POLITECNICO DI TORINO

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SYNTHESIS AND CHARACTERIZATION OF A NOVEL SULFOBETAINE COPOLYMER FOR ANTIBIOFOULING SURFACES

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Sommario

Sintesi e caratterizzazione di un nuovo copolimero con base sulfobetanica per superfici antiadesive

INTRODUZIONE

Nel corso degli ultimi anni è stato riscontrato sempre più frequentemente un fenomeno potenzialmente molto pericoloso per l'uomo: alcuni batteri hanno sviluppato resistenza agli antibiotici usati per combatterli. Questo fenomeno è strettamente legato allo sviluppo di infezioni. Le infezioni, che già erano uno dei maggiori rischi in ambito biomedico, con questa evoluzione dei batteri sono tornate a fare grossi danni. Ricerche hanno mostrato che i pazienti con infezioni resistenti hanno probabilità più alte di avere tempi di ricovero più lunghi, recuperi più lenti, disabilità a lungo termine e, nel peggiore dei casi, di morire.

Ci sono quattro sistemi principali per combattere queste letali infezioni:

- Prevenire l'infezione e la diffusione della resistenza agli antibiotici
- Tracciare i batteri resistenti
- Migliorare l'uso degli antibiotici
- Sviluppare nuovi antibiotici e nuove analisi diagnostiche per i batteri resistenti

Il primo sistema richiede che venga fatto un passo indietro e che l'infezione, invece che essere combattuta, venga prevenuta. Ciò di cui si necessita è un particolare tipo di biomateriali. Quando un biomateriale interagisce con un ambiente biologico è interessante investigare la sequenza di eventi che porta alla risposta del sistema e scoprire in che modo modificare questa risposta.

Esistono diverse classi di biomateriali, con funzioni anche molto diverse tra loro. In molte di queste applicazioni, l'obbiettivo è lo studio dell'adesione cellulare e la sua proliferazione, tuttavia le cellule non interagiscono direttamente con la superficie, bensì attraverso le proteine che si sono precedentemente legate alla stessa. L'adesione proteica è il primo passo in molti processi biologici che portano allo sviluppo di un'infezione, quindi l'adesione proteica dovrebbe essere evitata. L'adesione biologica è basata sull'adesione organica e consiste di tre fasi: adesione reversibile, adesione irreversibile e proliferazione. La strategia più semplice è bloccare il processo prima che l'adesione irreversibile cominci.

Le proteine sono complessi biopolimeri derivanti da 20 aminoacidi. Sono solitamente zwitterioniche e spesso solubili in acqua, hanno una catena idrofobica e teste polari o non polari. Quando entrano in contatto con una superficie deteriorano: avviene la distruzione della conformazione e la perdita delle molecole d'acqua integrate con conseguente aumento dell'energia libera di Gibbs. Per cercare di ridurla la proteina tenta di stabilire nuovi legami (Van der Waals, legami idrogeno o di natura elettrostatica) con la superficie.

L'adesione di proteine su impianti biomedici riduce l'efficacia di questi e può inoltre provocare effetti secondari dannosi quali trombosi. Per di più, l'adesione proteica può innescare adesione di particelle, batteri o cellule che possono promuover cascate di infiammazioni o processi di danneggiamento. Per finire, l'adesione di proteine sulla superficie crea uno strato favorevole alla colonizzazione e alla formazione di biofilm, il quale può portare ad infezioni. Un ulteriore possibile risultato dell'adesione proteica è l'adesione

tissutale, temuta in ambito chirurgico perché porta dolore intenso, ostruzione funzionale degli organi e difficili interventi chirurgici perché venga rimossa.

Alcuni dei fattori che controllano l'adesione delle proteine sono parametri esterni quali pH, temperatura, forza ionica e composizione dell'ambiente. Il contributo principale è dovuto a un aumento di entropia e la minimizzazione dell'energia libera di Gibbs, come accennato in precedenza. Altri fattori sono da considerare nell'adesione di proteine ad una superficie: una di queste è la taglia delle proteine: l'effetto Vroman suggerisce che le proteine più piccole siano le prime a legarsi alla superficie, ma che nel tempo queste vengano rimpiazzate da quelle di taglia superiore, le quali si legano più forte. Le proprietà della stessa superficie sono importanti: energia superficiale, polarità, carica e morfologia sono solo alcune.

In conclusione, è di fondamentale importanza ottenere superfici che combattano l'assorbimento di proteine per evitare la loro proliferazione e i successivi passi che portano a conseguenze molto pericolose.

Design di superfici antiadesive

Ci sono diversi meccanismi per ottenere superfici antiadesive: repulsione sterica, repulsione elettrostatica, strutture a microdomini o idratazione del layer superficiale. In tutti questi meccanismi l'energia libera gioca comunque un ruolo fondamentale.

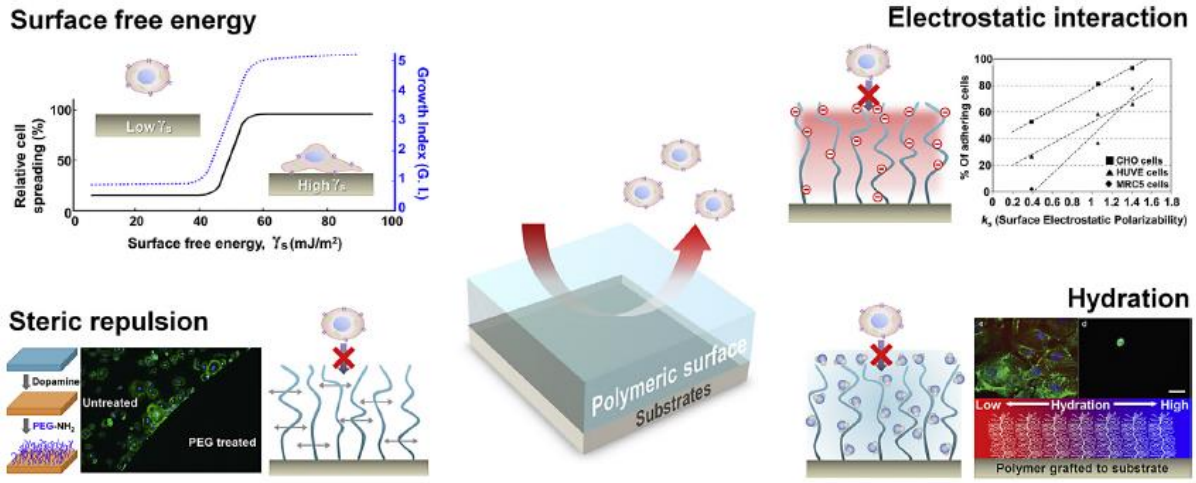


Figura 1: meccanismi di antiadesione proteica.

La repulsione sterica è il meccanismo generalmente sfruttato da coating e polimeri graffati alla superficie per mantenere la superficie pulita; quando la proteina si avvicina alla superficie, le catene polimeriche sono compresse, comportando un aumento dell'energia libera di Gibbs. La diffusione della proteina nello strato polimerico è energeticamente sfavorevole e il polimero tende a recuperare il suo stato esteso. Il secondo meccanismo è la repulsione elettrostatica: è più difficile che le proteine aderiscano a superfici neutre. Costruire una struttura basata su domini idrofobici e idrofilici alternati di dimensione micrometrica è un terzo meccanismo utilizzato per prevenire la proliferazione di proteine sulla superficie di un materiale. L'ultimo meccanismo considerato, nonché quello più efficace, è l'idratazione superficiale. L'inclusione di molecole d'acqua sia da parte della superficie che della proteina è il primo passo obbligatorio per impedire l'adesione proteica riducendo la barriera di energia libera. Il legame con le molecole d'acqua può formarsi per legami idrogeno, ma questo tipo di legame non è sufficientemente forte e la sua efficacia non è assicurata, oppure tramite interazione elettrostatica tra la catena polimerica e le molecole d'acqua.

L'idratazione superficiale è ritenuto un contributo chiave nello sviluppo di materiali con proprietà antiadesive.

Immobilizzare glicole polietilenico (PEG) sulla superficie mediante grafting è un comune metodo per impartire resistenza proteica alla superficie grazie al notevole volume libero e la mobilità delle catene. Il meccanismo comporta inoltre la formazione di un guscio di molecole d'acqua formato tramite legami idrogeno. Il PEG, tuttavia, non è perfetto a causa dell'eccessiva mobilità delle catene e per la sua tendenza ad auto ossidarsi e formare aldeidi ed eteri in presenza di ossigeno e metalli di transizione, perdendo le proprietà antiadesive. Un'alternativa al PEG è data dai polimeri zwitterionici grazie al loro bilanciamento di carica e dipolo minimizzato. I polimeri zwitterionici sono la scelta più comune quando si parla di idratazione superficiale per proprietà antiadesive e sono considerati ottimi materiali antiadesivi.

Polimeri zwitterionici

I polimeri zwitterionici fanno parte della famiglia di materiali che contengono gruppi ionici. Tra questi esistono i polielettroliti, che possiedono solo il gruppo cationico o anionico, e i polianfoliti, che invece li presentano entrambi. Tra questi, poi, i polimeri zwitterionici sono una sottoclasse la cui caratteristica è il bilanciamento tra i gruppi carichi e quindi la neutralità di carica. Sono tipicamente idrofili.

Negli anni '80 questi polimeri sono stati identificati come analoghi ad importanti strutture biologiche, rendendoli interessanti in ambito biomedico. Molti polimeri zwitterionici mostrano temperatura critica di solubilità inferiore (LCST) in ambienti acquosi, quindi sono spesso utilizzati come sistemi di risposta a stimoli, essendo reversibilmente sensibili a cambiamenti in temperatura, forza ionica, reazioni chimiche o pH.

Il tipico comportamento dei polizwitterioni in risposta a un cambiamento nella forza ionica è opposto rispetto a quello dei polielettroliti: mentre per gli ultimi l'aggiunta di un elettrolita a basso peso molecolare comporta il restringimento della catena, per i polizwitterioni la catena si estende, riducendo la viscosità e il punto critico. Questo è dovuto all'effetto di schermatura che i sali hanno nei confronti dei gruppi carichi sul polimero ed è chiamato *effetto antipolielettrolita*.

Per finire, un'importante caratteristica dei polizwitterioni, nonché la loro principale limitazione, è la difficile solubilità in molti solventi, che rende la loro sintesi complicata nella maggior parte dei solventi e con le tecniche più comuni.

Polibetani

I polibetani sono uno specifico gruppo di polimeri zwitterionici. Possiedono, a differenza di altre molecole zwitterioniche, i gruppi positivo e negativo sulla stessa unità monomerica. In queste specie il gruppo cationico è solitamente formato da uno ione ammonio o da uno fosfonio. Il gruppo funzionale anionico invece classifica i polimeri in diverse categorie:

- Gruppo solfonato → sulfobetani
- Gruppo carbossilato → carbossibetani
- Gruppo fosfonato → fosfobetani

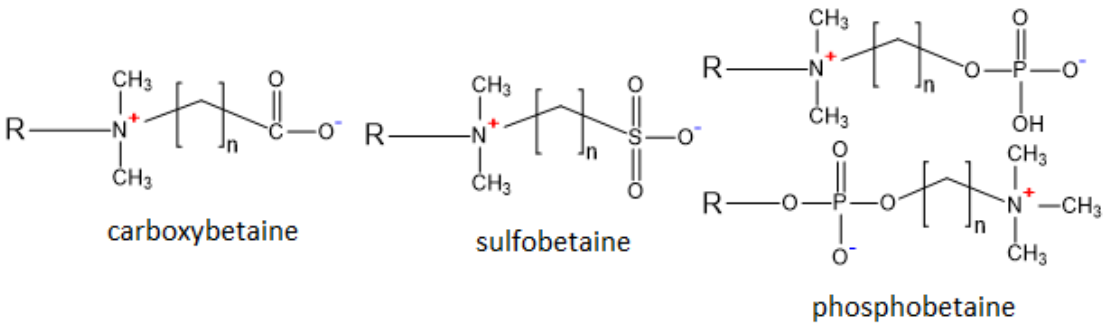


Figura 2: strutture di carbossibetani (sinistra), sulfobetani (centro) e fosfobetani (destra).

Ci sono diversi polibetani utilizzati con fini antiadesivi, come il *2-Methacryloyloxyethyl phosphorylcholine* (MPC) o varie strutture di poli sulfobetan metacrilati (PSBMA), tuttavia la loro applicazione è sempre limitata dai loro problemi di solubilità e di sintesi.

In generale, la sintesi di polizwitterioni deve far fronte alle difficoltà dovute alla presenza sia di gruppi elettrofili che nucleofili. I monomeri zwitterionici sono tipicamente polimerizzati in soluzione acquosa; una possibile soluzione al problema della sintesi è data da modificazioni post-polimerizzazione dei precursori, tuttavia queste sono raramente complete e possono cambiare il bilancio stechiometrico, causando anche la perdita della neutralità di carica.

I polibetani mancano di solubilità in acqua pura, a causa della formazione di contatti ionici intra- e intercatena risultanti in una struttura ionicamente reticolata. Anche la solubilità in solventi organici è limitata, in particolare per quanto riguarda i sulfobetani. La loro solubilità è limitata a solventi con grande abilità di donatori idrogeno, come gli alcoli. L'idrolisi è un problema in più da considerare e in particolare la stabilità del legame tra i gruppi ionici e la catena polimerica.
I composti zwitterionici sono spesso assemblati tramite una complessa sintesi a più fasi. Per ottenere una migliore solubilità e miscibilità e per evitare reazioni parassite, il gruppo anionico è spesso introdotto in forma protetta, come un'apertura d'anello con eterocicli (propan-/butansultone per gli ammoniosolfonati).

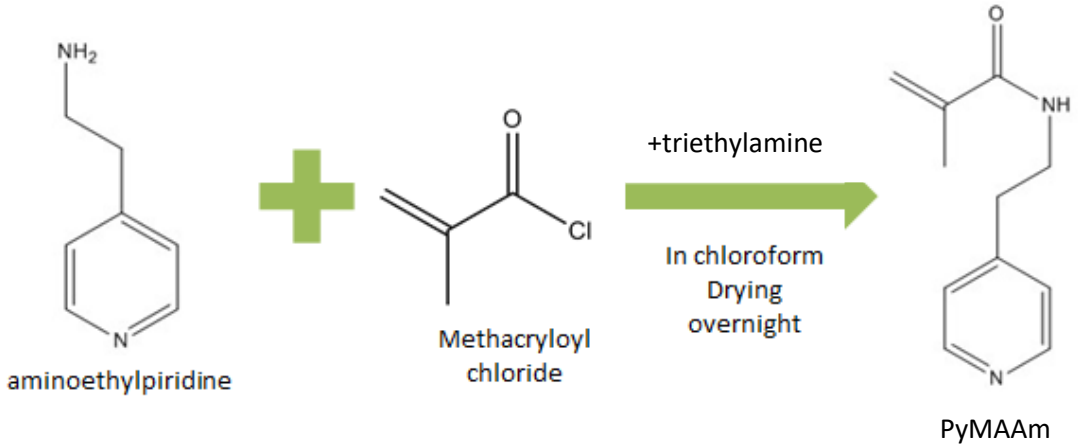
Il più comune meccanismo di sintesi per ottenere polimeri zwitterionici è la polimerizzazione a catena. La polimerizzazione radicalica libera, in particolare, è estremamente popolare grazie all'alta tolleranza dei radicali verso elettrofili e nucleofili. Questo tipo di polimerizzazione è consigliata per ottenere un copolimero random.
Allo scopo di migliorare la sintesi e solubilità dei polimeri zwitterionici, una strategia diffusa è di copolimerizzare i monomeri zwitterionici con altri monomeri in grado di migliorare le performance desiderate.

PySMAAm, HEMA e BMA

Questa ricerca è basata su uno specifico monomero sulfobetano, un monomero sulfobetano metacrilamidico con un catione piridinico (PySMAAm). Il monomero era stato selezionato per un precedente studio riguardante polimeri con proprietà stimoli dipendenti. Tra questi, vi sono i polimeri che presentano una temperatura critica di solubilità inferiore (LCST) e quelli che hanno una temperatura critica di solubilità superiore (UCST). Quest'ultimi, in particolare, hanno attirato l'attenzione perché possono mostrare una temperatura di transizione in condizioni fisiologiche.

I sulfobetani sono candidati promettenti per UCST in ambiente acquoso perché i loro gruppi zwitterionici possono formare forte attrazione inter- e intrapolimeri mediante collegamenti elettrostatici a bassa temperature risultanti in solubilità.

Il monomero PySMAAm è stato sintetizzato in due forme, a seconda che si facesse reagire un'aminoetilpiridina o un'aminometilpiridina. La molecola finale differiva solo per la lunghezza della catena alchilica, espressa con una lettera all'inizio della molecola: ePySMAAm per la catena etilica, mPySMAAm per quella metilica. Il PySMAAm è ottenuto da una reazione in due fasi. Inizialmente il precursore (aminoetilpiridina) era mischiato con cloruro metacrilico in diclorometano in presenza di trietilammina, usata per rimuovere l'acido cloridrico condensato nella reazione. La reazione era condotta per tutta la notte, per ottenere l'intermedio chiamato PyMAAm.

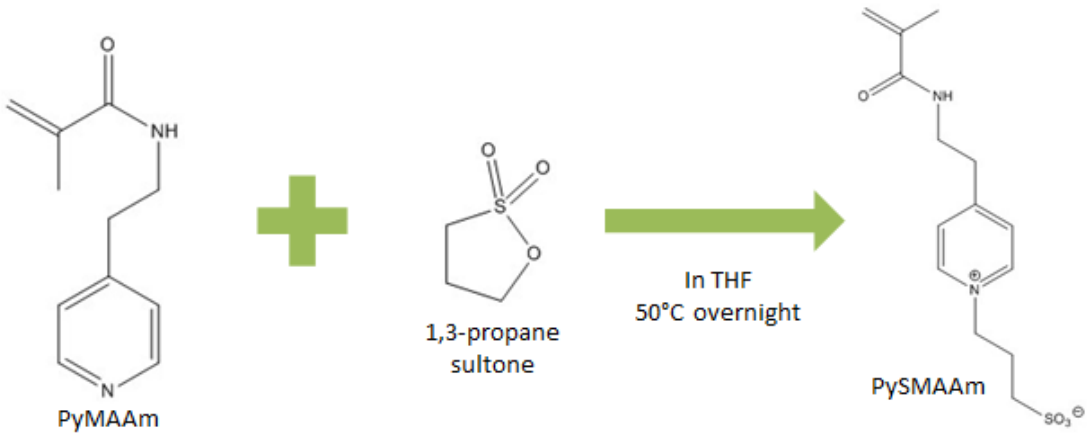


La reazione veniva eseguita seguendo la seguente stechiometria (Tabella 1):

Tabella 1

	Aminoetilpiridina	Cloruro metacrilico	Trietilammina
Equivalenti	1	1	1
Moli [mol]	0.016	0.016	0.033
Massa [g]	2	1.71	3.31
Volume [mL]	1.942	1.584	4.563

La seconda parte della reazione consisteva nell'addizione di 1,3-propan sultone al PyMAAm in quantità pari all'1.5 volte il numero di moli di intermedio. La reazione era fatta a 50°C in tetraidrofurano (THF) e lasciata per la notte.



