UNIVERSITY OF SOUTHAMPTON

Synthesis and Application of Polystyrene Latex Microspheres

by

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Master of Philosophy

Department of Chemistry
Faculty of Science

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To Bordin,

to my family,

to my friends,

to my land,

with all my love

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

CHEMISTRY

Master of Philosophy

SYNTHESIS AND APPLICATION OF POLYSTYRENE LATEX MICROSPHERES

By Nutcha Chitkul

The aims of this project were the synthesis of polystyrene latex microspheres, within a size range capable of being able to be taken up by cells, and their subsequent functionalization with biomolecules, such as oligonucleotides or oligopeptides.

Polystyrene latex microspheres possessing hydroxy, carboxylate and amino functionalities were synthesized by emulsifier-free emulsion co-polymerization. The polystyrene latex microspheres were characterized by IR and titration methods. The size distribution was determined by scanning electron microscopy and photon correlation spectroscopy.

The synthesized microspheres were labelled with dyes such as 4(5)-carboxyfluorescein and studies with ND7 cells indicated cellular uptake of these microspheres, offering potential for development as a reporter or carrier system. Successful solution phase studies with microsphere bound olgionucleotides and a FRET peptide showed the possibility of use as a real time probe.

Table of Contents

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Finally, special thanks to my mother for her love and allowing me to stay away from home for so many years. Now its time to go back to my land.

ABBREVIATIONS

aa	amino acid
Abz	anthranilic acid
Boc	t-butyloxycarbonyl
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DIC	*N*, *N*-diisopropylcarbodiimide
DITC	1,4 phenylene diisothiocyanate
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's Modified Eagles Medium
DMF	dimethylformamide
DVB	divinyl benzene
Eq	equivalent
EtOH	ethanol
ES+	electrospray positive ion
FAM	4(5)-carboxyfluorescein
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
Fmoc	9-fluorenylmethoxycarbonyl
FTIR	fourier transform infrared
s	strong
w	weak
m	medium
br	broad
h	hour
HEMA	hydroxyethyl methacrylate
HMPA	4-hydroxymethylphenoxyacetyl
HOSu	*N*-hydroxysuccinimide
HOBt	*N*-hydroxybenzotriazole
HPLC	high performance liquid chromatography
MAA	methacrylic acid
NMR	nuclear magnetic resonance

δ	chemical shift (ppm)
δ_C	chemical shift ^{13}C
δ_H	chemical shift ^{1}H
d	doublet
dd	doublet of doublet
dt	doublet of triplets
J	coupling constant (Hz)
s	singlet
t	triplet
O^{t}Bu	t-butoxy
RP-HPLC	reverse-phase high performance liquid chromatography
RT	room temperature
R_t	retention time
SPPS	solid phase peptide synthesis
^{t}Bu	tertiary butyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropylsilane
TLC	thin layer chromatography
Tris	*tris* (hydroxymethyl) aminomethane
VBAH	vinylbenzylamine hydrochloride

Amino acid are abbreviated using standard 3-letter codes and all are configured L as follows:

Asp (D)	aspartic acid
Ala (A)	alanine
Glu (E)	glutamic acid
Gly (G)	glycine
Lys (k)	lysine
Ser (S)	serine
Tyr (Y)	tyrosine
Val (V)	valine

CHAPTER 1

POLYMER MICROSPHERES

1.1 INTRODUCTION

The bioavailablity of drugs or molecular probes directed at intracellular targets depends significantly on their being sufficiently polar for administration yet sufficiently non-polar for passive diffusion through the relatively non-polar lipid bilayer of the cell. Several techniques have been developed to ensure effective cellular uptake, such as incorporation into cationic liopsomes[1a] attachment to dendrimers[1b] and siderophores[1c], all of which have their drawbacks.

Other methods of cellular penetration involve harnessing the cells' own transport mechanisms. Cells actively engulf and internalize compounds from the extracellular space in a process known as endocytosis. The engulfed material is actively transported within the cellular system, though the exact mechanism of transport is not fully understood. Neuroanatomists have exploited this phenomenon since the 1960's to study the regional connectivity of axonal tracts in the central and peripheral nervous systems. One type of tracer that falls in this category are latex microspheres labelled with fluorescent markers[1d]. Once engulfed, these tracers are actively transported throughout the cell and, due to their relative inertness, possess low cytotoxicity. In fact they can remain within cells without adverse effects for several months.

The remainder of this chapter will focus on microspheres: their biological applications and synthesis.

1.2 BIOLOGICAL APPLICATION OF LATEX MICROSPHERES

Microspheres have been used in numerous biological and medical applications. Their sizes vary from 10 nm to 100 μm, depending upon the requirement of the application. Table 1.1[2] summarizes some of the biomedical applications of microspheres according to their size.

Table 1.1 Biomedical applications of polymer microspheres.

Biocompound	Size (micron)	Use of microspheres
Protein	0.01	
Virus	0.1	Cell label Particle for phagocytosis assay Latex diagonostics Protein separator
Bacterium	1.0	Drug carrier Blood flow indicator Cell separator
Cell	10.0	Column packing reagent Embolum
	100	Heterogeneous immunoassay support Cell culture carrier

1.3 SYNTHESIS OF POLYMER MICROSPHERES

Polymer microspheres can be described as polymeric particles that are in the order of sub-micron to several microns in diameter. Originally the term *latex* referred to a dispersion of microspheres from natural rubber whereas the term *polymer colloid* was associated with a suspension of synthetically produced microspheres. Nowadays the terms latex and polymer colloid are used interchangeably and have essentially the same meaning.

Functionalized microspheres have received a great deal of interest in two main areas:

(i) Firstly, they can provide useful models for fundamental studies in colloid science, physics and rheology, establishing synthesis-structure-properties in emulsion polymers;

(ii) Secondly, they are currently used in a wide range of applications e.g. binders (paints, paper coating, textile) or as solid supports in medical and biological fields[2] (Table 1.1).

In both cases, various methods and strategies to prepare these micro-particles in various sizes and with a variety of surface group functionalities have been reported and can be classified in two main categories:

(i) Physical methods, such as spray-drying and emulsification;

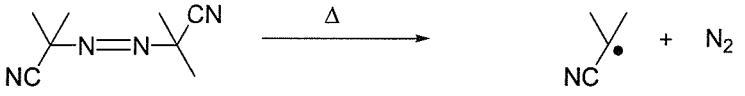
(ii) Chemical methods, such as heterogeneous polymerization (emulsion, dispersion, etc). Most microspheres are prepared by emulsion polymerization.

The efficient production of functional latex microspheres requires the synthesis of monodisperse microspheres (a ratio of weight–average diameter to number–average diameter less than 1.005) that will allow reliable and reproducible results from their applications. In order to produce particle bearing these properties it is necessary to understand the principles of particle nucleation and growth in particle-forming polymerization.

1.4 MODES OF EMULSION POLYMERIZATION

Two types of emulsion polymerization have been identified: Firstly, emulsion polymerization in the presence of an emulsifier,[3-5] and secondly, emulsifier–free emulsion polymerization.[6-10] The features of latex microspheres prepared by emulsion polymerization is dependent on the monomer, emulsifier and initiators used. To date persulfate has been used as initiator in which case the polymer chains are terminated by sulfate, hydroxyl, or carboxyl groups[4,11,12] resulting from hydrolysis of the sulfate end groups which are not completely stable under the reaction conditions.

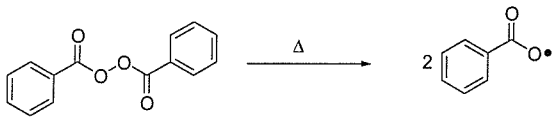
Other initiators[13] include azo compounds. One of the most commonly used initiators is 2,2′-azobisisobutyronitrile (AIBN). The thermal decomposition of AIBN is shown in Equation 1.1.



(1.1)

Other azo-compounds commonly used as initiators include 2,2′-azobis(2-methylpropionamide) dihydrochloride ($(NH_2)(NH)(CH_3)_2$-N=N-$(CH_3)_2(NH)NH_2$ ·2HCl) and dimethyl-2,2′-azobisisbutyrate (MAIB) $(CH_3)_2C(C(=O)OCH_3)N=N$-$C(C(=O)OCH_3(CH_3)_2$

Peroxides also constitute a class of initiator. Benzoyl peroxide is an organic peroxide widely use as a thermal initiator. Its primary decomposition represented in Equation 1.2.



(1.2)

The radical formed subsequently decomposes according to Equation 1.3.

(1.3)

Thus in the polymerization processes involving benzoyl peroxide as an initiator, the polymerization is initiated by both C_6H_5-C(=O)-O· and C_6H_5· radicals.

1.4.1 Emulsion polymerization in the presence of an emulsifier

Emulsion polymerization, also known as latex polymerization, is a heterogeneous process and generally requires two immiscible phases in the presence of a water soluble initiator and an emulsifier, such as a surfactant (e.g. sodium lauryl sulfate ($CH_3(CH_2)_9CH_2OSO_3^-Na^+$)) or a detergent in order to stabilize the emulsion. A batch emulsion polymerization process can be divided in to three distinct intervals[3] as exemplified in Figure 1.1.

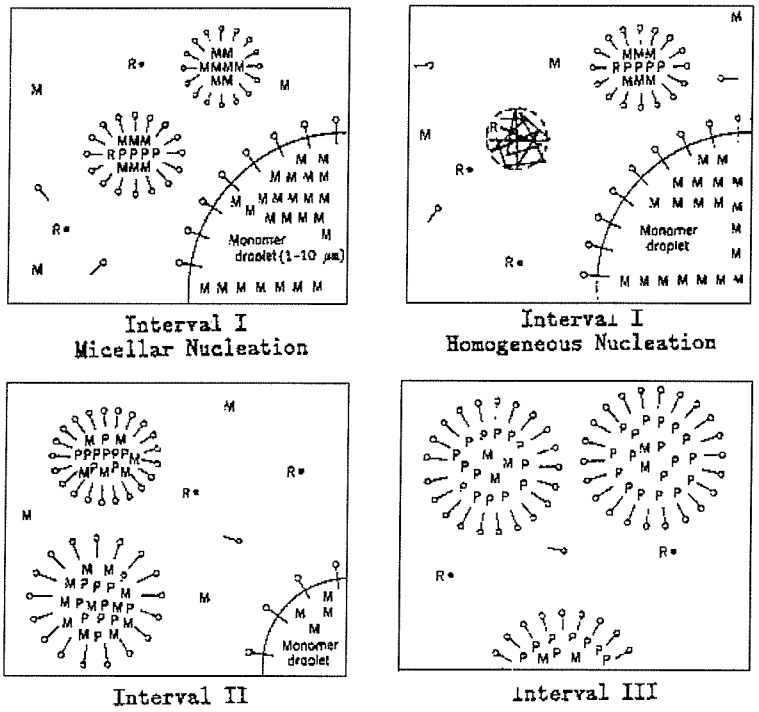


Figure 1.1: Representation of emulsion polymerization in batch.[3]

R˙ represents an initiator radical, M a monomer molecule, P a polymer molecule and —O a surfactant molecule. Interval I is the particle formation period, interval II is the contact growth period and interval III is the period in which monomer concentration decreases sharply.

The surfactant gives rise to micelles, each incorporating the organic monomer represented in Interval I (Figure 1.1, upper left). The monomer residues mainly in larger size (>1 μm) droplets, which act as reservoirs of monomer rather than taking a direct part in the polymerization process, as represented in the lower right corner of upper left Interval I. When reaction starts, the initiator decomposes to from radicals which react with the monomer (M) dissolved in the aqueous phase and become oligomeric radicals (R˙) which are then adsorbed onto monomer micelles and start polymerization. The resulting growth of the chain is represented in Interval I (top right of Figure 1.1). As the polymer forms, more M enters the micelle from the large reservoir droplets after diffusing through the aqueous phase. This process is continuous: the large monomer reservoir droplets being depleted whilst the micelles swell as they accumulate polymer chains as the reaction time passes in the batch reaction process. This is evident by the two left-hand parts in Interval II of Figure 1.1. Thereafter, the large droplets will have disappeared when 50-75% of the initial M has

been incorporated into chain of polymer. This is represented by the lower right-hand part in Interval III of Figure 1.1.

It is clear that emulsion polymerization is a heterogeneous process, with active species traversing phase boundaries. The mode of polymerization can be use to produce spherical particles in the order of 0.1-0.5 μm.

1.4.2 Emulsifier–free emulsion polymerization

In the emulsifier–free emulsion polymerization process, polymerization occurs in a similar manner to emulsion polymerization except it is conducted in the absence of an emulsifier. The basic materials are water, monomer, with or without ionic co-monomer and initiator. The process of emulsifier-free emulsion polymerization proceeds through a complex mechanism,[6-8] a simplified mechanism is shown in Figure 1.2.

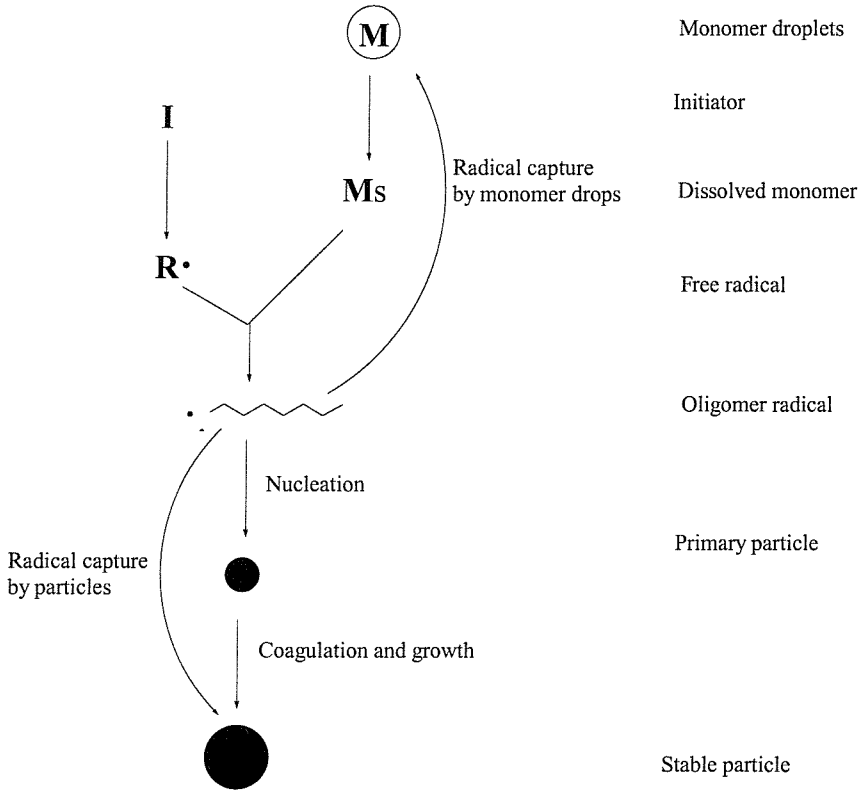


Figure 1.2: Schematic of particle-formation mechanism [6]

Water is the continuous phase and the co-monomer and initiator are dissolved in water. Initially the monomer is present either as dissolved monomer or monomer droplets. It is generally considered that the formation of particles occurs through homogeneous nucleation mechanism containing three sequential steps. Firstly the reaction starts in water phase; the initiator decomposes to form radicals which react with the dissolved monomer to form oligomeric radicals. The polymerization process mainly takes place in these particles and then stabilized by collapsing on itself or by coalescing with other oligomeric radical to form primary particles. At this time the number of polymer particles remains constant while the monomer diffuses from the droplets into the particles. The reaction rate is relatively rapid and constant until the monomer droplets disappear. Finally, primary particles in the system may coagulate and coalesce to from a colloidally more stable particles. The end of the nucleation stage in this system occurs when stable particles are sufficiently large and numerous to capture all newly formed oligomeric radicals as shown in Figure 1.2.

1.5 COMPARISON BETWEEN EMULSIFIER-FREE EMULSION POLYMERIZATION AND EMULSIFIER EMULSION POLYMERIZATION

The preparation of polymer microspheres by emulsion polymerization containing emulsifier is generally suitable for particles ranging in diameter from 0.1-0.5 μm, with solids contents as high as 50 %. However, for many cases there are certain drawbacks of this technique, e.g. the polymer particles tend to be partly stabilized by adsorbed surfactant[14] and removal of the surfactant can lead to floculation.[15] In other cases, the surfactant may affect the properties of the latex, e.g., an adsorbed surface layer may cause an apparent increase in diameter in suspension and, in electrophoretic measurements, it will affect the magnitude of the surface charge.[11] The addition of a small amount of nonionic emulsifier caused a bimodal distribution of particle size.[16] An example of a surfactant having an adverse effects includes surfactants that are used to disperse the human serum albumin microspheres. These have the potential of influencing tissue interactions and drug activity.[17] Removal of the emulsifier can prove to be difficult and whether complete removal can ever be achieved is debatable.[4,14] To overcome these potential problems, emulsifier-free emulsion polymerization is often preferred.

