding to the ray parameter(s) it coincided with, as summarized in table below.

Table 4.5. Weightage factors for ray parameters possessing dissimilar intervals.

Ray parameter	Weightage factor
$yPos = 0$	0.5
$\theta_{\text{START}} = \theta_{\text{Crit}} - (\theta_{\text{Crit}} + 0.09^\circ)$	0.01
$\theta_{\text{START}} = (\theta_{\text{Crit}} + 0.1^\circ) - (\theta_{\text{Crit}} + 0.9^\circ)$	0.1
$\theta_{\text{START}} = 88.0^\circ - 89.9^\circ$	0.1

4.5 Results and Discussion

Results from the ray-tracing model were first contrasted with that from experiments. This was performed for both the dcPCF and its solid core counterpart. Following which, the various computed components were individually analyzed to relate and substantiate any observed trends or peculiarities.

4.5.1 In-fiber fluorescence spectroscopy of carboxyfluorescein

4.5.1.1 Defected core PCF

Figure 4.6. Comparison between calculated (solid line) and experimental (dotted line with crosses) values of $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for dcPCF under both bent (in blue) and straight (in red) orientations.

Comparison of the plots for the bent dcPCF in Figure 4.6 (in blue), showed good agreement between the ray-tracing model's computed $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ and that obtained experimentally. This was however only valid for low enough concentrations of CF, where the assumption of an n_m equivalent to water's holds. As depicted in the plot, deviation between the two began at a concentration of 80 μM .

Computed results for the straight orientation, on the other hand, were performed for a varied range of θ_{START} . This was based on the numerical aperture calculation described in [207], that constrained θ_{START} to begin from 85.7°,

more closely concurring with its single-mode operation. This resulted in the slower rate of change in $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ and reflected a lower sensitivity.

4.5.1.2 Solid core PCF

Figure 4.7. Comparison between calculated (solid and dashed lines) and experimental (dotted line with crosses) values of $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for scPCF under both bent (in blue) and straight (in red) orientations. Note that the blue dashed line represented a computation of $R_{\text{bend}} = 12.5$ mm with an adjusted θ_{START} range of 67.1° to 89.9° .

The model was similarly applied for the scPCF studied in Chapter 3 for refractive index measurements. This was performed with identical definitions and parameters as listed in Table 4.4, with the exception of D_{core} and $D_{\text{def-core}}$ that were set to $13.8 \mu\text{m}$ and $0 \mu\text{m}$ respectively. In this case, however, the calculated results differed drastically from the experimental data as shown in Figure 4.7. It only tended towards the latter upon a shortening of its θ_{START} span. Specifically, commencing θ_{START} from 67.1° , instead of the original 66.1° (θ_{crit}). Interestingly, back calculating n_m from $\theta_{\text{crit}} = 67.1^\circ$, gave a larger refractive index of 1.3474, in contrast to that of water. This larger n_m could however be attributed to the larger effective refractive index proximal to the PCF core, relative to the refractive index of the air holes' contents. Upon further inspection of the scPCF's cross-section (shown in Figure 3.2), the more significant presence of the fused silica material between said air holes does concur with the above deduction. Essentially, this was due to an assumption-linked limitation, where the PCF core was assumed to be a cylinder with the medium forming a concentric and continuous layer around it. In short, an

increased presence of the inter-air hole (fused silica) regions degraded the accuracy of said assumptions and hence reduced the viability of the model.

On the other hand, similar numerical aperture calculations set the θ_{START} range to begin from 87.6° , which was employed in the computation for the straight configuration as shown by the red solid line in Figure 4.7.

4.5.2 In-fiber light-fluorophore interactions

4.5.2.1 Comparison between defected core and solid core PCFs

Figure 4.8. Breakdown of calculated components for bent dcPCF (in blue) and scPCF (in grey) at $\psi = 0^\circ$ with respect to (a, b, c) θ_{START} and (d, e, f) CF concentration. Solid and dotted lines for (c) and (f) corresponded to that at λ_{ems} and λ_{exc} .

In general, the contrast between the various computed components revealed broad similarities in trends as well as notable differences between and within the two PCFs.

First, from Figure 4.8a, it was observed that a smaller θ_{START} resulted in a higher N_{Refl} . This was essentially a result of a smaller θ_{START} leading to a smaller α , which hence brought about a shorter L_z and thus more frequent reflections. Comparing between the PCFs revealed a much higher N_{Refl} for the defected core, a consequence of its more confined volume of propagation. This stemmed from the smaller D_{core} and presence of a hollow center. Essentially,

both factors restricted propagation in the x-direction, resulting in a shorter L_z . On the other hand, the initial jump in N_{Refl} depicted in the blue curve, corresponded to a lost in launched rays due to refraction. This was consequent of a small θ_{START} leading to a α smaller than α_{Crit} , hence the ray not satisfying the conditions of TIR. Since α_{Crit} was calculated from a $n_{\text{co,bent}}$ -dependent n_{rel} , the smaller $n_{\text{co,bent}}$ found at the inside of the bend (positive x-direction) resulted in a larger α_{Crit} that made the TIR criteria more difficult to meet. Therefore, despite the ray bending away from the normal after each layer-layer interface as it propagated in the positive x-direction, its α at the core-medium interface still failed to be sufficiently large.

The effects of N_{Refl} proceeded to dominate in $I_{\text{Fl,norm}}$ (via Equations (4.26) and

(4.27)) and $1-f_{\text{abs}}$ (Equations (4.29) and (4.30)), where both scaled to the power of it. This was reflected in the non-linear changes presented in Figure 4.8b and c, correspondingly. Specifically, $I_{\text{Fl,norm}}$ is large while $1-f_{\text{abs}}$ is small at higher N_{Refl} values. Essentially, these implied that fluorescence emission and absorption were more significant in the dcPCF due to its higher N_{Refl} . Likewise, the eventual behaviors of $I_{\text{Fl,norm}}$ and $1-f_{\text{abs}}$ were still governed by effects of N_{Refl} as shown by non-linear curves in Figure 4.8e and f, correspondingly. This was despite CF concentrations just linearly scaling S_{ray} and f_{abs} in Equations (4.18) and (4.25) respectively.

Lastly, the difference between the $1-f_{\text{abs}}$ plots in Figure 4.8c and f for λ_{ems} (solid) and λ_{exc} (dotted) was due to the larger value of ε for the latter, which resulted in lower-positioned λ_{exc} curves. This translated to a more rapidly decreasing $1-f_{\text{abs,exc}}$ value at higher CF concentrations, which coupled with the inherently smaller $I_{\text{Fl,norm}}(\lambda_{\text{exc}})$ resulted in an exponentially increasing $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ as described by Equation (4.17).

Comparison of the two PCFs highlighted two primary advantages for dcPCF: (1) higher sensitivity and (2) higher repeatability. The significantly higher N_{Refl} values accounted for dcPCF, as substantiated by its dominating influence on $I_{\text{Fl,norm}}$ and $1-f_{\text{abs}}$ that translated to increased amounts fluorescence emission and absorption. On the contrary, the lower repeatability in scPCFs were deduced to

be a result of its lower radial homogeneity, consequent of the more significant presence of fused silica in the air hole array surrounding its core. This implied that upon bending, the x' -axis (axis that defined the direction of bending) had a much higher probability of crossing a fused silica segment (found between two air holes), instead of a fluorescent dye-filled air hole. For the same reason, it extended to explain why the ray-tracing model produced a less coherent fit for the scPCF. Nevertheless, the obvious difference between the bent and straight configurations was still observed for both PCFs, with the former being the preferred option for fluorospectroscopy.

4.5.2.2 Convergence study on mesh density

Figure 4.9. $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for bent dcPCF calculated with various mesh densities defined by $\text{Lay}_{\text{Tot}} = 21$ (grey dotted line), 51 (black dashed line) and 101 (blue solid line).

A study was also conducted on the effects of mesh density, which was described to approximately equivalent to $\text{Lay}_{\text{Tot}}^2$. As would be expected, increasing the mesh density via a larger Lay_{Tot} , brought the calculated $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ curve closer to the experimentally obtained results as observed in Figure 4.9. This was consequent of the concomitant increase in N_{Ref} as Lay_{Tot} was raised, which as established in the earlier section is a dominant factor of both the extent of absorption and fluorescence generation. This increase in N_{Ref} was attributed to the cylindrical structure of the PCF core being more accurately approximated by the cuboidal layers as the mesh density increased. Intuitively, this could be understood as a result of the larger t_{Lay} (for smaller Lay_{Tot}) causing the point of reflection at the core-medium interface to reside

further from the actual limits of the PCF core. This increased the volume for propagation, eliciting a larger L_z and hence a correspondingly smaller N_{Refl} . This was a result of the discretization method, where as highlighted at the end of Section 4.4.3.1 necessitated that reflections occur only at the edges of each cuboidal unit. Nevertheless, from the sufficiently low discrepancy observed, a Lay_{Tot} of 101 was considered large enough as to not incur additional unrequired computations.

4.5.2.3 Discussion on arbitrarily determined fluorescence quantum yield

Figure 4.10. $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for bent dcPCF calculated with $\Phi_{\text{FI}} = 0.1$ (grey dotted line), 0.5 (blue solid line) and 0.9 (black dashed line).

Fluorescein and its derivatives are sensitive to pH changes [208, 209]. This sensitivity results in corresponding changes to its Φ_{FI} , where a reduction is generally created at lower pH values. Although the typical Φ_{FI} under the experiment's buffered conditions has been reported to be 0.93 [208, 210], a deviation from the experimental results was observed (as shown by the black-dashed curve in Figure 4.10). Only upon an arbitrary reduction to 0.5, did the model-derived results concur with the experimental. This reduction could be attributed to the lower pH of the region proximal to the air hole surface, a result of counter-ion accumulation on the silica surface [211].

As illustrated in Figure 4.10, smaller Φ_{FI} values led to a steeper $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ curve. This was a result of the denominator ($I(\lambda_{\text{exc}})$), decreasing more rapidly with increasing CF concentrations. Specifically, at low Φ_{FI} values, the Φ_{FI} -

dependent $I_{\text{Fl,norm}}$ components in Equation (4.17) became more negligible, while the remaining $1-f_{\text{abs}}$ components consequently gain dominance. Since the denominator depicted intensity changes at the wavelength of maximal absorption, it's $1-f_{\text{abs}}$ would correspondingly be smaller than that in the numerator. Therefore, a higher $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ results.

4.5.2.4 Computational study of defected core diameters and bending radii

Further studies on $D_{\text{def-core}}$ and R_{bend} were also made using the model. All $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ curves were computed with the same $I_0(\lambda)$ from the dcPCF.

Figure 4.11. (a) N_{Refl} at $\psi=0^\circ$ and (b) $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for bent dcPCF calculated with $D_{\text{def-core}} = 0 \mu\text{m}$ (grey dotted line), $1.55 \mu\text{m}$ (blue solid line) and $3.1 \mu\text{m}$ (black dashed line) for $D_{\text{core}} = 10 \mu\text{m}$ and $\text{Lay}_{\text{Tot}} = 51$.

For a fixed D_{core} of $10 \mu\text{m}$, decreasing the $D_{\text{def-core}}$ resulted in a reduced sensitivity. This is illustrated in Figure 4.11b, where the curves become more gradual as $D_{\text{def-core}}$ decreases. This trend is consistent with the earlier conclusion that a defected core results in a more confined volume of propagation. As shown in Figure 4.11a, N_{Refl} decreases with the decreasing presence of a hollow center. Consequently, as N_{Refl} is the dominant factor influencing the change in $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$, its significance determines the sensitivity. Although it is observed that a larger $D_{\text{def-core}}$ results in a higher sensitivity, the trend would only apply if the microstructure were capable of supporting light guidance via TIR. When $D_{\text{def-core}}$ becomes too large, the dcPCF is transformed into a hollow

core PCF (shown earlier in Figure 2.1), where light is guided via PBG guidance.

Figure 4.12. (a) N_{Refl} at $\psi=0^\circ$ and (b) $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ for bent dcPCF calculated with $R_{\text{bend}} = 10.0$ mm (grey dotted line), 12.5 mm (blue solid line) and 15.0 mm (black dashed line) for $L_{\text{PCF}} = 12.5 \times \pi$ mm and $\text{Lay}_{\text{Tot}} = 51$.

In studies involving various bending radii, a fixed L_{PCF} of $12.5 \times \pi$ mm was used. This was to maintain a constant interaction length for a fair comparison. Results in Figure 4.12 show the expected relation between N_{Refl} and $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$, where the lower N_{Refl} for $R_{\text{bend}} = 10.0$ mm resulted in its lower sensitivity. It was noted that a smaller R_{bend} resulted in smaller α values. This increased the extent of light-fluorophore interactions per reflection, as defined by S_{ray} and f_{abs} in Equations (4.18) and (4.25) respectively. However, smaller α values did not result in the expected increase of N_{Refl} . This was because a larger number of rays were refracted, as their α values were below the required α_{Crit} for TIR. On the other hand, when R_{bend} was increased from 12.5 mm to 15.0 mm, no significant differences were observed. This was a result of a similar set of α values and extent of refraction. Nevertheless, it is possible that the range of θ_{START} in $R_{\text{bend}} = 15.0$ mm has to be shortened. This correction would make the ray-trace more closely resemble that of single-mode operation, as was performed for straight lengths of PCF in Section 4.5.1. In the regard of practicality, a smaller R_{bend} would be more ideal considering the benefits of compactness and faster rates of fluidic manipulation.

4.6 Conclusion

The developed ray-tracing model and accompanying ratiometric method was demonstrated to be effective in the computation of in-fiber fluorescence spectroscopy for index-guiding PCFs – solid core and defected core.

The ray-trace was attained via a simplification of said PCFs' cores into cylinders, followed by a tracking of individual ray trajectories as they axially-traversed the PCF via TIR. The consequent light-fluorophore interactions at each point of reflection were further accounted through calculations of absorption and collectable emission. This enabled the derivation of a ratiometric relation between the intensities at wavelengths that marked the peak emission and absorption, described by $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$. This dimensionless ratio not only mitigated the effects of intensity fluctuations, but also more importantly enabled the platform to operate under a filterless regime and thus retain its all-fiber configuration and low cost.