Il successo della sintesi è stato accertato tramite analisi ^{1}H-NMR.

Il risultato rilevante di questa precedente analisi è la valutazione della citotossicità dei polimeri derivanti dai due monomeri sintetizzati. La misurazione è stata attuata tramite incubazione per 24 h in cellule HeLa. I polimeri derivati da ePySMAAm e mPySMAAm presentano bassa citotossicità, una caratteristica fondamentale per un polimero biocompatibile; P(ePySMAAm) in particolare sembrava il candidato più promettente ed è stato quindi deciso di testarlo come agente adesivo per ricoprire superfici. Il problema principale, come menzionato precedentemente, era la solubilità del monomero in ambienti acquosi. La strategia adottata per superare questa complicazione è stata la copolimerizzazione del PySMAAm con un altro monomero che ne migliorasse la stabilità. La scelta è caduta su due monomeri diversi: 2-idrossietil metacrilato (HEMA) e n-butil metacrilato (BMA).

L'HEMA è un composto liquido. Quando polimerizzato è un comune polimero in ambito biomedico. Come monomero è idrofobico e solubile in acqua, tuttavia cambia le sue proprietà quando polimerizzato con peso molecolare >10k, diventando idrofilico. È un buon polimero antiadesivo ma le sue proprietà si limitano alla repulsione di proteine, mentre con le cellule non ha un comportamento soddisfacente.

Il BMA è un polimero idrofobico, scelto come alternativa all'HEMA nel tentativo di ottenere un radicale più stabile per la reazione di copolimerizzazione. È già stato usato in passato nella copolimerizzazione di monomeri betanici in ambito antiadesivo.

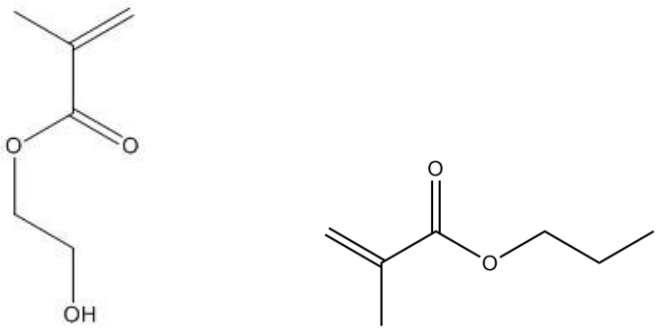


Figura 3: strutture di HEMA (sinistra) e BMA (destra).

MATERIALI E METODI

Dai risultati delle analisi sulla citotossicità dei due polimeri zwitterionici è stato deciso di concentrarsi sulla copolimerizzazione del monomero ePySMAAm. Alcuni campioni di copolimero di mPySMAAm sono stati preparati come confronto, ma l'utilizzo di un monomero piuttosto che l'altro non modifica in alcun modo il processo.

Copolimerizzazione

ePySMAAm è stato copolimerizzato con HEMA per polimerizzazione radicalica libera. La reazione è stata eseguita in solventi diversi cambiando la concentrazione iniziale di monomero. La scelta dell'iniziatore è legata alla scelta del solvente. Composizioni iniziali differenti dei monomeri sono state testate: 80-20, 50-50 e 20-80 di PySMAAm-HEMA rispettivamente.

Processo:

- ePySMAAm viene pesato e sciolto nel solvente
- la corretta quantità di HEMA è aggiunta alla miscela
- l'iniziatore è dissolto in 1 mL di solvente e la frazione adeguata è mescolata coi monomeri
- il resto del solvente viene aggiunto
- se necessario, la soluzione deve essere filtrata

- degasaggio con N_2 per 30 minuti
- la polimerizzazione è effettuata in un bagno di silicio alla temperatura di 60°C
- una volta completata la reazione, la soluzione polimerica è messa in un filtro e lasciata in acqua deionizzata per una settimana per rimuove le catene a più basso peso molecolare
- dopo una settimana, il polimero è stato raccolto mediante *freeze drying*

La copolimerizzazione di ePySMAAm e BMA segue la stessa procedura, senonché la soluzione non può essere filtrata in acqua a causa dell'idrofobicità del BMA. La soluzione in questo caso è concentrata per evaporazione e successivamente il copolimero è riprecipitato nell'agente precipitante adeguato, che è poi rimosso. Il copolimero è infine asciugato in vuoto per essere raccolto.
La resa della reazione è calcolata per ogni campione dall'equazione:

$$resa = \frac{m_{polimero}}{m_{ePySMAAm} + V_{HEMA(BMA)} * \delta_{HEMA(BMA)}}$$

Test di solubilità

Per poter eseguire le analisi successive è necessario identificare i solventi migliori per ogni campione. I test sono stati svolti dissolvendo pochi milligrammi del campione nel solvente con una concentrazione di 5 o 10 mg/mL.

^{1}H-NMR

Un campione di ogni copolimero è stato raccolto per essere analizzato tramite spettroscopia ^{1}H-NMR, con l'obbiettivo di confermare il successo della copolimerizzazione e di valutare la composizione finale del prodotto. Copolimeri con un contenuto di PySMAAm maggiore in composizione sono stati disciolti in soluzione 1M di NaCl in D_2O, gli altri campioni invece dissolti in 2,2,2-trifluoroetanolo (TFE) a causa della loro insolubilità nel solvente precedente.

Gel Permeation Chromatography

Il peso molecolare dei copolimeri è stato misurato tramite *Gel Permeation Chromatography*(GPC). L'analisi è stata condotta utilizzando la soluzione 0.4M $NaNO_3$ come solvente, in una colonna TOSOH PWXL3000+PWXL4000. La concentrazione del polimero scelta era di 5 mg/mL, la temperatura 25°C e la velocità di flusso 0.7 mL/min. Sono stati valutati peso molecolare e polidispersità.

Coating

I polimeri sono stati sintetizzati con l'idea di essere utilizzati come coating. Sono stati realizzati coating mediante due tecniche: immersione e spin coating. I polimeri sono stati tutti sciolti in TFE con una concentrazione di 5 mg/mL.
Il coating per immersione (*dip coating*) è stato effettuato su quadratini (1X1 cm^2) di polietilentereftalato (PEF). Il substrato, dopo essere stato pulito, è stato immerso 5 volte nella soluzione del polimero, quindi posto in un vetrino poggiato in obliquo, in modo che entrambe le facce potessero asciugarsi uniformemente. Dopo un'ora la procedura di immersione era ripetuta e i campioni erano quindi lasciati nel vetrino per la notte in presenza di una vaschetta contenente 2-3 mL di TFE. Il giorno successivo il TFE era rimosso e i campioni asciugati sotto vuoto.
Per eseguire lo spin coating è stato usato uno Spin Coater MS-B100 (Mikasa Opticoat). Il substrato selezionato era un wafer di silicio, tagliato in quadratini e pulito. 50 µL della soluzione del polimero sono stati versati sul substrato di silicio posto nella macchina, la quale è stata settata in un coating a due fasi:

inizialmente avrebbe rotato a 500 rpm per 5 s, poi 30 s a 3000 rpm. Il substrato col coating era poi asciugato sotto vuoto.

Analisi SEM

I campioni ottenuti per spin coating sono stati graffiati sulla superficie e poi spediti al laboratorio per essere studiati tramite *scanning electron microscopy* (SEM) per confermare la presenza del coating, vederne la superficie e per valutare lo spessore del coating.

Analisi FTIR

L'analisi tramite *Fourier Transformed Infrared Spectroscopy* (FTIR) è stata compiuta su campioni con coating e anche su campioni di copolimero non deposto su un substrato. La macchina usata è stata un IRPrestige-21. I campioni con *dip coating* sono stati studiati per confermare la stabilità del coating dopo immersione in una soluzione di *phosphate buffer saline* (PBS) per 20 h ca. Dopo l'immersione nella soluzione salina sono stati puliti con acqua deionizzata e asciugati. Per ogni campione sono stati analizzati due coating, uno prima e uno dopo immersione in PBS per valutare la differenza negli spettri, che poi sono stati confrontati con lo spettro del substrato di PET non rivestito. I campioni di copolimero non depositati sono stati investigati per confermare ulteriormente la copolimerizzazione, come fatto con la spettroscopia ^{1}H-NMR.

Misura dell'angolo di contatto

L'analisi principale che è stata condotta per confermare la stabilità dei coating dopo immersione in PBS è stata la misura dell'angolo di contatto statico. 1.0 µL di acqua deionizzata veniva posta in contatto con la superficie dei campioni e l'angolo di contatto era misurato da un software (programma FAMAS) legato a una fotocamera. Nuovamente sono stati preparati 2 campioni per ogni copolimero, in modo da avere l'angolo prima e dopo l'immersione in PBS. Per migliorare ulteriormente la stabilità dei coating, inoltre, è stato valutato l'effetto di un trattamento superficiale ad ozono sulla superficie di PET prima che il coating fosse effettuato.

Sono stati preparati i campioni dei seguenti copolimeri: P(PySMAAm-HEMA): 80-20, 50-50, 20-80, P(mPySMAAm-HEMA)50-50 e P(PySMAAm-HEMA)50-50 sintetizzato con concentrazione di monomero dello 0.1M. I campioni di P(PySMAAm-BMA) erano: 80-20, 50-50, 20-80, 15-85 e 50-50, il quale è stato ridissolto e *freeze dried*. Per finire, è stato misurato l'angolo di contatto anche di campioni di controllo quali PET e substrati rivestiti di P(HEMA) e P(BMA).

Valutazione dell'adesione proteica

L'analisi più importante che è stata effettuata sui campioni è quella di adesione proteica, mediante la *Micro BCA Microplate Procedure* (Thermo Fisher Scientific). L'analisi è usata per il riconoscimento e la misurazione colorimetrica delle proteine totali, basata sull'acido bicinconinico come reagente di riconoscimento per Cu^{+1}, che è formato quando Cu^{+2} è ridotto dalle proteine in un ambiente alcalino.

I campioni di PET rivestiti sono stati immersi in PBS approssimativamente 20h, dopodiché sono stati presi e immersi in una soluzione 50 mg/mL di *bovine serum albumine* (BSA) e incubata a 37°C per 1 h. Ogni campione è stato poi lavato in PBS per rimuovere le proteine in eccesso e posto in una cella e sonicato in presenza di una soluzione 5 mg/mL di laurilsolfato di sodio (SDS) in acqua deionizzata, per rimuovere dalla superficie del campione tutte le proteine adsorbite. 150 µL sono stati raccolti da ogni cella e posti in una cella nella quale sono stati aggiunti 150 µL di *Working Reagent* (WR), preparato seguendo le istruzioni del kit per l'analisi:

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

Sono stati preparati in questo modo i campioni dei copolimeri e degli standard usati per costruire la curva con la quale i risultati vengono analizzati. Il micropiatto con tutte le celle contenenti le soluzioni dei campioni è stato quindi incubato a 37°C per 2 h, dopo le quali è stato raffreddato a temperatura ambiente. Per finire, il piatto coi campioni è stato posto in un spettrofotometro UV-vis (Spectra Max M3) col quale è stata misurata l'assorbanza a 562 nm.

4 coating sono stati testati per ogni composizione di copolimero e per i campioni di controllo in modo da avere una buona misura statistica.

RISULTATI E DISCUSSIONE

Copolimeri PySMAAm-HEMA

La polimerizzazione è stata condotta inizialmente con concentrazione di monomero 0.1M in 30 mL di solvente, metanolo (MeOH). L'iniziatore, solubile in MeOH, era *azobisisobutyronitrile* (AIBN), in quantità pari all'1% delle moli di monomero (in questo caso 1% di 3 mmol=0.03mmol). La polimerizzazione è stata eseguita in un bagno di olio caldo a 60°C per 2 h tuttavia, al momento della raccolta, non è stato trovato quasi nulla. Il processo era quindi da modificare.

Il primo parametro sul quale si è lavorato è stato il tempo di polimerizzazione, aumentato da 2 a 20 ore. Questi nuovi campioni hanno dato i primi risultati: tutte e tre le composizioni del copolimero sono state raccolte sotto forma di polvere bianca. È stata studiata la solubilità di queste polveri in diversi solventi e si è ottenuto che:

- 1M NaCl è un buon solvente per i campioni ad alto contenuto di PySMAAm, ma non per il campione di composizione 20-80
- TFE è un valido solvente per tutti i copolimeri
- 0.4M $NaNO_3$ può essere usato come solvente per i campioni per le analisi GPC
- La stabilità dei campioni in PBS è risultata insoddisfacente; il campione 20-80 è l'unico a presentare qualche difficoltà a solubilizzarsi.

I risultati di composizione finale del copolimero valutata tramite ^{1}H-NMR, di peso molecolare e polidispersità e di resa sono riassunti in Tabella 2:

Tabella 2

Campione	Composizione finale	Mw	Mw/Mn	Resa [%]
P(PySMAAm-HEMA) 80-20	71-29	8.6k	1.62	31.6
P(PySMAAm-HEMA)50-50	40-60	9.5k	1.78	49.3
P(PySMAAm-HEMA)20-80	-	-	-	17.6

È stata notata una tendenza ad aumentare la quantità di HEMA nel copolimero, segno di un monomero più reattivo. Resa e peso molecolare erano entrambi troppo bassi e dovevano essere migliorati. La resa di P(PySMAAm-HEMA)20-80 era così bassa che il prodotto non è stato sufficiente per fare le altre analisi.

Per incrementare i risultati di peso molecolare e resa dei copolimeri, è stata provata la copolimerizzazione in diversi solventi:

- MeOH/1M NaCl 50/50 %vol; l'iniziatore era *4,4'-azobis(4-cyanovaleric acid)*;
- 1M NaCl con *2,2'-Azobis[2-(2-imidazolin-2-yl)propane]* (VA061) come iniziatore;

- TFE con AIBN come iniziatore.

Il solvente che ha dato i risultati migliori è stato la soluzione 50/50 di MeOH/1M NaCl, come riportato in Tabella 3.

Tabella 3

Campione	Composizione Finale	Mw	Mw/Mn	Resa [%]
P(PySMAAm-HEMA)80-20	67-33	16.7k	2.65	54
P(PySMAAm-HEMA)50-50	33-67	34.7k	2.07	75
P(PySMAAm-HEMA)20-80	-	17k	2.24	40.7

Lo spettro ^{1}H-NMR di P(PySMAAm-HEMA) 50-50 ottenuto con le condizioni di polimerizzazione appena descritte è usato per identificare i picchi relativi alle varie parti della molecola, sia dovuti al PySMAAm che all'HEMA.

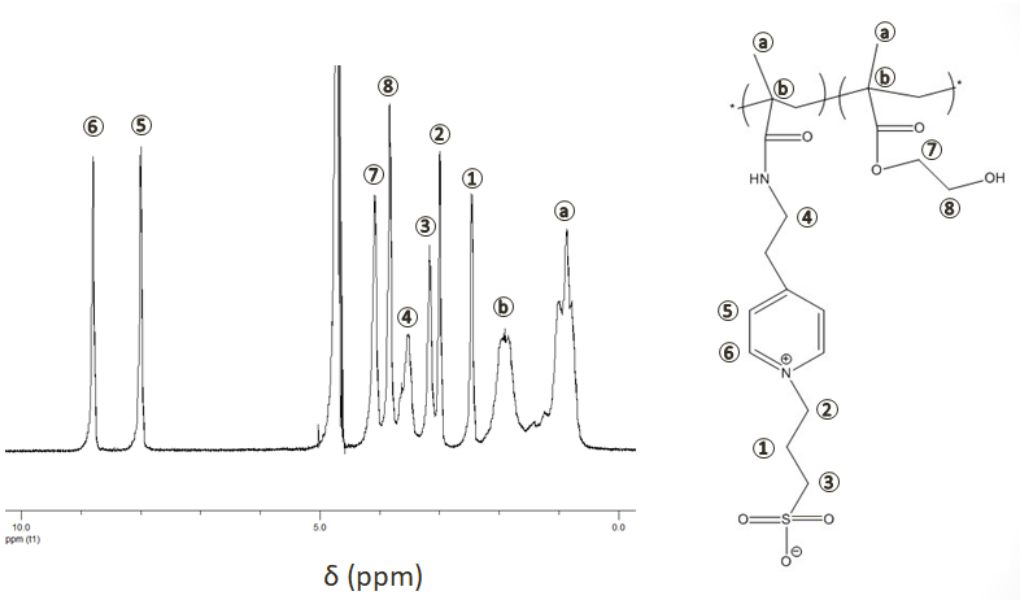


Figura 4: ^{1}H-NMR di P(PySMAAm-HEMA) 50-50 coi picchi assegnati.

Il nuovo processo di sintesi, anche se ha portato un aumento del peso molecolare, ha anche provocato un aumento dell'indice di polidispersità dei campioni. Dopo aver provato senza successo a modificare la quantità di iniziatore nella reazione, si è deciso di aumentare la concentrazione di monomero da 0.1 a 0.3M, ottenendo i risultati in Tabella 4.