CHAPTER 2

PREPARATION AND CHARACTERIZATION OF POLYSTYRENE LATEX MICROSPHERES

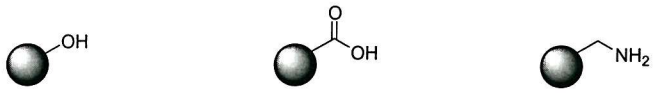
2.1 INTRODUCTION

In this chapter the preparation of a variety of functionalized polystyrene latex microspheres *via* emulsifier-free emulsion polymerization procedures are discussed.

2.2 RESULTS AND DISCUSSION

2.2.1 Synthesis of polystyrene latex microspheres

Three polystyrene latex microspheres **(1, 2** and **7)** having DVB as a crosslinking agent have been prepared possessing, hydroxyl, carboxyl and amino functionalization (Figure 2.1) using the emulsion polymerization emulsifier-free technique (Chapter 1, Section 1.4.2).

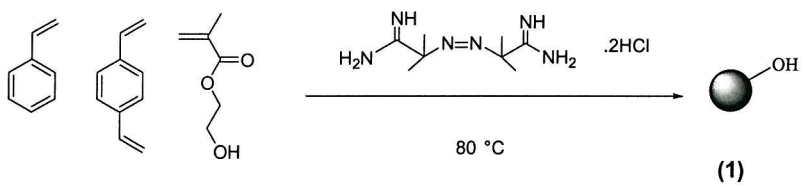


(1) **(2)** **(7)**

Figure 2.1: Representation of polystyrene functionalized latex microspheres.

2.2.1.1 *Synthesis of hydroxyl functionalized polystyrene latex microspheres (1)*

The hydroxyl functionalized polystyrene latex microspheres were synthesised using the emulsifier-free emulsion co-polymerization method described in Chapter 1 (Section 1.3.2). Hydroxyethyl methacrylate was added to give a theoretical loading of 2.6 mmol/g with 4.3 mol % divinyl benzene (with respect to styrene) as the cross linker and using 2-2′-azobis(2-methylpropionamidine) dihydrochloride[18] as the initiator (Equation 2.1) to give product **(1)** in 57% yield.

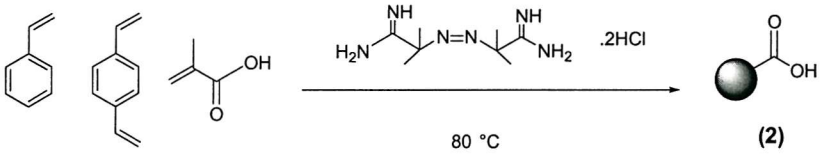


(1) (2.1)

The hydroxyl content was determined by titration with phthalic anhydride[19-21] to give a hydroxyl loading of 0.92 mmol hydroxyl/g.

2.1.1.2 *Synthesis of carboxyl functionalized polystyrene latex microspheres (2)*

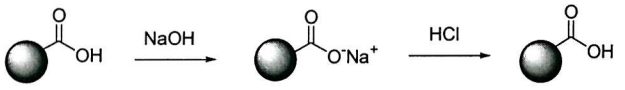
The carboxyl functionalized polystyrene latex microspheres were synthesised by the method described in Section 2.2.1.1. Methacrylic acid was added to give a theoretical loading of 1.6 mmol/g. Polymerization was conducted using 4.3 mol % divinyl benzene (with respect to styrene) as cross linker at 80 °C for 16 h (Equation 2.2) to give product **(2)** in 50% yield.



(2.2)

(2)

The presence of carboxylic groups were quantified by titration according to a protocol developed by Sivakumar *et al.*[22(b)] This involves the reaction of the polymer with NaOH and back-titration with HCl (Equation 2.3) to give loading of 1.35 mmol carboxyl/g.

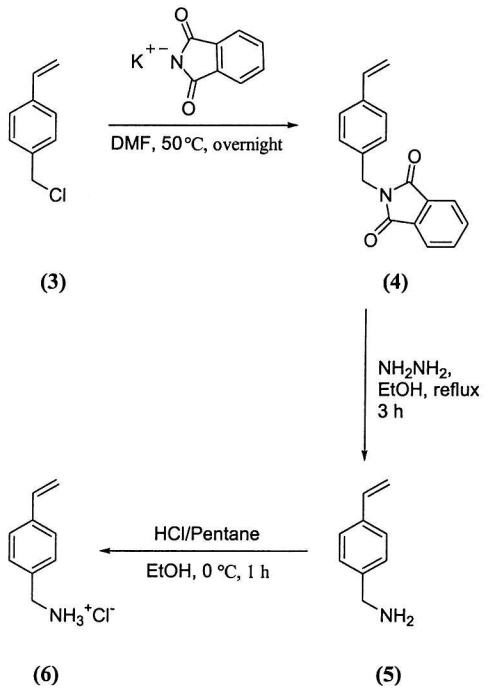


(2.3)

2.2.1.3 *Preparation of amino functionalized polystyrene latex microspheres (7)*

The amino functionalized polystyrene latex microspheres **(7)** were prepared in two steps.

(i). Preparation of the co-monomer **(6)**, vinylbenzylamine hydrochloride (VBAH), has literature precedence.[18] It has been shown to be a necessity to protect the amino group during polymerization and one possible protection method was found to be protonation to yield the hydrochloride salt **(6)**, which was obtained according to Scheme 2.1.

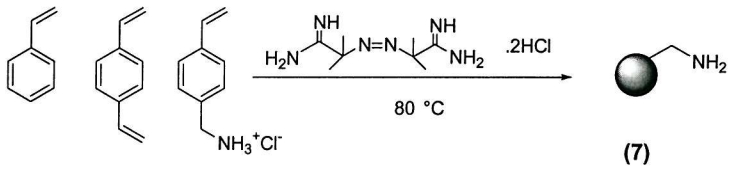


(3) (4) (5) (6)

Scheme 2.1: Synthesis of vinylbenzylamine hydrochloride (VBAH) (6).

The *p*-vinylbenzyl chloride **(3)** was converted to *p*-vinylbenzyl-*N*-phthalamide **(4)** in 88 % yield by nucleophilic displacement with potassium phthalimide. Subsequent hydrazinolysis afforded *p*-vinylbenzylamine **(5)**[23(a)] in 73 % yield and the free amino group was converted to the HCl salt using 1 N HCl in pentane to give vinylbenzylamine hydrochloride (VBAH) **(6)** in 96 % yield as a water-soluble monomer.

(ii). Co-polymerization by emulsifier-free emulsion polymerization of styrene was conducted using the method described in Section 2.2.1.1. VBAH **(6)** was added to give a theoretical loading of 0.3 mmol/g, using 2 mol % of DVB (with respective to styrene) as a crosslinker. Polymerization was carried out at 80 °C for 1.5 h (Equation 2.4) to give product **(7)** in 14 % yield.



(7) (2.4)

The amount of free amino groups on the resin was determined by a quantitative ninhydrin test developed for solid phase synthesis by Sarin, *et al.*[24] which gave a loading of 0.25 mmol/g.

Microanalysis gave a content of 88.36 % carbon, 6.99 % hydrogen and 0.42 % by mass of nitrogen. This corresponded to a loading of 0.3 mmol/g compared to the theoretical loading of 0.3 mmol amino/g.

2.2.2 Washing of the latex microspheres

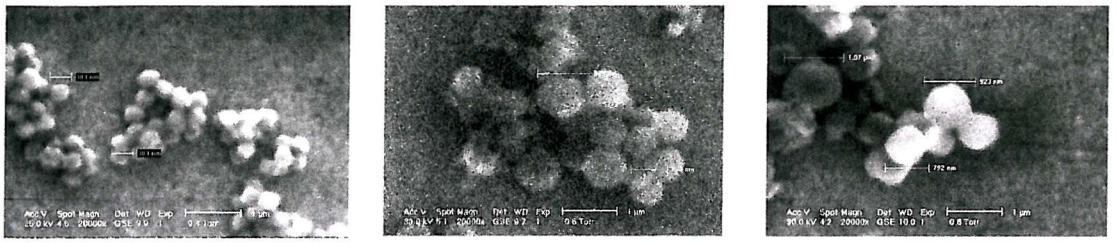
In all cases, before any characterization, the latex microspheres were washed using centrifugation or *via* a soxhlet with methanol and then water to eliminate any residual monomer and water-soluble polymers.

2.2.3 Particle size distribution and scanning electron micrographs of polystyrene latex microspheres

The particle size distribution of the polystyrene latex microspheres was determined using a 'COULTER® N_4 PLUS'counter (Figure 2.2a). The size of the particle ranged was found from 355 to 463 nm. However for run 2 (Figure 2.2b) and run 3 (Figure 2.2c) the determination of particle size distribution using this method was difficult to calculate. This may be attributed to a bigger particle size being formed during the polymerization step, which results in an aggregation of the beads. It was therefore necessary to use scanning electron microscopy as a method of particle size distribution. For run 2 particle sites were to range from 600 to 800 nm and for run 3: 500 to 800 nm. The results indicate that the synthesized polystyrene latex microspheres obtained particles ranging from 0.3 – 0.8 μm in diameter. The results obtained were extremely encouraging as these particles were adequate in diameter to allow internalization by cells (on average a diameter ≤1.8 μm was found to be sufficient).[23b] The low latex microspheres yield may be attributed to the low radical production rate in the continuous phase during nucleation and growth stage.

Table 2.1 Functionalization of polystyrene latex microspheres

Run	Functional monomer (g)			Co-monomer (g)	%Covn
	HEMA	MAA	VBAH	Styrene	
1	4.50	-	-	8.55	57
2	-	1.50	-	8.55	50
3	-		0.45	10.00	14

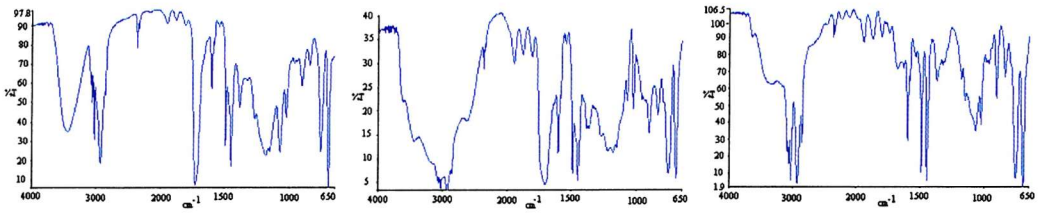


(a) **(b)** **(c)**

Figure 2.2: Particle size distribution of polystyrene latex microspheres. Scanning electron micrographs: (a) run 1; b) run 2; a) run 3

2.2.4 FT-IR spectra of polystyrene latex microspheres

The FT-IR spectra of the polystyrene latex microspheres **(1)**, **(2)** and **(7)** are shown in Figure 2.3. Microspheres **(1)** showed strong and sharp absorption bands at 3448 and 1723 cm^{-1} indicating the presence of hydroxyl and ester groups. Likewise, presences of hydroxyl and carbonyl groups were evident from the absorption bands at 3300-2500 and 1701 cm^{-1} for the carboxylate microspheres **(2)**. Finally the amino functionalized microspheres **(7)** show a broad band at 3360 cm^{-1} representative of the amino functionality.



(1) **(2)** **(7)**

Figure 2.3: FT-IR of polystyrene latex microspheres (1), (2), (7)

2.2.5 Solubility of latex microspheres

In addition, it is of worth to note that the microspheres prepared are not soluble in any organic solvent (DMF, acetone, THF) this may be attributed to, the divinyl benzene[25] crosslinking agent.

2.3 CONCLUSION

These results show that polystyrene latex microspheres incorporating DVB as a crosslingking agent can easily be prepared. Three polystyrene latex microspheres types (hydroxyl, carboxyl and amino functionalized) were prepared in which the size distribution was from 0.3 μm to 0.8 μm in diameter which is within the range required for cellular uptake.

CHAPTER 3

APPLICATIONS OF POLYSTYRENE LATEX MICROSPHERES

3.1 INTRODUCTION

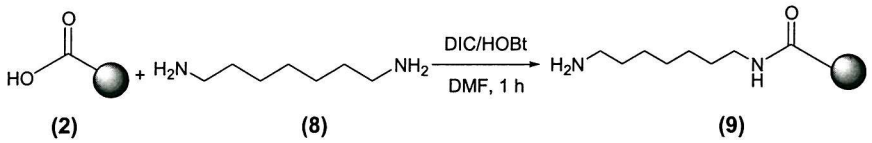
This chapter describes some of the chemical reactions of the various functionalized polystyrene latex microspheres and their applicability in biological assays.

3.2 INITIAL EXAMINATION OF MICROSPHERES AS POTENTIAL CELL TRANSPORT VEHICLES

3.2.1 Preparation of amino functionalized latex microspheres (9)

In order to introduce an amino functionalized spacer arm as shown in Equation 3.1 carboxylate polystyrene latex microspheres **(2)** were reacted with diaminoheptane **(8)** using the DIC/HOBt coupling strategy. The suspension was then washed *via* centrifugation with DMF and MeOH. A qualitative ninhydrin test indicated a positive result and a quantitative ninhydrin test gave a loading of 0.97 mmol/g for **(9)**. Which represents a 72 % conversion from the initial carboxyl polystyrene latex microspheres.

Microanalysis of compound **(9)** gave a content of 81.43 % carbon, 8.41 % hydrogen and 2.46 % of nitrogen. This corresponded to an amino loading of 0.87 mmol/g.

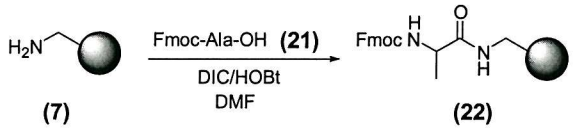


(2) (8) (9) (3.1)

Monitoring of the reaction was followed by FT-IR spectroscopy resulting in an almost complete disappearance of the C=O stretch at 1723 cm^{-1} of the carboxylic acid, and the appearance of an amide (C=O) stretch at 1635 cm^{-1}.

3.2.2 Attachment of Fmoc-Ala-OH to amino functionalized polystyrene latex microspheres (7)

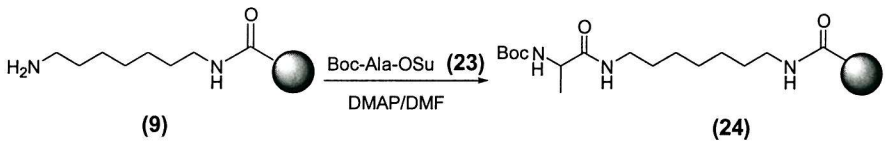
Amino functionalized polystyrene latex microspheres **(7)** were reacted with Fmoc-Ala-OH **(21)** using the DIC/HOBt coupling strategy to give compound **(22)** as shown in Equation 3.2. The coupling was driven to completion by using an excess of reagents and was monitored qualitatively by the Kaiser test.



(7) (22) (3.2)

3.2.3 Attachment of Boc-Ala-OSu to amino fucntionalized polystyrene latex microspheres (9)

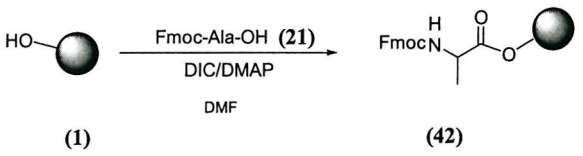
Amino functionalized polystyrene latex microspheres **(9)** were reacted with Boc-Ala-OSu **(23)** under mild conditions to give compound **(24)** (Equation 3.3). The reaction being monitored by the Kaiser test.



(9) (24) (3.3)

3.2.4 Attachment of Fmoc-Ala-OH to hydroxyl functionalized polystyrene latex microspheres (1)

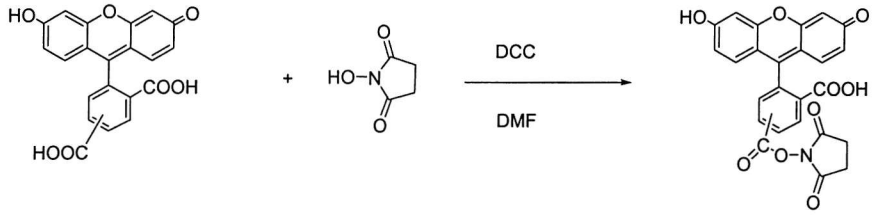
Hydroxyl functionalized polystyrene latex microspheres **(1)** were reacted with Fmoc-Ala-OH **(21)** using DIC/DMAP in DMF to give a negative ninhydrin test, producing compound **(42)** as shown in Equation 3.4. A quantitative Fmoc test gave a resin substitution 0.77 mmol/g. Monitoring of the reaction was followed by FT-IR, resulting in a disappearance of the hydroxyl band at 3448 cm^{-1}.



