Free Solution Capillary Electrophoresis for Purity and Identification of Synthetic Oligonucleotides on Polymer Modified Capillary Surfaces

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Kerrilee E. Allan on _____/_____/_____

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Abstract

This thesis describes a capillary electrophoretic method to determine the identity and purity of oligonucleotides (ODNs) and phosphorothioate antisense oligonucleotides (PS-ODNs) using cationic copolymers as a dynamic coating, based on monomers of ethylpyrrolidine methacrylate (EPyM) and methyl methacrylate (MMA). ODNs are short fragments of single-stranded (ss) or double-stranded (ds) deoxyribonucleic acid (DNA) and their uses range from biological systems to drug delivery and pharmaceutical analysis. In particular, antisense oligonucleotides (AS-ODNs) have a role as therapeutic molecules which inhibit expression of target genes by binding to a specific RNA sequence, blocking the translation process. AS-ODNs exist in various modified forms of which PS-ODNs are the most common. It is therefore necessary to have methods in place to determine the identity and purity of these modified AS-ODNs.

Capillary electrophoresis (CE) has been extensively used for separation of DNA fragments focusing on large strands. Consequently, there is a significant gap in the literature regarding the analysis of short strands of DNA (such as ODNs). DNA strands below the DNA persistence length (p_{DNA}) are rod-like and non-free draining, and exhibit different migration patterns in free solution compared to larger strands which cannot be separated in this way. Operating in free solution has the advantage of only requiring surface confined capillary modification for electroosmotic flow (EOF) suppression allowing for charge-based separation in which larger, more negatively strands exhibit a greater mobility.

In this thesis, homopolymers and copolymers based on EPyM and MMA were prepared by conventional free radical (CFR) and reversible addition-fragmentation chain transfer (RAFT) polymerisation. This work reports for the first time the use of a RAFT block copolymer for the separation of ODNs. Polymer solutions were prepared for surface-confined capillary modification via physical adsorption and the adsorptive properties (onto silicon (Si) wafers) were investigated via atomic force microscopy (AFM). It was observed that the effectiveness of these polymers for capillary surface modification via adsorption was highly dependent on the polymer concentration, tacticity and hence, stereochemistry. A dilute polymer solution of CFR poly(ethylpyrrolidine methacrylate-*co*-methyl methacrylate)

(PEPyM-*co*-PMMA) random copolymer (1 mg mL^{-1}) provided a homogenous surface coating owing to the polymer chains being hydrodynamically separated allowing for adsorption as individual globules. Whereas the RAFT poly(ethylpyrrolidine methacrylate-*block*-methyl methacrylate) (PEPyM-*b*-PMMA) block copolymer provided an uneven and incomplete surface coverage owing to the rigidity of the polymer chains.

Enhanced CE separation of ODNs (ds and ss) ranging from 16 - 20 base pairs (bp) (containing the same 16 base (b) sequence) was achieved in free solution on a capillary dynamically coated with CFR PEPyM-*co*-PMMA random copolymer (21/79). Fast and efficient surface modification was achieved on bare fused-silica capillaries activated with hydrochloric acid (HCl), H_2O, sodium hydroxide (NaOH) and Tris(hydroxymethyl)aminomethane (Tris)-borate (100 mM)/urea (7M) buffer, by flushing with polymer solution and H_2O followed by a final treatment with running buffer. 1 bp resolution (R_s) and was seen for long (30 cm) and short (8 cm) capillaries with partial R_s of the 16 bp and 17 bp mixture. Co-migration of some strands was attributed to ODN-ODN interactions during migration. These interactions were confirmed by the 16 bp peak migration time (t_m) not being additive within each mixture. The 1 bp R_s achieved for the complementary sequence strands was improved by up to 37 % for separation of dsODNs containing non-complementary sequences. Interestingly, separation of a dsODN mixture containing two 16 bp strands of different sequences resulted in partial R_s (0.52) suggesting that the free solution mobility of dsODNs was sequence dependent. Differential mobilities for ssODN fragments of the same length were also observed.