Comparison between the experimental and model-derived values validated the described model, particularly for the dcPCF. A limitation of the model was also noted. In particular, the assumption of PCFs' cores as cylinders was only appropriate when there is a sufficiently low presence of silica in the core-adjacent layer of air holes. Further analysis of the model, revealed N_{Refl} as the dominant factor in influencing the rate of change in $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$. Consequently, the significantly higher N_{Refl} values for the dcPCF – a result of its more confined volume of propagation – substantiated its superiority over its solid core counterpart. In addition, the absence of small launch angles also resulted in a correspondingly lack of high N_{Refl} components. This was reflected in the lower sensitivities observed in the straight configurations and the scPCFs, where θ_{START} was deduced to have commenced from larger angles.

In conclusion, a ray-tracing model and method of ratiometry were developed to enable in-fiber fluorospectroscopy with minimal instrumentation. This method of fluorospectroscopy would provide a means of characterizing PCF contents based upon fluorophore concentrations. The fundamental understanding of light-fluorophore interactions gained here would also facilitate subsequent exploitation in photochemical processes.

5

In-fiber Photo-immobilization of Bioactive Surfaces

5.1 Introduction

This chapter would demonstrate the formation of bioactive surfaces within PCFs. By exploiting in-fiber light-fluorophore interactions, studied in the preceding chapter, a photochemical method would be used. This would likewise be performed using the devised optofluidic platform. The earlier developed method of in-fiber fluorospectroscopy would be used in the characterization of the bioactive surface. This in-fiber bioactive surface would provide a foundation for the construction of biomolecular assemblies within PCFs, as would studied in the next chapter.

5.2 Background

Bioactive surfaces are surfaces engineered to interact specifically with biomolecules [212-214]. These surfaces have been demonstrated to be efficient in the attachment and capture of biomolecules. This has enabled them to be employed in a myriad of applications such as directed cell-growth [215], tissue engineering [216], improved biocompatibility of implants [217, 218], chromatography [219, 220], filtration [221] and biosensing [222, 223]. Exploiting the selectivity and the localization of the interactions at the surface, a notable form of biosensing is that in surface-specific optical biosensors.

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5.2.1 Surface-specific optical biosensors

The immobilization of biomolecules onto optical waveguide surfaces conforms to the localization in bioactive surfaces, where light-matter interactions only occur within several hundred nanometers from the waveguide surface. Two schemes of operation prevail, namely fluorescence-based detection [79] and label-free detection [80]. The former, as its name suggests, entails the labeling of the interacting molecules with fluorescent tags, which hence serve as the targets for optical probing. In contrast, label-free methods do not require any prior tagging, instead they often operate on the detection of changes in refractive index, absorption and Raman scattering.

Optical fibers, boasting the advantages of miniaturization, flexibility and extended interaction lengths, have stood out among the various forms of optical waveguides as the ideal substrate and have been extensively reviewed [224-228]. However, the conventional immobilization of bioactive molecules on their outer surfaces entailed the incorporation of flow or sample cells [229, 230], which essentially overshadow the miniaturization offered. On the other hand, as highlighted in the earlier chapters, PCFs intrinsically offer a self-contained system. This comprised microfluidic channels adjacent to a source of guided light that enabled light-matter interactions to occur within a mere 125 μm thick strand of fiber.

5.2.2 In-fiber bioactive surfaces

The further miniaturization offered by PCFs have hence seen to the development of several in-fiber immobilizations of bioactive surfaces. These methods involved the negatively charged in-fiber silica surfaces coupling to a base layer of anionic polyelectrolytes [68, 139] or silane molecules [138, 231, 232] via electrostatic coupling and chemical condensation, correspondingly. Successive layers of other alternately charged polyelectrolytes may also follow in the former. Functional groups on polyelectrolytes and silanes thus provide sites for the subsequent covalent attachment of biomolecules. Nevertheless, the listed methods were extremely elaborate, involved hazardous chemicals and often required the separate activations of the surface and bioactive element.

5.2.3 Adopting a photochemical method

Notably, there have also been literature reporting a photochemically induced functionalization with biomolecules in microfluidic channels [233] and on planar substrates [234-238]. Of these methods, several comprised entirely of biomolecules [233-236] and involved a simple two-step protocol: (1) protein passivation and (2) photobleaching of a biotin-conjugated fluorophore – i.e. fluorescein in biotin-4-fluorescein (B4F). The photobleaching process comprised the radicalization of fluorescein and its consequent binding to the base layer of protein, hence immobilizing its attached biotin. This photo-immobilization process is simpler and does not involve any toxic chemicals or pre-activation reactions. Furthermore, with the recent demonstrations of in-fiber photochemical processes [31-35], an in-fiber light-induced immobilization method was deliberated to be highly feasible. The mechanisms for each step are detailed in the following subsections.

5.2.3.1 Protein passivation

The adsorption of proteins on solid surfaces posed advantages related to biocompatibility as well as disadvantages associated with reduced concentrations. The implications of this fundamental phenomenon across numerous fields have generated much interest and therefore have been outlined in several reviews [239-242]. The largely accepted mechanism that accounts for this adsorption process is a thermodynamic interpretation [243-245]. Three primary interactions were often discussed, namely electrostatic effects, dehydration effects and protein structural changes. Electrostatic and dehydration effects comprised changes to the surface properties of proteins, altering charges and redistributing hydrophilic/phobic groups respectively. These resulted from environmental changes such as pH and ionic strength or were dependent on the solid surface's hydrophilicity/phobicity, correspondingly. However, these interactions were inferred to not dominate as bovine serum albumin (BSA), a soft globular protein, was experimentally demonstrated to adsorb on electrostatically repelling surfaces [246] and on both hydrophilic and hydrophobic surfaces [242]. Moreover, the redistribution of groups experienced during dehydration effects essentially constituted to a structural change. Protein structural changes were hence deduced to be the

overall driving force. In particular, this restructuring entailed a loss in alpha-helix content [244], resulting in increased rotational mobility in the protein and consequently a larger conformational entropy – the driving force for adsorption. The ease and spontaneity of BSA adsorption onto silica surfaces was hence employed as a means of passivation in the methods of surface-functionalization. This thus minimized interactions between the silica surface and subsequently introduced chemicals.

5.2.3.2 Photochemistry of biotin-immobilization

Since its first demonstration in 2003 [234], an abundance of literature have reported their utilization of the photo-immobilization method as a means of functionalizing surfaces with biotin. Nevertheless, a mechanism of the process has yet to be detailed. A collation of literature discussing the interaction between B4F and BSA under optical excitation yielded the following.

The B4F to BSA coupling process fundamentally involved the fluorescein (Fl) component of B4F and a Tyrosine residue (Tyr) of BSA [247]. This chemical conjugation was enabled by their respective prior radicalizations. Following the photo-excitation (represented by $h\nu$) of Fl, it is radicalized during the electron transfer from its excited triplet state [97] (detailed in Section 2.2.5):

Here, S and T are the singlet and triplet ground states of Fl respectively, with * denoting their excited counterparts; R and X, represent the semi-reduced and -oxidized Fl radical (Fl•), correspondingly. These quenching reactions essentially create radicals that eventually become unable to fluoresce, hence photobleached [97]. The process of photobleaching hence constituted a prolonged duration of photo-excitation that resulted in Fl's eventual inability to participate in absorption and emission.

Accompanying this photobleaching, is the radicalization of Tyr [248] by photosensitized singlet oxygen ($^1\text{O}_2$), a consequent product when T^* is quenched by triplet oxygen ($^3\text{O}_2$) [98]:

The subsequent radical-radical reaction results in the formation of a Tyr-Fl bond and therefore a linkage between BSA and biotin:

It should also be noted that BSA has three other sites, isolated to peptide sequences, that non-specifically and weakly bind Fl [249]. Furthermore, as it was highlighted that the interaction was most favourable when BSA was in its globular form, its contributions to the immobilized biotin population could be ignored.

5.2.3.3 Streptavidin-biotin interactions

Possessing one of the highest affinities for non-covalent interactions, with dissociation constants (K_d) reported between 4×10^{-14} M [250] and 10^{-15} M [251, 252], the streptavidin-biotin pair has been extensively used [253, 254]. The ease of conjugating biotin or streptavidin with fluorophores, phospholipids, antibodies, antigens and genetic sequences has further led to a broad spectrum of biomolecular applications [254]. This coupled with the four biotin-binding sites on streptavidin would largely facilitate the formation of biomolecular assemblies or multiplexes.

5.3 Scope

This chapter focuses on: (1) a rigorous experimental description of the bioactive surface formation within PCFs, which comprised three main steps – protein passivation, biotin photo-immobilization and streptavidin-biotin conjugation (2) the spectral characterization and discussion of each step of the bioactive surface formation; (3) a quantification of the bioactive surface coverage density using the in-fiber fluorospectroscopy method discussed in Chapter 4 and [255], where the spectral data of surface-bound dye-tagged streptavidin was matched with model-derived calculations to determine its extent of coverage; (4) a study to determine the optimal protocol with considerations of repeatability and experimental feasibility.

5.4 Methodology

5.4.1 Materials

Bovine serum albumin (BSA), biotin-4-fluorescein (B4F), Atto 488-streptavidin (AttoSA) and phosphate buffered saline at pH 7.4 (PBS) were purchased from Sigma-Aldrich. All other chemicals were of reagent grade. PCFs were manufactured by Yangtze Optical Fibre and Cable Company Ltd.

5.4.2 Set-up for photo-immobilization and spectral acquisition

Figure 5.1. Configuration of the simplified platform. It comprised a 105 mm length of PCF bent at 12.5 mm coupled to an LED source (input) and a computer-linked spectrometer (output) at opposite ends. Each end was inserted into a FC fiber connector that was connected to a SMA905 fiber cable via a mating sleeve. Inset: SEM Micrograph of the employed PCF's microstructure. © 2015 IEEE

dcPCFs were cut to lengths of 105 mm and functionalized internally with biotin via a photochemical process. Reagents were infiltrated and flushed from lengths of PCFs via a customized 50 ml syringe pump system, external to the optofluidic platform. Each length of PCF had one end dipped into the respective reagents and the other within the syringe pump, at various pressures. Spectral acquisitions and photochemical procedures were, on the other hand, conducted using a simplified version of the platform (shown in Figure 5.1). Each length of PCF was stripped at both ends, of 10 mm of its polymer jacketing, and inserted into a FC fiber connector coupled to either the input or output SMA905 fiber

cables. Similar to the configuration in Chapter 4 the excitation input was a 2.3 mW 490 nm fiber-coupled LED (Thorlabs, M490F1), while the spectral output was acquired through a computer-linked spectrometer (Ocean Optics, Maya2000). Spectral acquisition was likewise performed at 80% of the LED's maximum power.

5.4.3 In-fiber bioactive surface formation

Figure 5.2. Schematics for in-fiber photo-immobilization of bioactive surfaces summarized into three key steps. (BSA: Bovine Serum Albumin; B4F: Biotin-4-fluorescein; pbB4F: photobleached B4F; SA: Streptavidin; AttoSA: Atto 488-conjugated SA)

In brief, formation of the in-fiber bioactive surface comprised three principal steps – (1) a prior BSA passivation of the air hole surfaces; (2) a photochemically-induced functionalization with biotin (primary bioactive surface); and (3) a conjugation of streptavidin (secondary bioactive surface). The procedure is detailed as follows:

Step 1. BSA passivation

- i. A fresh length of PCF was continuously infiltrated with 2% BSA* for a duration of 30 min. This was performed with a 30 μ l aliquot of the BSA solution, with the syringe pump drawn at 6 ml of pressure.
- ii. Flushing with distilled ultra-filtrated (DIUF) water was done promptly under 50 ml of drawn pressure for 15 min.

- iii. Steps (i) and (ii) were repeated, and the PCF's output spectrum post BSA passivation was recorded.

**2% BSA was prepared by dusting 10 mg of BSA onto 0.5 ml of DIUF water in a 2.0 ml centrifuge tube. The BSA was dissolved by gently rocking so as to minimize the formation of bubbles, and subsequently left under refrigeration for at least 30 min to equilibrate before use. Storage under refrigeration allowed for usage over a week. Aliquots were discarded after each two-cycle passivation.*

Step 2. Biotin-functionalization via photo-immobilization

- i. The earlier BSA-passivated PCF was infiltrated for 5 min with 77.6 μ M of B4F in 0.01 M of PBS*.
- ii. The B4F-filled PCF was next inserted into the platform for B4F photobleaching under maximum LED power for 10 min. The pre- and post-photobleaching spectra were likewise acquired.
- iii. Flushing was again performed with DIUF water for 10 min under 50 ml of drawn pressure.
- iv. Step (iii) was repeated to ensure maximal removal of unbound and excess B4F. Its corresponding output spectrum was again recorded.

**Stock B4F was prepared at a concentration of 3.88 mM by dissolving 5 mg of B4F in 2.0 ml of 0.05 M PBS. Prolonged storage was allowed under higher buffer concentrations. 5 μ l of the stock was aliquoted and diluted to 77.6 μ M in 0.01 M PBS with the addition of 20 μ l of DIUF water and 225 μ l of 0.01 M PBS. This diluted form, however, possessed a shelf life of only two weeks.*

Step 3. SA conjugation with immobilized biotin

- i. 1 mg/ml of AttoSA* was infiltrated into the biotin-functionalized PCF under 50 ml of drawn pressure for 5 min.
- ii. Flushing with DIUF water was immediately performed for 10 min under 50 ml of drawn pressure.
- iii. Step (ii) was repeated to similarly remove unbound AttoSA.
- iv. The PCF was next allowed to sit in the DIUF water for 15 min under no applied pressure, and its output spectrum was collected after.