(1) (42) (3.4)

3.2.5 Synthesis of 4(5)-carboxyfluorescein active ester (12)

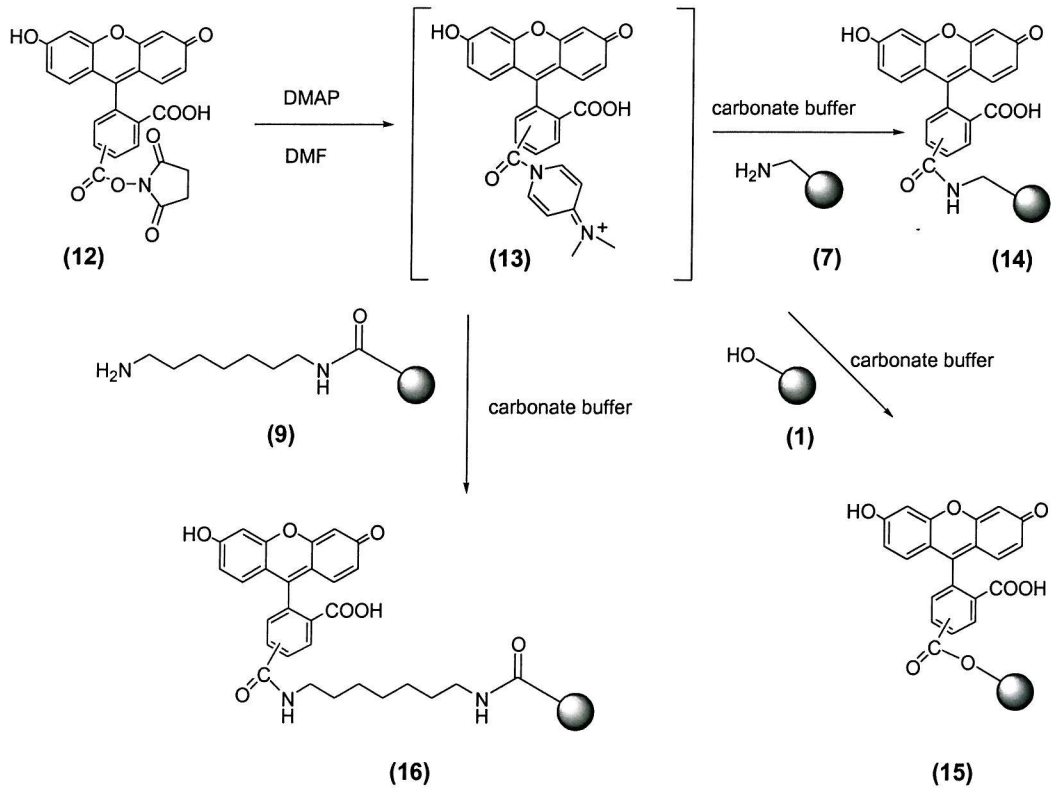
4(5)-Carboxyfluorescein active ester **(12)** was synthesised by reacting 4(5)-carboxyfluorescein **(10)** with HOSu **(11)** in the presence of DCC (Equation 3.5).[26] After elimination of DCU by filtration, the DMF was removed *in vacuo* and product dried under vacuum. The crude product used in subsequent reactions.



(10) (11) (12) (3.5)

3.2.6 Preparation of fluorescein latex microspheres

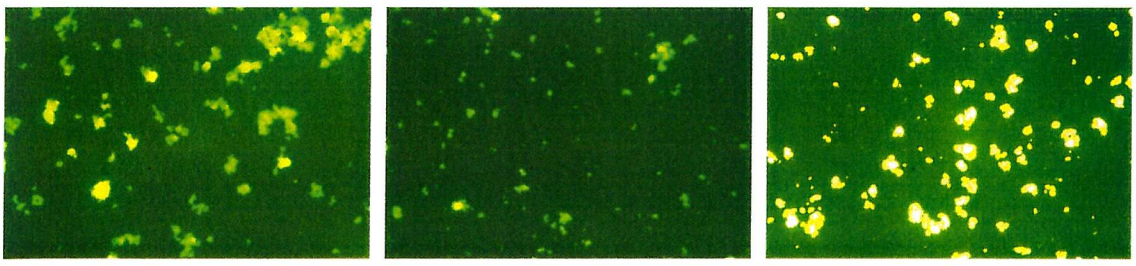
Using the procedure reported by Bologna *et al.*[26] the polystyrene latex microspheres (**(1)**, **(7)** and **(9)**) were labelled with a 4(5)-carboxyfluorescein by reacting with **(12)** in the presence of DMAP in carbonate buffer (0.1 M $NaHCO_3$, 1 M NaCl, pH 9.30) (Scheme 3.1). The reaction between the latex microspheres (**(1)**, **(7)** or **(9)**) and activated carboxyfluorescein **(13)** was rapid, occurring within 5 minutes at room temperature. The polystyrene latex microspheres were then washed with DMF and MeOH using centrifugation to furnish fluorescein-labelled polystyrene latex microspheres **(14, 15** and **16)**.



Scheme 3.1: Labelling of latex spheres.

3.2.6.1 *Analysis of the fluorescently labelled microspheres by fluorescence microscopy*

Fluorescently labelled microspheres were analysed under a fluorescence microscope using a FITC filter (Figure 3.1). Analysis indicated that the polystyrene latex microspheres **(1, 7 and 9)** were labelled.



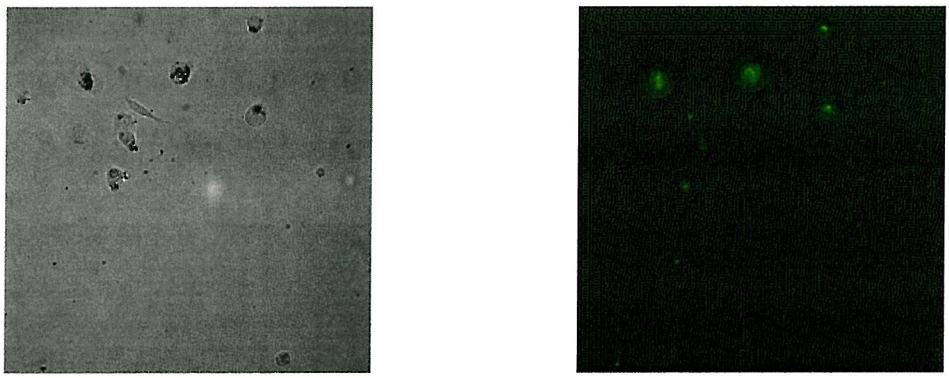
(a) **(b)** **(c)**

Figure 3.1: Representation of fluorescently labelled microspheres a: (14); b: (15); c: (16).

3.2.6.2 Uptake of fluorescently labelled polystyrene latex microspheres by ND7 cells

As previously discussed latex microspheres are able to penetrate and provide a means of delivering biological molecules into cells (Chapter 1, Section 1.2). Our first priority was to determine whether the fluorescently labelled latex microspheres **(14, 15** and **16)** were able to pass into cells. For this study the ND7 cell line, a mouse-rat neuronal hybrid, was used. The cells were incubated at 37 C° with 2.5-10 % labelled latex microspheres in growth medium for 24 h. After this time period, the cells were washed with fresh growth medium to remove any residual fluorescently labelled polystyrene latex microspheres remaining in the medium. Cells then analysed using a fluorescence microscope using a FITC filter. Analysis indicated that all three types of fluorescein-labelled microspheres **(14, 15** and **16)** are taken up by the ND7 cells, resulting in green fluorescence. Figure 3.2 is representative of all three types of polystyrene microspheres, Figure 3.2 (a) shows the ND7 cells under normal light and Figure 3.2 (b) is after incubation with labelled polystyrene latex microspheres. Control experiments (minus the polystyrene latex microspheres) showed no fluorescence.

These results verify that these microspheres may be used as a potential carrier or transporter system.



(a) **(b)**

Figure 3.2: The uptake of fluorescently labelled polystyrene latex microspheres (14) by the ND7 cells. (a) represents cells under normal light and (b) the same cells analysed under a FITC filter.

3.3 USE OF MICROSPHERES AS POTENTIAL BIOLOGICAL PROBES

Functionalization of the microsphere beads with a number of specific compounds, which are able to target specific sites within a cell, will allow the visualization of particular biological functions *in situ*. The aims of this section will be the custom functionalization of novel "sensor based" micro-bead probes designed to measure specific cellular events, such as protease activation, using fluorescence microscopy.

3.3.1 Use of FRET peptide probes

FRET peptide substrates are based on fluorescence resonance energy transfer (FRET). These substrates require the synthesis of a specific oligopeptide which contains a donor (fluorescent) group at one end of the substrate and an acceptor (quencher) moiety at the other end. It is customary to study the activity and specificity of proteases by measuring the level of products resulting from the enzymatic digestion of a suitable peptide. The intact substrates are poorly fluorescent whereas the cleaved substrates, liberated by enzymatic hydrolysis, are highly fluorescent. The hydrolysis of such an oligopeptide can be followed by a number of methods, such as HPLC[27], NMR spectroscopy[28] and spectrometric detection, but spectrometric analysis is usually preferred because of high sensitivity and convenience. Fluorescence monitoring of proteolytic activity is possible only if a change in emission occurs during the cleavage of a specific bond linking a suitable chromophore to the rest of the molecule. A variety of chromophores-bearing oligopeptide derivatives have been successfully developed and utilized in assaying proteolytic enzymes. In order to use spectrophotometric methods it has been necessary for development of fluorogenic substrates quenched by resonance energy transfer techniques.

3.3.1.1 *Principles of fluorescence resonance energy transfer (FRET)*

There are two types of intramolecularly quenched substrates according to the nature of the quenching interaction:

(1) quenching through collision between the fluorophore and the quencher;

(2) quenching through non-radiative electronic excitation energy transfer between a fluorescent donor and a suitable acceptor.

3.3.1.1.1 *Fluorogenic substrates quenched by intramolecular collision*

The spectral properties of a fluorescent molecule can be affected by interactions with the environment around the fluorophore. An interaction with another molecule can result in a change in the fluorescence intensity by a variety of mechanisms. In dynamic quenching, the encounter with a quencher during the fluorescence lifetime competes with the emission for depopulation of the excited state. Static quenching,[29] on the other hand, occurs when a fluorescent group forms non-fluorescent complex by interaction with a quencher prior to excitation. Both cases, dynamic and static quenching, require short-range interactions and are termed "collision" or "contact" quenching.

3.3.1.1.2 *Fluorogenic substrates quenched by resonance energy transfer (RET)*

Resonance energy transfer, also known as non-radiative energy transfer, is a type of long-range quenching acting at distances of approximately 10 Å. The mechanism of action involves the transfer of energy from an exited fluorophore (donor) to another chromophore (acceptor). This transfer of energy is not mediated through direct contact of the donor-acceptor groups but as a result of an effective spectral overlap of the emission spectrum of the fluorophore and the absorption spectrum of the acceptor. If the acceptor is also a fluorophore, then excitation at the wavelengths of the donor, where the acceptor is not excited, will result in an emission characteristic of the acceptor. The efficiency of energy transfer (E), as developed by Förster, may be expressed by Equation 3.6.[30]

$$E = 1 - \frac{F}{F_0} = \frac{r_0^6}{r^6 + r_0^6} \qquad (3.6)$$

Where:

F = fluorescence intensity of donor in presence of acceptor

F_0 = fluorescence intensity of donor in absence of acceptor

r = distance between the centres of the two chromophores

r_0 = distance between the donor and acceptor when the efficiency is 50

r_0 may be defined by equation 3.7.

$$r_o = (8.78 \times 10^{-25} k^2 q J / n^4)^{1/6} \quad (3.7)$$

Where:

k^2 = an orientation factor

q = donor quantum yield in the absence of transfer

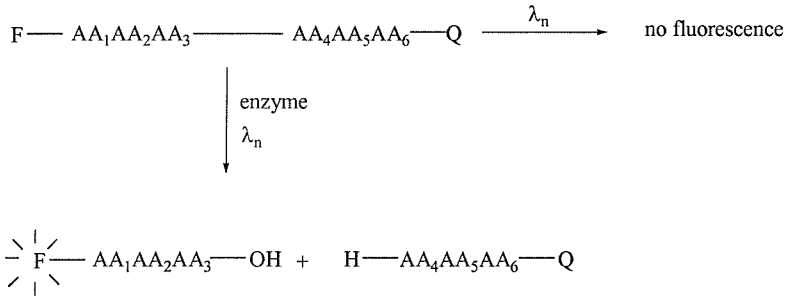
J = spectral overlap integral

n = refractive index of the solvent.

The main consequence of the FRET theory is that the efficiency of energy transfer directly correlates to the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor and this efficiency decreases with the sixth power of the distance between the chromophores. The latter dependence of energy transfer on distance raises the possibility that energy donor-acceptor pairs might be used to reveal proximity relationships in biological macromolecules. This relationship of energy transfer-distance dependence has been experimentally verified and there exist numerous models that substantiate the predictions according to Förster.[31-34]

3.3.1.2 *Proposed research using FRET peptides*

There exist a number of suitable donor-quencher pairs for use in FRET studies. The *o*-aminobenzoyl (Abz) group is a small fluorophore with high quantum yield and 3-nitrotyrosine is a well-known quencher. The pair possesses excellent spectra overlap properties resulting in an efficient energy transfer and they lend themselves very well to SPPS.[35-36] The aim of this research was the synthesis of an intramolecularly quenched peptide on solid phase incorporating the Abz and 3-nitrotyrosine donor-acceptor pair. It was decided to focus our attention on a peptide substrate containing a pepsin scissile bond and initially evaluate the substrate in solution (Scheme 3.2) before connection to the functionalized microspheres and subsequent evaluation as a probe.

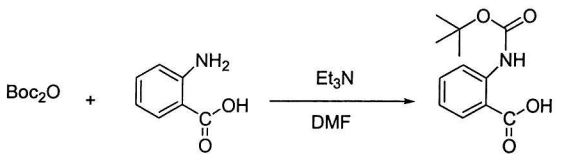


Scheme 3.2: Principle of FRET peptides
AA_n = amino acid residues; F = fluorophore; Q = quencher;
λ_n = excitation wavelength of fluorophore

3.3.1.3 *Synthesis of the fluorophore and quencher moieties*

3.3.1.3.1 *Synthesis of Boc-anthranilic acid (27)*[31]

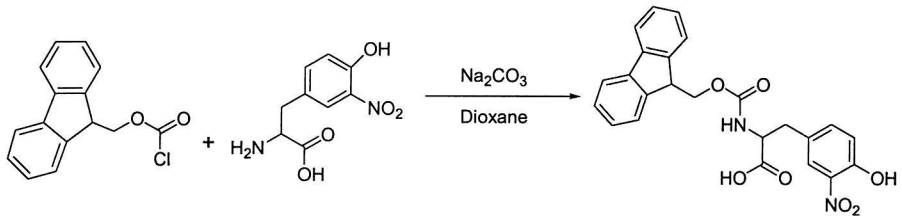
Boc-anthranilic acid (**27**) was prepared by reacting Boc_2O with anthranilic acid in the presence of triethylamine in DMF (Equation 3.8). Recrystallization of the crude material using 50% aqueous ethanol gave compound (**27**) in good yield (65%).



(25) (26) (27) (3.8)

3.3.1.3.2 *Synthesis of Fmoc-3-nitrotyrosine (30)*[31]

Fmoc-3-nitrotyrosine **(30)** was prepared in quantitative yield by the condensation of Fmoc-chloride **(28)** and 3-nitrotyrosine in dioxane in the presence of sodium carbonate (Equation 3.9).

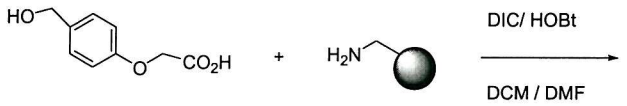


(28) (29) (30) (3.9)

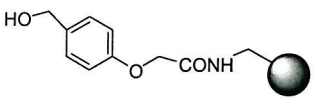
3.3.1.4 *Solid phase synthesis of FRET peptide (34)*

3.3.1.4.1 *Synthesis HMPA-resin (33)*

The linker **(31)** was coupled to an amino methyl polystyrene resin **(32,** 200-400 μm) using DIC/HOBt coupling method. The reaction was monitored by a qualitative ninhydrin test to give resin **(33)** as shown in Equation 3.10.