Tabella 4

Campione	Composizione Finale	Mw	Mw/Mn	Resa [%]
P(PySMAAm-HEMA)80-20	68-32	60.4k	2.61	64.2
P(PySMAAm-HEMA)50-50	35-65	61.2k	2.69	43.7
P(PySMAAm-HEMA)20-80	7.5-92.3	-	-	73.5

La composizione dei campioni è in accordo con quella delle analisi precedenti, in tutti gli altri risultati è stato osservato un incremento, notevole per quanto riguarda il peso molecolare ma sempre a spese della polidispersità. Il peso molecolare di P(PySMAAm-HEMA)20-80 non è stato calcolato a causa di problemi con la strumentazione. Una polidispersità così elevata può avere effetti negativi sulla stabilità dei coating. Un ulteriore tentativo di diminuire la polidispersità dei copolimeri è stato fatto riducendo di nuovo la quantità di iniziatore allo 0.8% in numero di moli di monomero. Il campione, un P(PySMAAm-HEMA) 50-50 ha ottenuto il peso molecolare più alto registrato (85.7k), ma l'indice di polidispersità è ulteriormente aumentato (2.88). Tutti i campioni analizzati fino ad ora sono sintetizzati usando ePySMAAm, tuttavia è stato prodotto come confronto anche un copolimero P(mPySMAAm-HEMA) 50-50 con le condizioni che ottimizzavano il processo. Il campione ottenuto aveva Mw=81k e Mw/Mn=1.90 ma la composizione finale era più sbilanciata verso il monomero HEMA (26-74 di mPySMAAm-HEMA).

La stabilità dei copolimeri in PBS sembra migliorare all'aumentare del peso molecolare, in particolare per i copolimeri con quantità di HEMA in composizione maggiore, come ci si aspettava.

Tutte le analisi eseguite su campioni di P(PySMAAm-HEMA) seguenti sono state fatte usando campioni ad alto peso molecolare.

Le prime valutazioni sulla stabilità dei copolimeri erano promettenti, tuttavia è stato sintetizzato un altro copolimero utilizzando un monomero diverso dall'HEMA, il BMA.

Copolimeri PySMAAm-BMA

Poiché questo copolimero è stato prodotto dopo gli altri, è stato possibile sintetizzarlo subito coi parametri che ottimizzano il processo di sintesi dei copolimeri di PySMAAm-HEMA:

- Concentrazione di monomero: 0.3M
- Concentrazione di iniziatore: 1% moli di monomero
- Tempo di polimerizzazione: 20h
- Temperatura di polimerizzazione: 60°C

La scelta del solvente è stata controllata dall'insolubilità del BMA in soluzioni acquose, di conseguenza MeOH è stato scelto, portando con sé AIBN come iniziatore. Sono stati prodotti campioni con percentuali di PySMAAm e BMA rispettivamente di 80-20, 50-50, 20-80 e 15-85.

I campioni raccolti non si presentavano più come una polvere bianca bensì come uno slime giallognolo molto viscoso. La valutazione di peso molecolare e indice di polidispersità di questi copolimeri non è stata potuta effettuare a causa della loro tendenza ad aggregarsi in 0.4M $NaNO_3$. È stato fatto un tentativo di ridurre questo comportamento, dovuto anche alla riprecipitazione del polimero, dissolvento un campione di P(PySMAAm-BMA) 50-50 in 1M NaCl dopo riprecipitazione, per poi essere filtrato e *freeze dried*. È stata ottenuta una polvere bianca che però ha mantenuto le proprietà di aggregazione.

La solubilità dei campioni è stata testata e la stabilità dei campioni in PBS sembrava promettente.

Dagli spettri ^{1}H-NMR la copolimerizzazione è stata confermata e la composizione dei copolimeri è stata valutata (Figura 5). In generale questi mostrano più stabilità in confronto ai campioni di P(PySMAAm-HEMA).

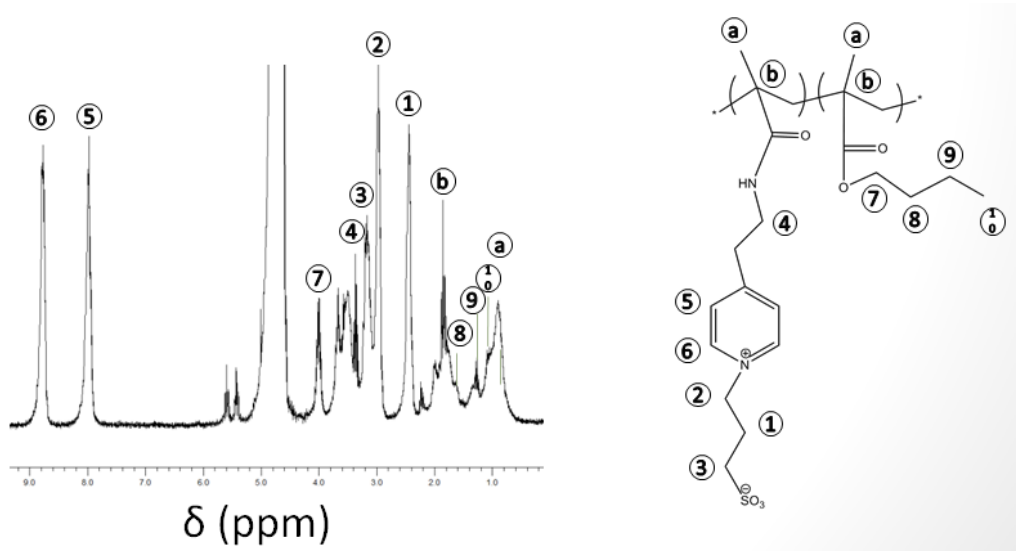


Figura 5: ^{1}H-NMR di P(PySMAAm-BMA) 80-20 con picchi assegnati.

Analisi SEM

Sono stati osservati 3 campioni: P(PySMAAm-HEMA) 50-50, P(PySMAAm-BMA) 50-50 e P(PySMAAm-BMA)20-80. Nonostante la superficie dei campioni fosse nitida, non è stato possibile valutare lo spessore del coating, probabilmente perché troppo sottile. La superficie appare granulare e uniforme.

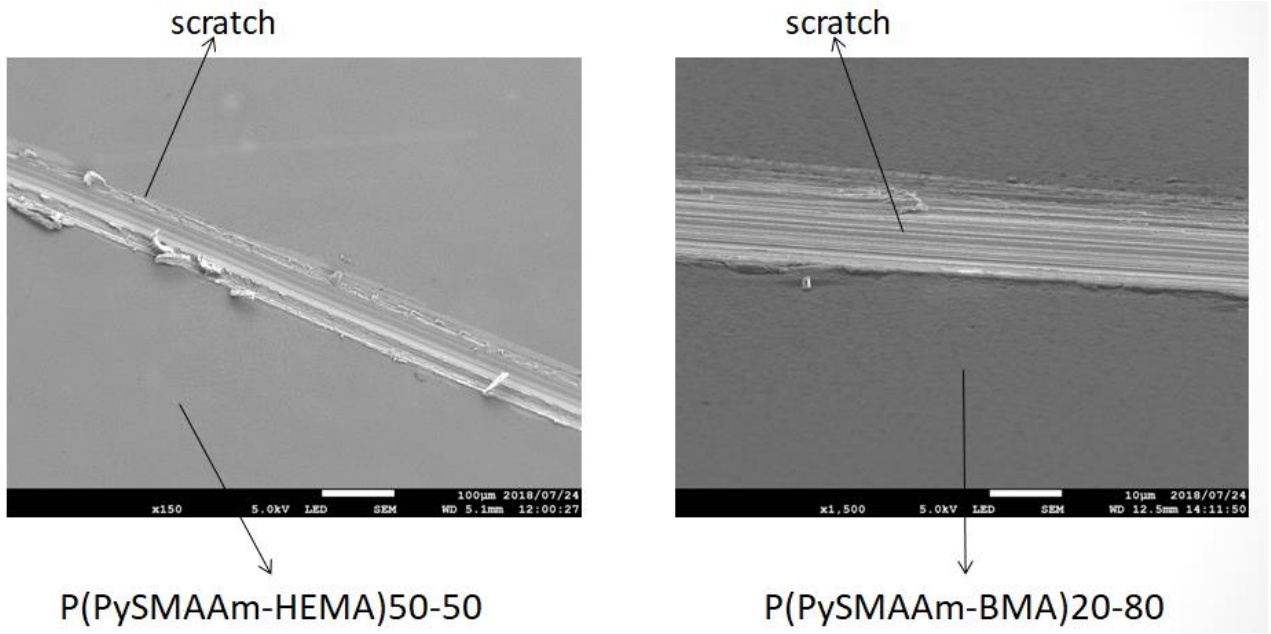


Figura 6: Immagini SEM di due campioni di coating.

Analisi FTIR

L'analisi è stata condotta per confermare la stabilità dei coating dopo l'immersione in PBS, tuttavia confrontando lo spettro del substrato di PET non rivestito con quello dei campioni di coating, non si osservano sostanziali differenze (Figura 7), si pensa nuovamente a causa dello spessore troppo ridotto del coating.

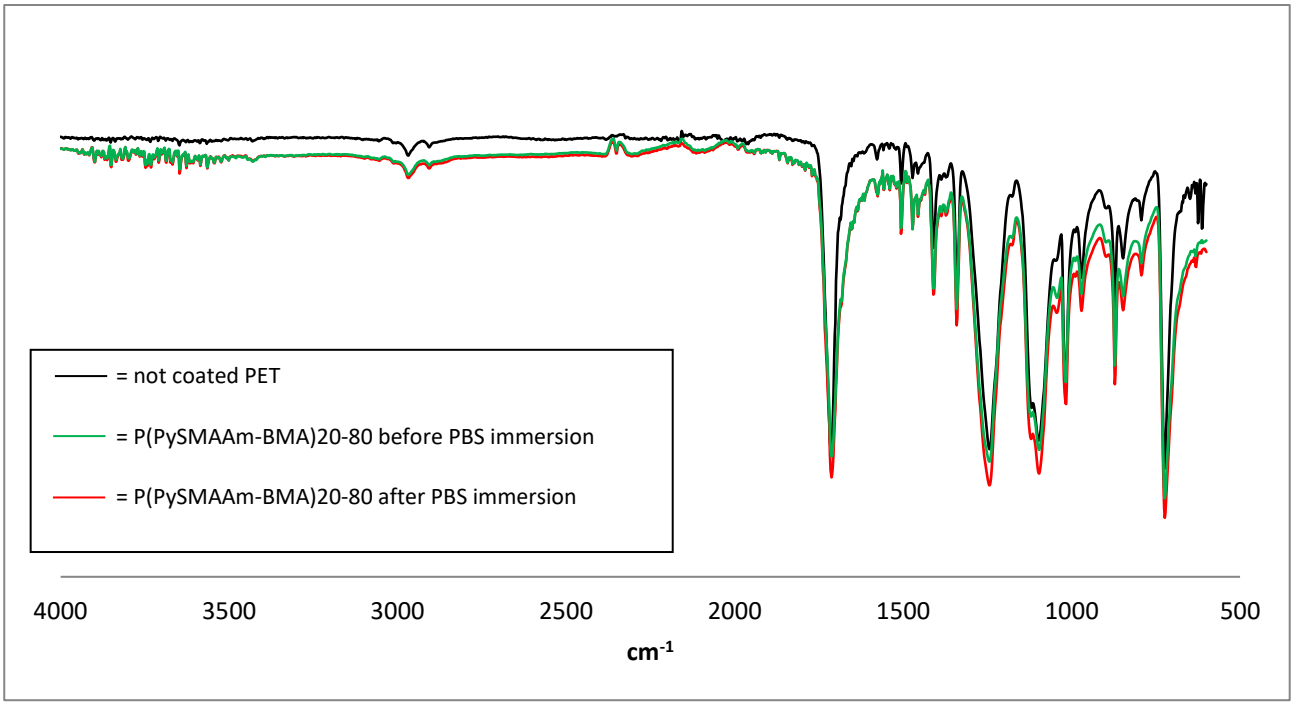


Figura 6: Spettri FTIR sovrapposti di PET (nero), P(PySMAAm-BMA) 20-80 prima (verde) e dopo (rosso) immersione in PBS.

L'analisi quindi è stata ripetuta analizzando non i coating bensì i campioni di copolimero (polvere e slime) per identificarne i picchi.

Gli spettri riportati sono di P(PySMAAm-HEMA) 80-20 (Figura 7) e di P(PySMAAm-BMA) 20-80 (Figura 8). A parte la differenza di intensità, i due spettri presentano gli stessi picchi e confermano l'avvenuta copolimerizzazione dei monomeri.

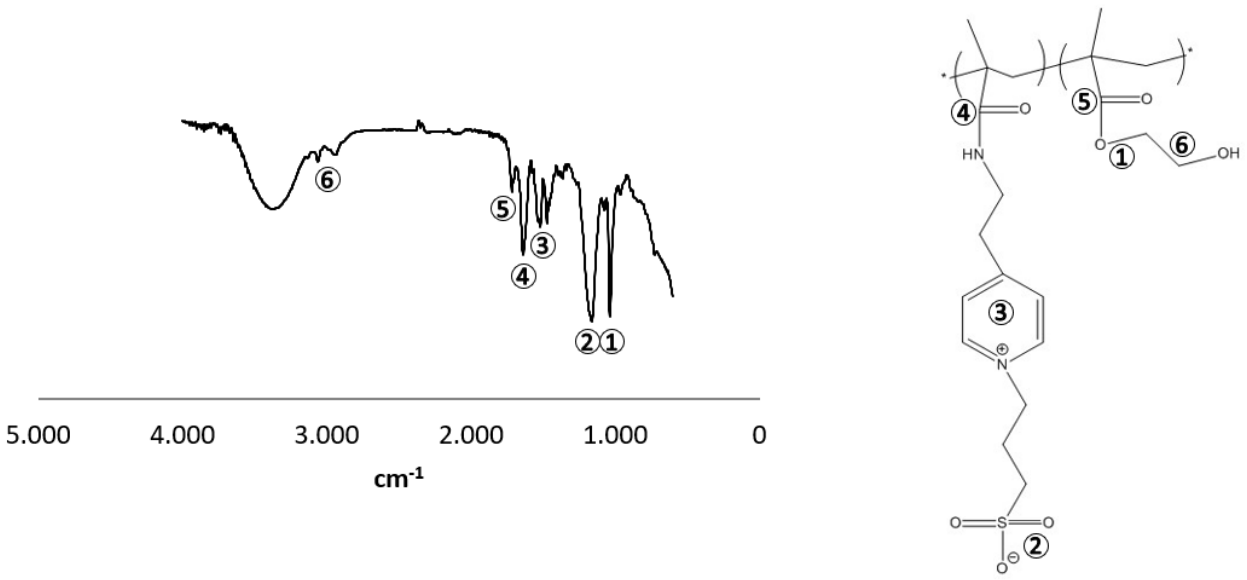


Figura 7: Spettro FTIR di P(PySMAAm-HEMA)80-20 con picchi assegnati.

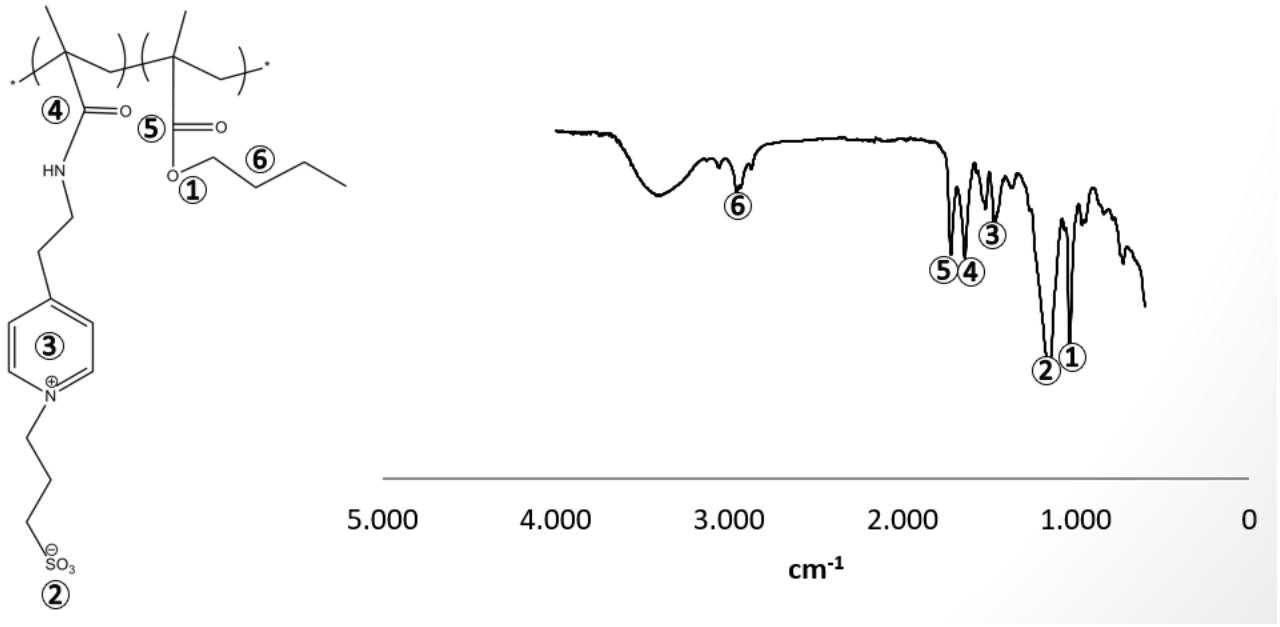


Figura 8: Spettro FTIR di P(PySMAAm-BMA)20-80 con picchi assegnati.

Misura dell'angolo di contatto

L'analisi era volta alla determinazione della stabilità dei coating confrontando campioni immersi in PBS per 20 h con campioni che non sono stati equilibrati. I campioni di controllo analizzati sono (Tabella 5):

Tabella 5

Campione	Non immersi in PBS	Immersi in PBS
Substrato di PET	70°	-
Coating di P(HEMA)	26°	26°
Coating di P(BMA)	75°	46°

L'effetto dei coating di P(PySMAAm-HEMA) sui substrati ha comportato una generale diminuzione dell'angolo di contatto prima dell'immersione in PBS. Gli angoli di contatto erano minori o uguali a quello del coating di P(HEMA). Dopo l'immersione in PBS però molti dei campioni sono tornati ad avere angoli di contatto ampi. Il problema di questi campioni è che, essendo il P(HEMA) di per sé idrofilico, non si può dire con certezza che il coating sia stabile. I campioni che però sembravano dare buoni risultati sono stati selezionati per le prove di adesione proteica (Figura 9). Questi campioni sono: P(PySMAAm-HEMA) 80-20, 50-50, 20-80 e il P(mPySMAAm-HEMA) 50-50.