**AttoSA was prepared by dissolving 1 mg of it in 1 ml of 0.01 M PBS. This was separated in to 50 μ l aliquots and frozen for prolonged storage. Thawed aliquots could be used for a maximum period of two weeks.*

5.4.4 Quantification of coverage density

Using the model detailed in Section 4.4.3 and [255], the bioactive surface's coverage density could be determined. Since the AttoSA coverage density was proportional to the concentration of its dye conjugate, Atto488, the former could be derived by means of in-fiber fluorospectroscopy. This was accomplished using the ratio between the intensities at the peak absorption and emission wavelengths of the Atto488 – $I(\lambda_{\text{Atto488,ems}})/I(\lambda_{\text{Atto488,exc}})$. From Step 3(iv) of Section 5.4.3, this ratio could be calculated and matched with the model-computed values to acquire a corresponding [Atto488]. The following lists the fluorescent dye definitions required for the computation.

Table 5.1. Fluorescent dye definitions used in ray-trace algorithm for the computation of $I(\lambda_{\text{Atto488,ems}})/I(\lambda_{\text{Atto488,exc}})$.

Fluorescent dye definitions
$c = [\text{Atto488}] = 0 - 50 \mu\text{M}$ (at 5 μM intervals)
$\lambda_{\text{Atto488,exc}} = 501 \text{ nm}$; $\lambda_{\text{Atto488,ems}} = 525 \text{ nm}$
$I_0(\lambda)/I_0(\lambda_{\text{Atto488,exc}})$ was obtained from water-filled BSA-passivated PCF
Acquired from ATTO-TEC GmbH (www.atto-tec.com) for Atto488 in water:
$\varepsilon(\lambda)$; $\Phi_{\text{Fl}} = 0.8$; $f_{\text{Fl}}(\lambda_{\text{Atto488,exc}}) = 0.00276$; $f_{\text{Fl}}(\lambda_{\text{Atto488,ems}}) = 0.0191$

Assuming each AttoSA possessed an average of two Atto488 molecules (reported by Sigma-Aldrich as 1 to 3 Atto488 per AttoSA), its concentration would effectively be half that of Atto488's. Further considering only a monolayer of AttoSA, an accurate approximation under conditions of specific binding, the coverage density (in μm^{-2}) could be derived as:

$$\begin{aligned}
\text{coverage density} &= \frac{\text{number of AttoSA}}{A_{\text{tot}}} \\
&= \frac{\frac{1}{2}[\text{Atto488}] \times N_A \times V_{\text{exc}}}{A_{\text{tot}}} \quad \left| \quad V_{\text{exc}} \approx A_{\text{tot}} \times d_p \right. \\
&= \frac{1}{2}[\text{Atto488}] \times N_A \times d_p
\end{aligned} \tag{5.1}$$

Here, V_{exc} is the excitation volume, approximated to be a thin layer (of thickness d_p) enveloping the PCF's core over a total surface area of A_{tot} . N_A is Avogadro's constant ($N_A = 6.022 \times 10^{23} \text{ mol}^{-1}$) and d_p is the penetration depth. This coverage density was then computed for each of the triplicate repetitions.

5.4.5 Optimization study

The extent of BSA passivation was varied to determine the optimal protocol for bioactive surface formation within dcPCFs. This was achieved by varying the number of BSA passivation cycles, described in Section 5.4.3 by Step 1(i) and (ii). This was performed for 0x (uncoated), 1x, 2x and 3x repetitions of BSA cycles. Each variation was performed in triplicate and the corresponding coverage densities were computed.

5.5 Results and Discussion

Spectral results collected at each step of the bioactive surface formation served as a means of characterization as well as an indicator on the success of the in-fiber coating. Next, the eventual bioactive surface coverage density was quantified using the method of in-fiber fluorospectroscopy discussed in Chapter 4. Lastly, results from the optimization study were discussed so as to deduce the optimal bioactive surface formation protocol to adopt in subsequent studies.

5.5.1 In-fiber spectral characterization of bioactive surface formation

This section would discuss the spectral results and characterization methods employed in each step. The following summarizes the different in-fiber surfaces resulting from experimental variations.

Figure 5.3. Summary of different in-fiber surfaces with the corresponding sections discussing them indicated in parenthesis. Experimental variations are likewise denoted.

5.5.1.1 BSA passivation

Figure 5.4. Normalized output spectra for Uncoated (black solid) and BSA-passivated (blue dotted) dcPCFs filled with DIUF water. Inset: Magnification of peak region with black arrows indicating the peaks. Experiment was done in triplicate.

The output spectrum of a DIUF water-filled BSA-passivated dcPCF was compared with its similarly filled and Uncoated counterpart. As shown in Figure 5.4, the presence of BSA has negligible spectral influence, with only an approximate +0.1 nm shift in the peak (depicted in the inset). This shift could be attributed to BSA's absorption in the UV-region [256], which hence encroached onto the left tail of the blue LED excitation. Regardless, since the BSA-passivated PCF's output spectrum served as the reference ($I_0(\lambda)/I_0(\lambda_{\text{Atto488,exc}})$) in computations, the mentioned spectral differences could simply be ignored.

As BSA effected no discernable spectral changes, it was difficult to spectrally characterize the amount of passivation at this step. Nevertheless, two experimental observations, namely (1) the extent of flushing and (2) the peak output intensity, provided indications to the absence or extent of choking – a common problem when performing in-fiber coating procedures. This was inferred from (1) a substantial flow of DIUF water through the PCF and (2) a sufficiently high output intensity, correspondingly.

5.5.1.2 Biotin-functionalization via photo-immobilization

Figure 5.5. Output spectra of BSA-passivated dcPCF filled with B4F. Spectra before and after the 10 min duration of photobleaching are represented in blue dotted and red dotted-dash lines respectively. Experiment was done in triplicate.

Upon infiltration with 77.6 μM B4F in 0.01 M PBS, a distinctive spectral change, pertinent to the presence of fluorophores, was observed. Specifically, the obvious hump between 500 and 525 nm of the blue-dotted plot in Figure 5.5 was representative of the fluorescence emission from the fluorescein component of B4F. Upon a prolonged 10 min excitation under maximum LED power, photobleaching resulted. This was similarly reflected by a decrease in the hump, corresponding to a decrease in emission due to photobleaching [97]. However, the concomitant decrease in absorption was not observed. This should have resulted in an increase in the remaining excitation represented by the left region of the spectrum, which was instead observed to have decreased. As biotin has been reported to not show UV absorption [257], this decrement could only be attributed to the surface scattering losses [258, 259] induced by increased irregularities on the in-fiber surfaces – a result of the photobleached B4F (pbB4F) binding randomly to the base layer of BSA. The biotin-functionalization hence creates the primary bioactive surface, simplified as BSA-pbB4F.

Figure 5.6. Output spectra of Uncoated dcPCF filled with B4F. Spectra before and after the 10 min duration of photobleaching are represented in black solid and red dotted-dash lines respectively. Experiment was done in triplicate.

On the other hand, the same B4F photobleaching process performed in an Uncoated dcPCF yielded contrasting results, as illustrated in Figure 5.6. Aside from the expected drop in fluorescence emission upon photobleaching, the remaining excitation likewise behaved in the predicted manner. In particular, the remaining excitation increased due to the lowered absorbing population of fluorescein, elicited from photobleaching. Additionally, Controls (performed without photobleaching) showed no spectral differences after the 10 min incubation of B4F within the PCFs. This substantiated that the spectral changes observed were solely a result of prolonged excitation.

Further comparing the pre-photobleaching output spectra, it could be observed that the fluorescence emission was less prominent in the BSA-passivated dcPCF. This was a consequence of the BSA passivation limiting the light-fluorophore interaction, a result of the surface-bound BSA occupying a fraction of the excitation volume. Accordingly, reduced absorption and fluorescence emission collection followed. The prominence of B4F's emission thus served as an indicator to the extent of BSA passivation. Additionally, from experimental observations, accompanying the less prominent emission was the choking of the PCF in subsequent steps. This was inferred to be a result of more BSA being

present, but instead of forming a monolayer on the in-fiber surfaces were adsorbed as aggregates [260, 261].

5.5.1.3 SA conjugation with immobilized biotin

Figure 5.7. Normalized (to intensity at $\lambda = 501$ nm) output spectra of dcPCFs with various in-fiber surfaces after flushing out an earlier infiltration of AttoSA. The Uncoated in-fiber surface is represented in black; while BSA-passivated surfaces with and without (Control) biotin-functionalization are indicated in red dotted-dash and blue dotted lines respectively. Experiment was done in triplicate.

Following the photochemically-induced biotin-functionalization, AttoSA was introduced for conjugation and hence formation of a secondary bioactive surface (BSA-pbB4F-AttoSA). The dye component, Atto 488, provided an additional means of characterizing and quantifying (detailed in the next section) the bioactive surface.

First, contrasting between the output spectra of dcPCFs with and without biotin-functionalization in Figure 5.7, a shoulder of varied prominence was observed beyond the wavelength of 500 nm. Since this shoulder corresponded to the emission of Atto 488, its prominence was indicative of the amount of AttoSA present. Although the biotin-functionalized dcPCF presented the highest shoulder, a result of AttoSA specifically binding to the immobilized biotin, considerable amounts of Atto 488 emission were also observed in the dcPCFs where biotin was absent. Since there were supposedly no binding sites for AttoSA in the Uncoated and Control (identically prepared with the

exception of photobleaching) dcPCFs, the detected AttoSA was attributed to its non-specific binding directly onto the in-fiber silica surface [262].

Further comparison of the plots in Figure 5.7 revealed the presence of a higher amount of AttoSA in the Uncoated dcPCF, relative to the Control (BSA-passivation only). This could be understood as the BSA-passivation limiting the amount of non-specific AttoSA binding to the silica surface due to its own occupancy of the in-fiber surface. Additionally, attempts made to reduce the extent of non-specific AttoSA binding via more BSA-passivation, almost always resulted in undesired choking.

5.5.2 Coverage density of bioactive surface

Figure 5.8. Calculated values of $I(\lambda_{\text{Atto488,ems}})/I(\lambda_{\text{Atto488,exc}})$ for dcPCF under the bent orientation using the water-filled BSA-passivated dcPCF as the reference (i.e. $[\text{Atto488}] = 0 \mu\text{M}$), and its polynomial fit is indicated by a red-dotted plot. Inset: Peak normalized output spectra for the reference (black solid) and a typical dcPCF flushed of its earlier infiltrated AttoSA (red dotted-dash), with intensities represented by I_0 and I respectively. The subscripts “ems” and “exc” refer to the wavelengths $\lambda_{\text{Atto488,ems}} = 525 \text{ nm}$ and $\lambda_{\text{Atto488,exc}} = 501 \text{ nm}$, correspondingly.

The $I(\lambda_{\text{Atto488,ems}})/I(\lambda_{\text{Atto488,exc}})$ values shown in Figure 5.8 were computed using the model detailed in Section 4.4.3 and [255]. Intensity ratios were also calculated for each output spectrum as indicated by $I_{\text{ems}}/I_{\text{exc}}$ in the inset. From the model-computed values, the corresponding $[\text{Atto488}]$ for each experimentally obtain ratio could be derived. With these $[\text{Atto488}]$ values, the extent of coverage by AttoSA was calculated from Equation (5.1), where a d_p of

76.46 nm was used. This d_p was an average of all the launched ray's d_p , computed from Equation (4.20), with consideration of the weightage factors listed in Table 4.5. From the triplicate repetition of the BSA-pbB4F-AttoSA formation, a coverage density of $616 \pm 59 \mu\text{m}^{-2}$ (2.1% surface coverage) was obtained from an intensity ratio of 0.610 ± 0.077 . The data and calculations are summarized in Table 5.2, below.

Table 5.2. Summary of experimentally obtained $I(\lambda_{\text{Atto488,ems}})/I(\lambda_{\text{Atto488,exc}})$ with corresponding calculations for [Atto488] and coverage density. Each set of results comprised a Biotin-functionalized and Control pair.

In-fiber Surface	Set	$\frac{I(\lambda_{\text{Atto488,ems}})}{I(\lambda_{\text{Atto488,exc}})}$	[Atto488] (μM)	Coverage Density (μm^{-2})
Biotin-functionalized	1	0.547	24.6	567
	2	0.696	29.6	682
	3	0.586	26.0	599
Control	1	0.401	18.9	436
	2	0.451	21.0	485
	3	0.392	18.6	427

However, this computed coverage comprised not only the biotin-bound AttoSA, but also included those bound non-specifically, as briefly discussed in Section 5.5.1.3. Employing an identical method of quantification, the extent of non-specific coverage by AttoSA was calculated. For each of the earlier repetitions, a Control was concurrently performed – all reagents and conditions were one and the same, but without photobleaching. These, on the other hand, were calculated to possess a coverage density of $449 \pm 31 \mu\text{m}^{-2}$ from an intensity ratio of 0.415 ± 0.032 . Similarly, the data and calculations are listed in Table 5.2.

Despite this considerable amount of non-specifically bound AttoSA, their effectiveness in functioning as a bioactive surface was expected to be severely

hindered by: (1) a one to five order reduction in SA's binding affinity to biotin upon its prior direct surface attachment [263, 264]; (2) steric hindrance resulting from (i) SA's "buried" position amongst the base layer of BSA and (ii) lost of biotin binding sites at the SA interface with silica and adjacent BSA; (3) a relatively weaker attachment of SA due to its potentially smaller footprint (SA native profile: 4.5 nm x 4.5 nm x 5 nm) in contrast to its BSA-bound counterparts (BSA native profile: 4 nm x 4 nm x 14 nm), resulting in easier dissociation under continuous shear forces from flushing. Therefore, it was appropriate to assume these non-specifically bound AttoSA were unable to provide enduring and stable biotin binding sites in the secondary bioactive surface. In addition, although a direct attachment of SA within a polymer MOF has been reported [81], its target biomolecule was a SA-antibody and interactions were hence very different from that of SA and biotin. Moreover, brief attempts made in directly attaching SA to the in-fiber surfaces were difficult to repeat and very often resulted in choking – two crucial experimental woes not discussed in literature.