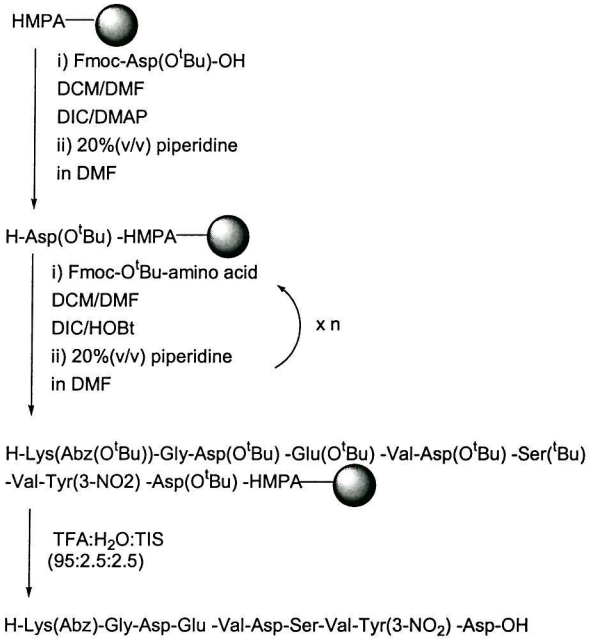
(31) (32) (3.10)



(33)

3.3.1.4.2 *Construction of the peptide on solid phase*

Esterification of the linker with the first amino acid Fmoc-Asp(O^tBu)-OH was carried out using DIC and DMAP. The subsequent Fmoc protected[37] amino acids were added sequentially *via* DIC/HOBt mediated coupling. The extent of each coupling reaction was assessed qualitatively by the ninhydin test until the final step, whereupon Boc–anthranilic acid was coupled. The peptide derivative was cleaved from the resin and simultaneously deprotected by treating the resin for 4 h with a cocktail of TFA:H_2O:TIS (95:2.5:2.5) to furnish the desired peptide **(34)** in 27% overall yield (Scheme 3.3).



(34)

Scheme 3.3: Synthesis of internally quenched fluorogenic substrate (34).

3.3.1.5 *Evaluation of internally quenched fluorogenic substrate (34)*

Peptide **(34)** was purified by semi-preparative RP-HPLC (Figure 3.3). Figure 3.4 shows analytical HPLC profile of the purified material at λ = 220 nm. The peptide was characterized by ES-MS (ES+ve), giving the desired $[M+H]^+$ ion (Figure 3.5).

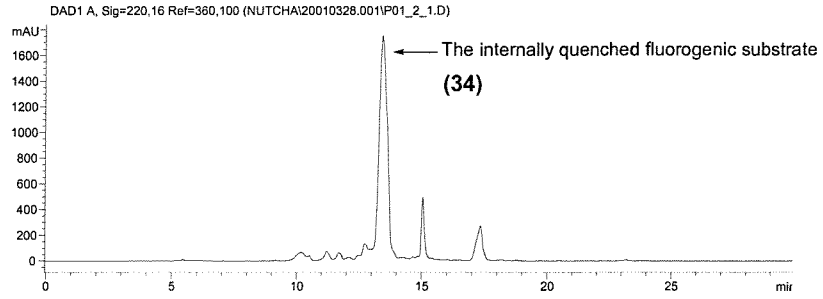


Figure 3.3: Semi-preparative RP-HPLC profile of crude material (34).

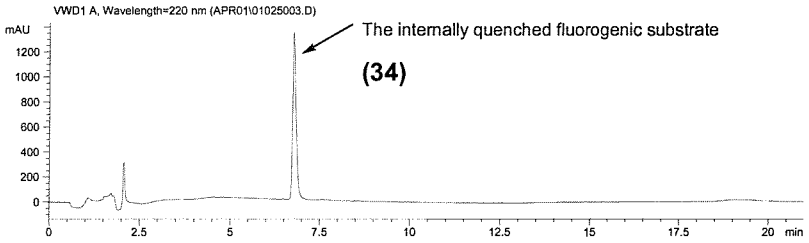


Figure 3.4: Analytical RP-HPLC profiles of the purified internally quenched fluorogenic substrate (34)

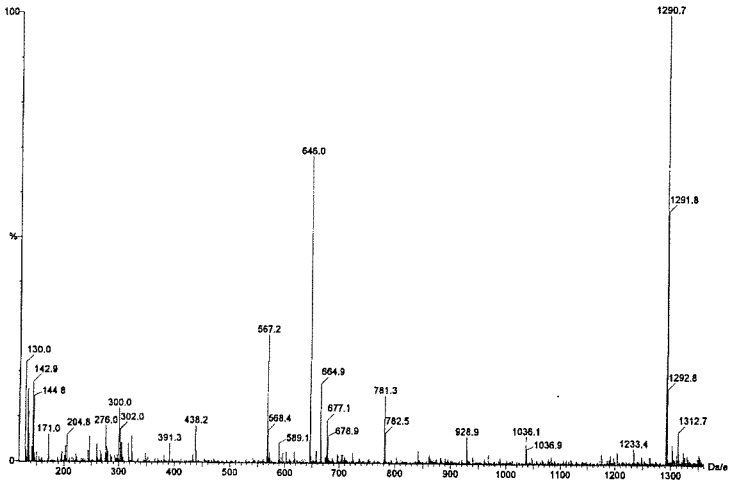


Figure 3.5: ES-MS of internally quenched fluorogenic substrate (34)

3.3.1.5.1 *Enzymatic hydrolysis of FRET peptide (34).*

FRET peptide **(34)** contains both pepsin and chymotrypsin sensitive bonds (Figure 3.6).[38] In this study, pepsin was the enzyme of choice. Pepsin was incubated with the FRET peptide in formic acid buffer (0.017 mg/mL, 50 mM, pH 3.4)

according to literature protocol.[31] Cleavage was monitored *via* 2 methods: HPLC and fluorimetric analysis.

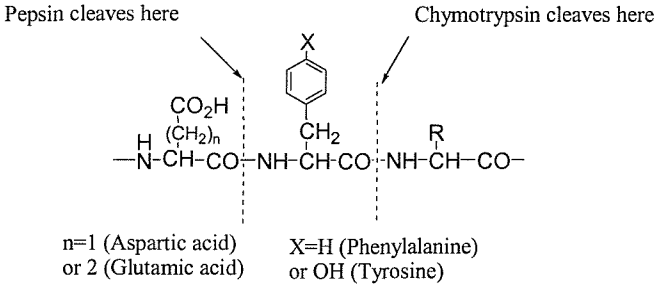
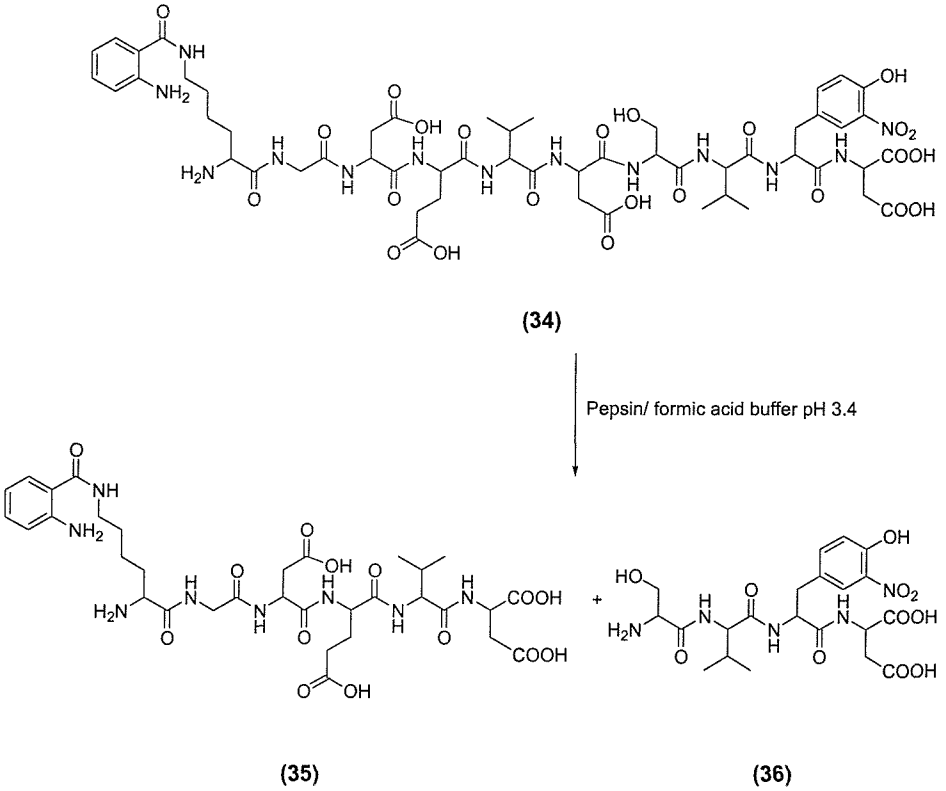


Figure 3.6: The representative of pepsin and Chymotrypsin hydrolysed peptide.

3.3.1.5.1.1 *HPLC analysis*[31]

Analytical HPLC analysis showed the hydrolysis of the substrate, producing 2 fragments **(35, 36)** (Equation 3.11 and Figure 3.7).



(3.11)

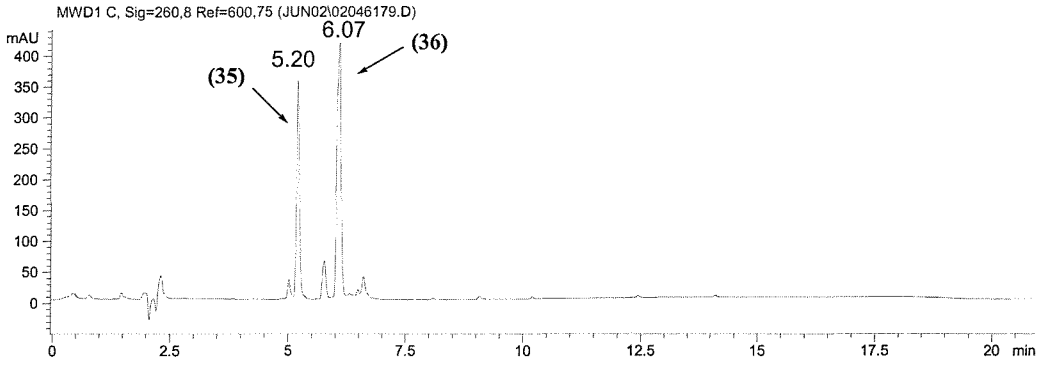
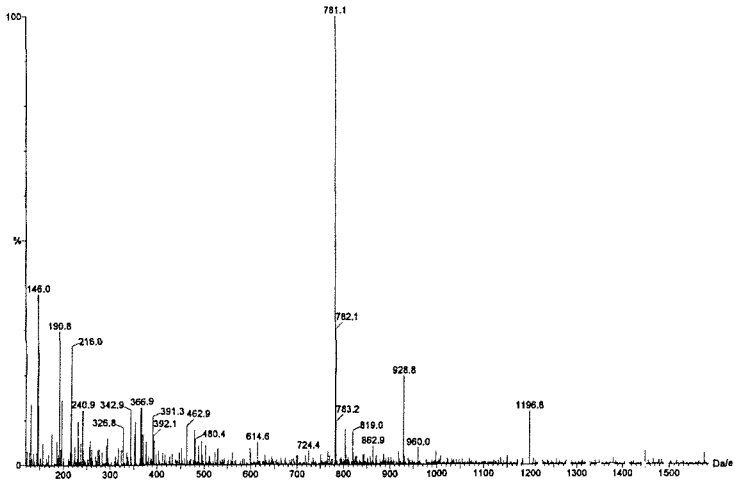
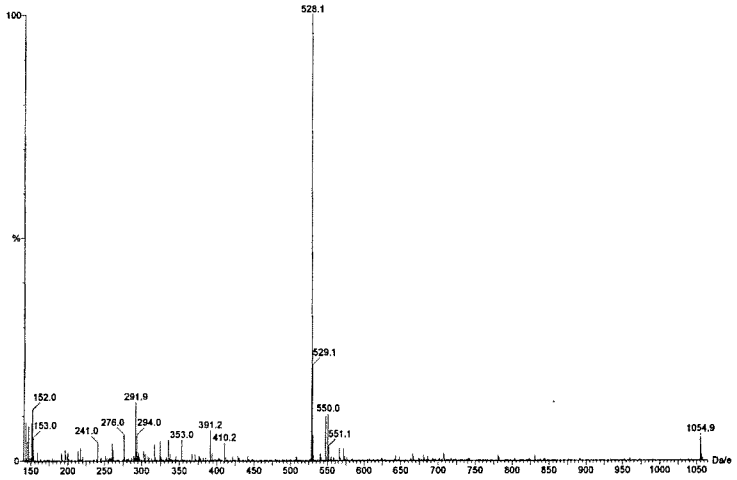


Figure 3.7: RP-HPLC profiles of the hydrolysed FRET peptide (34).

ES-MS (ES +ve) analysis of the 2 fragments **(35, 36)** correlated perfectly with the predicted cleavage, cleaving between the Asp-Ser residues of peptide **(34)** (Figure 3.8)



(35)



(36)

Figure 3.8: ES-MS spectra of peptide (35) (36).

3.3.1.5.1.2 *Fluorimetric analysis*[31]

Cleavage of peptide **(34)** by pepsin was also monitoring by fluorescence spectroscopy. A gradual increase in fluorescence intensity should be observed due to the separation of the fluorescent moiety (the anthranilic group) from the quencher moiety (3-nitro tyrosine) over time. Indeed this was the case: incubation of pepsin with the FRET peptide produced an increase in fluorescence when monitored at the emission wavelength of the anthranilic group (λ_{ex} = 320 nm, λ_{em} = 420 nm) (Figure 3.9), indicating proteolytic activity.

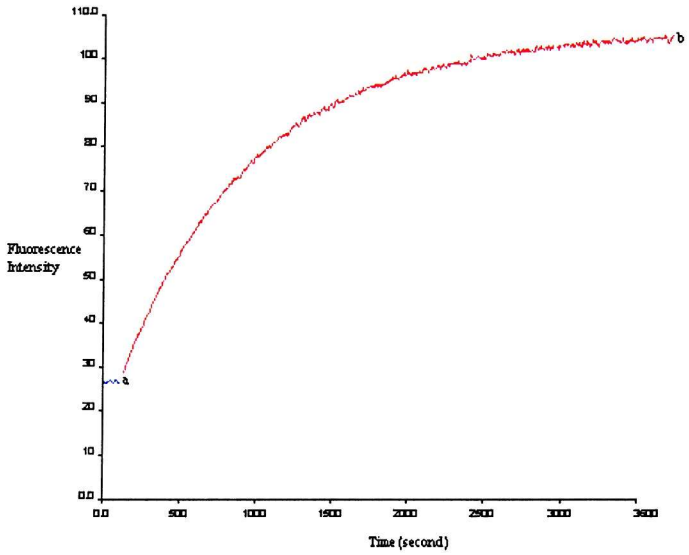
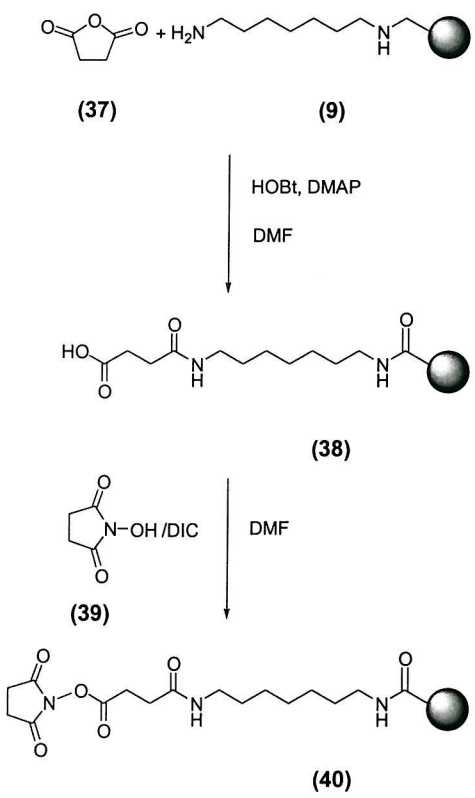


Figure 3.9: Progress curves for pepsin hydrolysis of FRET peptide (34) (a) the fluorescence of (34) in buffer (b) the fluorescence of (34) in buffer containing pepsin.