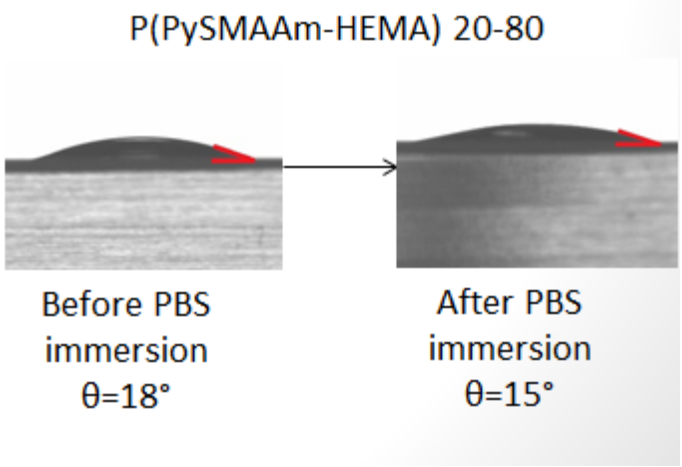


Figura 9: Differenza di angolo di contatto per un campione di P(PYSMAAm-HEMA)20-80.

Per quanto riguarda il trattamento superficiale ad ozono, si è valutato che questo non comporta nessun miglioramento nella stabilità dei coating.

L'analisi dei risultati di questa analisi per i copolimeri di PySMAAm e BMA è stata più facile rispetto al copolimero precedente per l'idrofobicità del BMA: se dopo l'immersione in PBS l'angolo di contatto diminuisce, è segno di idratazione del coating e quindi di stabilità dello stesso. Tutte le composizioni dei coating (80-20, 50-50, 20-80, 15-85) si sono dimostrate stabili (Figura 10).

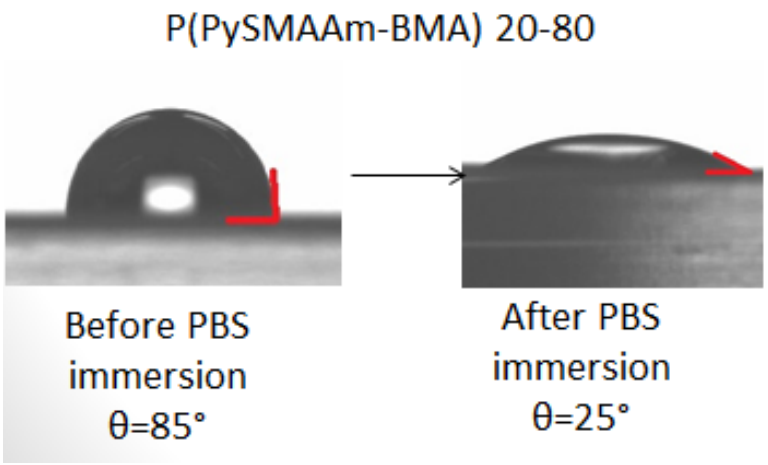


Figura 10: Differenza di angolo di contatto per un campione di P(PySMAAm-BMA)20-80.

Come prima, l'effetto del trattamento superficiale è stato valutato ma non ha dato frutti. Tutti i campioni analizzati sono quindi buoni candidati per la prova di adesione proteica.

Valutazione dell'adesione proteica

Per ottenere una superficie antiadesiva, l'adsorbimento di proteine deve essere ridotto il più possibile. L'analisi *Micro BCA* forniva i valori di assorbanza dei campioni rivestiti e delle soluzioni standard, le quali hanno concentrazione di BSA in PBS crescente: 0 (Blank)-0.5-1-2.5-5-10-20-40-200 µg/mL. I risultati delle analisi degli standard sono stati presi e dopo essere stati corretti con l'assorbimento a 562 nm dello standard Blank, sono stati plottati e ne è stata ricavata una retta di tendenza, grazie alla quale i valori di assorbimento per i vari campioni sono stati calcolati tenendo conto di tutti i valori statistici. È stata calcolata media e deviazione standard e i risultati sono stati convertiti in µg/cm^2, l'unità di misura comunemente usata per queste analisi.
P(PySMAAm-HEMA) e P(PySMAAm-BMA) sono stati confrontati coi rispettivi omopolimeri P(HEMA) e P(BMA).

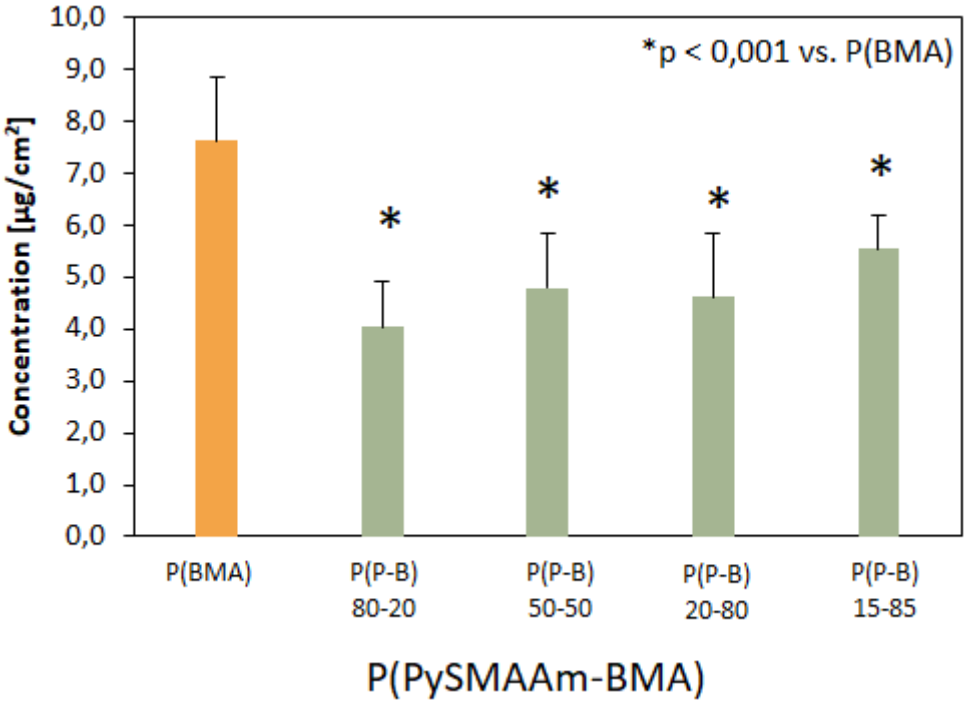


Figura 11: Risultati della prova di adesione proteica di P(PySMAAm-BMA).

Il P(BMA) non è una superficie antiadesiva, di conseguenza la sua resistenza alle proteine non è considerevole, tuttavia è impiegato in ambito biomedico. In tutti i campioni è stata osservata una notevole diminuzione nell'adsorbimento proteico, con un effetto maggiore per contenuti di PySMAAm più elevati (Figura 11).

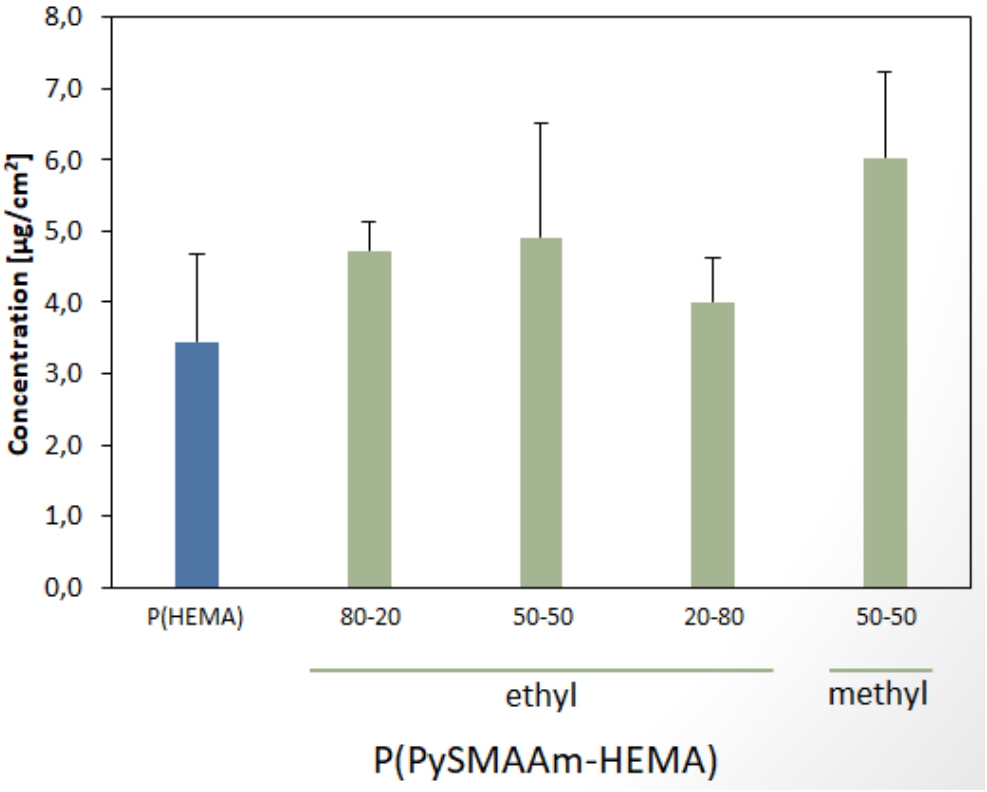


Figura 12: Risultati della prova di adesione proteica di P(PySMAAm-HEMA).

P(HEMA), invece, è un comune polimero antiadesivo e i suoi valori di adsorbimento sono generalmente bassi. Dall'istogramma di Figura 12 si può osservare che i campioni sintetizzati a partire da ePySMAAm hanno valori confrontabili con P(HEMA) e mostrano quindi buona resistenza alle proteine. Il campione P(mPySMAAm-HEMA) 50-50, al contrario, presenta valori più elevati di adsorbimento e quindi un risultato peggiore, come in effetti erano peggiori anche i suoi effetti di citotossicità.

I risultati dell'analisi di questi copolimeri sono confrontabili con quelli di un altro famoso copolimero usato nel settore, P(MPC-co-BMA). I copolimeri studiati sono promettenti le loro proprietà antiadesive dovrebbero essere ulteriormente studiate ed approfondite.

CONCLUSIONI

L'obbiettivo di questo progetto di ricerca era la sintesi e caratterizzazione di un nuovo copolimero a base sulfobetanica per superfici antiadesive.

La sintesi del processo è stata migliorata, il peso molecolare dei copolimeri incrementato quasi di un fattore 10 e anche la resa è migliorata.

Il successo della copolimerizzazione è stato confermato da analisi ^{1}H-NMR, che hanno permesso inoltre di valutare la composizione finale dei copolimeri, evidenziando che i copolimeri P(PySMAAm-HEMA) tendono ad aumentare la quantità di HEMA nel prodotto finale e che i P(PySMAAm-BMA) sono invece più stabili.

La stabilità in ambiente acquoso è stata valutata mediante misure dell'angolo di contatto, che hanno dato risultati soddisfacenti per la maggior parte dei campioni, e in particolare per i copolimeri del monomero BMA.

Tutti i copolimeri prodotti hanno esibito ridotta adesione proteica sulla superficie, di conseguenza si presentano come buoni candidati da essere utilizzati per superfici antiadesive.

Stabilità e assorbimento proteico dei copolimeri sono tuttavia da approfondire ulteriormente, in particolare sarà necessario identificare la composizione dei copolimeri che ottimizza i risultati.

Un aspetto del processo di sintesi che è ancora da migliorare è la riduzione dell'indice di polidispersità.

Sviluppi futuri del progetto possono coinvolgere l'analisi della resistenza all'adesione cellulare.

1. INTRODUCTION

Something that has always inspired humanity to do research is the desire to improve the quality of life. This aim can be reached in different ways: it could be a new machine, a new technology, a new medical treatment,…

Out of all these possibilities there is one field that will never stop to be researched: the human body, its development and its health. In order to do that there are different approaches. The first and most obvious is medicine, then biology, but one that is gaining increasing importance with time is the field of biomedical engineering and biomaterials. As a science, biomaterials are relatively recent, only about fifty years old. There are three main classes of biocompatible materials, materials that are compatible with the human body: bioinert, for which there is no reaction with the body; bioactive, that create a connection between implant and tissue; biomimetic, materials that can exchange signals with the organism.

The situations in which biomaterials are used are not the same as a few years ago. Implantable medical devices are still important, but now drug and gene delivery systems, tissue engineering, cell therapies, organ printing, nanotechnology based imaging, diagnostic systems and microelectronic devices are considerable application fields too. All the big families of materials (ceramics, metals and polymers) are researched in the biomaterials field.

One thing that is fundamental for many of these applications is the contact between the implant of biomaterial and the human body; it's something that can't be underestimated and that influences all the following steps in the functionality of the biomaterial. A material interacts with the human body following several steps, the first of which is protein adsorption. After that, everything else follows. That's why a lot of effort is put in the development of biomaterials that can control the protein adsorption: enhance it or stop it. These materials can be applied on the surface of the implant or can be the implants themselves. Antibiofouling surfaces, therefore, are an emerging trend.

The purpose of the present work is the development of a novel sulfobetaine copolymer for antibiofouling surfaces. The synthesis of this copolymer will be reviewed, focusing on the parameters to optimized the process and the choice of monomer to be copolymerized with. Following this, the copolymers will be characterized by several analysis to assess the success of the polymerization, their stability and antifouling properties.

2. BIOFOULING AND ANTIBIOFOULING

Bacteria, like all living organisms, tend to evolve in order to survive. Some of these organisms, dangerous for the human body, developed for this purpose resistance to what was more harmful for them: antibiotics. Antimicrobial resistance is a major health threat.

Infections from resistant bacteria are now too common, and some pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA) or Vancomycin-resistant *Enterococcus* (VRE) have even become resistant to multiple types or classes of antibiotics (antimicrobials used to treat bacterial infections). The loss of effective antibiotics will undermine our ability to fight infectious diseases and manage infectious complications. Research has shown that patients with resistant infections are often much more likely to die, and survivors have significantly longer hospital stays, delayed recuperation, and long-term disability.
Infection rates related to implants are as follows:

- 0.8-1.2% for orthopedic surgery (hip replacement)
- 3.6-8.1% for closed fractures
- 17.5-21.2% for open fractures in traumatological surgery

Even only 1% would be a high percentage, meaning that it is necessary to act somehow to prevent the infection.

Each year in the United States, at least 2 million people acquire serious infections with bacteria that are resistant to one or more of the antibiotics designed to treat those infections. At least 23000 people die each year as a direct result of these antibiotic-resistant infections. Many more die from other conditions that were complicated by an antibiotic-resistant infection.

The use of antibiotics is the single most important factor leading to antibiotic resistance around the world. Antibiotics are among the most commonly prescribed drugs used in human medicine. However, up to 50% of all the antibiotics prescribed for people are not needed or are not optimally effective as prescribed. There are four core actions that will help fight these deadly infections:

- preventing infections and preventing the spread of resistance
- tracking resistant bacteria
- improving the use of today's antibiotics
- promoting the development of new antibiotics and developing new diagnostic tests for resistant bacteria

The first action says that to avoid complications the infection itself should be prevented to start and to spread [1]. How can this be obtained? Antibiotics are often administered after surgery or when an implant or any kind of subcutaneous device is placed in the human body (artificial bones, diabetes sensors, artificial organs,…) hence, it is a foreign object that is added to a delicate environment. When a foreign body is inserted in the human body, we're talking about biomaterials.

When a biomaterial interacts with the biological environment it is interesting to understand the sequence of steps that leads to a response of the system. A generic definition of biocompatibility used to be: the ability of a material to perform with an appropriate host response in a specific application. Even if now it's more difficult to provide a precise definition, because the concept of biocompatibility is different in different environments, this one is still acceptable. The desired conclusion is the acceptance of the foreign object in the system.

There are different types of biomaterials with different functions: in the field of tissue engineering and regenerative medicine, bioactive materials have been required to encourage cell adhesion, spreading and

proliferation and to supplant cell-intrusive synthetic extracellular matrices as cell supporting scaffold. In an opposite field, bioinert materials suppress nonspecific adhesion that induces thrombosis and immunological responses. They are required for most biomedical devices and implants such as blood- or tissue-contact devices. In many of these applications the purpose is to study cells adhesion and proliferation, however cells do not interact directly with the surface but via the proteins that they secrete, which are adsorbed on the surface adhesive [2]. Protein adsorption is the first step in many biological processes. Therefore, to prevent infections, protein adsorption should be avoided. Biological fouling is based on organic fouling and it consists in three steps: reversible adsorption, irreversible adsorption and proliferation. The last one refers to biofilms, that are hard to be removed. The easiest approach is to stop the process before irreversible adsorption begins. Protein adsorption is "a common but very complicated phenomenon", as professor Nakanishi once said [3].

Proteins are complex biopolymers composed of 20 naturally occurring amino acids as monomeric units plus possible additional side chains like phosphates, oligosaccharides or lipids introduced after translation. A classification of proteins with respect to their interfacial behavior can be achieved by considering properties like size, structural stability and composition. Proteins usually are amphiphilic, zwitterionic and often dissolvable in water molecules. They have hydrophobic backbone, polar or non-polar residues and at least one active region. In aqueous solution they keep conformation with water molecules inside and around via hydrogen bonds and electrostatic interactions. When in contact with the surface they deteriorate: destruction of conformation and loss of integrated water molecules, which translates into an increase of Gibbs free energy (Figure 2.1). Hence the protein tries to establish new interactions (Van der Waals, hydrogen bond, electrostatic,…) with the surface [4].

Protein fouling on biological implants reduces the efficacy of the devices and may also result in harmful side effects such as thrombosis. Furthermore, protein adsorption can trigger adhesion of particles, bacteria or cells possibly promoting inflammation cascades, or fouling processes. Moreover, protein adsorption provides a conditioning layer for microbial colonization and subsequent biofilm formation. Biofilm formation on medical implants can lead to infection. Tissue adhesion, another result of protein adsorption, is an undesirable result after surgery and is considered a big challenge in clinical fields. Tissue adhesion could lead to severe pain, functional obstruction of organs and difficult reoperative surgery [5]. Fouling is a crucial danger mainly in the biomedical field, however it is also important in the case of surfaces subjected to aquatic environments where marine microorganisms can bind to a surface and form a conditioning layer. Issues associated with biofouling are increased operational and maintenance cost and increased degradation. Fouling is a fundamental aspect to be considered in filtration membranes too: a cake layer can form on the surface of the membrane and shrink and block the membrane pores, reducing permeation and separation. Further applications, that are still at the first steps, are the application of antibiofouling properties for electric power plants or vascular tubes electrospinning.