The method described herein was capable of resolving mixtures of PS-ODNs and ODNs, (including R_s of ss impurities from ds fragments) indicating that this method is suitable for determining ODN and PS-ODN purity. Both ODN and PS-ODN mobility was found to be proportional to an increase in bp length, suggesting the separation mechanism is based on the free solution mobility and ion-pairing between the surface and the analyte. The developed method has the advantage of fast and simple preparation, easy regeneration, extended capillary life-time, unrestricted polymer solubility, and enhanced stability under harsh conditions (high voltage and temperature, and extreme pH).

Publications

The publications originating from the work within this thesis are as follows:

Papers

Kerrilee E. Allan; Claire E. Lenehan; Dmitriy A. Khodakov; Hilton J. Kobus; Amanda V. Ellis, High-performance capillary electrophoretic separation of double-stranded oligonucleotides using a poly(ethylpyrrolidine methacrylate-*co*-methyl methacrylate) coated capillary, *Electrophoresis* **2012**, *33*, 1-10

Conference Proceedings

Kerrilee E. Allan, Polymeric Surface Modification of Capillaries for the Separation of DNA by Capillary Electrophoresis. Oral presentation at the *European Polymer Congress (EPF2011) and XII Congress of the Specialized Group of Polymers (GEP)*, Granada, Spain, **2011**

Kerrilee E. Allan, Random versus block copolymers for dynamic coatings for capillary electrophoretic separation of DNA. Oral presentation at the *32nd Australasian Polymer Symposium (APS)*, Coffs Harbour, NSW, **2011**

Kerrilee E. Allan, Capillary electrophoretic separation of double-stranded DNA oligonucleotides using a PEPyM-*co*-PMMA dynamically coated capillary. Poster presentation at the *ANZFSS 20th International Symposium on the Forensic Sciences*, Sydney, NSW, **2010**

Kerrilee E. Allan, Investigation of PEPyM-*co*-PMMA copolymer as a dynamic coating for capillary electrophoretic separation of DNA fragments. Oral presentation at the *ARNAM/ARCNN 2010*, Adelaide, SA, **2010**

Kerrilee E. Allan, Capillary electrophoretic determination of double-stranded DNA through an intercalating dye using the zone-passing technique, Poster presentation at the *ANZFSS 19th International Symposium on the Forensic Sciences*, Melbourne, Vic., **2008**

Table of Contents

List of Figures

List of Tables

List of Schemes

List of Abbreviations and Symbols

[M]	Concentration of monomer (via integration)
[M_0]	Initial concentration of monomer (via integration)
[η]	Intrinsic viscosity
$\sum_k$	Particle (k) surface area
μ_{app}	Apparent electrophoretic mobility
μ_{BRF}	BRF mobility
μ_{BRM}	BRM mobility
μ_{CR}	CR mobility
$\mu_{CR(tot)}$	Total CR mobility
μ_{EOF}	Electroosmotic mobility
μ_{ep}	Electrophoretic mobility
μ_o	Free solution mobility
μ_{obs}	Observed electrophoretic mobility
μ_{rep}	Reptation mobility
A	Adenine
a_0	Contour length of one DNA bp
ACN	Acetonitrile
ACPA	4,4'azobis(4-cyanovaleric acid)
AFM	Atomic force microscopy
AIBN	Azo-*bis*-isobutyrylnitrile
APS	Ammonium persulphate
AS-ODN	Antisense oligonucleotide
b	(DNA) base
b	Kuhn length
BGE	Background electrolyte
bp	(DNA) base pair
BRF	Biased reptation with fluctuations
BRM	Biased reptation model
Bu_3SnH	Tributylstannane
C	Cytosine
c	Concentration
c*	Overlap concentration
C16-HEC	C16-derivatised 2-hydroxyethyl cellulose
cat-HEC	Cationised hydroxyethylcellulose