As can be seen from Figure 3.9, cleavage of the FRET peptide produces an approximately four-fold increase in fluorescence intensity in 50 mins. Also, it is noteworthy to indicate that the intact FRET peptide possesses a little residual background fluorescence due to incomplete quenching.

3.3.1.6 *Activation of the amino functionalized polystyrene latex microspheres (9)*

It was necessary to incorporate a spacer arm onto the polystyrene latex microspheres in order to minimize any undesirable steric interactions when coupling the FRET peptide. Amino functionalized polystyrene latex microspheres **(9)** were reacted with succinic anhydride **(37)** to give carboxyl functional microspheres **(38).** This carboxyl functional was activated using *N*-hydroxysuccinimide in DMF to give the corresponding succinimide active ester **(40)** (Scheme 3.4).



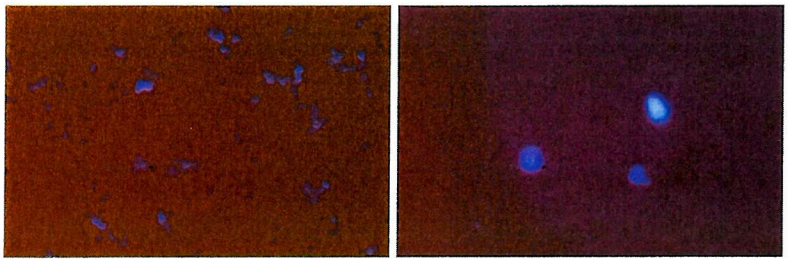
Scheme 3.4: Activation of the amino functionalized polystyrene latex microspheres (9).

3.3.1.7 *Attachment of the FRET peptide to the activated functionalized polystyrene latex microspheres (40)*

The FRET peptide **(34)** was reacted with active ester **(40)** in DMF for 3days. The resin was washed with DMF (3 x 1 mL) and methanol (3 x 1 mL), air dried to give compound **(41)** then analyzed under fluorescence microscope (Figure 3.10).

3.3.1.8 *Enzymatic hydrolysis of the immobilized FRET peptide by pepsin*

Fluorescence microscopy of the resin loaded FRET peptide **(41)** indicated little background fluorescence (Figure 3.16). This peptide, which contains a pepsin cleavable bond (Asp-Ser), was hydrolysed by pepsin in formic acid buffer (0.017 mg/mL, 50 mM, pH 3.4). Analysis under a fluorescence microscope indicated an increase in fluorescence intensity compared to the uncleaved FRET peptide (Figure 3.10).



(a) (b)

Figure 3.10: Fluorescence image of the immobilized FRET peptide (a) immobilized intramolecularly quenched fluorogenic substrate, (b) after cleaved by pepsin.

3.3.2 Use of molecular beacon probes

3.3.2.1 *Background into molecular beacons*

Molecular beacons are single-stranded oligonucleotide probes that possess a stem and loop hairpin structure (Figure 3.11). The loop portion of the molecule is a probe sequence that is complementary to a predetermined sequence in a target nucleic acid.[39-43] The stem is formed by annealing two short complementary arm sequences of nucleotides attached to the 3′ and 5′ ends of the probe sequence, the arm sequences being unrelated to the target sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm, as shown in Figure 3.11. When the unhybridized probe is in solution, the stem adopts a hairpin structure, keeping these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer (FRET).[39-47] The fluorescence is restored when the loop binds to a target nucleic acid allowing separation of the fluorophore from quencher.

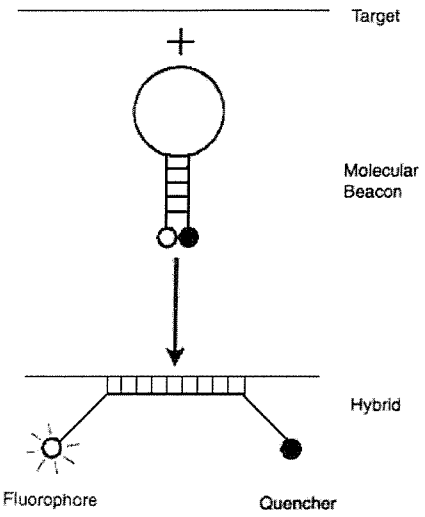


Figure 3.11: Operation of molecular beacons[39]

3.3.2.2 *Proposed research using molecular beacons*

Molecular beacons have been used for the detection of specific nucleic acids in homogeneous assays in living cells and have received great interest in DNA hybridization studies.[39-43, 45-47] However the real-time study of molecular beacons poses major drawbacks: generally, the probe is introduced *via* micro-injection which is an extremely tedious and laborious task. In order to eliminate this problem, it was proposed to covalently link the probe to functionalized microspheres in order to allow the possibility of real-time monitoring of DNA hybridization

3.3.2.3 *Structure of molecular beacons*

The molecular beacons are shown in Table 3.1.

Table 3.1 ***DNA oligonucleotides employed***

Sequence Name[a,b]	Type of modification[c,d]	Sequence 5′-3′	Sequence 3′-5′	Length (nt)[e]
Series A **MB 1**	5′-Amino 3′-**T**=ME RED **8**=dR FAM	**8**CGGCTTTTCTTAT GGCTCTGAGAGAC CTGACTTGGCCG**T**		40
Series A **OT 1**	Normal		AAAAGAATACCGA GACTCTCTGGACT GAAC	30
Series B **MB 2**	5′-Amino 3′-**T**=ME RED **8**=dR FAM	**8**CGGCCAAGTCAG GTCTCTCAGAGCC ATAAGAAAAGCCG **T**		40
Series B **OT 2**	Normal		GTTCAGTCCAGAG AGTCTCGGTATTC TTTT	30

[a]MB = Molecular Beacon, [b]OT = Oligonucleotide Target, [c]ME-RED = Methyl Red, [d]FAM = 4(5)-carboxy fluorescein, [e]nt = Nucleotide.

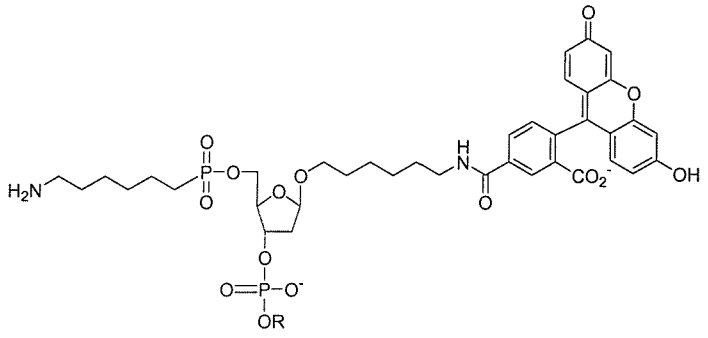


Figure 3.12: dR-FAM Monomer (5′ end of oligonucleotide).

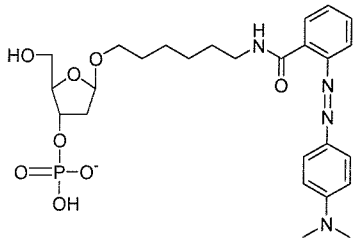


Figure 3.13: dR Methyl Red Monomer (3'end of oligonucleotide).

3.3.2.4 *Solution phase hybridization studies*[39b]

Before surface immobilization of the molecular beacons onto the microspheres, the molecular beacons were assayed in solution against their complementary targets.

3.3.2.4.1 *Fluorescence profile with complementary oligonucleotide target strand*

Initial fluorescence studies were conducted in order to determine the residual background fluorescence (Figure 3.14). Then hybridization reactions were performed using **MB 1** and **MB 2** (1 μM) and a ten-fold molar excess of the corresponding oligonucleotide target. The increase in fluorescence intensity was measured until a stable level was reached (λ_{ex} = 480 nm, λ_{em} = 515 nm) The results indicated that in the case of **MB 1**, hybridization with the complementary strand oligonucleotide resulted in a 18-fold increase in fluorescence intensity compared to the stem-loop structure and **MB 2** resulted in a 23-fold increase in fluorescence intensity when hybridized to its complementary oligonucleotide target strand (Figure 3.14).

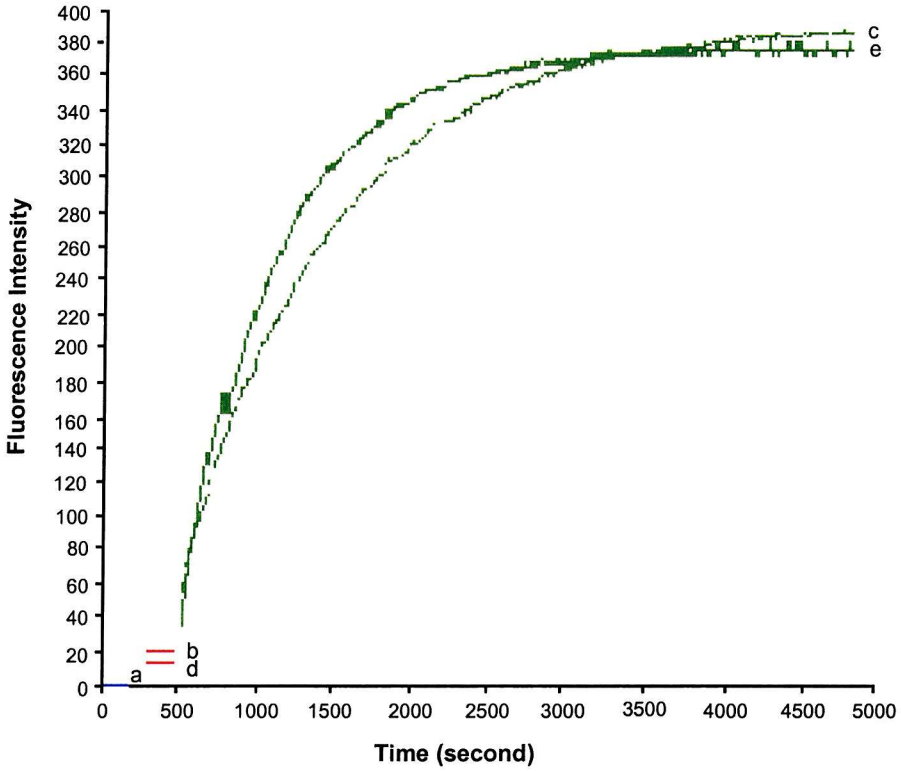


Figure 3.14: The fluorogenic response of the molecular beacon upon the addition of the target: (a) the fluorescence of the buffer, (b) the fluorescence of the buffer containing the molecular beacon (MB 1), (c) the increase in fluorescence that occurs upon the addition of target oligonucleotide (OT 1), (d) the fluorescence of the buffer containing the molecular beacon (MB 2), (e) the increase in fluorescence that occurs upon the addition of target oligonucleotide (OT 2).

3.3.2.4.2 *Thermal denaturation profiles*

Thermal denaturation profiles were performed to ascertain the thermal stability of the oligonucleotide probes. **MB 1** and **MB 2** (40 nM) was dissolved in molecular beacon buffer (3.5 mM $MgCl_2$, 10mM Tris-HCl, pH 8.0) and the oligonucleotide target added to make a final concentration of 64 nM. The fluorescence of each solution was determined as a function of temperature using a thermal light cycler. The results are shown in Figure 3.15 and Figure 3.16. Results indicate that the probe-target hybrid denatured at 62 °C and the stem of the molecular beacons denatured at 65 °C. In the range 27 °C to 50 °C, the free probe has very little fluorescence, whereas the target-bound form is fluorescent. The profiles indicate that

these two molecular beacons can be used below 50 °C, which make them ideal candidates as cell based probes in which experiments will be conducted at 37 °C.

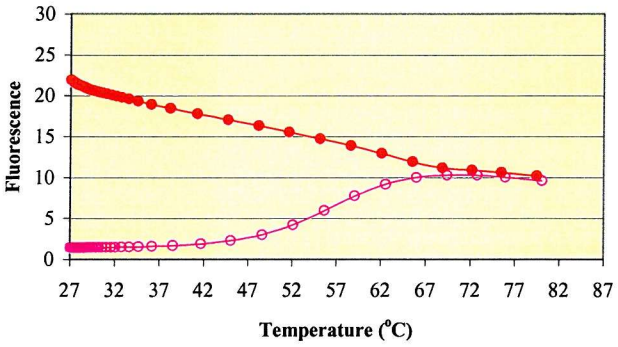


Figure 3.15: Thermal denaturation profiles of MB 1 (open circles) and the hybrid formed between the MB 1 and its oligonucleotide target (OT 1) (filled circles).

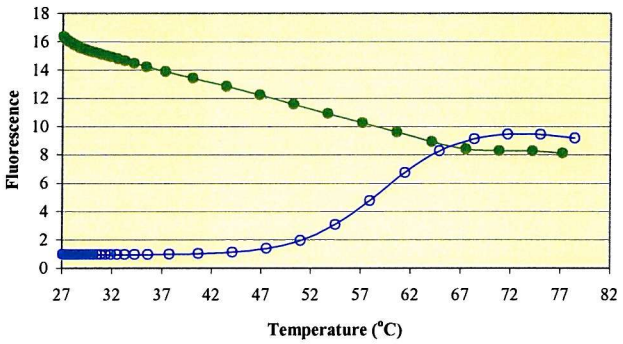


Figure 3.16: Thermal denaturation profiles of MB 2 (open circles) and the hybrid formed between the MB 2 and its oligonucleotide target (OT 2) (filled circles).

3.3.2.5 *Immobilization of molecular beacons to amino functionalized polystyrene latex microspheres (7)*

Immobilization of the molecular beacon **(17)** to the amino functionalized polystyrene latex microspheres **(7)** was performed *via* activation of the molecular beacon **(17, MB 1** or **MB 2)** using 1,4-phenylene diisothiocyanate (DITC) **(18)** in carbonate buffer (0.1 M $NaHCO_3$, 1.0 M NaCl, pH 9.3) to give immobilized molecular beacon **(20a** (for **MB 1**), **20b** (for **MB 2))** (Figure 3.17) following literature protocol.[48] Fluorescence analysis of the immobilized molecular beacons using a FITC filter showed a little background fluorescence (Figure 3.18).

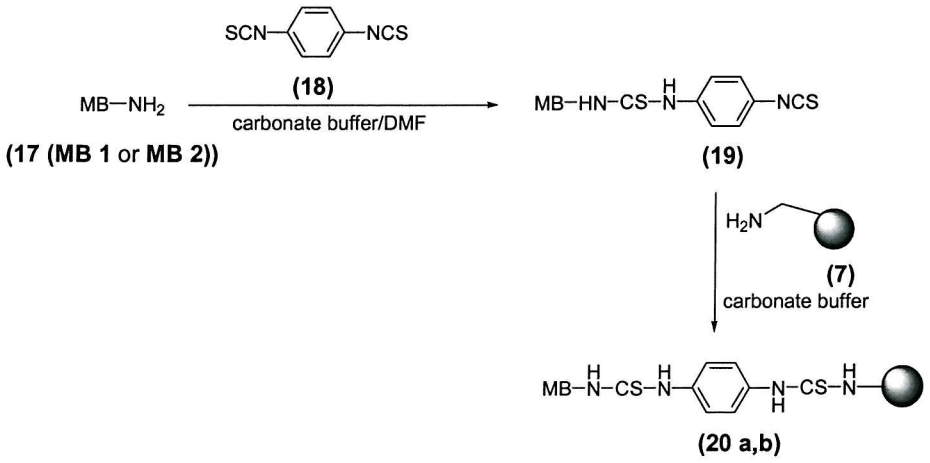


Figure 3.17: Immobilzation of Molecular beacon onto polystyrene latex microspheres.

3.3.2.6 *Hybridization studies of immobilized molecular beacons*

The hybridization of the immobilized molecular beacons to their target oligonucleotides was performed in hybridization buffer[49] (20mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 8.0) and mixed for 3 h at room temperature while monitoring the change in fluorescence using a FITC filter. Figure 3.18 shows the increase in fluorescence compared to the residual background fluorescence, indicating an opening of the stem of the molecular beacon.

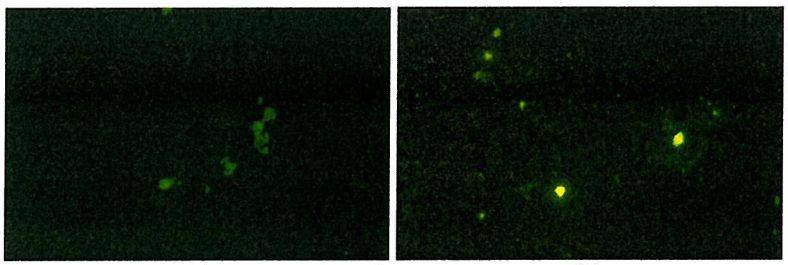


Figure 3.18: Fluorescence image of the immobilized molecular beacon (MB 1) (a) immobilized molecular beacons (b) after hybridized with oligonucleotide target.