It should also be highlighted that the reference spectrum used, was that of the water-filled BSA-passivated dcPCF. Although BSA-pbB4F was the in-fiber surface present prior to the introduction of AttoSA, its spectrum only differed marginally from that lacking the bound pbB4F (i.e. BSA-passivated only) – $I(\lambda_{\text{Atto488,ems}})/I(\lambda_{\text{Atto488,exc}}) = 0.221$ and 0.194 , correspondingly. Moreover, as the Control was only BSA-passivated, this reference could more accurately accounted for the non-specifically bound AttoSA. In addition, while Φ_{Fl} was arbitrarily determined for CF, as discussed in Section 4.5.2.3, the manufacturer-reported value for Atto 488 was used. This was with the further knowledge that the Atto 488 molecules conjugated to SA were not significantly altered with regards to their spectral properties, as reported by Sigma-Aldrich. Moreover, their conjugation to SA, would effectively shield the Atto 488 molecules from effects of environmental changes. These hence substantiated the non-arbitrary choice of Atto 488's Φ_{Fl} .

5.5.3 Optimization study

Figure 5.9. Calculated coverage densities of AttoSA for varying number of BSA cycles. In-fiber surfaces with and without (Control) biotin-functionalization are indicated by red diamonds and blue squares respectively. Note that the 0x BSA cycle corresponded to Uncoated in-fiber surfaces.

In order to reduce the extent of non-specifically bound SA highlighted in the previous section, an optimization study on BSA passivation was conducted. The effect of varying the number of BSA cycles is illustrated in Figure 5.9 by their resulting AttoSA coverage densities.

The blue-coloured plot represented the Control where no photo-immobilization of B4F was performed. The detected presence of AttoSA hence corresponded to the contributions of non-specifically bound SA. This non-specific binding was deduced to have stemmed from the interactions between SA and exposed (not covered by BSA) in-fiber silica surfaces. More repetitions of the BSA passivation were hence expected to increase the BSA coverage and consequently reduce these non-specific interactions. In accordance, the coverage density of AttoSA was observed to decrease with the increasing number of BSA cycles.

On the other hand, the red-coloured plot represented biotin-functionalized in-fiber surfaces. Here, the presence of AttoSA reflected the summation of both specifically and non-specifically bound SA. Accordingly, the former could be deduced from the difference between the red and blue plots. For the 1x to 3x

repetition of BSA cycles, no distinctive deviation in the amount of specific binding was observed. This was attributed to the effects of steric hindrance where biotin-bound SA reduced the accessibility of proximal biotin to free SA. In contrast, for the Uncoated (0x BSA cycle) in-fiber surface, the overlap of its blue and red data points indicated the absence of specific binding altogether. This was in accordance to the fact that no BSA was present for the immobilization of B4F. Additionally, the observation of a general decrease in SA coverage density could likewise be attributed to the earlier discussed reduction in non-specific binding.

Notably, the standard deviation in results was observed to decrease up to 2x repetition of the BSA cycle, and was ascribed to the BSA's increased uniformity. This uniformity of BSA was crucial to subsequent phases of the bioactive surface formation, accounting for the repeatability of results and reduced frequency of choking. On the contrary, an increased deviation was observed for the 3x repetition of the BSA cycle. This was inferred to be a result of BSA aggregate formation [260, 261], evident during the experiments as a frequent experience of choking. In sum, the 2x repetition of the BSA cycle was concluded to be most suitable.

5.6 Conclusion

A photochemical means was demonstrated to be efficient in the biotin-functionalization of the air hole surfaces within PCFs. This formed a primary bioactive surface that was subsequently conjugated with streptavidin to generate the secondary bioactive surface.

The method of in-fiber bioactive surface formation consisted of three main steps: (1) a protein passivation of the in-fiber surfaces with BSA; (2) the functionalization of this base layer of BSA via the binding of B4F, enabled by the photo-induced radicalization of its fluorescein conjugate and a Tyr residue in BSA; and (3) the conjugation of SA to the BSA-bound B4F. In comparison to other techniques, this was simpler and did not involve any toxic chemicals or tedious pre-activation reactions. During the process, each step was characterized spectrally and contrasted with its uncoated (in Steps 1 through 3) and non-biotin-functionalized (in Step 3) counterparts, substantiating the validity of the respective steps of coating.

The coverage density of SA was subsequently quantified by the earlier developed method of in-fiber fluorospectroscopy. This involved firstly the acquisition of experimental ratiometric values, corresponding to the intensity ratios between the peak emission and absorption of the fluorescent dye-conjugate of SA. Next, these ratiometric values were matched with the model-derived calculations to determine their respective SA concentrations and hence, coverage density. Observations of considerable amounts of non-specifically bound SA were also deliberated to possess negligible contributions as part of the bioactive surface. A further study on the optimal protocol for minimal non-specific binding was also made, with strong considerations for high repeatability and low frequency of choking.

In conclusion, the photo-immobilization of a bioactive surface was both experimentally demonstrated and quantified within dcPCFs. This would serve as a foundation for the subsequent construct of in-fiber biomolecular assemblies.

6 In-fiber Tethering of Liposomes

6.1 Introduction

This chapter would demonstrate and validate the tethering of liposomes within PCFs. The tethering of liposomes is enabled by the presence of in-fiber bioactive surfaces, as described in the previous chapter. Their successful immobilization would be verified using means of in-fiber fluorospectroscopy, and would require the loading of said liposomes with fluorescent dyes. Additional studies on the optical behaviour of these dye-loaded liposomes would also be performed outside PCFs to create a baseline. This chapter aims to explore the potential of using liposomes in bio-integrated photonic devices.

6.2 Background

Leveraging on the advantages of in-fiber light-matter interactions, a photochemically-induced functionalization of air hole surfaces within PCFs was demonstrated in Chapter 5 and [265]. The final conjugation of streptavidin formed a bioactive surface that provided three additional biotin binding sites [266]. Since it is considerably easy to biotinylate biomolecules – eg. antibodies, antigens, genetic sequences, fluorophores and phospholipids [254] – a host of biomolecules could be bound to the in-fiber bioactive surface. This further indicated the potential of immobilizing or even constructing biomolecular nano-

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architectures [267] within PCFs. Their integration would hence allow for optical probing and potentially the generation of optical effects, all of which could be performed within the microscopic dimensions of PCFs. An ideal nano-architecture to start with would be liposomes comprising biotinylated phospholipids. This choice was substantiated by their ease in fabrication as well as versatility in size and encapsulated contents.

6.2.1 Liposomes

Figure 6.1. Schematics of a unilamellar liposome. The detailed chemical structure of a naturally occurring phospholipid (L- α -phosphatidylcholine) highlights the hydrophobic and hydrophilic regions and their respective compositions.

Liposomes or lipid vesicles were first described in 1964 by Bangham and Horne [268]. They are vesicular structures consisting of self-assembled bilayers of phospholipids encasing an aqueous core, as shown in Figure 6.1. The ability to self-assemble stemmed from the amphiphilic nature of phospholipids, comprising a hydrophilic head and a hydrophobic tail. Each phospholipid comprised two fatty acid residues linked to a phosphorylated alcohol (choline is the alcohol in Figure 6.1) via a glycerol backbone. The degree of saturation (or hydrogenation) and length of the fatty acid residues determines the phase-transition temperature and fluidity of the formed bilayer [269]. Variation of the polar head group, on the other hand, determines its surface charge and thus governs membrane-membrane or -environment interactions [270, 271]. Two general structural properties allow for the classification of liposomes, namely size and lamellarity [272-275]. Large unilamellar vesicles (100 – 1000 nm with

single bilayer) have been of particular interest in research, primarily due to their relatively simpler preparation and better-characterized membranes.

Since such liposomes largely resemble naturally occurring membranes, they have been frequently utilized as membrane models [276-281]. This included the study of membrane permeability, dynamics and interactions as well as the mimicry of cellular environments for analysis of cellular events. On top of which, liposomes possess the capacity of confining water- and lipid-soluble substances in their cores and phospholipid bilayers respectively. This has enabled numerous applications in controlled drug [282-288] and dye [289, 290] delivery as well as gene therapy [291-293]. Research in these areas have essentially been encouraged by their low intrinsic toxicity, weak immunogenicity, bio-degradability and -inertness [294]. In addition to applications as transport vesicles, the isolated and controllable environments within their cores and bilayers have further enabled their use as nanoreactors [295] and bio-analytical systems [296]. To further facilitate the manipulation and study of liposomes and their contents, their immobilization on solid surfaces is often performed.

6.2.2 Surface-tethered liposomes

The tethering of liposomes primarily offered the additional control of localization for easier isolation and probing of interactions. This has enabled applications such as: (1) single biomolecule detection by the encapsulation of close to one molecule per liposome, where the immobilized liposome would allow for extended observations without the drawbacks of the molecule undergoing diffusion [297]; (2) chromatography for the study of membrane interactions with the passing solute [298-302]; (3) drug release (i) at site of biomedical implants to aid the initial wound healing response [303] or (ii) at the substrates of cell cultures for research purposes [304]; (4) two-dimensional arrays (i) for the study of membrane interactions and diffusion dynamics [305, 306] and (ii) simultaneous analysis of liposomes with varied contents [307, 308]; (5) nanoreactors for confined reactions and their subsequent isolation as individuals or groups, which could further be investigated via surface sensitive techniques [309]; (6) surface-specific biosensing of (i) changes within

liposomes [310] and (ii) gain or loss in the number of stacked liposomes [311] as well as (iii) serving as a means of signal amplification [115]. Amongst the host of listed applications, the two most prevalent methods of tethering liposomes were via complementary oligonucleotides and the biotin-avidin (or avidin-like) pair. The latter stood out as the more popular choice, likely due to its ease in conjugation with other biomolecules and lower cost.

6.2.3 Liposomes in optics

Despite the myriad of applications, liposomes have been less explored in the field of optics – particularly for their optical properties. Apart from serving as markers upon encapsulating fluorophores [289, 290, 312, 313], there have been no other reports of liposomes being applied in optics. Lipid-based constructs, on the other hand, have been demonstrated to be capable of forming optical diffraction gratings – lipid multilayers deposited on surfaces by dip-pen nanolithography in a periodic manner [314, 315].

Its inherent ability to scatter light [316, 317], however, has thus far been only utilized for its own characterization. Additionally, there is a compositional likeness of dye-loaded liposomes to dye-doped crystalline [318] and polymeric [319-322] nanoparticles that have been used in achieving random lasing. This combination of scatterer and laser gain medium, being likewise present in dye-loaded liposomes, indicated their potential as optical manipulators. Notably, there have also been remarkable demonstrations of biological lasing from green fluorescent protein (GFP)-expressing eukaryotic cells [323, 324]. The authors, however, highlighted the need for miniaturization of its external cavity in order to realize the concept *in vivo*. Liposomes being biological in nature implied minimal interference with cellular structures or activities as well as no biotoxicity. These therefore further substantiated the need for exploring the potential of liposomes in achieving lasing.

In order to facilitate this study, liposomes were tethered within PCFs. This combination of immobilization and optofluidic control offered the advantages of: (1) elimination of diffusive effects, which retained the same population of liposomes under excitation throughout the span of study; (2) easily changeable medium surrounding liposomes, without the need for external time-consuming

methods like size-exclusion chromatography and re-suspension after centrifugation; (3) integrated waveguide component, implying no need for external optical characterization. In addition, the utilized dcPCF possessed the further advantages of nanoliter volume requirements and surface-specificity, which minimizes sample wastage and restricted light-matter interactions to only surface-bound liposomes respectively.

6.3 Scope

This chapter focuses on: (1) a preliminary spectroscopic study on free-floating dye-loaded liposomes under continuous excitation by a laser source; (2) accounting for and explaining any observed phenomena (i) by considering changes to the encapsulated dye's properties upon photobleaching; and (ii) by hypothesizing a scheme; (3) the in-fiber immobilization of dye-loaded liposomes followed by an optical probing and comparison with results in the preliminary study, both of which serve to verify their successful attachment and structural integrity.

6.4 Methodology

6.4.1 Materials

1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (15:0 PC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (biotin-DPPE) were purchased from Avanti Polar Lipids, Inc. 5(6)-Carboxyfluorescein (CF), bovine serum albumin (BSA), biotin-4-fluorescein (B4F), streptavidin (SA), Atto 488-streptavidin (AttoSA) and phosphate buffered saline at pH 7.4 (PBS) were obtained from Sigma-Aldrich. 1.0 M Tris buffer at pH8.8 was purchased from 1st Base. All other chemicals were of reagent grade.

Whatman Nucleopore track-etched polycarbonate membrane filters with pore size of 0.2 μm and PD-10 desalting columns (packed with Sephadex G-25M) were acquired from GE Healthcare. PCFs were manufactured by Yangtze Optical Fibre and Cable Company Ltd.

6.4.2 Preparation of dye and buffer solutions

100 mM CF was prepared by dissolving 564 mg of CF in 10.5 ml of distilled ultra-filtrated (DIUF) water and 4.5 ml of 1.0 M sodium hydroxide. The resulting 100 mM CF had an osmolarity of 900 mOsm and pH value of 12.5. In order to match its osmolarity, the 1.0 M Tris buffer was diluted to 321 mM with DIUF water.

6.4.3 Fabrication and characterization of liposomes

This section details the fabrication and optical characterization of both liposomes and biotinylated-liposomes encapsulating CF.