Some of the factors controlling protein adsorption are the influence of external parameters, specifically pH, temperature, ionic strength and buffer composition. The major driving force of protein adsorption is an entropy gain arising from the release of surface adsorbed water molecules and salt ions and from structural rearrangement inside the protein and the minimization of Gibbs free energy. A temperature increase results in an increase of protein adsorption. The isoelectric point minimizes repulsion, which translate to a higher packing; proteins have a higher adsorption rate when there is charge opposition between substrate and protein. Another effect to be considered is that of ionic strength: the resulting effect is screening, therefore a higher density could be obtained, leading to aggregation phenomena. The size of the proteins is relevant too: small proteins diffuse faster and are dominating in early adsorption stages, while larger proteins, even though slower, bind stronger to the surface. Surface properties themselves are important:

surface energy, polarity, charge and morphology are only some of them. Proteins tends to adhere more strongly to non-polar surfaces rather than polar ones: non-polar substrates destabilize proteins, therefore facilitate conformational reorientation, which leads to strong inter protein and protein- surface interactions. Proteins prefer higher surface tension than low surface tension, and charged substrates are more favorable to protein adsorption compared to uncharged substrates. Uniformity of charge distribution and charge neutrality affect the antifouling properties. Finally, affinity of proteins increases on hydrophilic substrates and decreases on hydrophobic substrates [3]. It has to be said, though, that different surface properties may induce similar responses *in vivo* because of non-specific protein adsorption. The adsorbed protein layer will influence the subsequent biological reaction including platelet adhesion and activation, protein adsorption, in fact, provides a conditioning layer for microbial colonization and biofilm formation.

In conclusion, it is important to obtain surfaces that fight the adsorption of proteins to avoid their proliferation and all the subsequent steps leading to harmful consequences.

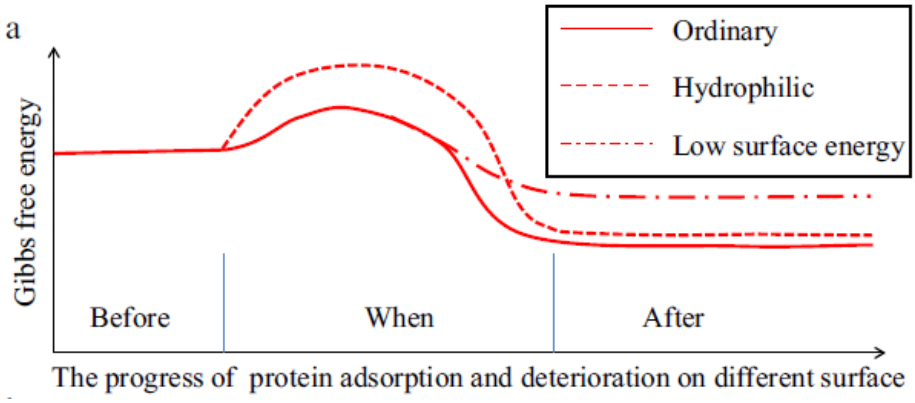


Figure 2.1: The Gibbs free energy changes before, when and after the adsorption and deterioration of proteins on ordinary, hydrophilic and low surface energy membrane [4]

2.1 DESIGN OF ANTIBIOFOULING SURFACES

How can these antibiofouling surfaces be obtained? Historically, biomaterials are designed to control the contact with the biological environment. The class of materials which is used in this field is polymers. For some applications, like in the field of tissue engineering and regenerative medicine, the bioactive polymers have been used. Other times, instead, bioinert polymers are preferred, as mentioned before.

The design of these antibiofouling surfaces can be of different types using different mechanisms: steric repulsion, electrostatic repulsion, microdomain structure or hydration of the surface layer (Figure 2.2). All these mechanisms are strictly connected with surface free energy: the adsorption of proteins is completely governed by the substratum's one.

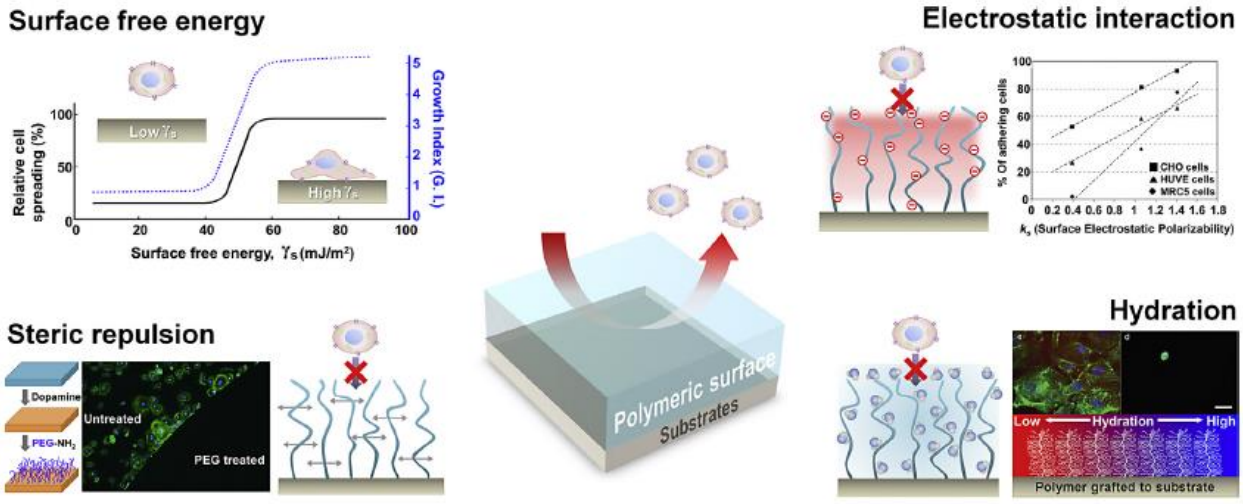


Figure 2.2: overview of the antibiofouling surfaces mechanisms.

Polymer brushes and coatings generally use the mechanism of steric repulsion to keep the surface clean. As the protein approaches the surface the polymer is compressed, leading to a decrease in entropy and an increase of Gibbs free energy: diffusion of the protein into the polymeric layer would be energetically unfavorable and the polymer tends to recover the swelling state and stop the foulants.

The second mechanism used is electrostatic interaction: cell-surface adhesion is deterred by electrostatic repulsion in mediators of low-ionic strength such as physiological media and grafted polymer brushes on substrates. Electrostatic repulsion between charged surfaces and cells is screened by a counterion release model, which correlates protein adsorption on weak polyelectrolyte brushes. On the substrate, cell attachment to surfaces tends to decrease rather than increase as the surface charge density is raised. The foulants would need a lot of energy to break through.

Microdomain structure is based on alternate hydrophobic and hydrophilic domains with the size of micrometers. Different proteins are allowed to adsorb on the surface but the alternation of different types inhibits their proliferation (Figure 2.3). Nevertheless, this mechanism is not safe because the proteins could denature the surface and change its properties.

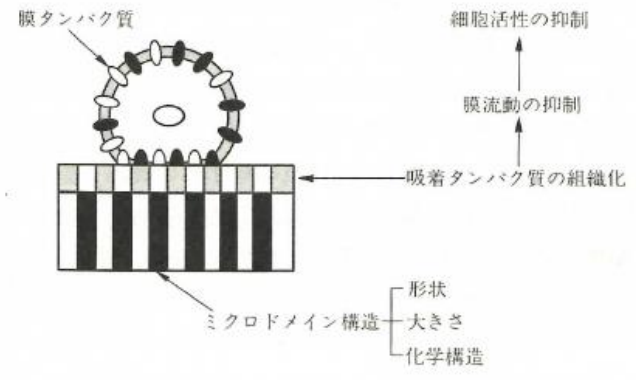


Figure 2.3: Microdomain structure for antifouling surfaces

Finally, a mechanism that is gaining more and more interest in the field is surface hydration to prevent the proteins to approach and attach to the surface. Low fouling or nonfouling polyhydrophilic materials have in common hydrophilic nature, electrical neutrality and that they all are hydrogen bond

acceptors/donors. Inclusion of water molecules from both surface and protein is the obligatory first step to impede protein adsorption by reducing the free energy barrier (Figure 2.4). Strength of the surface hydration is determined by physico-chemical properties of polymers (molecular weight, density,…) and by their surface packing (coating thickness, packing density and chain conformation). The hydration shell acts as a barrier that would require a lot of energy from the foulants to be broken. Hydration can be obtained by hydrogen bond formed between the polymer chain and the water molecules (like the following example of PEG chains), but this connection isn't strong enough and its efficacy is not assured. A much more stable bond is formed by electrostatic interaction between the polymer chain and the water molecules, the perfect example of which are zwitterionic polymers: a denser and tighter water molecule layer is adsorbed on the surface to prevent the fouling of the surface.

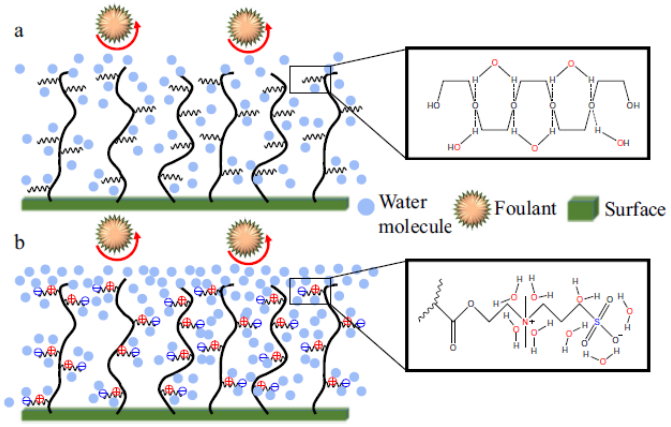


Figure 2.4: Schematic illustration for the formation of hydration shell. (a) Each unit of the representative PEG materials is integrated with one water molecule. (b) Each unit of the zwitterionic materials is integrated with eight water molecules. [4]

It is widely believed that strong surface hydration is a key contribution to their nonfouling properties: a highly hydrated polymer surface can bind water molecules so strongly that other molecules and organisms can't replace these interfacial water molecules for fouling to occur [4].

Immobilizing polyethylene glycole (PEG), a well-known biocompatible polymer, on the surface by grafting is a common approach to impart protein resistance to a surface because of its large excluded volume and mobility that stops the proteins from reaching the surface (Figure 2.5). The mechanism also implies an hydration shell formed by hydrogen bond between PEG chains and water molecules in addition to steric repulsion effect due to the flexible chain. PEG, however, is not perfect: its efficacy is influenced by water solubility and flow properties, physical adsorption can't reduce the fouling under a certain point because of steric issues that limit the density of the polymer chains, and PEG has a tendency to auto-oxidize and form aldehydes and ethers in the presence of oxygen and transition metal ions, leading to the loss of protein resistance ability. Protein adsorption cannot be reduced below a certain limit because of steric issues that limit the density of attached polymer chains.

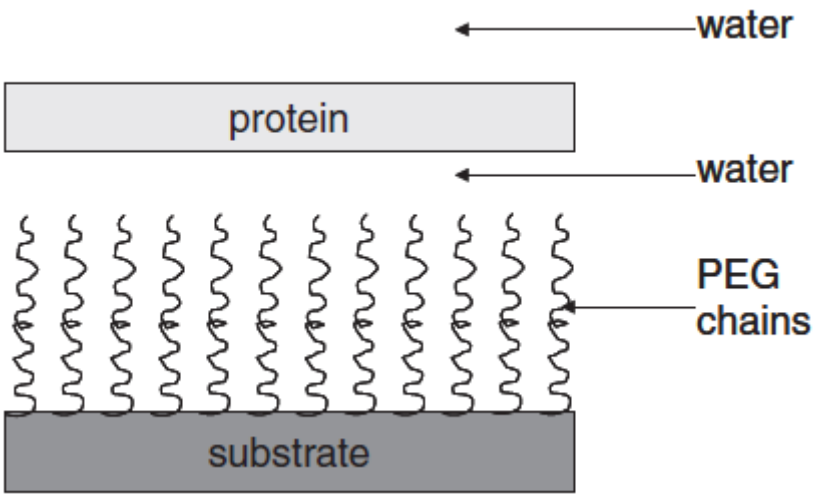


Figure 2.5: Mechanism of antifouling by steric repulsion of PEG chains

Zwitterionic polymers can be used as an alternative to PEG thanks to their balanced charge and minimized dipole, that allow them to bind water molecules on the surface and prevent protein adsorption by hydration as it will be explained later [2].

Polyzwitterions are the most common choice when it comes to hydration of surfaces with antibiofouling purposes; strong hydration, moderated self-association and few protein interactions make them good and common antifouling materials.

3. ZWITTERIONIC POLYMERS

Polymers containing ionic groups are among the most important classes of macromolecules. There are mainly two types of these polymers, those that bear only cationic or anionic group, the polyelectrolytes, and those that bear both of them: the polyampholytes.

A characteristic of polyelectrolytes is chain extension, and thus large hydrodynamic volume, in deionized water at low concentrations. This is due to coulombian repulsions between charged groups along the polymer chain, forcing the polymer into an extended rodlike conformation. The addition of low molecular weight electrolyte or changes in solution pH screens the repulsive electrostatic forces, and the polymer coil shrinks, adopting a more entropically favored conformation. This is known as the *polyelectrolyte effect*.

Polyampholytes carry simultaneously cationic and anionic groups, however these can be scattered randomly along the polymer chain and one charged species may outnumber the other, therefore polyampholytes often bear an overall net charge, positive or negative. In fact, they act mainly as polycationic or polyanionic species. Moreover, they are extremely sensitive to pH changes and ionic strength of the system, considering both the absolute amount and the change of the sign. Polyampholytes are interesting for numerous reasons, not the least the fact that they are synthetic analogues of naturally occurring biological molecules such as proteins and find applications in areas such as lithographic film, emulsion formulations, and drag reduction [6].

Polyzwitterions are a subclass of polyampholytes in which the charged groups are balanced and the polymers therefore show charge neutrality (Figure 3.1). The charges may be located either on pendant side chains of different monomer units or the same monomer unit, or one or both of the charges may be located on the polymer backbone. They are typically hydrophilic, thanks to strong Coulomb interactions.

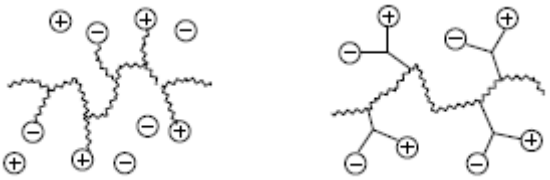


Figure 3.1: Simplistic model of polyampholytes (left) and polyzwitterions (right). [7]

Polyzwitterions have a wide variety of applications that include ion exchange, chelation to bind trace metals (Hg, Cd, Cu, and Ni) from drinking water, sewage treatment, soil conditioning, paper reinforcement, pigment retention, and formulation in shampoos and hair conditioners. In the ‘80s they were recognized as analogs of important biological structures (i.e.: phospholipids), a feature that makes them interesting for mimicking cell membranes and for preparing tailored surfaces, as they can confer excellent lubrication and excellent resistance to (bio)fouling. Many zwitterionic polymers show a lower critical solution temperature (LCST) in aqueous media. Hence, stimuli-responsive polymer systems were designed from polyzwitterions, being reversibly sensitive to changes in temperature, ionic strength, specific ion airing, and chemical reactions, or, more rarely, to changes in pH.

A further specification that should be made is to distinguish polyzwitterions from other polymer classes like mesoionic polymers and polymeric ylides (Figure 3.2). The first ones formally contain cationic and anionic sites, yet they do not carry separate charges but only show a high dipole moment and are not good hydrophilic groups. Ylides are 1,2-dipolar compounds with a semi-polar bond, which may be formally represented with separate positive and negative charges on neighbouring atoms, yet these polymers usually exhibit a high double bond character and low hydrophilicity [7].

The typical behavior of zwitterionic polymers in response to a change in the ionic strength is opposite to that of polyelectrolytes: chain expansion occurs upon the addition of low molecular weight electrolyte (=increasing the ionic strength), leading to an increase in the reduced viscosity and a lowered cloud point. This is due to the screening effect of salts towards the charged groups on the polymer and it's called *antipolyelectrolyte effect*.

Finally, an important feature and the main limitation in the use of polyzwitterions is their difficult solubility in many solvents, which makes their synthesis challenging in the most common solvents and with the most common techniques.

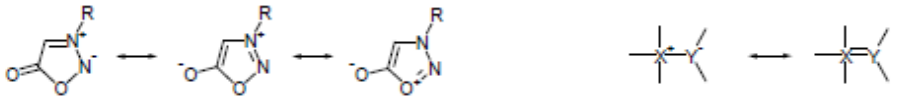


Figure 3.2: Models for mesoionic (left**, here: a sydnone) and ylide structures (**right**) [7].**

3.1 POLYBETAINES

Polybetaines are a specific group of zwitterionic polymers. These, compared to other zwitterionic molecules, possess the anionic and cationic groups on the same monomer unit (Figure 3.3). In these species the positively charged cationic group is usually a quaternary ammonium or a phosphonium. The anionic functional group classify the polymers in different categories:

- Sulfonate group → sulfobetaines
- Carboxylate group → carboxybetaines
- Phosphonate group → phosphobetaines

Betaines application space from fungicides to fire resistant polymers, from lubricating oil additives to emulsifying agents, from wetting agents in the cleaning industry to cryoprotectants, and in the biomedical field they are well known antibiofouling polymers.