3.4 CONCLUSION

Experimental results indicate that the polystyrene latex microspheres are readily labeled with fluorescein *via* standard coupling methodology. Initial studies of the fluorescently labeled particles confirm that the microspheres are readily taken up by the ND7 cells and may be used as potential delivery vehicles.

Solution phase studies on a FRET peptide and molecular beacons show the possibility of combining these with the microspheres to develop potential "real-time" probes.

CHAPTER 4

EXPERIMENTAL

4.1 GENERAL INFORMATION

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX400 (400 and 100 MHz, respectively), or Bruker AM300 (300 MHz) spectrometer at 298 K. All chemical shifts are quoted in ppm on the δ scale using the residual hydrogen in the solvent as the internal standard. Coupling constants (*J* values) were measured in Hz.

ESI mass spectra were recorded using a VG Platform Quadrupole Electrospray Ionisation mass spectrometer, measuring mono-isotopic masses (mode ES+ or ES-). GC-MS mass spectra were record on a Thermoquest Trace GC-MS, source: Electron /Chemical Ionisation, column: Optma Delta 3 (0.25 μm, 30 m x 0.25 mm). High resolution accurate mass measurements were recorded on FT-MS Bruker Apex III Fourier Transfrom Ion Cyclotran Resonance-mass spectrometer with a Electronspray source.

Analytical RP-HPLC was performed on a HP1100 system equipped with a Phenomenex Prodigy C_{18} reverse phase column (150 x 4.6 mm i.d.) with a flow rate of 1 mL/min, monitoring at a wavelength of 220 nm and eluting with (A) 0.1% TFA in H_2O and (B) 0.042% TFA in acetonitrile, with a gradient of 0% (B) to 100% (B) over 20 minutes.

Semi-preparative RP-HPLC were performed on a HP1100 system equipped with a Phenomenex Prodigy C_{18} reverse phase column (250 × 10.0 mm i.d., flow rate 2.5 mL/min), monitoring at a wavelength of 220 nm and eluting with (A) 0.1% TFA in H_2O and (B) 0.042% TFA in acetonitrile, with a gradient of 0% (B) to 100% (B) over 40 minutes.

IR spectra unless stated otherwise were obtained on a BioRad FTS 135 spectrometer with a Goldengate ATR accessory with neat compounds, a FTIR perkin-Elmer 2000 Spectrometer (Beaconsfield, Bucks, England) coupled with an AutoIMAGE FTIR microspectrometer (Beaconsfield, Bucks, England) was use to acquire the IR spectrums of the samples (32 scans with resolution of ± 8cm^{-1}).

Electron microscopy images were recorded on a Philips, XL 30 ESEM scaning microscope.

Particle-size distributions were recorded on a COULTER® N_4 PLUS.

Thermal denaturation profiles were recorded on a Roche LightCycler.

Fluorescence measurements and enzymatic were recorded on Perkin-Elmer LS 50 B Luminescence spectrometer.

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected.

All fluorescent images were captured on an inverted Leica DM-IRBE epifluorescence microscope (Milton Keynes, UK) fitted with a 100 W Hg lamp and standard rhodamine optics (excitation 510-560 nm; dichroic mirror 620 nm; emission > 590 nm). Images were acquired with a 5X NA 0.12 lens and a cooled Hamamatsu digital camera and digitised at 12 bit resolution for analysis with OpenLab 2.1 software (Improvision, Coventry, UK) running on a Macintosh G4/400.

ND7 cells were grown in full medium, which consisted of Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum and 1 mM L-glutamine. Cell cultures were incubated at 37 °C in an atmosphere of 5% CO_2.

4.2 GENERAL EXPERIMENTAL PROCEDURES

4.2.1 Quantitative ninhydrin test[50]

A known mass of resin (<5mg) was treated in a small test tube with 6 drops of reagent A (described below) and 2 drops of reagent B (described below) and heated at 110 °C for 10 mins. The tube was cooled and 60 % ethanol (2mL) added to the tube. The contents of the tube were filtered through a pipette charged with a plug of glass wool and the blue filtrate collected in a 25 mL volumetric flask. The resin was washed using a solution of tetraethyl ammonium chloride (NEt_4Cl) (0.5M in CH_2Cl_2, 2 x 0.5 mL) and the sample made up to 25 mL using 60% aqueous ethanol. The absorbance at 570 nm was measured against a reagent blank. The amount of primary amine present on the resin was calculated using the equation:

$$\text{loading (mmol/g)} = (A \times V) / (\epsilon_{570} \times W) \times 1000$$

ϵ_{570} = Molar extinction co-efficient (15000 $M^{-1}cm^{-1}$).
V = diluted volume (25 mL).
W = mass of resin (mg).
A_{570} = absorbance measured at 570 nm

Reagent A

Solution-1: Phenol (40 g) was dissolved in absolute EtOH (10 mL) with warming and then stirred over an Amberlite mixed-bed resin MB-3 (4 g) for 45 min. The mixture was then filtered.

Solution-2: Potassium cyanide (65 mg) was dissolved in water (100 mL). A 2 mL aliquot of this solution was diluted with pyridine (100 mL) (freshly distilled from ninhydrin) and stirred over an Amberlite mixed-bed resin MB-3 (4 g). The solution was filtered and mixed with solution-1 to give reagent A.

Reagent B

Ninhydrin (2.5 g) was dissolved in absolute ethanol (50 mL).

4.2.2 Quantitative Fmoc test[51]

To a known mass (<5mg) of resin was added a solution of 20% piperidine/ DMF (1.5 mL). The resin was allowed to stand for 15 min and the solution filtered through a glass pipette with a glass wool plug and filtrate diluted to 25 mL with 20% piperidine/DMF. The absorbance at 302 nm was recorded, measured against a blank of 20% piperidine/ DMF. The loading was calculated from the following equation:

$$\text{loading (mmol/g)} = (A_{302} \times V) / (\epsilon_{302} \times W) \times 1000$$

A_{302} = absorbance of the piperidyl-fulvene adduct

V = volume of the volumetric flask (25 mL).

W = mass of the resin sample (mg

A_{302} = molar extinction co-efficient of the adduct at 302 nm ($7800/M^{-1}cm^{-1}$).

4.2.3 Monomer treatment

Styrene (50 mL) and DVB (10 mL) were washed with 25% NaOH (aq) (2 x 100 mL, then 2 x 20 mL) and distilled water (5 x 100 mL, then 5 x 20 mL). HEMA and MAA were distilled under reduced pressure, dried over molecular sieves and stored at 4 °C.

4.2.4 Solid content of the emulsion[52]

A known mass of suspension of polystyrene latex microspheres (1-2 g, suspended in water) was placed in a petri dish, covered with aluminium foil and dried at 80 °C overnight and reweighed.

4.2.5 Preparation of methanolic sodium hydroxide[22(a)]

A solution of NaOH (2.0 g) in a minimum amount of water was made up to 1 litre by the addition of methanol and was allowed to stand overnight. The methanolic sodium hydroxide solution was standardized against primary standard grade potassium hydrogen phthalate (0.25 g, 0.05 N, 25 mL) using phenolphthalein as an indicator (6 drops, 0.25 g, 50% hot ethanol (aq)) to the first pink end point.

4.2.6 Acid group test[22]

To a known mass (mg) of the latex microspheres was added DMSO (1.0 mL). The suspension was stirred for 10 min. A known excess of 0.05 N methanolic NaOH was added and stirred for 10 min, water (10 mL) was added and mixed. The unreacted NaOH was back-titrated with standard 0.05 N HCl. A blank titration was also performed.

$$\text{Acid loading (mmol/g)} = (V_b - V_S) \times \text{Conc} / W \times 1000$$

V_b = Volume of HCl (mL) for blank
V_S = Volume of HCl (mL) for sample
Conc = Concentration of HCl (N)
W = Weight in (mg) solid of polystyrene latex microspheres

4.2.7 Quantitative hydroxyl group test[19-21]

Hydroxy-functionalized polystyrene latex microspheres (200 mg) were treated with reagent A (250 μL) (described below) and pyridine (1 mL) in a capped glass vial. The mixture was then swirled for 2 mins. The sample and blank (reagent A and pyridine) were place in an oven at 95 °C for 3 h. The vials were removed from the oven and allowed to cool in air to room temperature. Water (10 mL) was added and swirled for 2 mins before the addition of phenolphthalein indicator (1.0% solution in pyridine, 6 drops). The mixture was then tritrated against NaOH (0.025 N).

Loading) (mmol/g) = $(V_b - V_S)$ x Conc / W x 1000

V_b = Volume of NaOH (mL) for blank

V_S = Volume of NaOH (mL) for sample

Conc = Concentration of NaOH (N)

W = Weight in (mg) solid of polystyrene latex microspheres

Reagent A

In a brown bottle was added dry pyridine (100 mL), phthalic anhydride (16 g) and immidazole (2.5 g), swirled carefully to dissolve, and allowed to stand overnight before use (once prepared the solution can be kept up to 3 months at room temperature).

4.2.8 General procedure for the washing of the latex microspheres

The latex microspheres were washed using centrifugation in an eppendorf tube (1.5 mL capacity). Carboxyl and amino latex microspheres were centrifuged at 12000 rpm for 2 mins while hydroxyl latex microspheres were centrifuged at 12000 rpm for 10 mins. In each case the supernatant was removed and discarded. Before reaction in DMF the latex microspheres were centrifuged, the water removed and the beads washed with DMF twice before use.

4.2.9 Preparation of polystyrene latex microspheres for scanning electron microscopy

For scanning electron microscopy, polystyrene latex microspheres were deposited onto a gold slide by placing the gold slide into a suspension of the polystyrene latex microspheres (0.5 mL, 1wt. % solid), covering with parafilm and stirring overnight. The gold slide was then quickly washed with water and air-dried for 2 days.

4.2.10 Preparation of polystyrene latex microspheres for measurement of particle size distribution

Polystyrene latex microspheres (0.1 % solid) in polyoxyethylenesorbitan monolaurate (2% in water) were sonicated for 30 mins. Approximately 10 μl of this solution was diluted with water (2.40 mL) before measurement.

4.2.11 General procedure for amino acid coupling

The resin was pre-swollen in CH_2Cl_2 for 30 mins and the solvent drained. The Fmoc protected amino acid (3 eq.) and HOBt (3 eq.) were dissolved in CH_2Cl_2:DMF (10mL, typically 9:1) and stirred for 10 mins. DIC (3 eq.) was added and the mixture stirred for a further 10 mins. This mixture was added to the pre-swollen resin and shaken for 3 h. The reaction was filtered, washed with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL), ether (3 x 5 mL) and dried *in vacuo*. Fmoc group removal was performed using 20% (v/v) piperidine in DMF (2 treatments x 10 mins). The resin was filtered, washed with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL), ether (3 x 5 mL) and dried *in vacuo*.

4.3 EXPERIMENTAL TO CHAPTER 2

4.3.1 General procedure for emulsifier-free emulsion co-polymerization of hydroxyl functionalized polystyrene latex microspheres (1)

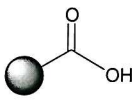
(1)

The reaction was performed batchwise in a thermostated reactor under a nitrogen atmosphere. Water (180 g), styrene (inhibitor-free, 8.55 g), DVB (inhibitor-free, 0.45 g), magnesium sulphate (0.14 g) and HEMA (inhibitor-free, 4.50 g) were stirred together at room temperature with nitrogen bubbling through the solution for 30 mins. The reaction was heated to 80 °C with mechanical stirring (350 rpm) for 20 mins at this temperature. 2-2′-Azobis(2-methylpropionamidine) dihydrochloride in water (2 mL, 1.0 mM) was added and the mixture stirred at 80 °C for 16 h. The latex microspheres were washed by centrifugation with methanol (2 x 200 mL) and water (2 x 200 mL) for 2 h and stored in water at 5 °C.

Yield 7.80 g, 58 %.

IR (υ_{max}/cm^{-1}): 3347 O-H stretching (br), 3026 Aryl C-H stretching (m), 1723 C=O (br), 1602, 1493, 1453 C=C ring stretching (m), 1183 C-O stretching (s).

4.3.2 General procedure for emulsifier-free emulsion co-polymerization of carboxyl functionalized polystyrene latex microspheres (2)



(2)

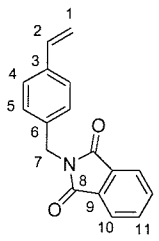
Water (180 g), styrene monomer (inhibitor-free, 8.55 g), DVB (inhibitor-free, 0.45 g), magnesium sulphate (0.14 g) and MAA (inhibitor-free, 1.5 g), were stirred together at room temperature with nitrogen being bubbled through the solution for 30

mins and then heated to 80 °C and stirred (350 rpm) for 20 mins at this temperature. 2-2′-Azobis(2-methylpropionamidine) dihydrochloride in water (2 mL, 1.0 mM) was added and the mixture stirred at 80 °C for 16 h. The latex microspheres were cleaned *via* a soxhlet with methanol and water overnight and stored in water at 5 °C.

Yield 2.91 g, 50 %.

IR (υ_{max}/Cm^{-1}): 3300-2500 O-H stretching (br) 3026 Aryl C-H stretching (m), 1701 C=O stretching (br) 1602, 1493, 1452 C=C ring stretching (m).

4.3.3 Synthesis of *N-p*-Vinylbenzylphthalimide (4)[23]



(4)

p-Vinylbenzyl chloride (1.53 g, 10 mmol) and potassium phthalimide (1.85 g, 10 mmol) were dissolved in DMF (5 mL) and heated at 50 °C overnight. The mixture was treated with 0.2 N sodium hydroxide (200 mL). The aqueous phase was extracted with ethyl acetate (2 x 100 mL) and the combined ethyl acetate extraction washed with water (2 x 100 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel, eluting with (hexane/ethyl acetate) (5:1) to give a white solid.

Yield 2.31 g, 88 %.

IR (υ_{max}/cm^{-1}): 1702 –CONCO (s).

R_F: 0.45 (3:1, hexane/ethyl acetate).

m/z (EI): 263 (100 %, [M^+]).

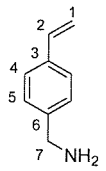
HRMS (ESI): $C_{17}H_{13}NO_2Na$ Calc.286.0844, Found 286.0838.

m.p: 105-106°C (Lit[2]. 107-108 °C).

δ_H ($CDCl_3$): 4.76 (2H, s, $^7CH_2$); 5.13 (1H, d, *J* 11, $^1CH_2$-cis); 5.62 (1H, d, *J* 18, $^1CH_2$-trans); 6.59 (1H, dd, *J* 18, 11, ^2CH); 7.27 (2H, d, *J* 8, ^4CH); 7.32 (2H, d, *J* 8, ^5CH); 7.62 (2H, dd, *J* 6, 3, ^{11}CH); 7.76 (2H, dd, *J* 6, 3, ^{10}CH).

δ_C ($CDCl_3$): 41.8 (C^7); 114.5 (C^1); 123.7 (C^{10}); 126.9 (C^4); 129.3 (C^5); 132.6 (C^9); 134.4 (C^{11}); 136.3 (C^6); 136.7 (C^2); 137.6 (C^3); 168.4 (C^8).

4.3.4 Synthesis of *p*-vinylbenzylamine (5)[23]



(5)

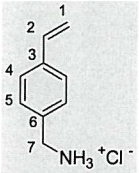
A solution of hydrazine hydrate (0.66 g, 10 mmol) and *N-p*-vinylbenzylphthalamide **(4)** (1.84 g, 7 mmol) in ethanol (25 mL) was refluxed with vigorous mechanical stirring for 3 h. A white gelatinous side product was removed by filtration and the filtrate was concentrated to dryness. The solid was treated with aqueous potassium hydroxide (60 mL, 1.5 M) and the aqueous mixture was extracted with ether (1 x 100 mL and 4 x 50 mL). The combined ether solutions were washed with 2% potassium carbonate solution (4 x 50 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield a yellow oil.

Yield 0.68 g, 73 %.
IR (υ_{max}/cm^{-1}): 3368 N-H (m) stretch, 1628 N-H bend (m).
m/z (ES$^+$): 134.0 (85%, $[M+H]^+$).