6.4.3.1 Fabrication of dye-loaded liposomes

Liposomes were fabricated using an adaptation of the typical reverse-phase evaporation vesicles (REV) preparation method [325-327]. Modifications were made to accommodate the lack of standard equipment (e.g. rotatory evaporator) and smaller volume required. The protocol is detailed as follows:

Step 1. Creation of an aqueous-in-organic emulsion

- i. 2 mg (2.83×10^{-6} mol) of 15:0 PC was dissolved in 1.0 ml of the water-washed diethyl ether* (organic phase) in a 2.0 ml centrifuge tube. It should be highlighted that all subsequent processes were performed in the said tube unless otherwise specified. Small amounts of chloroform were added drop-wise to completely dissolve the phospholipids. This was coupled with vortexing (Scientific Industries, Vortex Genie 2), until a clear solution was obtained.
- ii. 200 μ l of 100 mM CF (aqueous phase) was pipetted and added drop-wise, creating an aqueous-in-organic emulsion that would be observed to sink to the bottom of the tube.
- iii. The resulting mixture was vortexed and next sonicated for 10 min in a water bath, using a sonic dismembrator (Fisher Scientific, 505 Sonic Dismembrator). The initial vortexing would break up the larger emulsions, while the sonication would form a homogenous cloudy dispersion.

** 2.0 ml of diethyl ether was pipetted into at least 5.0 ml of DIUF water and shaken. The mixture was allowed to stand until a distinctive separation could be seen, where the less dense water-washed diethyl ether could subsequently be carefully pipette from the top.*

Step 2. Removal of organic phase via evaporation

- i. The cloudy dispersion was next vortexed, with the dial set at a maximum of speed 3, under a gentle stream of nitrogen gas directed into the mouth of the tube. This would evaporate the organic phase and gradually reduce the mixture's volume.
- ii. Upon reaching a remaining volume of under 0.5 ml, the vortexing speed and nitrogen gas flow rate could be increased. These should be done carefully and gradually so as to avoid spillage. It should also be noted that the emulsion would often be observed to settle to the bottom of the tube at this stage.

- iii. When the bulk of the organic phase is removed a thick gel would remain, the vortexing speed could then be further increased to facilitate its break-up into an aqueous suspension.
- iv. The resultant suspension was made up to 700 μ l with 100 mM CF and placed overnight in a vacuum chamber at -400 mmHg at room temperature. This would ensure complete remove of any remnant traces of the organic phase.

Step 3. Resizing liposomes through extrusion

- i. The mixture was then transferred to a 50 °C water bath for a 15 min period of incubation. The bath temperature, being higher than the 33 °C phase-transition temperature of 15:0 PC, allowed for a fluidization of the lipid membrane [269] and hence facilitating the subsequent extrusion process.
- ii. This heated mixture was passed 10 times through a polycarbonate membrane* of 0.2 μ m pore size. An additional 5 passes were made into a fresh centrifuge tube to minimize contamination from non-extruded liposomes.
- iii. An additional extrusion was then made with 300 μ l of similarly heated 100 mM CF to collect any membrane-trapped liposomes.

** The polycarbonate membrane was first equilibrated with 100 mM CF. This was achieved by passing approximately 0.5 ml of similarly heated 100 mM CF through it.*

Step 4. Removal of non-encapsulated dye via size exclusion

- i. The resulting 1.0 ml suspension (comprising of small unilamellar vesicles encapsulating and suspended-in 100 mM CF), was first put through a gravity-protocol of size exclusion. It was transferred into a pre-equilibrated PD-10 desalting column* and allowed to completely enter the column before an additional 1.5 ml of Tris buffer was added. 3.5 ml of Tris buffer was added and the same volume of eluate was collected. This eluate would appear orangey-brown with a tinge of bright yellow.

- ii. The 3.5 ml eluate was equally separated into two other pre-equilibrated PD-10 desalting columns** and centrifuged (Thermo Scientific, Heraeus Megafuge 16) at 1000×g for 2 min. The eluate following this cycle would appear orangey-brown. Each 1.75 ml of eluate should immediately be transferred to a fresh centrifuge tube and iced.

** Gravity-protocol PD-10 desalting columns were equilibrated prior to each use, by filling with refrigerated 321 mM Tris buffer and allowing the buffer to pass through the column. This was performed for a minimum of 2 repetitions.*

*** Spin-protocol PD-10 desalting columns were equilibrated prior to each use in an identical manner to the gravity-protocol. Following the 2 repetitions, the column was filled again with the buffer and spun down at 1000×g for 2 min.*

Step 5. Further homogenization and purification with centrifugation

- i. In order to remove the liposomes sized far below the extrusion limit (i.e. 0.2 µm pore size), both 1.75 ml volumes of eluate were centrifuged (Hettich, Mikro 22R) for 3hr at 18000 rpm and 5 °C.
- ii. Following centrifugation, the supernatant was carefully pipetted and disposed. The remaining pellets were re-suspended in 175 µl of refrigerated 321 mM Tris buffer and combined into a single fresh centrifuge tube. This yield a stock suspension of liposomes encapsulating 100 mM CF, hereon referred to as Lipo(CF).

6.4.3.2 Fabrication of dye-loaded biotinylated-liposomes

Biotinylated-liposomes encapsulating 100 mM CF, bLipo(CF), were prepared using the method in the above section, with modifications made to only the phospholipid composition described in Step 1(i).

Specifically, 7.9 mg (1.08×10^{-5} mol) of DPPC and 0.1 mg (1.06×10^{-7} mol) of biotin-DPPE were dissolved in 6.0 ml of water-washed diethyl ether and 2.0 ml of chloroform, resulting in a molar ratio of 99:1. Following solvation into a clear solution, the mixture was aliquoted into four 2.0 ml centrifuge tubes. This yielded a molar content per tube (2.69×10^{-6} mol DPPC and 2.66×10^{-8} mol biotin-DPPE) similar to that in the section above (2.83×10^{-6} mol). Each tube

was then processed accordingly with the remaining steps in the detailed method.

Following the vacuum-oven-assisted removal of the organic phase in Step 2(iv), the contents could be stored under refrigeration for later processing. These have yielded comparable results, despite having been stored for a month.

6.4.3.3 Size and concentration measurements

Aliquots of liposomes, Lipo(CF) or bLipo(CF), were diluted 400,000x in 321 mM Tris buffer to a total volume of 1.0 ml. This was loaded through a syringe into the sample chamber (approximately 0.3 ml in volume) of the NanoSight LM10 system. Its coupled Nanoparticle Tracking Analysis software, allowed for the subsequent computation of liposomal concentration by tracking each liposome as it scatters the excitation laser within the field of view.

Size measurements, on the other hand, were performed at 1,000x dilution to a total volume of 0.5 ml – similarly achieved with 321 mM Tris buffer. This was loaded into a UV semi-micro plastic cuvette (Latech Scientific Supply) and placed in the Zetasizer Nano ZS (Malvern Instruments). The liposomes were then sized via means of dynamic light scattering.

All measurements were made in triplicate at room temperature.

6.4.4 Spectroscopic measurements of dye-loaded liposomes

Figure 6.2. Schematics (not drawn to scale) of set-up comprising the NanoSight viewing unit and an externally positioned spectrometer-linked 10x objective lens. Lipo(CF) were excited by the viewing unit's 405 nm laser source. Blue and green arrows represent scattered light and CF's fluorescence emission, correspondingly. © 2014 IEEE

A spectroscopic study of the Lipo(CF) was performed with the set-up shown in Figure 6.2. It comprised the NanoSight viewing unit, in which the Lipo(CF) sample was loaded and subsequently excited; and a computer-linked spectrometer (Ocean Optics, Maya2000) fiber-coupled to a 10x objective lens, where the spectral information was collected. The loaded Lipo(CF) was excited by the viewing unit's 405 nm continuous-wave laser, with maximum output power of 65 mW. The objective lens's focal point was centered along the path of the excitation laser through the sample chamber. Spectral acquisitions were made with it positioned approximately 5 mm away from the transparent window – point of entry for the excitation laser that approximates 100 μm in beam width.

1.0 ml of Lipo(CF) was filled into a syringe, where approximately 0.5 ml would be injected into the sample chamber and allowed to equilibrate to room temperature. The excitation laser was next switched on and allowed to stabilize.

Following which, the laser-excited fraction of Lipo(CF) in the chamber is replaced with a fresh amount by further injection from the filled syringe. Concurrently, the spectral acquisition was commenced at intervals of 30 s over a span of 10 min. The behavior of these spectra over time was subsequently studied. Acquisitions were made for varied dilutions from the stock 350 μl of Lipo(CF), specifically at dilution factors of 5, 10, 20 and 200.

6.4.5 In-fiber tethering and fluorospectroscopy of dye-loaded liposomes

Figure 6.3. Schematics of a biotinylated liposome tethered to an in-fiber bioactive surface. (CF: Carboxyfluorescein; SA: Streptavidin; pbB4F: photobleached Biotin-4-Fluorescein; BSA: Bovine Serum Albumin) © 2015 IEEE

An in-fiber bioactive surface was prepared using the method detailed in Section 5.4.3, with 1 mg/ml of SA used in Step 3(i) instead of its fluorophore-conjugated counterpart.

Using the customized 50 ml syringe pump system, bLipo(CF) was infiltrated at a 10x dilution for 5 min under 50 ml of drawn pressure. This was immediately flushed under the same pressure with 321 mM Tris for two 10 min cycles, to remove non-bound and excess bLipo(CF). The resulting in-fiber surface-tethered bLipo(CF) (shown in Figure 6.3) were next probed using the set-up described in Section 5.4.2 and its output spectrum was acquired.

Following which, a further 30 min duration of excitation under maximum LED power was performed and the corresponding output spectra were logged at 1 s intervals. The ratiometric change, $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$, was subsequently studied over time. Here, $\lambda_{\text{ems}} = 510$ nm and $\lambda_{\text{exc}} = 490$ nm corresponded to that used in Chapter 4, as the probed fluorophores were identical – carboxyfluorescein (CF).

6.5 Results and Discussion

Spectral results of dye-loaded liposomes under continuous exposure to a laser excitation were logged over a 10 min span. The evolution of their peak emission intensities were subsequently acquired and studied over time. The various trends observed for different liposome concentrations would hence be discussed.

Similar dye-loaded liposomes tethered onto in-fiber bioactive were excited with an LED source over 30 min. Liposomes would then be characterized based upon their fluorescence behaviour analyzed via the method of in-fiber fluorospectroscopy discussed in Chapter 4. Trends observed in the preceding study would further be employed in accounting for results here.

6.5.1 Spectroscopic measurements of dye-loaded liposomes

During the spectroscopic study of Lipo(CF), a counterintuitive phenomenon was observed upon a extended exposure of the Lipo(CF) under a laser excitation. In brief, instead of the typical decrease in fluorescence emission upon photobleaching, an increase was observed. This section hence discusses and explains the phenomenon. Lipo(CF) was sized at 139.33 ± 7.08 nm and at a stock concentration of $(2.13 \pm 0.32) \times 10^{14}$ particles/ml. Dilution of the stock yielded the concentrations 1.07, 10.7, 21.4 and 42.8×10^{12} particles/ml – subsequently referred to as 1x, 10x, 20x and 40x Lipo(CF), correspondingly.

6.5.1.1 Spectral results over span of laser excitation

Figure 6.4. Spectral outputs of Lipo(CF) over 10 min span of excitation within NanoSight viewing unit, for 1x, 10x, 20x and 40x concentrations. Plots are coloured from blue to red, corresponding to the duration of exposure from 0 to 10 min. © 2014 IEEE

The evolution of Lipo(CF)'s output spectra over exposure time are illustrated in Figure 6.4. Unlike previous spectral results that also comprised the remaining excitation, output spectra here corresponded to only the emission of CF. The emission could be isolated here as the excitation laser had a narrow bandwidth centered at 405 nm, significantly away from the emission band. Before the observed spectral changes are discussed, encapsulated CF had to be verified as the source of this collected emission.

6.5.1.2 Discussion on source of fluorescence emission

In order to establish that the Lipo(CF) population under illumination would not change during the span of study, their displacement within the sample chamber had to be computed. Considering a two-dimensional diffusion regime, the liposomes' mean squared displacement,

$$\langle \Delta x^2 \rangle = 4Dt, \quad (6.1)$$

was calculated to be $2.16 \times 10^3 \mu\text{m}^2$ – using a diffusion coefficient, $D = 0.9 \mu\text{m}^2/\text{s}$ for dilute samples of liposomes [328] after a duration of $t = 600$ s. This being a 1/1000 fraction of the field of view implied that the liposomal population was unchanged – i.e. any spectral changes observed over time were a result of changes experienced by the same Lipo(CF)s at $t = 0$ s.

Next, to rule out emission contributions of any non-encapsulated CF, the supernatant of a centrifuged volume of Lipo(CF) was injected into the set-up's sample chamber. The absence of detectable emission in this investigation implied that all observed emission originated from CF within liposomes.

Additionally, a continuous monitoring of the sample chamber's temperature was conducted to exclude contributions of photothermal effects, which could result in the phase-transition of the liposomal membrane [269] and consequently leakage of liposomal content. Since a temperature increment of up to only 1 °C was recorded, photothermal effects were considered negligible. This was further verified through a fluorescence characterization of the post-lased Lipo(CF)'s suspending medium. The supernatant of a centrifuged volume of post-lased Lipo(CF), was measured to only emit at 0.3% the emission intensity of completely release CF (from lysed Lipo(CF)). This minute amount of emission could in fact be ascribed to the imperfect separation of Lipo(CF) from its suspending medium during the centrifugation process. Moreover, size and concentration measurements of post-lased Lipo(CF), revealed changes within the standard deviation of the freshly prepared Lipo(CF). This further verified the stability of the fabricated liposomes.

Figure 6.5. Spectral outputs of externally heat-treated 1x Lipo(CF) over 10 min span of excitation within NanoSight viewing unit. Plots are coloured from blue to red, corresponding to the duration of exposure from 0 to 10 min. Inset: Corresponding peak emission intensity over exposure time. © 2014 IEEE

Lastly, to further verify that the CF encapsulated at a self-quenching concentration [94] of 100 mM experienced no irreversible changes, heat-treated aliquots of 1x Lipo(CF) were studied. Heating resulted in the release and equilibration of CF across the liposomal membrane. This efflux of encapsulated CF was reflected in the 20-fold increment of the initial emission intensity relative to 1x Lipo(CF) without heat treatment. Prolonged exposure to the excitation laser yielded a negative exponential profile (shown in Figure 6.5(inset)) typical of photobleaching [97]. This implied the ability of self-quenched CF to fluoresce upon a reduction of quenching effects.