$CDCl_3$	Chloroform, deuterated
CE	Capillary electrophoresis
CEP	Capillary electrophoresis phase
CFR	Conventional free radical
CGE	Capillary gel electrophoresis
c_k	Bulk concentration of '*k*' ions
CMS	Current monitoring system
c_{opt}	Optimum polymer concentration
CR	Constraint release
CTA	Chain transfer agent
D	Tangential distance of chain
$\bar{d}$	Mean difference
d6-DMSO	Dimethylsulphoxide, deuterated
DEAEMA	2-(diethylamino)ethyl methacrylate
DMF	Dimethylformamide
DMF	Diethylmalonate
DNA	Deoxyribonucleic acid
ds	Double-stranded
dsAS-ODN	Double-stranded antisense oligonucleotide
dsDNA	Double-stranded DNA
dsODN	Double-stranded oligonucleotide
dsPS-ODN	Double-stranded phosphorothioate antisense oligonucleotide
D_t	Translational diffusion coefficient
E	Total field strength
EDL	Electric double layer
EDTA	Ethylenediaminetetraacetic acid
EOF	Electroosmotic flow
epoxy-PDMA	Poly(dimethylacrylamide-*co*-allylglycidyl ether)
epoxy-poly(AG-AA)	Poly(acrylamide-*co*-allyl-β-D-glucopyranoside-*co*-allylglycidy ether)
EPy	*N*-(2-hydroxyethyl)-2-pyrrolidine
EPyM	Ethylpyrrolidine methacrylate
ez_k	Charge of ion '*k*'
F	Faradays constant
F_{drag}	Average drag force acting on the DNA due to one polymer
FTIR	Fourier transform infrared

G	Guanine
g	Grams
g	Polymer globule
GPC	Gel permeation chromatography
h	Hours
H_3PO_4	Hydrogen phosphate
HCl	Hydrochloric acid
HEC	Hydroxyethylcellulose
HMC	Hydroxypropylmethylcellulose
HPLC	High performance liquid chromatography
I_B	Bjerrum length
id	Capillary inner diameter
k	Boltzmann constant
K	Constant of proportionality
K*	Mark-Houwink-Sakurada constant
kDA	Kilodaltons
L	Litres
L	Molecular chain length
L_c	DNA contour length
L_d	Length to the detector (effective capillary length)
LIF	Laser induced fluorescence
L_p	Polymer contour length
L.O.D	Limit of detection
LPA	Linear polyacrylamide
L_t	Total capillary length
m	Metres
M	Moles per litre (mol L^{-1})
MALDI	Matrix-assisted laser desorption/ionisation
MALLS	Multi-angle laser light scattering
MC	Methylcellulose
M_{DNA}	Base pair length of DNA
min	Minutes
MMA	Methyl methacrylate
M_n	Average number molecular mass
mol	Moles
MPTS	γ-methacryloxypropyltrimethoxysilane
M_w	(Average weight) molecular mass

N	Molecular length
n	Mean number of polymer chains in contact with the DNA
N	Theoretical plates
N_A	Avogadro's number
NaCl	Sodium chloride
NaDM	Sodium diethylmalonate
NaH_2PO_4	Dihydrogen sodium phosphate
NaOH	Sodium hydroxide
N_{bl}	Number of globules per chain
N_k	Total number of Kuhn segments
NMR	Nuclear magnetic resonance
N_p	Number of polymer molecules
$N_{polymer}$	Degree of polymerisation of the polymer
od	Capillary outer diameter
ODN	Oligonucleotide
OHP	Outer Helmholtz plane
p	Persistence length
Pa	Pascals
PAM	Polyacrylamide
Pd(A)	Polydeoxyadenylic acid
Pd(T)	Polydeoxythymidylic acid
PDEA	Polydiethylacrylamide
PDEAEMA	Poly(diethylaminoethyl methacrylate)
PDEAEMA-*co*-PMMA	Poly(diethylaminoethyl methacrylate-*co*-methyl methacrylate)
PDI	Polydispersity index
PDMA	Polydimethylacrylamide
PDMA-*co*-PEPyM	Poly(*N*,*N*-dimethylacrylamide-*co*-ethylpyrrolidine methacrylate)
PDMA-*co*-PMAEM	Poly(*N*,*N*-dimethylacrylamide-*co*-4-(ethyl)-morpholine methacrylamide)
p_{DNA}	Persistence length of DNA
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PEPyM	Poly(ethylpyrrolidine methacrylate)
PEPyM-*b*-PMMA	Poly(ethylpyrrolidine methacrylate-*block*-methyl methacrylate)
PEPyM-*co*-PMMA	Poly(ethylpyrrolidine methacrylate-*co*-methyl methacrylate)
PHEA	Poly-*N*-hydroxyethylacrylamide