δ_H (CD_3OD): 4.11(2H, s, $^7CH_2$); 5.30 (1H, d, *J* 11, $^1CH_2$-cis); 5.82 (1H, d, *J* 18, $^1CH_2$-trans); 6.74 (1H, dd, *J* 18, 11, ^2CH); 7.36 (1H, d, *J* 8, ^5CH); 7.49 (1H, d, *J* 8, ^4CH).

δ_C (CD_3OD): 46.8 (^7C); 114.1 (^1C); 127.8 (^5C); 129.3 (^4C); 138.2 (^3C); 138.3 (^2C); 143.5 (^6C).

4.3.5 Synthesis of vinylbenzylamine hydrochloride (VBAH) (6)[18]



(6)

Vinylbenzylamine **(5)** (0.53 g, 4 mmol) was dissolved in methanol (2 mL) and cooled to 0 °C. 1 N HCl solution in pentane (6 mL, 1.5 eq.) was slowly added. The mixture was stirred for 1 h then the methanol and pentane were removed *in vacuo* to give an oil. The product was precipitated by the addition of ether until no more solid precipitated. The precipitate was collected by filtration and washed with ether (20 mL) to give the title compound as a white solid.

Yield 0.65 g, 96 %.

IR (υ_{max}/cm^{-1}): 3150-2618 N-H stretch (br), 1594 N-H bend (s), 1627 N-H bend.

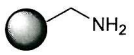
Analytical Result: Calc. For $C_9H_{12}NCl$ C, 63.72; H, 7.13; Cl, 20.90; N, 8.26, Found C, 63.12; H, 7.09; Cl, 21.66; N, 8.04.

m.p: decomposes 186 °C.

δ_H (300 MHz, DMSO-d_6): 4.09 (2H, s, $^7CH_2$); 5.40 (1H, d, *J* 11, $^1CH_2$-cis); 6.00 (1H, d, *J* 18, $^1CH_2$-trans); 6.85 (1H, dd, *J* 18, 11, ^2CH); 7.56 (1H, d, *J* 8, ^5CH); 7.62 (1H, d, *J* 8, ^4CH); 8.60 (3H, s, NH_3^+).

δ_C (D_2O): 43.2 (^7C), 115.7 (^1C), 127.2 (^4C), 129.3 (^5C), 132.5 (^3C), 136.4 (^2C), 138.6 (^6C).

4.3.6 General procedure for emulsion co-polymerization of amino functionalized polystyrene latex microspheres (7)



(7)

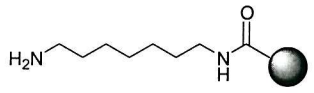
Water (180 g), styrene monomer (inhibitor-free, 10 g), DVB (inhibitor-free, 0.26 g), magnesium sulphate (0.14 g) and VBAH **(6)** (0.53 g) were stirred together at room temperature with nitrogen being bubbled through the solution for 30 mins and then heated to 80 °C and stirred (350 rpm) for 20 mins at this temperature. 2-2′-Azobis(2-methylpropionamidine) dihydrochloride in water (2 mL, 1.0 mM) was added and the mixture stirred at 80 °C for 1.5 h. The latex microspheres were collected by centrifugation, washed successively with methanol (2 x 200 mL) and water (2 x 200 mL) by centrifugation at 12,000 rpm for 2 h and stored in water at 5 °C.

Yield 1.5 g, 14 %.

IR (υ_{max}/Cm^{-1}): 3360 (br) NH_2, 3026 Aryl C-H stretching (m), 1602, 1493, 1452 C=C ring stretching (m).

4.4 EXPERIMENTAL TO CHAPTER 3

4.4.1 Synthesis of amino functionalized latex microspheres (9)

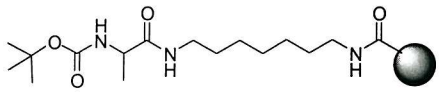


(9)

To a solution of 1,7-diaminoheptane (0.05 g, 0.4 mmol) in DMF (0.5 mL) was added HOBt (0.05 g, 0.4 mmol) and the resulting solution agitated for 10 mins. DIC (0.06 mL, 0.4 mmol) was added and the mixture added to the carboxyl functionalized polystyrene latex microspheres in DMF (0.5 mL, 2.5 % wt solids). The suspension was mixed overnight and washed by centrifugation with DMF (5 x 1mL) and MeOH (5 x 1mL).

IR (υ_{max}/cm^{-1}): 3378 NH_2 stretching (br), 3026, 2920,2853 C-H stretching (m), 1635 N-H bending (s), 1602, 1493, 1453 C=C ring stretching (m).

4.4.2 Attachment of Boc-Ala-OSu to amino functionalized polystyrene latex microsphere (9)



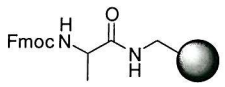
(24)

To a suspension of amino fucntionalized polystyrene latex microspheres **(9)** in DMF (0.1 mL, 2.5 wt. % solid, 3.2 x 10^{-2} mmol) was added a solution of Boc-Ala-OSu (18 mg, 6.4 x 10^{-2} mmol) and DMAP (4 mg, 1.6 x 10^{-2} mmol) in DMF (0.9 mL). The mixture was mixed for 3 days to give a negative ninhydrin test after resin washing with DMF (3 x 1 mL) and MeOH (3 x 1mL). The Boc group was removed by treatment with 50% TFA in CH_2Cl_2 (0.9mL) for 4 h. Diethyl ether (300 µl) was added and the mixture centrifuged, washed with DMF (3 x 1 mL) and MeOH (3 x 1mL). The TFA salt of the resin was subsequently neutralised by treatment with

aqueous ammonium hydroxide (10%, 0.9 mL) and mixed for 5 mins. The mixture was washed with water by centrifugation until pH 7.0 to give a positive ninhydrin test.

IR (υ_{max}/cm^{-1}): 3461 N-H stretching (br), 3026, 2925,2852 C-H stretching (m), 1740 C=O ester (s), 1660 N-H secondary amide (s), 1602, 1493, 1453 C=C ring stretching (m).

4.4.3 Attachment of Fmoc-Ala-OH to amino functionalized polystyrene latex microspheres (9)

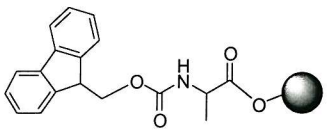


(22)

Fmoc-Ala-OH (7 mg, 22.5 x 10^{-3} mmol) was dissolved in DMF (0.4 mL) and HOBt (3 mg, 22.5 x 10^{-3} mmol) and DIC (4 μL, 22.5 x 10^{-3} mmol,) were added. The mixture was allowed to stand for 10 mins and then transferred to the suspension of latex microspheres (0.1 mL, 2.5 wt. % solid, 7.5 x 10^{-3} mmol in DMF). DMF was added to give a final reaction volume of 1 mL. The mixture was mixed for 4 h and the latex microspheres were then washed with DMF (3 x 1 mL) and MeOH (3 x 1mL). A qualitative ninhydrin test was negative.

IR (υ_{max}/cm^{-1}): 3418 N-H stretching (w), 1715 C=O (w), 1681 N-H secondary amide (w).

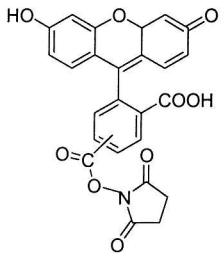
4.4.4 Attachment of Fmoc-Ala-OH to hydroxyl functionalized polystyrene latex microspheres (1)



(42)

Fmoc-Ala-OH (98 mg, 0.27 mmol) was dissolved in DMF (0.4 mL) and DIC (48 μL, 0.27 mmol) was added. The mixture was mixed for 10 mins and then transferred to a suspension of hydroxy-fucntionalized polystyrene latex microspheres **(1)** in DMF (0.5 mL, 7.6 wt. % solid, 0.03 mmol). DMF was added to make up a final volume of 1 mL. The mixture was mixed overnight and the latex microspheres were washed with DMF (3 x 1 mL) and MeOH (3 x 1 mL). A quantitative Fmoc test gave a resin substitution 0.77 mmol/g.

4.4.5 Preparation of *N*-hydroxy succinimide active ester of 4(5)-carboxyfluorescein (12)[26]



(12)

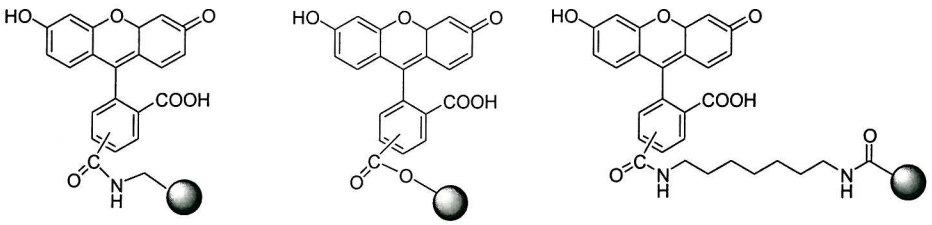
4(5)-Carboxyfluorescein (50 mg, 0.13 mmol, 1 eq.), DCC (25.48 mg, 0.13 mmol, 0.95 eq.), and HOSu (15.3 mg, 0.13 mmol, 1eq.) were stirred overnight at room temperature in DMF (1.30 mL). Upon filtration, the solvent was removed under vacuum to give an orange powder. The crude product was used in subsequent reactions.

IR (υ_{max}/cm^{-1}): 3500-2500 O-H stretching (br), 1775 C=O stretching (s), 1300-1000 C-O stretching (br).

R_F: 0.50 (1:5, MeOH/ CH_2Cl_2).

m/z (ES^-): 472.1 (100% $[M-H]^-$).

4.4.6 General procedure for preparation of polystyrene fluorescein-labelled latex microspheres (14, 15 or 16)[26]



(14) (15) (16)

The 4-dimethylaminopyridine active ester of 4(5)-carboxyfluorescein was prepared by adding 4-DMAP (0.8 mg, 6.55 x 10^{-3} mmol, 0.5 eq) in DMF (13 μL) to 4(5)-carboxyfluorescein active ester **(12)** (0.6 mg, 0.01 M) in dry DMF (0.13 mL) and stirring for 30 mins. This was added to a suspention of polystyrene latex microspheres (2.5 wt % solid) in carbonate buffer (0.85 mL, 0.1 M $NaHCO_3$, 1 M NaCl, pH 9.3) and the volume adjusted to 1 mL with DMF. The mixture was mixed for 5 mins. The latex beads were washed with DMF (3 x 1 mL) and MeOH (3 x 1 mL) by centrifugation and analysed under fluorescence microscope (see Chapter 3, Section 3.2.6.1, Figure 3.1). The fluorescent latex microspheres were stored in water at 5 °C.

4.4.7 Uptake of fluorescently labelled polystyrene latex microspheres by ND7 cells

ND7 cells in full medium were plated out into the wells of a 6-well plate. To the cells was added a known volume (2.5-10 μL, 0.25 wt% solid) of a suspension of the fluorescently labelled polystyrene latex microspheres (**14**, **15** or **16**) in sterile water. The cells were incubated at 37 °C, 5 % CO_2 for 24 h, the medium was removed, the cells washed with DMEM (1 x 1 mL) and then resuspended in full medium prior to the cells being visualised under a fluorescence microscope (see Chapter 3, Section 3.2.6.2, Figure 3.2).

4.4.8. Fluorescence profile with complementary oligonucleotide target strand [39(b)]

Fluorescence (F_{buffer}) was determined using a solution of the molecular beacon buffer (3.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.0, 450 μL) as a blank (λ_{ex} = 480 nm, λ_{em} = 515 nm). A solution of the molecular probe (**MB 1** or **MB 2**) (details in results and discussion, Chapter 3, Section 3.1.5) in water was added and the new level of fluorescence (F_{closed}) recorded. A ten-fold molar excess of the oligonucleotide target was added and the increase in fluorescence intensity monitored until a stable level was reached (F_{open}). The results are presented in Table 4.1.

Table 4.1: Signal to background ratio

Probes 1 μM		Target 10 μM		Fluorescence intensity		Signal to noise ratio
				$F_{(closed)}$	$F_{(open)}$	$(F_{open}-{}^{*}F_{buffer})/(F_{close}-F_{buffer})$
MB 1	25μL	T1	25 μL	20	355	18
MB 2	25μL	T2	25 μL	15	345	23

* $F_{buffer} = 0$

4.4.9 Thermal denaturation profiles[39(b)]

Molecular beacon (**MB 1** or **MB 2**), (25 μL, 40 nM) in molecular beacon buffer (3.5mM $MgCl_2$, 10 mM Tri-HCl, pH 8.0) was treated with the oligonucleotide target to a final concentration of 64 nM. The fluorescence of each solution was determined as a function of temperature (using a Roche lightcycler). The temperature was held to 80 °C for 5 mins then decreased to 27 °C in 5 mins steps. Each hold step lasting 5 mins, and the fluorescence was monitored after each hold (Table 4.2).

Table 4.2: Fluorescence intensity of MB 1 and MB 2 using lightcycler.

MB 1				MB 2			
T (°C)	a*	T (°C)	b#	T (°C)	c*	T (°C)	d#
80	10	80	10	78	9	77	8
75	10	76	11	75	9	74	8
72	10	72	11	68	9	70	8
69	10	68	11	64	8	67	8
65	10	66	12	61	7	64	9
59	8	62	13	58	5	60	9
55	6	59	14	55	3	57	10
52	4	55	15	51	2	53	11
49	3	52	15	48	1	50	12
45	2	48	16	44	1	46	12
42	2	45	17	41	1	43	13
39	2	41	18	38	1	40	13
36	2	36	19	34	1	37	14
33	1	33	20	32	1	33	15
30	1	30	20	30	1	30	15
29	1	29	21	29	1	29	16
27	1	27	22	27	1	27	16

* Fluorescence intensity of molecular beacon without oligonucleotide target.
# Fluorescence intensity of molecular beacon with oligonucleotide target.

4.4.10 Covalent binding of the molecular beacons to amino functionalized polystyrene latex microspheres (7)[48]

The binding reaction was performed in two steps. The molecular beacon (1.0 nmole) was dissolved in borate buffer (0.05 mL, 0.1 M boric acid, pH 9.3) then mixed with DITC (1,4 phenylene diisothiocyanate (1.6 mg, 8.3 x 10^{-3} mmole) in DMF (0.5 mL) and mixed for 2 h in the dark. The mixture was extracted with *n*-butanol:water (1:1, 6mL) and the aqueous phase was lyophilized.

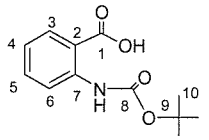
In the second step, the activated molecular beacon was attached onto the latex surface. For each sample, the latex spheres (amino functionalized polystyrene latex microspheres, 0.1 mL, 2.0 wt % solid) were added to carbonate buffer (0.85 mL, 0.1 M $NaHCO_3$, 1 M NaCL, pH 9.3). The activated molecular beacon (1.0 nmole) was dissolved in borate buffer (0.05 mL, 0.1 M boric acid, pH 9.3) and added to the

suspension of latex microspheres. Carbonate buffer (0.1 M $NaHCO_3$, 1 M NaCl, pH 9.3) was added to make up to a final volume of 1mL. After mixing for 4 h in the dark at room temperature the sample was centrifuged, the supernatant was removed and the microspheres washed with carbonate buffer (2 x 1mL) and water (2 x 1mL) by centrifugation. Hybridization buffer[49] (20 mM Tri-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 8) was then added to a total volume of 1 mL.

4.4.11 Hybridization study of immobilized molecular beacon probes[48]

The immobilized molecular beacon probe (**MB 1** or **MB 2**) in hybridization buffer (1.0 mL, 0.2 wt %, 20mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 8) was treated with its complementary DNA strand (14.0 nmol) and mixed for 3 h. The immobilized molecular beacon probe was then centrifuged and washed with hybridization buffer (2 x 1 mL) and water (2 x 1 mL) by centrifugation and monitored by fluorescent microscopy, (see Chapter 3, Section 3.3.2.6, Figure 3.18).

4.4.12 Synthesis of Boc-anthranilic acid (27)[31]



(27)

Boc_2O (5.16 g, 23.6 mmol), anthranilic acid (2.6 g, 18.9 mmol) and triethylamine (5 mL, 36 mmol) were dissolved in DMF (5 mL). The mixture was stirred for 24 h then treated with charcoal and filtered. The DMF was removed *in vacuo* and the residue was dissolved in CH_2Cl_2 (200 mL) and extracted with 10% sodium carbonate solution (100mL). The dark-brown aqueous phase was extracted with CH_2Cl_2 (100 mL). The aqueous extractions were acidified to pH 2 and extracted with diethyl ether (3 x 100 mL). The organic phase was dried over sodium sulfate, filtered and the volume was reduced to ~50 mL. Hexane was added until no more product precipitated. The crude product was recrystallized from 50% aqueous ethanol to give the little compound as a white solid.