All in all, the above discussed bolsters the fact that any collected emission originated from the encapsulated CF in Lipo(CF).

6.5.1.3 Experimental observation of photobleaching dequenching

Figure 6.6. Peak emission intensities of Lipo(CF) over 10 min span of excitation within NanoSight viewing unit, for 1x, 10x, 20x and 40x concentrations. A repetition was performed and the difference in peaking events is observed within 30 s. © 2014 IEEE

To better observe the spectral evolution, the peak emission intensities were acquired and traced over the duration of exposure, as shown in Figure 6.6. An initial analysis of the peak emission intensities, across the various Lipo(CF) concentrations as well as their behaviour over time, highlighted the following.

Firstly, a general increment in emission intensities was observed with increasing concentrations of Lipo(CF). This was as expected, considering the concomitant increase in the amount of CF at higher Lipo(CF) concentrations.

This increase was however non-linear, and could be attributed to the simultaneous increase in re-absorbance and scattering effects from the additional Lipo(CF).

Next, changes in the peak emission over time illustrated two broad behaviors. For lower concentrations of Lipo(CF), namely 1x and 10x, a general decrease in peak emission over time that closely resembles the negative exponential profile of photobleaching [97]. It should be highlight here that as CF was encapsulated at concentration of 100 mM, it experienced a self-quenching (or concentration quenching) effect. This effect primarily stemmed from resonance energy transfers (RET) between monomers and eventually to non-fluorescent dye aggregates as well as included some contributions from collisional quenching [94]. Hence, if photobleaching were to have an effect on the self-quenched CF, briefly discussed as dequenching [329], an increase in the peak emission intensity should have instead been observed.

Interestingly, dequenching presented itself at higher Lipo(CF) concentrations, specifically 20x and 40x. Here, an increase in the peak emission was observed with elapse of exposure time to the excitation laser. This observed dequenching could be attributed to the encapsulated CF becoming photobleached, resulting in their inability to undergo excitation-emission cycles. These photobleached CF molecules were hence also not able to participate in the process of RET. In particular, the photobleached acceptor in the acceptor-donor pair eliminated its ability in receiving energy from the excited donor. This was largely similar to the processes in a technique known as Förster resonance energy transfer (FRET) acceptor photobleaching [102, 103]. Furthermore, considering the effects of chemical equilibrium, a decrease in the CF concentration due to photobleaching would inevitably lead to a decrease in its aggregate concentration. This hence reduces the contributions of quenching via RET to CF aggregates.

The presentation of dequenching only at higher concentrations of Lipo(CF), could be ascribed to the increase in absorbing and scattering ability of the more concentrated sample. This resulted in a lower excitation laser power reaching the volume of detection and consequently a slower rate of photobleaching. To

elucidate this phenomenon of photobleaching dequenching, a mathematical representation of the encapsulated CF would be discussed in the following section.

6.5.1.4 Mathematically accounting for photobleaching dequenching

First, quenching of CF could be described by the quantum yield equation that governs its fluorescence [95, 330]:

$$\Phi_{\text{Fl}} = \frac{k_r}{k_r + k_{\text{nr}} + k_{\text{qM}}[\text{CF}_\text{M}] + k_{\text{qD}}[\text{CF}_\text{D}] + k_{\text{qT}}[\text{CF}_\text{T}]} \quad (6.2)$$

This is similar to the quantum yield expression in Equation (2.4) when a quencher is present. Here, k_r and k_{nr} are the radiative (fluorescence) and non-radiative rate constants respectively, while k_{qX} and $[\text{CF}_\text{X}]$ represent the quenching rate constant and quencher concentration, correspondingly. The subscript “X” denotes the aggregation state of the quencher, where M, D and T correspond to the monomeric, dimeric and trimeric forms of CF aggregates. Hence, from Equation (6.2), a higher quencher concentration would increase the dominance of its denominator and therefore result in a lower Φ_{Fl} . Additionally, the dimer and trimer concentrations could be expressed in terms of aggregation constants and monomer concentration, K_X and $[\text{CF}_\text{M}]$ respectively. These could be expressed as:

$$[\text{CF}_\text{D}] = K_\text{D}[\text{CF}_\text{M}]^2 \quad (6.3)$$

$$[\text{CF}_\text{T}] = K_\text{T}K_\text{D}[\text{CF}_\text{M}]^3 \quad (6.4)$$

Considering chemical equilibrium, the total CF concentration could thus be written as:

$$[\text{CF}]_{\text{tot}} = [\text{CF}_\text{M}] + 2K_\text{D}[\text{CF}_\text{M}]^2 + 3K_\text{T}K_\text{D}[\text{CF}_\text{M}]^3 \quad (6.5)$$

In general, the observed changes in emission intensities could be described by a combination of two effects – photobleaching dequenching and photobleaching itself. Their respective dominance hence results in a rise and a fall of emission intensities, correspondingly. Since photobleaching essentially affects $[\text{CF}_\text{M}]$ over time, it could be expressed as a negative exponential function of time [331, 332]:

$$[CF_M] = [CF_M]_0 \exp(-k_{pb} t) \quad (6.6)$$

Here, $[CF_M]_0$ is the initial CF concentration while k_{pb} is the rate constant of photobleaching. However, as $[CF_M]_0$ is not constant but varies according to the chemical equilibrium distribution in Equation (6.5), a better expression would be the change in $[CF_M]$ per quantized duration of photobleaching, Δt_{pb} , derived as:

$$\Delta[CF_M]_i = [CF_M]_i \left(\exp(-k_{pb} \Delta t_{pb}) - 1 \right) \quad (6.7)$$

Here, $[CF_M]_i$ represents the $[CF_M]$ at that instance in time, calculated from the $[CF]_{tot}$ at said instance, $[CF]_{tot,i}$, using Equation (6.5). Consequently, following this period of Δt_{pb} , a new $[CF]_{tot}$ is obtained:

$$[CF]_{tot,i+1} = [CF]_{tot,i} + \Delta[CF_M]_i \quad (6.8)$$

It should be noted here that based on Equation (6.6), $\Delta[CF_M]_i$ is negative. A new $[CF_M]$, $[CF_M]_{i+1}$, could thus be obtained from $[CF]_{tot,i+1}$ and reinserted back into Equation (6.7). The iteration hence cycles between Equations (6.7) and (6.8) until the end of photobleaching.

The above hence illustrates how photobleaching affects $[CF]$, which in turn influences Φ_{Fl} . In order to further relate photobleaching to fluorescence emission, the expression relating emission intensity to Φ_{Fl} was next studied:

$$I_{Fl} = \Phi_{Fl} f_{Fl,\lambda} I_{exc} \left(1 - 10^{-\epsilon_{exc} c l} \right) \quad (6.9)$$

Here, $I_{exc}(1-10^{-\epsilon_{exc} c l})$ corresponds to the absorbed excitation intensity (I_{abs}) – derived from the Beer-Lambert law – where I_{exc} is the excitation intensity; ϵ_{exc} and c are the extinction coefficient at the excitation wavelength and concentration of CF respectively; and l the path length of excitation light. Lastly, $f_{Fl,\lambda}$ denotes the fraction of emission intensity at λ [333].

To better elucidate the effects of photobleaching on I_{Fl} , it is normalized over its non-photobleached counterpart, $I_{Fl,0}$, and expressed by a modified version of the Stern-Volmer equation in Equation (2.5):

$$\frac{I_{Fl}}{I_{Fl,0}} = \frac{\Phi_{Fl}}{\Phi_{Fl,0}} \cdot \frac{I_{abs}}{I_{abs,0}} \quad (6.10)$$

Where the similarly normalized Φ_{Fl} and I_{abs} are derived as:

$$\frac{\Phi_{\text{Fl}}}{\Phi_{\text{Fl},0}} = \left(\frac{k_r}{k_r + k_{\text{nr}} + k_{\text{qM}}[\text{CF}_{\text{M}}] + k_{\text{qD}}[\text{CF}_{\text{D}}] + k_{\text{qT}}[\text{CF}_{\text{T}}]} \right) \bigg/ \left(\frac{k_r}{k_r + k_{\text{nr}}} \right) \\ = \left(1 + k_{\text{qM}}\tau_0[\text{CF}_{\text{M}}] + k_{\text{qD}}\tau_0[\text{CF}_{\text{D}}] + k_{\text{qT}}\tau_0[\text{CF}_{\text{T}}] \right)^{-1} \quad \tau_0 = (k_r + k_{\text{nr}})^{-1} \quad (6.11)$$

$$\frac{I_{\text{abs}}}{I_{\text{abs},0}} = \frac{1 - 10^{-\epsilon_{\text{exc}}[\text{CF}]_{\text{tot}}I}}{1 - 10^{-\epsilon_{\text{exc}}[\text{CF}]_{\text{tot},0}I}} \quad (6.12)$$

In Equation (6.11), which expressed the fraction of quantum yield, $\Phi_{\text{Fl},0}$ represented the quantum yield in the absence of all quenchers (i.e. $[\text{CF}_{\text{M}}] = [\text{CF}_{\text{D}}] = [\text{CF}_{\text{T}}] = 0$), while τ_0 is the characteristic fluorescence lifetime. Equation (6.12), on the other hand, depicted the relative ability of absorption over the course of photobleaching, where $[\text{CF}]_{\text{tot}}$ and $[\text{CF}]_{\text{tot},0}$ denoted the CF concentration as photobleaching progressed and its initial value, correspondingly.

Figure 6.7. Calculated normalized I_{Fl} over 10 min duration, with varying inputs for k_{pb} , K_{D} and K_{T} . Relative to the black solid line, the blue dashed and red dotted-dashed lines correspond to an 5x increased rate of photobleaching (k_{pb}) and doubled aggregation constants (K_{D} and K_{T}), correspondingly. © 2014 IEEE

Normalized I_{Fl} was plot with respect to time using Equation (6.10) and the constants reported in [95, 330] – specifically, $\tau_0 = 3.6 \times 10^{-9}$ s; $K_{\text{D}} = 5$; $K_{\text{T}} = 10$; $k_{\text{qM}} = 1.7 \times 10^{10}$ dm³/(mol.s); $k_{\text{qD}} = 3.9 \times 10^{11}$ dm³/(mol.s); $k_{\text{qT}} = 1.7 \times 10^{12}$ dm³/(mol.s); and the experimentally determined extinction coefficient of the

prepared CF at $\lambda = 405\text{nm}$ – $\varepsilon_{\text{exc}} = 1.30 \times 10^3 \text{ dm}^3/(\text{mol.m})$. A series of plots was made with arbitrarily determined values of k_{pb} as well as varied values of K_D and K_T , as shown in Figure 6.7. The different behaviours observed would be discussed below.

From Equation (6.10), two counteracting changes occur as a result of photobleaching decreasing $[\text{CF}]_{\text{tot}}$, namely (1) an increase in the fraction of quantum yield ($\Phi_{\text{Fl}}/\Phi_{\text{Fl},0}$) as given by Equation (6.11); and (2) a decrease in the ability of absorption ($I_{\text{abs}}/I_{\text{abs},0}$) as implied in Equation (6.12). At the early phase of photobleaching, $I_{\text{abs}}/I_{\text{abs},0}$ has little dominance since it decreases from a starting value of 1. Therefore, the increment in $\Phi_{\text{Fl}}/\Phi_{\text{Fl},0}$ takes precedence, resulting in a rise in the normalized I_{Fl} – corresponding to the observation of photobleaching dequenching. Consequently, as $\Phi_{\text{Fl}}/\Phi_{\text{Fl},0}$ tended towards 1 and $I_{\text{abs}}/I_{\text{abs},0}$ towards 0, an inevitable decrease resulted in the normalized I_{Fl} – concurring with the dominating effects of photobleaching alone. These hence led to the occurrence of peaking in the emission intensity over time, as clearly shown by the black solid line in Figure 6.7.

6.5.1.5 Discussion on the observed two phases of photobleaching dequenching

Reexamining the changes in peak emission over time for 20x and 40x Lipo(CF) in Figure 6.6, two peaking events were discernible. Specifically, an earlier phase that occurred within the first 60 s and a later phase at 270 s and 330 s for the 20x and 40x Lipo(CF) respectively.

These two phases of dequenching would be discussed with a hypothesized scheme (shown in Figure 6.8) that simplified the encapsulated CF as a two-component system, namely the outermost and inner bulk regions. In addition, the behaviour of normalized I_{Fl} under different rates of photobleaching and extent of aggregation (shown in Figure 6.7) would serve to explain the time evolution of the peak emission intensity.

Figure 6.8. Hypothesized scheme of primary and secondary dequenching events within Lipo(CF) under prolonged exposure to the excitation laser. © 2014 IEEE

(a) Primary dequenching

The first peaking event (primary dequenching) was deduced to be a result of dequenching occurring for CF residing in the outmost regions – i.e. nearest the liposome’s phospholipid bilayer. These CF molecules, being the first molecules in the path of excitation light entering the liposomes, lack other “shielding” CF and consequently become the first to undergo photobleaching. Furthermore, the transient electrostatic interactions between these CF and the phospholipids bilayer’s polar headgroups reduced their interactions with neighbouring CF molecules [94]. This inhibited aggregate formation and led to a lowered probability of RET, which increased the excited CF monomer’s lifetime in the excited state and consequently their susceptibility to photobleaching. For the same reason, initial emission from Lipo(CF) was expected to have originated from said CF, albeit their encapsulation under self-quenching conditions. As a result, the rate of photobleaching would also be higher in this region, leading to the observed shorter and faster peaking event. This narrower peak was likewise illustrated in Figure 6.7 by the blue dotted plot, where a larger k_{pb} was used in its computation. Primary dequenching proceeded until the population of photobleached CF in the outermost region outweighed that of viable CF, where emission hence dropped due to the sole effects of photobleaching.