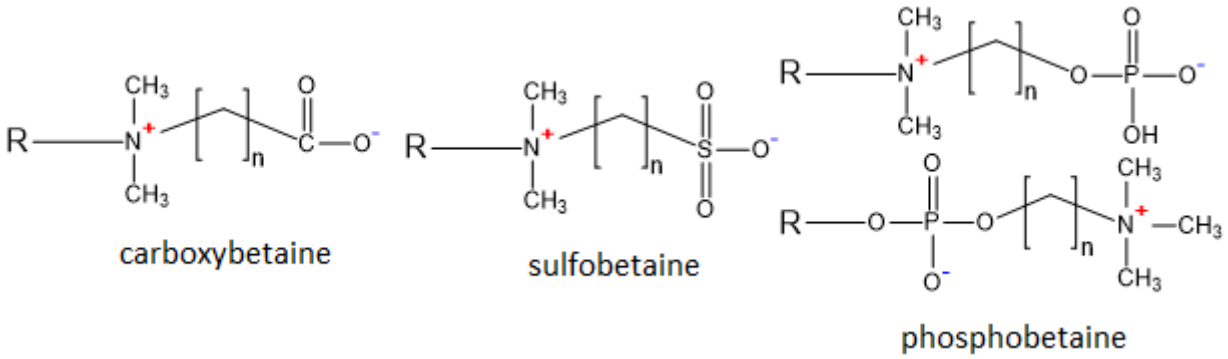


Figure 3.3: General structures of the three subclasses of betaine polymers

An interesting property of polymeric betaines is their bio-haemocompatibility due to their highly hygroscopic nature. Research has focused on phosphobetaine polymers in particular: they are thrombogenic and can improve the biocompatibility of ocular devices. Phosphobetaines present two different structures, however only the lower one in Figure 3.3, with the anionic functional group next to the radical, can create a successful antibiofouling surface. There are several widely used polybetaines used for antibiofouling applications, such as 2-Methacryloyloxyethyl phosphorylcholine polymer (PMPC) or various structures of poly (sulfobetaine methacrylate)s (PSBMA), however their application is always limited by their solubility and synthesis issues. Compared to MPC, SBMA and carboxybetaine methacrylate (CBMA) are more stable and easier to prepare (Figure 3.4) [4].

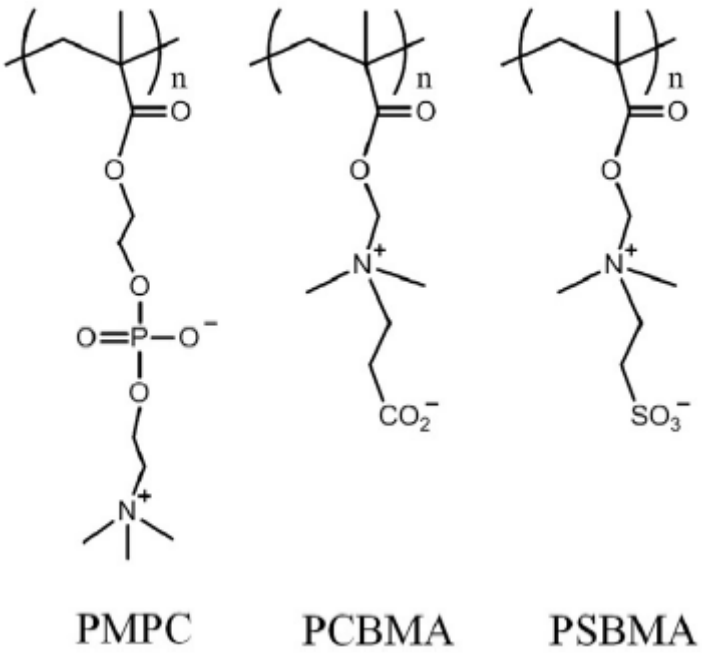


Figure 3.4: Representative structure of zwitterionic polymers: PMPC, PCBMA and PSBMA [4]

MPC was synthetized in the late 1980s, from that moment it has been widely used and has received a lot of attention in the biomedical field, as a homopolymer or copolymerized with long-chain alkyl methacrylates, to obtain copolymers that can mimic the structure of natural membrane lipids (a common comonomer for MPC is butyl methacrylate). As mentioned before, MPC is not the perfect antifouling polymer. This is because the phosphoester group is prone to hydrolysis and moreover MPC is moisture sensitive thus is not easily synthetized and handled. To overcome these problems, the other subclasses of polybetaines have been recently studied profusely since it is clearly desirable to develop novel materials with long-term antibiofouling properties for biomedical applications. Sulfobetaines have been utilized with various polymer substrates (polyurethane, silicone, and cellulose among the others). Protein adsorption and/or platelet adhesion on these surfaces is inhibited to some extent and both improved biocompatibility and anti-thrombogenicity are achieved. The results of PSBMA-based materials, however, are not as good as those of MPC polymers, but this could be attributed to the copolymerization of SBMA with other monomer units, that can influence the resulting packing and density of functional groups. According to this hypothesis the antibiofouling and biocompatibility capability of sulfobetaines may have been underestimated.

For what concerns PCBMA, these polymers show not only highly effective protein adsorption resistance, but also has abundant functional groups convenient for the immobilization of biological ligands. Thus, PCBMA with suitable decoration could both bind specific biomolecules desirably as well as resist the binding of the non-specific proteins. The unique dual-functional properties of carboxybetaine moieties have not been observed in other anti-biofouling moieties such as phosphobetaine and sulfobetaine [8].

3.2 SYNTHESIS AND SOLUTION PROPERTIES OF POLYBETAINES

In general, the synthesis of polyzwitterions has to face difficulties from sensibility or incompatibility of reactions towards the presence of both electrophiles and nucleophiles. The same problem is encountered when considering the solvents suited for zwitterionic compounds (alcohols, ionic liquids, other polar protic solvents,…). Zwitterionic monomers are typically polymerized in aqueous solution, which poses limitations for the incorporation of hydrophobic segments including co-monomers. A possible solution that was considered is post-polymerization modifications of precursors, however these are seldom complete and

could change the stechiometric balance, in the worst case resulting in the loss of overall charge neutrality [9].

Polybetaines lack of solubility in pure water, due to the formation of intra- and interchain ionic contacts resulting in an ionically cross-linked network structure. The polybetaines that are not soluble in pure water become soluble with the addition of low Mw electrolytes (i.e.: NaCl): the electrolyte penetrates the ionic network, screening the attractive interaction. Solubility of polybetaines in organic solvents is also limited. Sulfobetaines, in particular, have a smaller range of solubility compared to carboxybetaines and phosphobetaines. The solubility is limited to solvents with a high hydrogen-bond-donating ability, like alcohols. Hydrolysis is also a concern and especially the stability of the linkage between zwitterionic moieties and polymer chains, especially for molecules that carry the charged functional groups on the side chain. Zwitterionic polyvinylpyridinium derivatives are less stable in aqueous media than they might be, supposed due to the lack of any potentially fragile ester bond, etc., as strong nucleophiles tend to add to the activated aromatic ring in the 2- or 4- positions.

Zwitterionic compounds are often assembled via a complex multi-step synthesis. For better solubility and miscibility and to avoid side reactions, the anionic group is often introduced in a protected form, like ring opening alkylation with heterocycles (propane-/butanesultone for ammoniosulfonates).

The most common mechanism to obtain polyzwitterions is chain growth polymerization. Free radical polymerization, in particular, is extremely popular because of the high tolerance of radicals towards electrophiles and nucleophiles. Free radical polymerization is suited for statistical copolymerization, nevertheless it is handicapped by notorious poor solubility of zwitterions in most solvents, and additionally by their demanding molecular characterization and by the limited availability of convenient building blocks.

Other than the common free radical polymerization, a new technique that in the last 20 years have been developed and found more and more applications is reversible-deactivation radical polymerization (RDRP). RDRP techniques offer direct access to polymers bearing zwitterionic groups with previously unthinkable features, such as predefined molar masses and narrow molar mass distributions as well as well-defined functional end groups. Moreover, complex architectures, such as block copolymers, graft copolymers, hyperbranched or star polymers can be prepared conveniently and in good yields by RDRP. Thus, the past decade of polyzwitterion synthesis is marked by the extensive use of RDRP methods to create polyzwitterions with unusual or novel polymer architectures, let it be by nitroxide mediated polymerization (NMP), atom transfer radical polymerization (ATRP), reversible addition fragmentation chain transfer polymerization (RAFT), or other variants.

The other competitive mechanism in polymerization is the step growth however, in the case of zwitterionic polymers, it is rarely used, because the molecules are generally hygroscopic, and the preferential solubility in nucleophilic and protic solvents and the high melting points due to ionic nature are obstacles to the process.

In order to improve the synthesis and solubility properties of zwitterionic polymers, a diffuse strategy is to copolymerize them with monomers that can improve their performances.

4. PySMAA MONOMER, HEMA and BMA

The present research is based on a specific sulfobetaine monomer, a sulfobetaine methacrylamide monomer with a pyridinium cation (PySMAAm). The monomer was chosen for a previous project concerning stimuli-responsive polymers: these are polymers that respond to heat, salt, light, pH, electric or magnetic field,… changing their properties profile when the stimulus is applied; these polymers are applied to tissue engineering, drug delivery and several other fields. Among these, thermos-responsive polymers are well investigated: generally speaking, there are more polymers with Low Critical Solution Temperature (LCST) than with Upper Critical Solution Temperature (UCST). UCST and LCST are single distinct point in a phase diagram (Figure 4.1,4.2). LCST type polymers are soluble in cold water and become insoluble by heating above the faze transition temperature. There are many researches regarding these polymers because of their LCST near body temperature. On the other hand, UCST type polymers become insoluble by cooling to the faze transition temperature. In recent years, UCST polymers are a hot topic because they can show their fase transition temperature under physiological condition.

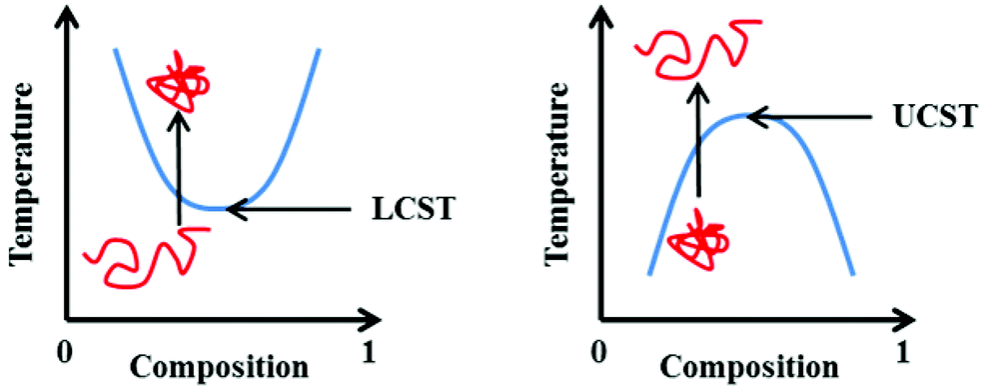


Figure 4.1: Representation of LCST (left) and UCST (right) in a temperature/composition diagram

The UCST behavior of polysulfobetaines is limited compared to that of LCST-type polysulfobetaines for two reasons:

- UCST transition shows a strong positive dependence on the molecular weight (Mw). Polymers with low Mw have lower critical temperatures or may be fully soluble.
- Antipolyelectrolyte effect of zwitterionic polymers: salts screen inter- and intraionic interactions, with a decrease of UCST and even temperature independent aqueous solubility.

Hence, the development of polymers with significant higher UCST is important.

Sulfobetaines are promising candidates for aqueous UCST behavior because their zwitterionic side groups can cause strong inter- and intrapolymer attraction through electrostatic interlocking at low temperature resulting in solubility [9].

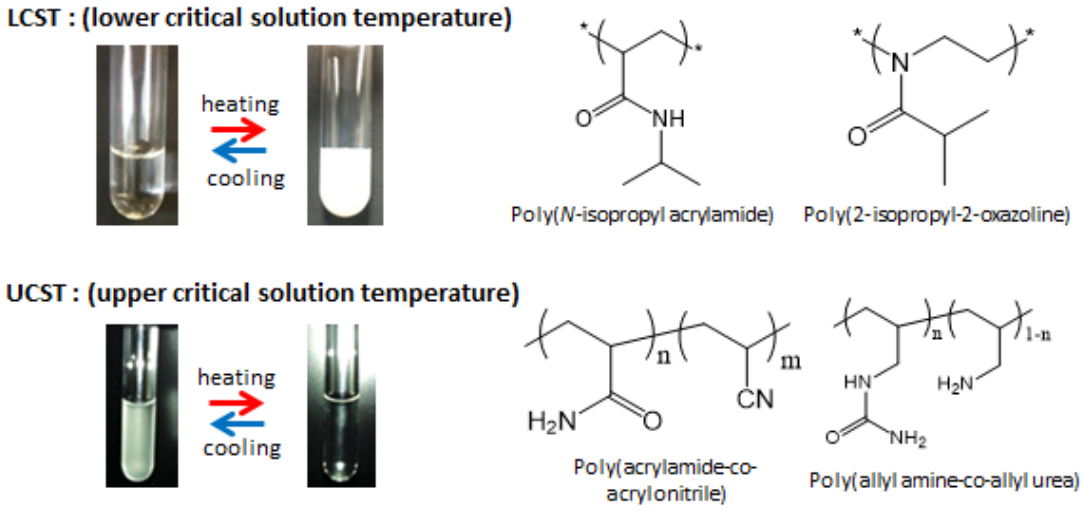


Figure 4.2: effect of LCST and UCST on solutions

Before the PySMAA monomer was taken into consideration, a different sulfobetaine monomer, 3-dimethyl(methacryloyloxyethyl) ammonium propane sulfonate (DMAPS) was studied in the laboratory. DMAPS polymer (PDMAPS), whose structure is shown in Figure 4.3 among those of other sulfobetaine monomers, is a type of sulfobetaine that shows biocompatibility, thermo-responsiveness and interaction with polyelectrolytes. However, PDMAPS is easy to aggregate in aqueous solutions. In a previous study, as it's often done to improve the stability of polysulfobetaines, a random copolymer of PDMAPS and poly(ethylene glycol) mono methacrylate (PEGMA) was designed and tested. The resulting P(DMAPS-ran-PEGMA), however, despite being successfully internalized in cell by membrane translocation and being successfully delivered to mitochondria, the copolymer didn't show any sign of thermo-responsiveness under physiological conditions.

Following the unsatisfying results of this research, a new sulfobetaine monomer was investigated and the PySMAA monomer was selected for the design of new sulfobetaine polymers with multiple stimuli-responsiveness for intracellular delivery. Its properties are strictly connected to the chemical structure:

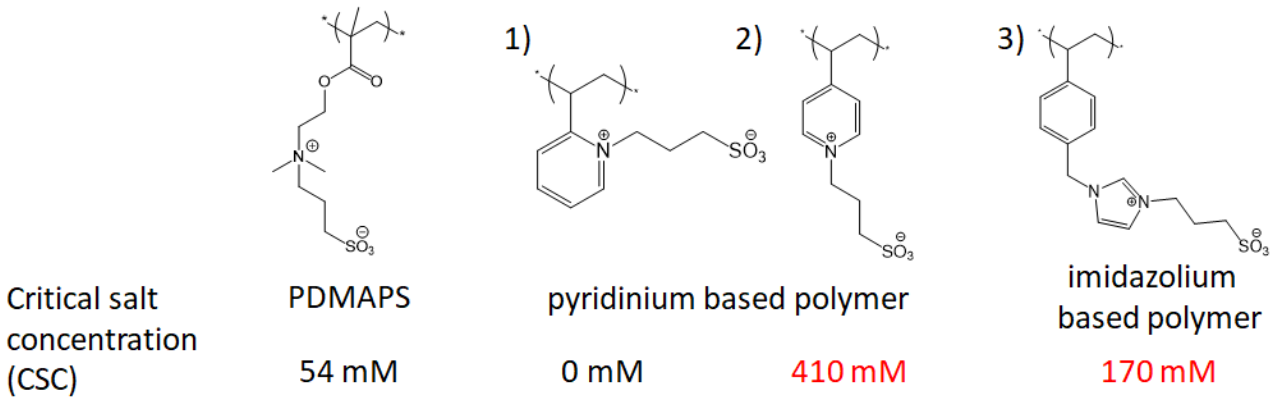


Figure 4.3: structures and CSC of sulfobetaine polymers

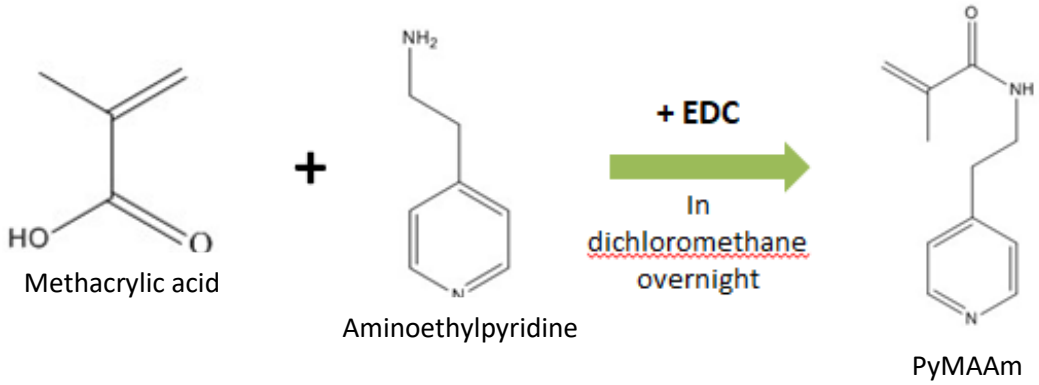
It can be seen that the pyridinium based polymers have very different critical salt concentration (CSC) depending on the position of the chain on the pyridine. The orto- position gives 0 mM of critical

concentration, whereas the para- conformation shows an impressive 410 mM of CSC, way higher than the previously evaluated PDMAPS and the other nitrogen-based molecules.

4.1 PySMAAm SYNTHESIS

Two different monomers were synthesized, starting from aminomethylpyridine or aminoethylpyridine, to obtain the final molecule with a different methylene chain length, a difference that is expressed in the name of the molecule by the initial of the chain length: ePySMAAm for aminoethylpyridine, mPySMAAm for aminomethylpyridine.

PySMAAm is obtained by a two steps reaction. Initially the pyridine precursor (aminoethylpyridine, Mw=122.17 g/mol) was mixed with methacrylic acid (Mw=86.06 g/mol) in dichloromethane in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Mw=155.25 g/mol), used to remove the condensed water molecules. The reaction was performed overnight, obtaining the intermediate called PyMAAm.