Yield 2.8 g 65%.

IR (υ_{max}/cm^{-1}): 1727 C=O carboxylic acid; 1664 –CONH- secondary amide.

R_F: 0.44 (ethyl acetate).

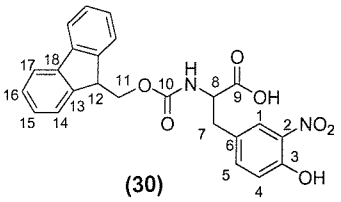
m/z (ES$^-$): 235.9 (100% [M-H]$^-$).

m.p: 154-155 °C (Lit.[13] 149-150 °C).

δ_H (300 MHz, DMSO-d_6): 1.48 (9H, s, $^{10}CH_3$); 7.07 (1H, t, *J* 8 ^{4}CH); 7.56 (1H, t, *J* 8 ^{5}CH); 7.96 (1H, d, *J* 8, ^{6}CH); 8.28 (1H, d, *J* 8, ^{3}CH); 10.55 (1H, s, NH).

δ_C (CD_3OD): 29.0 (^{10}C); 81.9 (^{9}C); 116.6 (^{2}C) 119.9 (^{3}C): 122.8 (^{4}C); 133.9 (^{6}C); 135.6 (^{5}C) 143.8 (^{7}C) 154.7 (^{8}C); 171.7 (^{1}C).

4.4.13 Synthesis of Fmoc-3-nitrotyrosine (30)[31]



(30)

To a solution of 3-nitro-L-tyrosine (4.30 g, 19 mmol) in dioxane (20 mL) and 10% sodium carbonate (50 mL) was slowly added a solution of 9-fluorenylmethyl chloroformate (4.92 g, 19.02 mmol) in dioxane (50 mL) at 0 °C. The mixture was stirred at 0 °C for 4 h followed by 8 h at room temperature. The mixture was poured into water (500 mL), acidified to pH 2 with 6 M HCl and extraced with ethyl acetate (2 x 200 mL). The organic layer was washed with water (2 x 200 mL), dried over sodium sulfate, filtered and the volume was reduced to ~50 mL *in vacuo*. Hexane was added until the product precipitated to give a yellow solid in quantitative yield.

Yield: 8.52 g, 100 %.

IR (υ_{max}/cm^{-1}): 1629 -CONH- (m) secondary amide; 1687 -O-CO-N urethane; 1719 C=O carboxylic acid.

R_F: 0.62 (85:13:0.5:1.5 CH_2Cl_2:MeOH:AcOH:H_2O).

m/z (ES$^-$): 447.2 (100% [M-H]$^-$).

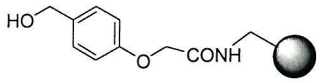
HRMS (ES$^+$): $C_{24}H_{20}N_2O_7$ Calc.471.1163, Found 471.1167.

m.p: 138-141 °C (Lit[13]. 145-148 °C).

δ_H (CD_3OD): 2.94 (1H, dd, *J* 14, 10, ^{7}C<u>H</u>H); 3.25 (1H, dd, *J* 14, 5, ^{7}CH<u>H</u>); 4.16 (1H, m, ^{12}CH); 4.25 (1H, dd, *J* 11, 7, $^{11}CH_2$); 4.36 (1H, dd, *J* 13, 11 $^{11}CH_2$); 4.45 (1H, dd, *J* 10, 5, ^{8}CH); 7.05 (1H, d, *J* 8, ^{4}CH); 7.26 (1H, t, *J* 8, ^{15}CH); 7.48 (1H, d, *J* 8, ^{16}CH); 7.55 (1H, d, *J* 8, ^{14}CH); 7.76 (1H, d, *J* 8, ^{17}CH); 7.98 (1H, s, ^{1}CH).

δ_C (CD_3OD): 37.6(^{7}C); 48.7 (^{12}C); 56.7 (^{8}C); 68.3 (^{11}C); 121.3 (^{4}C, ^{17}C); 126.5 (^{16}C); 126.9 (^{1}C); 129.1 (^{15}C); 129.4 (^{14}C) 131.4 (^{2}C); 135.7 (^{6}C); 139.5 (^{5}C); 142.9 (^{18}C); 145.5 (^{13}C); 154.8 (^{3}C); 158.8(^{10}C); 175.0 (^{9}C).

4.4.14 Synthesis of HMPA resin (33)



(33)

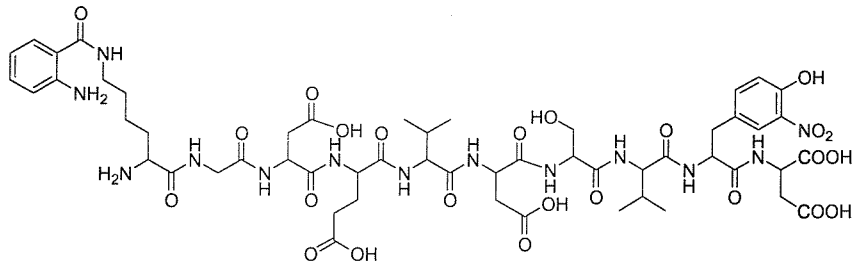
Linker **(31)** (0.55 g, 3.0 mmol) and HOBt (0.41 g, 3.0 mmol) were dissolved in CH_2Cl_2:DMF (9:1, 10 mL). The mixture was stirred for 10 mins, DIC (0.47 mL, 3.0 mmol) was added and stirring continued for a further 10 mins. The mixture was added to resin **(32,** 200-400μm) (1.0g, 1.0 mole/g) pre-swollen in CH_2Cl_2 (10 mL) and shaken overnight. Coupling was monitored by a qualitative ninhydrin test. The resin was filtered, washed with DMF (3 x 5mL), CH_2Cl_2 (3 x 5mL), MeOH (3 x 5mL), ether (3 x 5mL) and dried *in vacuo*.

4.4.15 Attachment of Fmoc-Asp(O^{t}Bu)-OH to Resin (33)

Resin **(33)** (1.20 g, 1.0 mmol) was swollen in CH_2Cl_2 (10 mL). (Fmoc–Asp(O^{t}Bu)-OH (1.23 g, 3.0 mmol) was dissolved in CH_2Cl_2:DMF (9:1, 10 mL) and DIC (0.78 mL, 3.0 mmol) was added. The mixture was stirred for 10 mins. DMAP (0.06 g, 0.50 mmol) was added and the mixture was added to the resin **(33)** and

shaken overnight, washed with DMF (3 x 5mL), CH_2Cl_2 (3 x 5mL), MeOH (3 x 5mL), ether (3 x 5mL) and dried *in vacuo*.

4.4.16 Synthesis of FRET peptide (34)[37]



(34)

The peptide was synthesized (0.2 mmol scale) on HMPA-amino methyl polystyrene resin using the Fmoc/^{t}Bu strategy until the last step whereupon Fmoc-Gly-OH was added followed by Boc-Lys(Fmoc)-OH. The Fmoc group was deprotected (20% pipperidine in DMF) and Boc-anthranilic acid was coupled under standard conditions. The peptide derivative was cleaved and deprotected by treating the resin with TFA/water/Tis (95 %: 2.5 %: 2.5 %) for 4 h, followed by concentrating *in vacuo* to give a yellow solid. The peptide was purified by semi-preparative HPLC to give product **(34)**.

Yield 0.07 g, 27 %.

IR (υ_{max}/cm^{-1}): 1654 –CONH-(s) secondary amide.

m/z (ES$^+$): 646 (70 %, $[M+2H]^+$); 1290.7 (100 %, $[M+H]^+$).

m.p.: decomposes 180 °C.

δ_H (DMSO-d_6): 0.78 (3H, d, *J* 4, Val CH_3), 0.84 (3H, d, *J* 5, Val CH_3), 0.90 (3H, d, *J* 7 Val CH_3), 0.94 (3H, d, *J* 7 Val CH_3), 1.43-1.54 (2H, m, Lys $C_\gamma H_2$), 1.55-1.68 (2H, m, Glu $C_\beta H_2$), 1.79-1.91 (2H, m, Glu $C_\gamma H$), 1.95-2.13 (2H, m, 2xVal $C_\alpha H$), 2.28-2.40 (2H, m, Lys $C_\delta H$), 2.55-2.70 (2H, m, Gly CH_2), 2.70-2.88 (6H, m, 3xAsp $C_\beta H_2$), 3.00-3.10 (2H, m, Lys $C_\beta H_2$), 3.24-3.25 (2H, m, Lys $C_\omega H_2$), 3.95 (1H, m, Tyr- $C_\alpha H$), 4.15-4.23 (1H, m, Val $C_\alpha H$), 4.23-4.43 (1H, m, Ser $C_\alpha H$), 4.36-4.43 (1H, m, Glu $C_\alpha H$), 4.44-4.51 (1H, m, Lys $C_\alpha H$),4.60-4.71 (3H, m, Asp $C_\alpha H$),6.64 (1H, t, *J* 8, Abz ArH),

6.81 (1H, d, *J* 8, Tyr ArH), 7.12 (1H, d, *J* 8, Abz ArH), 7.21 (1H, d, *J* 8, Abz ArH), 7.53 (1H, d, *J* 8.0,Tyr ArH), 7.57 (1H, d, *J* 8 Abz ArH), 7.8 (1H, Gly NH), 8.1 (Asp NH), 8.2 (1H,Lys NH), 8.3 (1H, Lys ω NH), 8.4 (1H, Asp NH), 8.8 (1H, Tyr NH).

δ_C (DMSO-d_6): 17.9 (Val CH_3); 18.2 (Val CH_3); 19.5 (Val CH_3); 19.7 (Val CH_3); 22.2 (Lys $C_\gamma H_2$); 27.5 (Glu $C_\beta H_2$); 29.1 (Glu $C_\gamma H_2$); 30.6 (Lys $C_\delta H$); 36.3 (Lys $C_\beta H_2$); 36.3 (Tyr $C\beta H_2$, 3xAsp $C_\beta H_2$); 38.9 (Lys $C_\omega H_2$); 42.2 (Gly $C_\alpha H$); 62.0 (Ser $C_\beta H_2$); 50.1, 50.2, 52.3 (Asp $C_\alpha H$); 55.0 (Glu $C_\alpha H$); 58.1 (Tyr $C_\alpha H$); 58.2 (Lys $C_\alpha H$); 58.5; (Ser $C_\alpha H$); 62.4 (Val $C_\alpha H$); 50.1,50.2,52.3 (Asp $C_\alpha H$); 55.0 (Glu $C_\alpha H$); 58.1 (Tyr $C_\alpha H$); 58.2 (Lys $C_\alpha H$); 58.5 (Ser $C_\alpha H$); 62.4 (Val $C_\alpha H$); 115.0 (Abz ArCH); 116.5 (Tyr ArCH); 118.6 (ArCH); 125.8 (Tyr ArCH); 128 (Abz ArCH); 134 (Abz ArCH); 135.4 Tyr ArCH); 169.8 (Abz C=O); 170.9 (Gly C=O); 171.29 (Gly C=O); 171.1 (Val CO); 171.4 (Lys C=O); 172.5-172.0 (2xAsp, Tyr, Ser C=O); 174.5 (Asp, Gly CO_2H).

4.4.17 Monitoring enzymatic hydrolysis of FRET peptide (34) by pepsin using analytical HPLC[31]

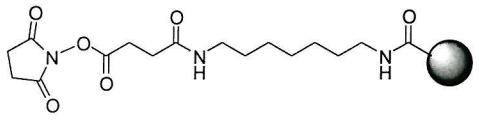
Intramolecularly quenched fluorogenic substrate **(34)** (0.44 mg, 3.41 x 10^{-4} mmol) was dissolved in DMF (32 μL). Formic acid buffer (468 μL, 50 mM, pH 3.4) and pepsin in formic acid buffer (500 μL, 0.083 mg/mL, 50 mM, pH 3.4) was added. The hydrolysis was monitored by analytical HPLC (λ = 260 nm). The cleaved products were collected and mass spectrometric (ES^+) analysis of isolated peaks, 5.20 mins **(35)** and 6.07 mins **(36)** was determined (see Chapter 3, Section 3.3.1.5.1.1 Figure 3.7 and 3.8).

4.4.18 Monitoring enzymatic hydrolysis of FRET peptide (34) by pepsin using spectrofluorimeter[31]

The intramolecularly quenched fluorogenic substrate **(34)** (0.65 mg, 5 x 10^{-4} mmol) was dissolved in DMF (100 μL) and water (25 mL) to give a concentration of 0.02 mM. This solution (200 μL) was added to formic acid buffer (2000 μL, 50 mM, pH 3.4) fluorescence of the solution was followed with time by monitoring the emission at 420 nm upon excitation at 320 nm for 120 second (fluorescence

background). Pepsin in formic acid buffer (250 μL, 0.168 mg/mL, 50 mM, pH 3.4) was added, new level of fluorescence recorded. The results are presented in Chapter 3, Section 3.3.1.5.1.2 Figure 3.9.

4.4.19 Activation of amino fuctionalized polystyrene latex microspheres (9)



(40)

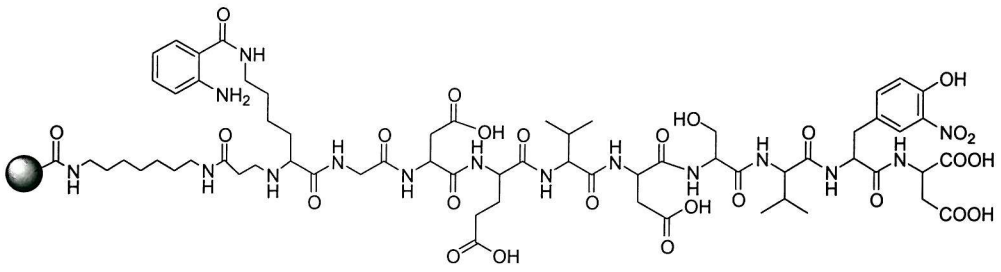
Succinic anhydride (0.02 g, 0.15 mmol) and HOBt (0.02 g, 0.15 mmol) were dissolved in DMF (0.5 mL). The solution was mixed for 10 mins and then transferred to a suspension of amino fucntionalized latex microspheres **(9)** (0.5 mL, 50 mg, 0.05 mmol) in DMF. DMAP (0.003 g, 0.025 mmol) was added and the mixture was mixed overnight. The microspheres were washed with DMF (3 x 1 mL) and MeOH (3 x 1 mL) to give a negative qualitative ninhydrin test yielding carboxyl functionalized polystyrene latex microspheres **(38)**.

IR (υ_{max}/cm^{-1}): 1700 C=O (s) carboxylic acid.

Latex microspheres **(38)** were swollen in DMF (0.5 mL). HOSu (0.02 g, 0.15 mmol), DIC (0.023 mL, 0.15 mmol) in DMF (0.5 mL) were added. The mixture was mixed for 3 h and the microspheres were washed with DMF (3 x 1 mL) and MeOH (3 x 1 mL) to give microspheres **(40)**.

IR (υ_{max}/cm^{-1}): 1740 C=O (s) ester.

4.4.20 Attachment of the FRET peptide to the activated amino functionalized polystyrene latex microspheres (40)



(41)

To a suspension of polystyrene latex microspheres **(40)** in DMF (0.1 mL, 0.25 wt.% solid) was added DMAP (4 mg, 0.03 mmol) and the solution mixed for 30 mins. The intramolecularly quenched fluorogenic substrate **(34)** (1 mg, 7.75 x 10^{-4} mmol) was dissolved in DMF (0.9 mL) and DMAP (4 mg, 0.03 mmol) added. The solution was mixed for 30 mins and then transferred to the suspension of polystyrene latex microspheres. The mixture was mixed for 3 days and the polystyrene latex microspheres were then washed by centrifugation with DMF (3 x 1mL) and MeOH (3 x 1mL) to give immobilized polystyrene latex microspheres **(41)** then analysed by fluorescence microscopy (see Chapter 3, Section 3.3.1.7, Figure 3.10 (a)).

4.4.21 Enzymatic hydrolysis of the immobilized FRET peptide (41) by pepsin

To a suspension of the immobilized intramolecularly quenched fluorogenic substrate **(41)** (0.1 mL, 0.2 wt.% solid) in formic acid buffer (0.1 mL, 50 mM, pH 3.4) was added pepsin in formic acid buffer (0.9 μL, 0.017 mg/mL, 50 mM, pH 3.4). The mixture was mixed overnight and the microspheres monitored by fluorescence microscopy (see Chapter 3, Section 3.3.1.8, Figure 3.10 (b)).

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