(b) Secondary dequenching

With continued exposure to the excitation laser, peaking was observed to occur again (secondary dequenching). This dequenching was deduced to be a result of photobleached CF in the outermost regions migrating towards the interior of the core, reducing the $[CF_M]$ and consequently the overall quencher concentrations of the inner bulk regions. It however happened at a much slower rate, in contrast to primary dequenching, as reflected in its broader span. This slower rate was attributed of two effects: (1) a slower rate of photobleaching due to the higher shielding effects experienced by CF in the inner bulk regions; and (2) an additional involvement of diffusion, which governed the mixing between photobleached and viable CF. The former concurred with that depicted in Figure 6.7, where a smaller k_{pb} generally resulted in a broader peak as shown by the broadening from the blue dotted to the black solid plots. The latter, on the other hand, was deduced to be largely similar to the phenomenon observed in fluorescence recovery after photobleaching [334, 335]. However, it should be noted here that due to the high encapsulation concentration, the mixing was expected to no longer follow the simple diffusion regime. Instead, photobleached and viable CF experienced anomalous diffusion due to the presence of obstacles [336] and binding events [337], a result of molecular overcrowding and electrostatic interactions with the phospholipid bilayer respectively. This slower rate of diffusion implied a slower equilibration between the various aggregated forms of CF and consequently larger aggregation constants – K_D and K_T . This is likewise shown in Figure 6.7, where upon a doubling of K_D and K_T , peak broadening was observed from the black solid to red dotted-dash plots. Nevertheless, following dequenching of the inner bulk regions, photobleaching subsequently prevailed as would be expected.

Furthermore, contrasting between the plots in Figure 6.6, the extent of secondary dequenching was deduced to be indicative of the competition between diffusion-dependent dequenching and photobleaching. In particular, a second peaking event of lower intensity than its first, as seen in 20x Lipo(CF), implied dominance of photobleaching. Conversely, the more obvious manifestation of secondary dequenching observed in 40x Lipo(CF), demonstrated the opposite. Additionally, the rapid rate of photobleaching in

lower concentrations of Lipo(CF) could explain the absence of any peaking events, where any instance of diffusion-dependent dequenching was largely overshadowed.

With the above-discussed understanding of photobleaching dequenching, this unique phenomenon would subsequently provide a means of characterizing dye-loaded liposomes. Specifically, it offers a simple optical method that verifies the high concentration of encapsulated dye, without the need for any heat-dependent content releases in conventional methods [289].

6.5.2 In-fiber fluorospectroscopy of tethered dye-loaded liposomes

Performing a similar spectroscopic study on in-fiber tethered bLipo(CF), the phenomenon of photobleaching dequenching was similarly observed. In addition, it served as an indicator on their structural integrity following surface tethering. Discrepancies from the earlier observations in directly laser excited Lipo(CF) would also be discussed. bLipo(CF) was sized at 224.73 ± 15.96 nm and at a stock concentration of $(1.10 \pm 0.22) \times 10^{14}$ particles/ml. bLipo(CF) was infiltrated with a 10 fold diluted concentration of the stock.

6.5.2.1 In-fiber spectral characterization of tethered dye-loaded liposomes

Figure 6.9. Normalized (to excitation peak) output spectra of dcPCFs with various in-fiber surfaces after flushing out earlier infiltrations of SA followed by bLipo(CF). BSA-passivated surfaces with and without biotin-functionalization (Control) are indicated in red dotted-dash and blue dotted lines respectively. Experiment was done in triplicate. © 2015 IEEE

Following the in-fiber formation of the bioactive surface, bLipo(CF) was introduced for conjugation. The encapsulated CF provided a fluorescent means of characterizing the bound liposomes using the fluorospectroscopic interpretation described in Chapter 4. This output spectra was compared with a Control – identically processed dcPCF with the exception of photobleaching – as shown in Figure 6.9.

Firstly, like earlier output spectra of CF-filled dcPCF, the right end of the spectra corresponded to the collected fluorescence emission. The extent of fluorescence emission was further indicated by the significance of this shoulder or hump. From the obvious hump in the red dotted-dash plot in Figure 6.9, CF was evidently present. This was in turn indicative of bLipo(CF) being successfully tethered to the in-fiber surfaces. In contrast, a weaker emission was presented in the blue dotted plot of the Control. This, on the other hand, could be attributed to the presence of non-specifically bound SA conjugating and trapping bLipo(CF). This existence of non-specific binding was deduced from the earlier similar observation (in Section 5.5.2) of fluorophore-conjugated SA remaining in dcPCFs, albeit them lacking biotin-functionalization.

Nevertheless, it should be highlighted that the significant difference observed here did not concur with the earlier calculations of SA coverage density. Specifically, contributions from non-specifically bound SA was calculated to comprise more than three-quarters the total bound SA (both specific and non-specific). This dominance of was however not reflected here by a correspondingly large extent of fluorescence emission for the Control. Hence, results here do support the earlier hypothesis that non-specifically bound SA possesses hindered functionalities that impede the efficient tethering of bLipo(CF).

This study yielded $I(\lambda_{\text{ems}})/I(\lambda_{\text{exc}})$ values of 1.405 ± 0.116 and 0.790 ± 0.293 for surface-tethered bLipo(CF) and the Control respectively. It was however not possible to quantify the concentration bLipo(CF) in this case, as the devised in-fiber fluorospectroscopy model is unable to account for the contributions self-quenching at high fluorescent dye concentrations.

6.5.2.2 In-fiber observation of photobleaching dequenching

Following the verification of successful in-fiber tethering of bLipo(CF), their structural integrity and thus content retention had to be validated. As discussed earlier in Section 6.5.1 and [338], the phenomenon of photobleaching dequenching could only be observed for fluorescent dye at self-quenched concentrations. Its observation here would hence served as an appropriate indicator to the presence of high CF concentrations within the bound bLipo(CF). Moreover, this could be simply achieved by adapting the optical trigger, required in the generation of dequenching, into the in-fiber regime that inherently already facilitated light-matter interactions.

Coherent with the phenomenon of photobleaching dequenching, an increasing prominence of the fluorescence emission was observed for up to the first 10 min. Like the two phases of dequenching discussed in Section 6.5.1.5, two peaking events were similarly observed here. In the triplicate repetitions, the first peaking was observed to occur between 1 and 5 min of excitation. This was followed promptly by a second that spanned the next 5 to 7 min of excitation. This largely corresponded with the observations in Lipo(CF) – an initial short-lived primary dequenching followed by a longer-lasting secondary dequenching. Hence, the events could be attributed to the same reason of photobleaching proceeding in an inhomogeneous manner within the liposomes. However, instead of the outside-in (towards the liposome's core) progression of photobleaching illustrated in Figure 6.8, a bottom-up (away from the air hole surface) progression would occur in the case of surface-tethered bLipo(CF). Specifically, the first peaking event could be attributed to the photobleaching of encapsulated CF closest to the air hole surface, reducing the self-quenching effects in their immediate vicinity. The second peaking event, on the other hand, resulted from a slower rate of photobleaching and diffusion-dependent mixing. The latter was between photobleached and still viable CF (those residing further away from the air hole surface). Both peaking events ended upon the overshadowing of dequenching effects by further photobleaching.

Comparing between the dequenching here and that from Lipo(CF) excited in the NanoSight viewing unit, the span of peaking events was observed to be

longer in the former. As discussed earlier, this could be ascribed to two reasons, namely a slower rate of photobleaching and anomalous diffusion. Since the in-fiber excitation here was from a LED of much lower power (approximately 1/28 that of the NanoSight laser's) and its mode of excitation was evanescent in nature, the former was deduced to be the fundamental cause. Additionally, this broadening of peaking events could also be attributed to the sustained supply of viable CF migrating from a reservoir residing outside the depth of excitation. Specifically, the BSA-to-encapsulated-CF distance was estimated to be approximately 8.3 nm – BSA thickness: 2.4 nm [339]; biotinylated phospholipid bilayer with bound SA: 5.9 nm [340]; SA-bound biotin: negligible [266]. Considering a liposome of 224.73 nm and penetration depth of 76.46 nm, the unexcited fraction of a surface-tethered liposome was more than half – approximately 66%. This majority hence substantiated the above postulation of an ample supply of viable CF being available to prolong and sustain the dequenching.

All in all, the observation of photobleaching dequenching reflected the presence of a high concentration of CF, which further implied no leakage and hence structural intactness. This also indicated that there was no direct interaction between the bLipo(CF) and the silica air hole surface, which would have resulted in their undesirable rupture [341, 342].

6.6 Conclusion

The continuous optical excitation of Lipo(CF), where CF was encapsulated at self-quenching concentrations, presented a notable counterintuitive phenomenon – photobleaching dequenching. The same phenomenon was then successfully employed in the characterization of in-fiber tethered bLipo(CF).

The above-mentioned phenomenon was observed as a peaking in emission intensity upon photobleaching by a laser excitation. This was very much contrary to the effects of photobleaching which would have resulted in decreased emissions. Using a mathematical approach, this phenomenon of dequenching was explained to stem from a photobleaching-induced reduction in the concentration of CF monomers. This consequently resulted in a higher Φ_{Fl} and a lower absorbance, which took precedence at the initial and later phases of exposure time respectively. The rise and fall thus accounted for a peaking event.

A hypothesized scheme was next used in the explanation of successive peaking events. The two peaks were attributed to dequenching occurring first in the outermost regions before proceeding to the bulk inner regions. Due to the combined effects of lower photobleaching rates and anomalous diffusion, the second peaking event was slower and spanned a longer duration.

Using the bioactive surface discussed in Chapter 5, bLipo(CF) was immobilized within PCFs. Its successful attachment was verified by means of in-fiber fluorospectroscopy detailed in Chapter 4. This highlighted a insignificant amount of non-specifically bound bLipo(CF), which further bolstered the negligibility of non-specifically bound SA mentioned in Section 5.5.2. In addition, the observation of photobleaching dequenching in the in-fiber tethered liposomes substantiated their structural integrity and content retention.

In conclusion, the successful tethering of liposomes within PCFs was demonstrated. Their intactness post tethering highlighted their feasibility as analogues to dye-doped nanoparticles. The integration of liposomes with PCFs hence shows potential as a bio-integrated photonic device.

7

Conclusion and Future Work

7.1 Conclusions

The inherent optofluidic advantages offered by photonic crystal fibers (PCFs) have provided the fundamentals required for in-fiber light-matter interactions. In this thesis, such interactions were exploited in fluorescence spectroscopy and the generation of photochemical reactions. The choice of index-guiding PCFs in these studies provided not only versatility by supporting a broad band of light, but more importantly surface-specificity via its evanescent field's interactions with the infiltrated matter. Additionally, selectivity of the target matter was enabled by the further immobilization of complementary biomolecules. The control achievable through these properties showed potential for the subsequent creation of bio-integrated photonic devices. The following summarizes the work presented in this thesis.

Firstly, prior to the conduct of in-fiber studies, an optofluidic platform was devised. The platform was demonstrated to be capable of infiltration and evacuating a length of PCF while maintaining alignment with optical fibers at both of its ends. Optical losses across connections were measured to be comparable to commercial connectors at within 3 dB, while the rate of filling was approximately ten-times faster than that achievable by the commonly used capillary action. Notably, the complete emptying of PCFs – something physically unattainable by capillary forces – was also achieved.

Next, the simple introduction of a bend to the PCF component created distinctive dips in the output spectrum. This occurred in solid core PCFs

(scPCFs) as a result of core-cladding coupling happening at specific wavelengths. Consequently, due to this involvement of the cladding region, which comprised the air hole array, any changes to its refractive index would alter the coupling condition and shift the dips. This hence provided a straightforward means of evaluating the platform's efficiency via refractive index measurements. Experimental results concurred well with simulations as well as demonstrated good repeatability and reusability with insignificant contributions from contaminations. The optofluidic platform was hence deemed practical for subsequent studies on in-fiber light-matter interactions.

Interestingly, bending was also observed to increase sensitivity in fluorescence spectroscopy. This was demonstrated experimentally with the fluorescent dye 5(6)-carboxyfluorescein (CF) in both scPCFs and defected-core PCFs (dcPCFs). From a ray-tracing model, developed to account for the in-fiber light-fluorophore interactions, the heightened sensitivity was ascribed to primarily result from a higher number of reflections at the core-air hole interface. Model-calculated results were further shown to correspond well with their experimental counterparts. Additionally, each spectrum for a specific concentration of infiltrated CF was described by a ratio between the peak emission and absorption of the fluorescent dye. This ratiometric method created a dimensionless ratio that mitigated the effects of intensity fluctuations. Furthermore, it enabled the platform to operate under a filterless regime and with just a broadband LED source. These thus allowed for a compact all-fiber configuration and low cost components.

Following the understanding of light-fluorophore interactions within PCFs, a photo-induced immobilization of fluorophore-conjugated biotin (biotin-4-fluorescein or B4F) was investigated. This entailed the passivation of in-fiber surfaces with a protein (bovine serum albumin or BSA); followed by the photo-induced radicalization of B4F's fluorescein conjugate and a tyrosine (amino acid) residue in BSA, where both radicals would react and consequently bind. This generated a primary bioactive surface consisting of immobilized biotin. Markedly, this was achieved without the use of any toxic chemicals or tedious pre-activation reactions that are commonly employed in silica coating

procedures. The subsequent conjugation of streptavidin (SA) to the immobilized biotin created the secondary bioactive surface that could bind further units of biotin or biotin-conjugated molecules. Using a fluorophore-conjugated SA, successful SA-biotin binding was verified and further quantified. Although the earlier-described method of fluorescence spectroscopy quantified a considerable presence of non-specific SA binding, they were discussed to possess negligible contributions as part of the bioactive surface. A systematic study further provided an optimal protocol with high repeatability and experimental feasibility.