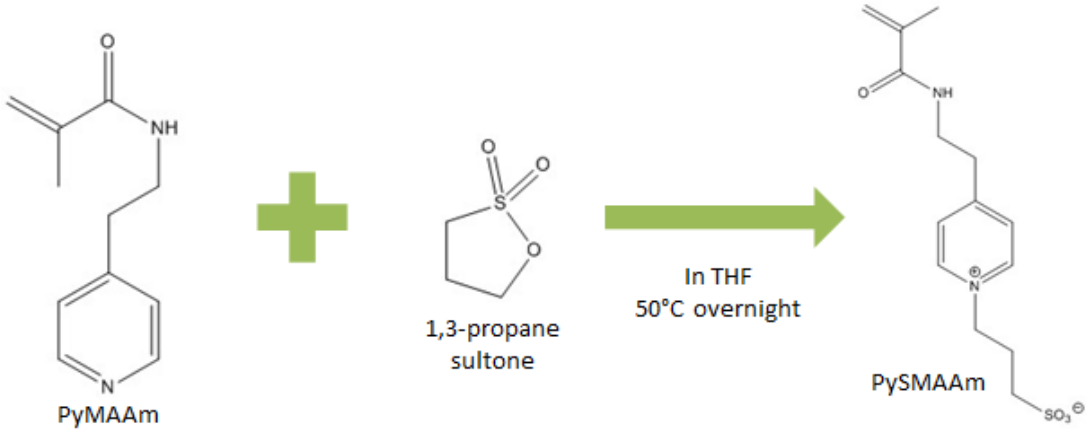
The reaction was performed according to the following stechiometry (Table 4.1):

Table 4.1: stechiometric parameters of the PyMAAm synthesis.

	Aminoethylpyridine	Methacrylic acid	EDC
Equivalents	1	0.8	1
Moles [mmol]	52.16	41.73	52.16
Mass [g]	6.372	3.59	10
Volume [mL]	6.21	3.55	-

After the first step, an ^{1}H-NMR sample of the intermediate was taken and analyzed to evaluate the quantity of impurities and whether the intermediate is suitable for the final product or not.

The second step of the reaction consists in the addition of 1,3-propane sultone (Mw=176.1 g/mol) to PyMAAm in a ratio of 1.5/1 moles. The reaction is made at 50°C in tetrahydrofuran (THF) overnight.



Again, the final product is analyzed by ^{1}H-NMR to assess the amount of impurities.

The process was subsequently modified in order to reduce the amount of impurities in the final product. Instead of using methacrylic acid, methacryloyl chloride (Mw=104.53 g/mol) was chosen, and EDC was substituted with trimethylamine (Mw=101.19 g/mol) to remove the hydrochloric acid condensed in the first step of the reaction. The reaction becomes like this (Table 4.2):

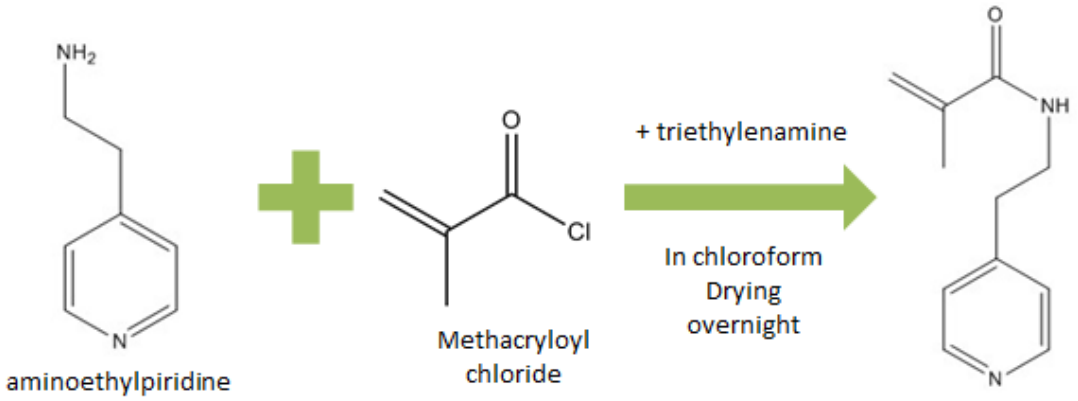


Table 4.2: stechiometric parameters of PySMAAm synthesis.

	Aminoethylpyridine	Methacryloyl chloride	Triethylamine
Equivalents	1	1	2
Moles [mmol]	0.016	0.016	0.033
Mass [g]	2	1.71	3.31
Volume [mL]	1.942	1.584	4.563

The second step of the reaction was not modified.

Figure 4.4 and 4.5 show the ^{1}H-NMR spectra of PyMAAm and PySMAAm for this new synthesis process respectively.

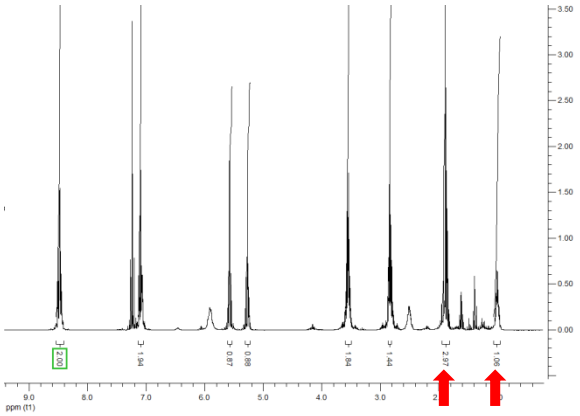


Figure 4.4: PyMAAm ^{1}H-NMR spectrum

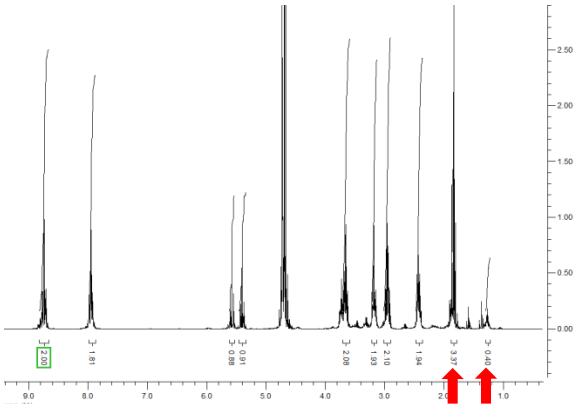


Figure 4.5: PySMAAm ^{1}H-NMR spectrum

The amount of impurities was calculated from the relative intensity of the peaks identified by the arrows for each of the spectra. The peak on the right represent the impurities, the one on the left identifies the monomer:

- PyMAAm:

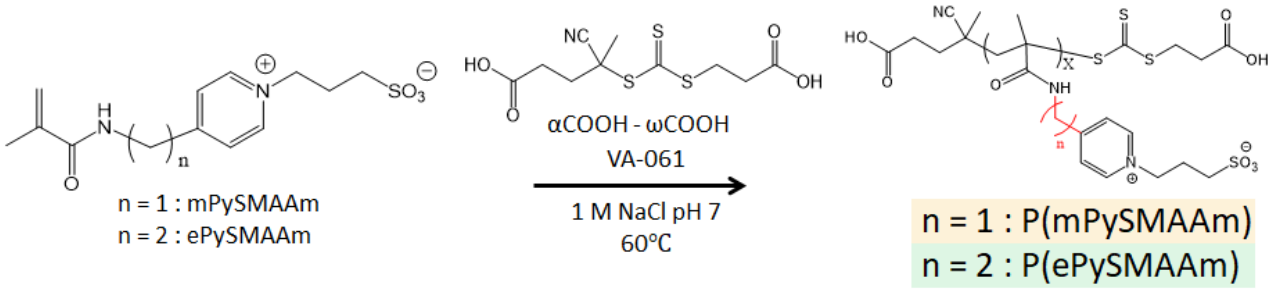
$$1.06 \div 2.97 = x \div 100$$
$$x = \frac{1.06 * 100}{2.97} = 35.69\%$$

- PySMAAm:

$$0.40 \div 3.37 = x \div 100$$
$$x = \frac{0.40 * 100}{3.37} = 11.86\%$$

The amount of impurities is still considerable and the process might have to be perfected.
^{1}H-NMR, however, confirmed the success of the sulfobetaine monomer synthesis.

The monomers were polymerized by reversible addition-fragmentation chain transfer polymerization (RAFT). The chain transfer was 4-[(2-carboxyethylsulfanylthiocarbonyl)sulfanyl-4-cyanopentanoic acid (αCOOH – ωCOOH), the selected initiator was 2,2'- azobis[2-(2-imidazolin-2-yl)propane] (VA-061):



However, the studies on UCST of P(PySMAAm)s do not concern the present research. What instead is important is that the cytotoxicity of the polymer was evaluated. The measurement was performed by 24h incubation in HeLa cells. Figure 4.6 shows cell viability.

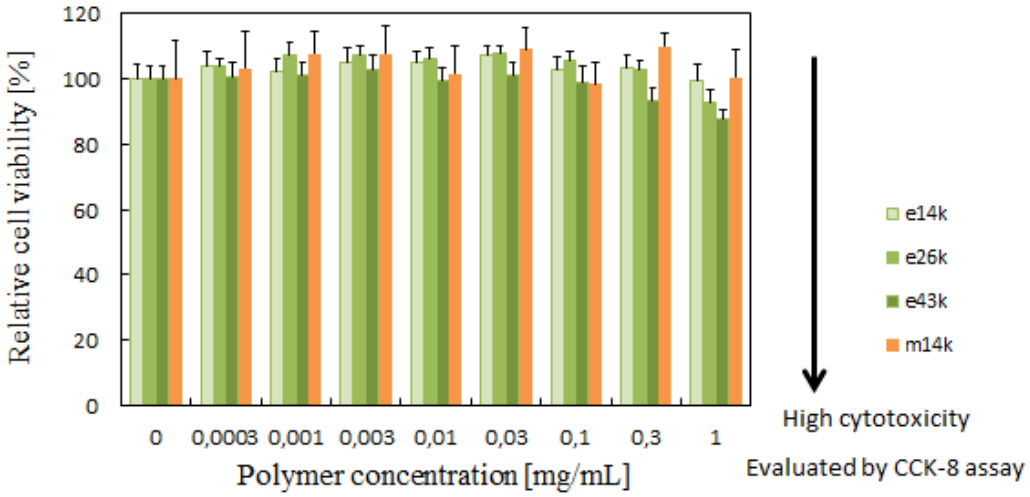


Figure 4.6: cytotoxicity of eP(PySMAAm) and mP(PySMAAm) with different molecular weights.

ePySMAAm and mPySMAAm polymers showed little cytotoxicity, a fundamental feature for biocompatible polymers. P(ePySMAAm) seemed to be the most promising of the samples.

Hence, it was decided to test this monomer as an antibiofouling agent to coat surfaces. The main problem, as mentioned before, was always the solubility in aqueous media of the sulfobetaines. The strategy adopted to overcome this issue was to synthetize a copolymer [10] [11] [12] [13], mixing PySMAAm with another more stable monomer. The choice fell on two different monomers: 2-hydroxyethyl methacrylate (HEMA) and butyl methacrylate (BMA).

4.3 2-hydroxyethyl methacrylate

2-hydroxyethyl methacrylate (Figure 4.7) is a clear, liquid compound obtained by reacting methacrylic acid (CH_2=C[CH_3]CO_2H) with ethylene oxide or propylene oxide. When polymerized it is a widespread polymer in the biomedical field. It's a soft, flexible, water-adsorbing plastic used to make soft contact lenses. It's been used to modify material surfaces [14]. As a monomer it is hydrophobic and water soluble, but it changes its properties once it polymerizes with a Mw>10k, becoming hydrophilic thanks to the hydrophilic pendant groups. It has good protein antiadhesion properties, but since its interaction with phospholipids, which are present on cell surfaces, is weak, it can't be used as a good call antiadhesion polymer.

4.4 Butyl methacrylate

n-butyl methacrylate (Figure 4.7) is a hydrophobic polymer, chosen as an alternative to HEMA in order to stabilize the radical and obtain a more stable copolymer. It is a clear liquid with a strong smell. It has been previously used, especially in the 1990s, to copolymerize MPC polymers in biomedical applications, especially for protein antiadhesion blood-contact situations [15] [16] [17].

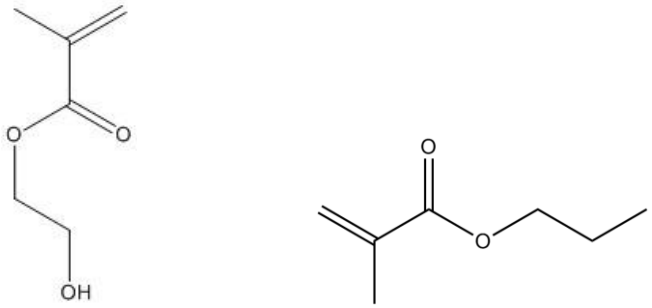


Figure 4.7: HEMA (left) and BMA (right) chemical structures

5. MATERIALS AND METHOD

5.1 COPOLYMERIZATION PROCESS

From the results of cytotoxicity analysis it was decided to focus on the synthesis of an ePySMAAm copolymer. Samples of copolymer with mPySMAAm were prepared as a comparison. All the following reactions and analysis are identical whether the monomer has a methylene or ethylene chain length.
ePySMAAm (312.3 g/mol) obtained from the two steps reaction described in section 4.1 was copolymerized with HEMA (130.14 g/mol) monomer by free radical polymerization. This polymerization was chosen because of the restrictions in solubility of sulfobetaine monomers and because it allows a good control over the molecular weight of the copolymer (which can't be obtained with RAFT polymerization, for instance).
The reaction was performed in different solvents changing the initial monomer concentration. The choice of the initiator depended on the solvent. Different initial composition of monomer in feed were tested: 80-20, 50-50 and 20-80 of PySMAAm-HEMA respectively.
Process:

- ePySMAAm was weighted and dissolved in the solvent
- The correct stechiometric amount of HEMA was then added to the mixture
- Initiator was dissolved in 1 mL of solvent and the adequate fraction was mixed with the monomers
- The rest of the solvent was added
- If necessary, the solution was filtered
- Degasing by N_2 was subsequently performed for 30 min
- Polymerization was performed in an oil bath at the temperature of 60°C
- Once the reaction was completed, the polymer solution was collected and put in a filtration paper for 1 week of dialysis in deionized water to remove polymer chains with lower Mw
- After 1 week, the solution was freeze dried and the copolymer was collected.

The copolymerization of ePySMAAm and BMA followed the same procedure, except the solution couldn't be dialyzed in water because of BMA's hydrophobicity. The solution was instead concentrated by evaporation and subsequently the copolymer was reprecipitated in an adequate precipitation agent on a magnetic stirrer. After reprecipitation the solution was left 30 minutes to stabilize, then the solvent was removed and the polymer was dried in vacuum to be collected.
The yield of the reaction was then calculated for each sample by the equation:

$$yield = \frac{m_{polymer}}{m_{ePySMAAm} + V_{HEMA(BMA)} * \delta_{HEMA(BMA)}}$$

5.2 SOLUBILITY TEST

In order to perform further analysis on the samples, it was necessary to identify the best solvents for each sample. The tests were performed by dissolving few milligrams of the sample in the solvents in a concentration of 5 or 10 mg/mL. In addition to that, it was desired to have an initial evaluation of the stability of the copolymers in aqueous environment such as 1M NaCl or phosphate buffer saline (PBS).

5.3 ^{1}H-NMR ANALYSIS

A sample of each copolymer was taken after collection to be evaluated by ^{1}H-NMR, in order to confirm the success of the copolymerization and to evaluate the final composition of the copolymer. Copolymers with a larger amount of PySMAAm in composition were dissolved in 1M NaCl in D_2O, the samples with a

higher amount of HEMA or BMA in composition were instead dissolved in 2,2,2-trifluoroethanol (TFE) because of their insolubility in the previous solvent.

5.4 SIZE EXCLUSION CHROMATOGRAPHY

The molecular weight of the copolymers was evaluated by GPC measurements. A higher Mw was desired in order to improve the stability of the copolymers. The analysis was performed with 0.4M $NaNO_3$ as the eluent, in a column TOSOH PWXL3000+PWXL4000. The concentration of the polymer was 5 mg/mL, the temperature 25°C and the flow rate 0.7 mL/min. Molecular weight and polydispersity were the analyzed data.

5.5 COATING

The purpose of these copolymers is the application as antibiofouling surfaces. From every sample was therefore obtained a coating. These coatings were performed with two different techniques: dip coating and spin coating. The solution used for the coating was a 5 mg/mL solution of the copolymer in TFE. TFE was found to be a good solvent for all the samples and it was chosen because its boiling point (74°C) is not too low to be difficult to use, nor too high, that would make it difficult to be removed.

5.5.1 Dip Coating

The substrate selected for the dip coating was polyethylene terephthalate (PET). PET sheets were manually cut into 1X1 cm^2 squares (Figure 5.1). A hole was then performed on the substrate to make the immersion of the substrate in the polymer solutions and in the analysis solutions easier. The samples were placed in ethanol and washed with ultrasonic (28kHz) for 30 minutes to clean the surface from atmosphere contamination, then dried in vacuum.

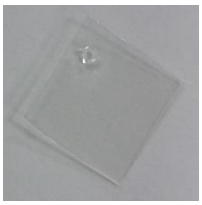


Figure 5.1: PET substrate

The substrate was dipped 5 times in the polymer solution, then the excess of liquid was removed from the surface and the coated substrate was placed in a glass leaning diagonally in order to dry evenly on both sides. After one hour the procedure of dipping was repeated and then the samples were left to dry overnight in the glass in the presence of 2-3 mL TFE. The following day the TFE glasses were removed and the samples were dried in vacuum. Figure 5.2 shows the process of dip coating.