PMMA	Poly(methyl methacrylate)
poly(AA-EE)	Poly(acryloylaminoethoxyethanol)
poly(AG-AA)	Polyacrylamide-*co*-allyl-*β*-D-glucopyranoside
poly(Agal-AA)	Poly(acrylamide-co-allyl-D-galactopyranoside)
polyE-323	Bis(3-aminopropylamino)ethane
PPS	Potassium persulphate
PS-ODN	Phosphorothioate oligonucleotide
PSS	Polystyrenesulfonate
PVP	Polyvinylpyrrolidone
Q	Total particle charge
R	Gas constant
r	Capillary radius
RAFT	Reversible addition-fragmentation chain transfer
R_g	Radius of gyration/migrating particle radius
RMS	(Roughness) root mean squared
RNA	Ribonucleic acid
R_p	Radius of the polymer chains
$\mathrm{R_s}$	Resolution (of peaks)
RSD	Relative standard deviation
s	Seconds
SCLRP	Surface-confined living radical polymerisation
$\mathrm{s_d}$	Standard deviation
SEC	Size exclusion chromatography
SECE	Size exclusion capillary electrophoresis
SEI	Short-end injection
Si	Silicon
SNP	Single nucleotide polymorphism
ss	Single-stranded
ssAS-ODN	Single-stranded antisense oligonucleotide
ssDNA	Single-stranded DNA
ssODN	Single-stranded oligonucleotide
ssPS-ODN	Single-stranded phosphorothioate antisense oligonucleotide
star-PEO	Star-polyethylene oxide
STRs	Short tandem repeats
T	Thymine
t	Time
T	Temperature

TA	Tris-acetate
TAE	Tris-acetate-EDTA
TAPS	*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TBE	Tris-borate-EDTA
t_{calc}	Calculated *t* value for *t*-test
TEA	Triethylamine
TEC	Transient entanglement coupling
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethane-1,2-diamine
THF	Tetrahydrofuran
$\mathbf{t_{int}}$	Injection time
$\mathbf{T_m}$	Melting temperature
$\mathbf{t_m}$	Migration time
TMS	Tetramethyl silane
Tris	Tris(hydroxymethyl)aminomethane
TrisCl	Tris chloride
t_{table}	*t* value from the table of *t*-distribution values for *t*-test
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V	Applied voltage
v	Volume
V_{DNA}	Velocity of the DNA
$\mathbf{V_{inj}}$	Injection voltage
$\mathbf{v_{int}}$	Volume of sample introduced/injected
V_n	Electrophoretic velocity of a DNA molecule with n polymers attached
V_{par}	Particle volume
V_p	Curvilinear velocity of the reptating polymer chains
V_s	Surface potential
$\mathbf{W_h}$	Peak width at half height
α_{MHS}	Mark-Houwink-Sakurada constant
β	Ratio of τ_p to τ_{DNA}
γ	$(\rho_{solvent}/\rho_{polymer})$ x $(N_{polymer})^{1+\nu}$
δ	Chemical shift
δ	Thickness of the EDL
ΔP	Change in pressure
ε	Reduced electric field
ε_o	Permittivity of vacuum

ε_b	Dielectric constant of the fluid
ζ	Zeta potential
η	Viscosity of solution
η_s	Viscosity of solvent
κ	Inverse Debye length
κ^{-1}	Debye screening length
μ	Micro
v	Velocity
v_c	Average number of chain centres/cm^3
v_{EOF}	EOF velocity
v_F	Flory's exponent
ξ	Polymer screening length
$\bar{\xi}$	Mesh size normalised to the DNA Kuhn length
ξ_b	Pore (mesh) size
ξ_f	DNA friction coefficient per bp
π	Pi
ρ	Density
σ	Surface charge density
τ_{DNA}	Release time of the polymer from the DNA
τ_p	Release time of the DNA from the polymer
ψ	Electric potential