Lastly, the in-fiber bioactive surface was explored for its ability in attaching biomolecular constructs. This was studied for the potential of creating bio-integrated photonic devices. Since in-fiber light-fluorophore interactions have been established in earlier studies, CF-loaded liposomes (Lipo(CF)) were chosen as the target construct for in-fiber tethering. Prior to investigating these liposomes within PCFs, they were first characterized externally. Intriguingly, a counterintuitive phenomenon was observed upon exposing Lipo(CF), encapsulating self-quenching concentrations of CF, to prolonged durations of optical excitation. This phenomenon was that of photobleaching dequenching, which presented itself as an increase in emission rather than the typical decrease due to photobleaching. A mathematical treatment of the observation explained it to be mainly a result of increased quantum yield and lower absorbance upon a reduction in the CF quencher concentration. The observation of successive peaking events was then explained by a scheme that attributed it to effects of photobleaching rates and diffusion. Following the conduct of in-fiber liposomal attachment, this observation of dequenching was utilized as a means of verifying the retention of liposomal content. Here, successful retention would imply their structural intactness and hence no undesired interactions with the in-fiber silica surfaces that would have otherwise resulted in their rupture. This highlighted their feasibility as analogues to dye-doped nanoparticles that have been explored in applications like random lasing.

In conclusion, the optofluidic platform has been demonstrated to efficiently facilitate studies on light-matter interactions within PCFs. The further

comprehension of in-fiber light-fluorophore interactions advanced the platform's application in fluorescence spectroscopy. In addition, it further inspired studies on photo-immobilizing biomolecules via the generation of in-fiber photochemical reactions. The subsequent successful in-fiber attachment of biomolecular constructs showed promise in creating bio-integrated photonic devices using PCFs.

7.2 Original Contributions of Thesis

The following highlights the original contributions of this thesis.

1. The successful development of a compact optofluidic platform, capable of completely infiltrating and evacuating a length of PCF while maintaining its alignment to other fiber optic components.
 - Multiple infiltration-evacuation cycles could be performed on a length of PCF for aqueous and viscous (oils) solutions, with negligible effects of contamination, using only a syringe pump.
2. The notable design of a ratiometric method of spectral interpretation and ray-tracing model to enable fluorospectroscopy under an all-fiber and low-cost configuration.
 - The ray-tracing model was demonstrated to be appropriate for for index-guiding PCFs, and required only the experimental data input of a reference spectrum and fluorescence parameters.
 - Ratiometry allowed fluorospectroscopy to be performed without bulky filters and with an inexpensive and broad-band LED excitation source.
3. The first experimental demonstration of biomolecular functionalization within PCFs via means of photochemical reactions.
 - Bioactive surfaces were formed within PCFs through a three-step protocol, utilizing only non-toxic chemicals and just a LED excitation source.
 - The immobilization of biomolecules was achieved within the micron-sized air holes in PCFs.
4. The first experimental demonstration of liposome tethering and optically excitation within PCFs as well as the first report of photobleaching-induced dequenching within dye-loaded liposomes.

- Dye-loaded liposomes, sized at approximately 200 nm, were successfully tethered within PCFs without any compromization of their structural integrity.

A devised mathematical model accounted for the phenomenon of photobleaching-induced dequenching, and its observation further served as an indication of high dye content in liposomes.

7.3 Future Work

7.3.1 Upgraded optofluidic platform

Figure 7.1. Schematics of the new custom-built optofluidic platform. It comprised two optofluidic adapters held at adjustable separations by a mount. Each optofluidic adapter would connect a FC fiber connector (for insertion of PCF) to a SMA905 fiber cable (for optical input or output) as well as provide a fluidic interface for either sample input or output.

Based on the design of the homemade optofluidic platform detailed in Chapter 3 and illustrated in Figure 3.1, an improved platform was devised. The new platform (shown in Figure 7.1) consisted of two components machined from blocks of stainless steel: (1) two optofluidic adapters that connected optical fiber components while providing an interface for sample input and output; and (2) a mount that could secure the adapters at adjustable separation distances. The configuration of optical fibers was essentially unchanged, where a bent

length of PCF was inserted into FC fiber connectors coupled to either the input or output SMA905 fiber cables via the optofluidic adapters. Similar to the homemade platform, “plugs” would be formed using the jacket-unjacketed interface of the PCF. These “plugs” would hence create a sealed chamber at the syringe-pump-connected adapter, allowing for a negative pressure to be applied across the PCF and consequently its infiltration and evacuation. On the other hand, the series of periodically drilled holes in the mount would allow the adapters to be secured at adjustable separation distances at 5 mm steps. This would allow the PCF component to be bent at different radii and thus facilitate further studies in that aspect.

Prior to application of the new optofluidic platform in studies, the optical losses in the platform would first be quantified. Since the adapter’s design was based upon a modification of commercially available mating sleeves, optical losses are expected to be within 3 dB. Subsequently, components required for fluidic manipulation would be incorporated. This would include flexible tubings for attachment of the syringe-pump; valves for pressure regulation; and o-rings for the formation of sealed chambers.

In sum, the new optofluidic platform would provide a more robust and versatile means of performing in-fiber light-matter interaction studies. This would facilitate the repetition of earlier work and provide ease to future studies.

7.3.2 Application in bio-integrated photonics

In more recent years, the burgeoning field of bio-integrated photonics has generated much interest in the research community for both application-driven bioengineering and curiosity-driven blue-sky science. Generally, the field encompasses the development and study of photonic devices that operate based on the properties of bio-derived or biological components. These components range from biomolecules to cells to tissues to organisms, boasting inherent bio-compatibility and -degradability. Such devices are hence frequently associated with biomedical applications, such as for sensing and therapeutics.

The achieved in-fiber bioactive surface (Chapter 5), which consisted of bio-derived proteins (bovine serum albumin and streptavidin) and vitamins (biotin),

thus places the optical fiber device into this category. The bioactive surface provided a foundation for the attachment of other biomolecules such as proteins and nucleic acids (genetic sequences), enabling immediate applications in sensing and study of biomolecular interactions. Moreover, as the fundamental scheme of detection is dependent on the common light-fluorophore interactions at a waveguide's surface, the established theoretical understanding (Chapter 4 would largely facilitate subsequent studies. This would be particularly pertinent to implantable waveguides, where the bulk of light-matter interactions inevitably occur at the waveguides' surfaces.

Notably, the further successful immobilization of biomolecular constructs (Chapter 5) indicated ability in integrating complex biological components. This offered a means of investigating the composition of constructs and could potentially provide a configuration for the generation of optical effects. Since the tethering of liposomes was achieved, it becomes rational that future work continues to explore their potential applications in photonics.

This section would hence discuss two areas for future studies: (1) light-matter interactions for implanted waveguides and (2) liposomes for photonics. These two areas essentially concur with the two wings of bio-integrated photonics, application- and curiosity-driven research respectively.

7.3.2.1 Light-matter interactions for implanted waveguides

Implantable waveguides offer a straightforward solution to the fundamental challenge of light delivery to and collection from deeper regions of the body during sensing and therapeutics. Considering the possible continued operation of such implants over extended periods of time, bio-compatible and -degradable materials have been fashioned into waveguides for research. These include bio-derived materials such as silk [343] and gelatin [344]; cells, namely *Escherichia coli* [345]; as well as the widely used polymer in biomedical applications – polyethylene glycol (PEG) [346]. PEG-based hydrogels, in particular, were integrated with cells and successfully demonstrated *in vivo* sensing and therapy via optical excitation guided by the hydrogel itself. Although this example elucidated light-matter interactions within the waveguide, a total internal reflection (TIR) mode of light guidance would unavoidably create evanescent

fields at the waveguide surface. Moreover, since the regions adjacent to the implanted waveguide are likely the bulk locations for sensing or therapy, deciphering the light-matter interactions at their interface becomes important. Future work could thus explore (1) means of transducing biomolecular reactions at the interface into detectable optical outputs for applications in sensing or (2) methods in enhancing the light-matter interactions for more efficient therapeutic effects.

7.3.2.2 Liposomes for photonics

The potential of using dye-loaded liposomes as analogues to dye-doped crystalline and polymeric nanoparticles for random lasing, was mentioned to be a motivator in Section 6.2.3. Although random lasing is conventionally achieved through the excitation of a scattering medium with a focused laser beam [347], there has been an interesting demonstration using excitation from just an evanescent field [348]. In that report, the evanescent field was introduced by means of TIR at a prism-medium interface. It also suggested that unlike in conventional excitation where a considerable portion of light was retroreflected, evanescent excitation provided a more efficient means of excitation. This was as only the required energy is coupled to the scattering medium for generation of random lasing. Since the waveguide-bound liposomes achieved in Chapter 6 very much resembled this configuration, its potential of attaining random lasing is substantiated. However, to realize random lasing using dye-loaded liposomes, a critical compositional change would have to be made, specifically their encapsulated content.

Following the remarkable demonstrations of lasing from cells expressing green fluorescent protein (GFP) [323, 324], there have been more recent reports of micro-lasers using GFP in the form of dried rings [349] and rod-shaped crystals [350]. This interest in GFP stemmed from two characteristics: (1) its biocompatibility and the ease in producing it biologically through genetic manipulation; and (2) its unique molecular structure, where a rigid cylindrical construct surrounds and protects its fluorophore component. The latter, in particular, suppresses effects of concentration quenching present in typical fluorescent dyes as depicted in the earlier chapters by carboxyfluorescein. This

hence allows for higher concentrations of GFP to be utilized, reducing the threshold for lasing. GFP is therefore an ideal choice as the lasing medium for incorporation into liposomes in future studies.

On the other hand, another optical phenomenon that utilizes a similar configuration for the coupling of light is that of whispering gallery modes (WGMs). WGMs are typically generated in resonators, such as microspheres, through their overlap with evanescent fields generated by TIR, which include waveguide surfaces [351]. Again, the waveguide-bound liposomes exemplify similarities, with the liposomes as potential WGM resonators. However, as the size of WGM resonators are often in the range of micrometers, larger liposomes would likely have to be fabricated for such studies. Markedly, there have also been reports where dielectric WGM resonators are functionalized with fluorophore-tagged phospholipids, forming biosensors [352] and micro-lasers [353]. These highlight that phospholipids are not only able withstand lasing but also serve as a support for the attachment of lasing medium. This hence presents an alternative configuration to the earlier dye-loaded liposome, where the lasing medium is not encapsulated but instead resides in the liposomal membrane.

In summary, two optical effects could potentially be achieved using liposomes, namely random lasing and WGMs. Additionally, these effects could likely be generated using the configuration presented earlier in this thesis, where liposomes are bound to the waveguide surfaces. GFP was also highlighted to be an ideal lasing medium that largely concurred with the concept of bio-integrated photonics.

List of Publications

Journal Articles

1. D. Yong, E. Lee, X. Yu, and C. C. Chan, "In-fiber tethering and fluorospectroscopy of dye-loaded liposomes," *Selected Topics in Quantum Electronics, IEEE Journal of*, vol. 22, pp. 4901006, 2016.
2. E. Lee, D. Yong, X. Yu, H. Li, and C. C. Chan, "In-fiber photo-immobilization of a bioactive surface," *Journal of Biomedical Optics*, vol. 19, pp. 120502, 2014.
3. D. Yong, E. Lee, X. Yu, and C. C. Chan, "In-fiber fluorospectroscopy based on a spectral decomposition method," *Optics Express*, vol. 22, pp. 23640-23651, 2014.
4. D. Yong, E. Lee, X. Yu, C. C. Chan, "Two-phase photobleaching dequenching in dye-loaded liposomes," *Selected Topics in Quantum Electronics, IEEE Journal of*, vol. 20, pp. 213-220, 2014.
5. D. Yong, W. L. Ng, X. Yu, C. C. Chan, "A compact opto-fluidic platform for chemical sensing with photonic crystal fibers," *Sensors and Actuators A: Physical*, vol. 191, pp. 22-26, 2013.
6. Y. Zhang, D. Yong, X. Yu, L. Xia, D. Liu, and Y. Zhang, "Amplification of surface-enhanced Raman scattering in photonic crystal fiber using offset launch method," *Plasmonics*, vol. 8, pp. 209-215, 2012.
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Conference Proceedings

1. D. Yong, E. Lee, K. Y. Lee, Y. Y. Tan, X. Yu, C. C. Chan, Q. Liu, C. Xu, "In-fiber Photo-immobilized Bioactive Surfaces for Liposome Tethering," in 2015 Conference on Lasers and Electro-Optics Pacific Rim, Busan, 2015, p. 28H2_4.
2. D. Yong, E. Lee, K. Y. Lee, X. Yu, C. C. Chan, Q. Liu, "In-fiber photo-immobilization of bioactive surfaces: An Optimization Study," *submitted as proceedings of ICMAT 2015*.
3. D. Yong, W. L. Ng, E. Lee, X. Yu, M. Bosman, and C. C. Chan, "Multi-layered liposomes as optical resonators," *Proc. SPIE 8598, Bioinspired, Biointegrated, Bioengineered Photonic Devices, 859803*, 2013.
4. D. Yong, E. Lee, W. L. Ng, X. Yu, and C. C. Chan, "Lab-in-fiber platform for plasmonic photothermal study," *Proc. SPIE 8615, Microfluidics, BioMEMS, and Medical Microsystems XI, 861504*, 2013.
5. D. Yong and M. Yu, "Infiltration-free Photonic Bandgap Fiber Sensor," in *Frontiers in Optics 2011/Laser Science XXVII, OSA Technical Digest (Optical Society of America, 2011), FWT3*, 2011.
6. D. Z. Y. Yong, X. Yu, C. C. Chan, Y. Zhang, and P. Shum, "Photonic crystal fiber integrated microfluidic chip for highly sensitive real-time chemical sensing," in *Proc. SPIE 7753, 21st International Conference on Optical Fiber Sensors, 775343*, 2011,
7. D. Yong, X. Yu, S. Zhang, C. C. Chan, Y. Zhang, H. P. Ho, and D. Liu, "Plasmonic-enhanced fluorescence emission using D-shape microstructured optical fiber," *Journal of Physics: Conference Series*, vol. 277, pp. 012044, 2011.

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