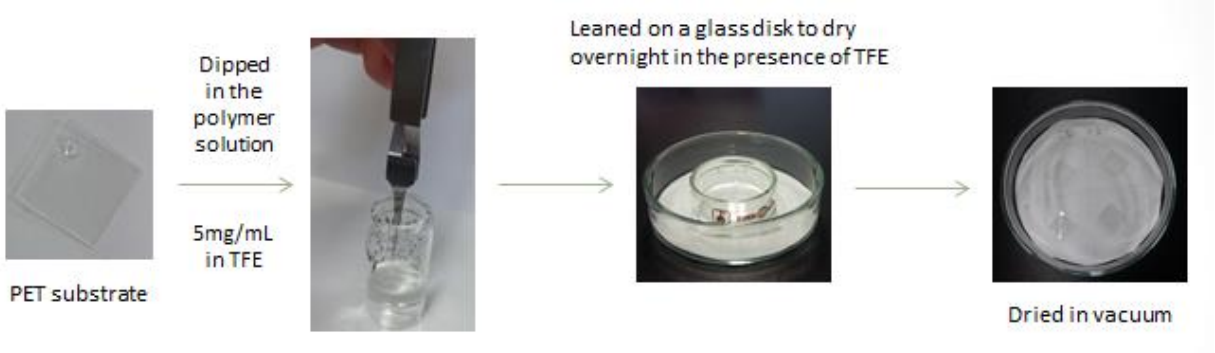


Figure 5.2: dip coating process

5.5.2 Spin Coating

Spin coating was performed using a Spin Coater MS-B100 (Mikasa Opticoat) (Figure 5.3). The selected substrate was a silicon wafer. After being cut with a diamond blade, the 1X1 cm^2 substrate was cleaned in ethanol with ultrasonic (28kHz) for 30 minutes and then dried in vacuum.

Figure 5.3: Spin Coater MS-B100 (Mikasa Opticoat)

50 µL of polymer solution were dropped on the silicon substrate placed in the spin coater, which was set to a two-steps coating: initially it would rotate at 500 rpm for 5 s, followed by 30 s at 3000 rpm. The coated substrate was then dried in vacuum for further evaluation.

5.6 SEM MEASUREMENTS

The spin coated samples were scratched on the surface and then sent to the SEM laboratory to be evaluated by scanning electron microscopy (SEM) to confirm the presence of the coating, to see the surface and to evaluate the thickness of the coating.

5.7 FTIR MEASUREMENTS

Dip coated samples and also not coated copolymer samples were evaluated by Fourier Transformed Infrared Spectroscopy (FTIR) with an IRPrestige-21 (Figure 5.4). The dip coated samples were studied in order to confirm the stability of the coating in aqueous environment by immersion of the coatings in PBS overnight. The immersed coatings were then dipped in deionized water to be cleaned and dried. Two samples for each copolymer and composition were prepared, one to be evaluated before PBS immersion, the other one after PBS immersion. The spectra of the two samples were then compared with the one of the not-coated PET substrate. Not coated copolymer samples were investigated too to further confirm the copolymerization, like already done by ^{1}H-NMR.

Figure 5.4: IRPrestige-21

5.8 CONTACT ANGLE MEASUREMENTS

The main analysis to confirm the stability of the coatings after PBS immersion was contact angle measurements. This was realized by static sessile drop method. 1.0 µL of deionized water was dropped on the surface of the samples and the contact angle was measured. Again, two samples for each copolymer composition were realized and analyzed. In order to improve the stability of P(PySMAAm-HEMA) and P(PySMAAm-BMA) coatings, the surface of some of the PET substrate was UV ozone treated before being coated and the effect of the treatment was evaluated.

Samples for the analysis were prepared of P(PySMAAm-HEMA): 80-20, 50-50, 20-80, P(mPySMAAm-HEMA)50-50 and P(PySMAAm-HEMA)50-50 synthetized with 0.1M monomer concentration. The P(PySMAAm-BMA) samples were: 80-20, 50-50, 20-80, 15-85 and 50-50, which was redissolved and freeze dried. Moreover, the contact angle was measured on control samples of uncoated PET, P(HEMA) and P(BMA) coated substrates.

5.9 PROTEIN ADSORPTION EVALUATION

The most important analysis performed, since the purpose is to obtain an antibiofouling surface, was the protein resistance assessment. This was made by Micro BCA Microplate Procedure (Thermo Fisher Scientific). The analysis is used for the colorimetric detection and quantitation of total protein, based on bicinchoninic acid as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment.

The coated PET samples were immersed in PBS overnight (approximately 20 h) (Figure 5.5), after that they were taken and immersed in a 50 mg/mL bovine serum albumin (BSA) solution in PBS and stored at 37°C for 1 h. Every sample was then washed in PBS to remove the excess of proteins and placed in a well of a 24 wells microplate filled with 1 mL of a 5 mg/mL sodium dodecyl sulfate (SDS) in deionized water solution, the microplate was the sealed and treated with ultrasonic (45kHz) for 10 minutes in order to remove all the adsorbed proteins from the surface of the substrate. 150 µL were collected from each well and transferred into two wells of a 96 wells microplate. 9 standard solutions (named from A to I) were prepared following Micro BCA assay kit instructions. Each standard had a different concentration of BSA in

PBS diluent, ranging from 0 mg/mL to 200 mg/mL. 150 µL of each standard solution were transferred in two wells too. A Working Reagent (WR) was prepared as instructed on the Micro BCA assay kit manual: the total volume of WR required was calculated as:

$$(\#\ \text{standards} + \#\ \text{unknowns}) \times (\#\ \text{replicates}) \times (\text{volume of WR per sample}) = \text{total volume WR required}$$

Where the composition of WR was obtained by mixing 25 parts of Micro BCA Reagent MA and 24 parts of Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC). 150 µL of the WR was added to each well and mixed for 30 seconds.

The microplate was then incubated at 37°C for 2h, after which the plate was cooled to room temperature.

The microplate with all the samples and the standard was finally placed in a UV-vis spectrophotometer (Spectra Max M3) where the absorbance at 562 nm at room temperature was measured (Figure 5.6).
4 coatings were tested for each copolymer composition in order to have a good statistical measure. Additionally, control samples of P(HEMA) and P(BMA) were tested.

Figure 5.5: Immersion of coated samples in BSA solution

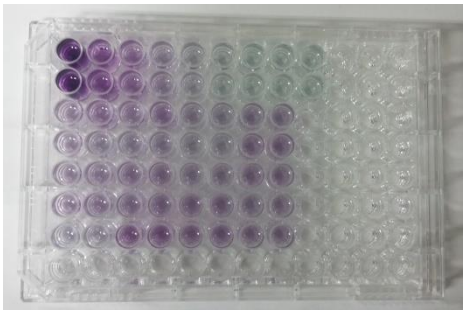


Figure 5.6: 96 wells microplate for Micro BCA assay

Results of the Micro BCA analysis were statistically evaluated by t-test in order to assess if the protein adsorpion mean value of the samples is significantly different from that of the control sample.

6. RESULTS AND DISCUSSION

6.1 PySMAAm-HEMA COPOLYMERS

The polymerization was initially performed with 0.1M monomer concentration in 30 mL of solvent, which was methanol (MeOH). The initiator was azobisisobutyronitrile (AIBN), which is soluble in MeOH. The quantity of initiator was 1% the number of moles of monomers (in this case 1% of 3mmol=0.03mmol). The polymerization was conducted in an oil bath at 60°C for 2 h however, after freeze drying, no polymer was detected. The result required for the process to be modified.

The first parameter that was changed was the polymerization time, increasing it from 2 to 20 hours. The new samples gave the first results: all three compositions of the copolymer were collected as a white powder (Figure 6.1), proving that the polymerization time was suitable.

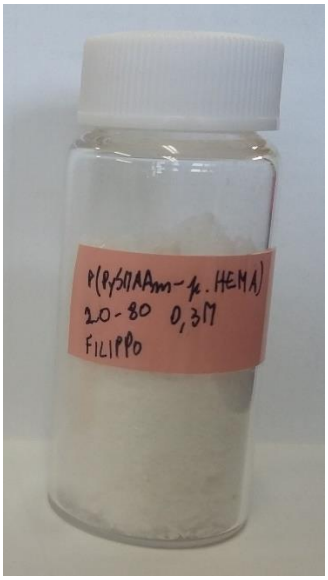


Figure 6.1: Sample of P(PySMAAm-HEMA) 20-80.

The solubility of the samples was evaluated and gave the results showed in the following table:

Table 6.1: Solubility of P(PySMAAm-HEMA) copolymers.

	P(PySMAAm-HEMA) 80-20	P(PySMAAm-HEMA) 50-50	P(PySMAAm-HEMA) 20-80
1M NaCl	o	o	x
HFIP	o	-	-
TFE	o	o	o
MeOH	x	x	x
0,4M $NaNO_3$	o	o	o
DMF	-	-	o
DMSO	-	x	o
PBS	o	o	x

1 M NaCl proved to be a good solvent for the samples with a higher content of PySMAAm, but not for the 20-80 sample. TFE was confirmed to be a good solvent for all the copolymers, as well as 0.4M $NaNO_3$. The stability of the polymers in PBS was evaluated but only the 20-80 sample showed some kind of stability.

The solubility of the samples in 0.4M $NaNO_3$ allowed GPC measurements for calculation of molecular weight and polydispersity. Copolymerization was confirmed by ^{1}H-NMR measurement of the 80-20 and 50-50 samples dissolved in 1M NaCl in D_2O. The analysis allowed the evaluation of the final composition of the samples too, by integration of the peaks resulting from PySMAAm and HEMA monomers. The results are expressed in table 6.2:

Table 6.2: Copolymerization results for reaction in MeOH for 20h at 60°C.

Sample	Final composition	Mw	Mw/Mn	Yield [%]
P(PySMAAm-HEMA) 80-20	71-29	8.6k	1.62	31.6
P(PySMAAm-HEMA)50-50	40-60	9.5k	1.78	49.3
P(PySMAAm-HEMA)20-80	-	-	-	17.6

The sample showed instability in the final composition, with a tendency to increase the HEMA quantity in the copolymer, sign of a more reactive monomer. The yield and the molecular weight were both low and one of the objective was to improve these properties. GPC measurements of P(PySMAAm-HEMA) 20-80 was not performed because the amount of polymer was not sufficient due to extremely low yield.

In order to improve molecular weight and yield of the copolymers, different solvents were tested for the polymerization process:

- MeOH/1M NaCl 50/50 %vol solution. The initiator was changed to 4,4'-azobis(4-cyanovaleric acid);
- 1M NaCl with 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] (VA061) as initiator
- TFE with AIBN as initiator

The solvent which gave the best results was the MeOH/1M NaCl 50/50 solution.

Table 6.3: Copolymerization results for reaction in MeOH/1M NaCl 50/50 solution for 20h at 60°C

Sample	Final Composition	Mw	Mw/Mn	Yield [%]
P(PySMAAm-HEMA)80-20	67-33	16.7k	2.65	54
P(PySMAAm-HEMA)50-50	33-67	34.7k	2.07	75
P(PySMAAm-HEMA)20-80	-	17k	2.24	40.7

The ^{1}H-NMR spectrum of P(PySMAAm-HEMA) 50-50 obtained with the polymerization conditions above is a good sample to show all the peaks assigned to the molecule, showing both PySMAAm and HEMA peaks in the copolymer (Figure 6.2).

Peaks 5 and 6, corresponding to the pyridinium are used for the evaluation of final composition comparing them to peaks number 7 and 8, derived from the HEMA chain. The ^{1}H-NMR spectra of the 20-80 copolymers, since they are not soluble in 1M NaCl in D_2O, was more difficult, but it was found out that TFE d3 could be used for monomers with lower PySMAAm content.

The other solvents didn't give any significant improvement in the polymerization. Even though the molecular weight of the copolymers was increased, this came at the cost of an increase in polydispersity of

the samples. Trying to reduce this parameter, new samples were prepared with the MeOH/1M NaCl solution as solvent, with the difference that the initiator, 4,4'-azobis(4-cyanovaleric acid), was 0.5% of the moles of monomers. The new synthesis, however, gave no result since no polymer was collected after freeze drying, supposedly because the quantity of initiator was insufficient for the polymerization to occur.

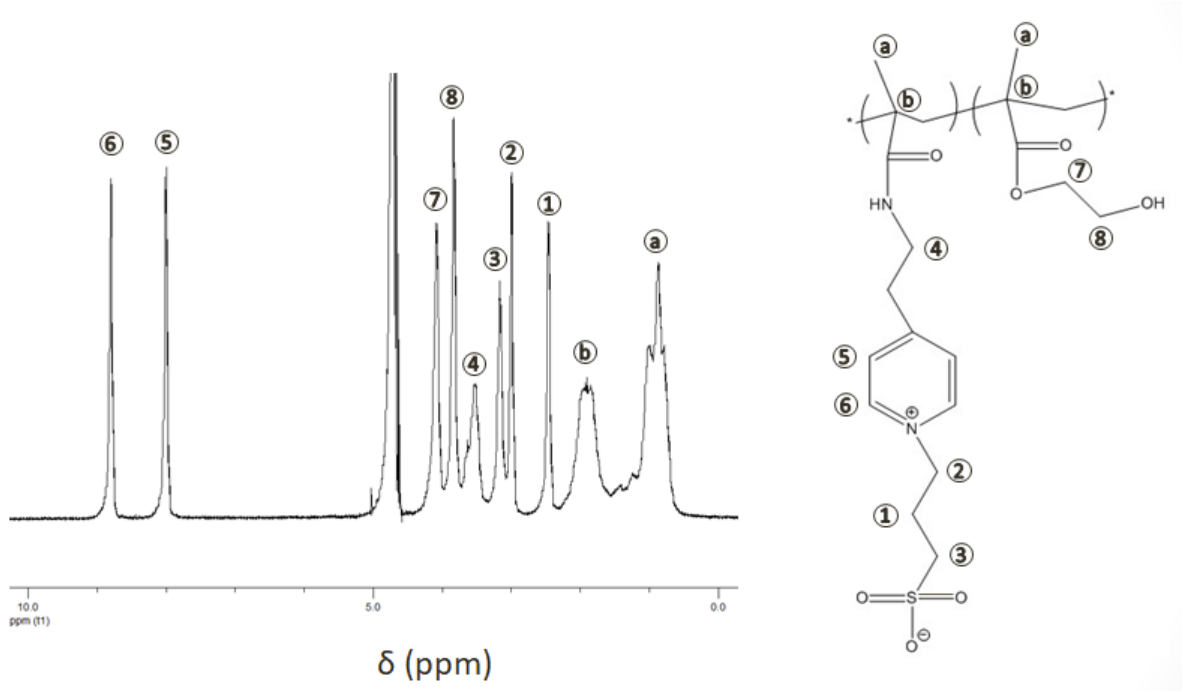


Figure 3.2: P(PyMAAm-HEMA)50-50 ^{1}H-NMR spectrum with peaks assigned.

Since the desired molecular weight of the copolymers was still much higher, it was decided to modify another parameter: the initial monomer concentration was kept low because of the solubility problems of the sulfobetaine monomer. It was however increased from 0.1M to 0.3M. The results are shown in table 6.4:

Table 6.4: Copolymerization results with 0.3M monomer concentration

Sample	Final Composition	Mw	Mw/Mn	Yield [%]
P(PySMAAm-HEMA)80-20	68-32	60.4k	2.61	64.2
P(PySMAAm-HEMA)50-50	35-65	61.2k	2.69	43.7
P(PySMAAm-HEMA)20-80	7.5-92.3	-	-	73.5

The composition of the copolymers was consistent with the previous synthesis, the yield too was comparable to the previous ones, except for the 20-80 sample, which was significantly boosted, proving that the new polymerization conditions improved the efficiency of the process. The molecular weight of the 20-80 sample could not be evaluated because of instrumentation issues, but that of the other two samples was definitely higher compared to the previous ones.

The downside of the synthesis with these parameters is the further increase of the polydispersity of the samples, which might affect the stability of the coatings. A high polydispersity means a big difference in the chain length of the polymers; smaller chains can act as points of weakness in the coating that can harm the stability of the whole. In order to reduce it, one sample of P(PySMAAm-HEMA)50-50 was synthetized at the

same conditions but using an amount of initiator that was 0.8% of the moles of monomer. The copolymer further improved the molecular weight to 85.7k but the polydispersity increased too to a value of 2.88.

All the copolymers synthetized were based on ePySMAAm, however one sample was prepared with mPySMAAm to be compared with the others: P(mPySMAAm-HEMA)50-50 sample was synthetized at the conditions that optimized yield, Mw and polydispersity (0.3 monomer concentration, 1% initiator concentration, MeOH/1M NaCl solvent). The obtained sample had Mw=81k and Mw/Mn=1.90, but showed more instability in the final composition, which was 26-74 (mPySMAAm-HEMA).

The stability of the copolymers in PBS seemed to have an increase with increasing molecular weight, especially for the samples with a higher amount of HEMA. The composition of the copolymers strongly influences the properties of the copolymers: the samples with more than 50% of sulfobetaine monomer in feed are expected to be the least stable in aqueous environment, but also the more susceptible to changes in the polymerization process. For these reasons, since the primary objective was to improve the polymerization, copolymers with a high amount of PySMAAm were synthetized. There have been previous researches, however, about copolymers of sulfobetaines (especially with acrylamides) where the content of sulfobetaine monomer did not exceed 25% of initial concentration [18] [19]. We expect the samples with higher HEMA content to be the more stable, without significant loss of antibiofouling properties due to PySMAAm.

All the analysis performed on PySMAAm-HEMA copolymers were made using only high molecular weight samples.

The solubility results of the copolymer of PySMAAm and HEMA, even if not entirely satisfying, were promising. Nevertheless, another type of copolymer was synthetized as an alternative using a hydrophobic monomer previously introduced: BMA.

6.2 PySMAAm-BMA COPOLYMERS

Since this copolymer was synthetized after all the others, most of the parameters were kept the same that optimized the process in the synthesis of P(PySMAAm-HEMA) copolymers:

- Monomer concentration: 0.3M
- Initiator concentration: 1% moles of monomers
- Polymerization time: 20 h
- Polymerization temperature: 60°C

The choice of solvent was controlled by the insolubility of BMA in aqueous solutions, therefore MeOH was chosen and subsequently, AIBN was the designed initiator. Four different composition in feed were tested: 80-20, 50-50, 20-80 and 15-85 (PySMAAm-BMA respectively).

The collected samples were not a powder like for the P(PySMAAm-HEMA) copolymers but they were a highly viscous yellow slime (Figure 6.3). These samples often showed aggregation behavior that made the evaluation of molecular weight and polydispersity by size exclusion chromatography in 0.4M $NaNO_3$ impossible. An attempt to reduce these properties (probably due to the reprecipitation process) was made by dissolving a P(PySMAAm-BMA)50-50 sample in 1M NaCl after reprecipitation, the new solution was then dialyzed and freeze dried. The result was a thick white powder which however kept the aggregation properties of the precipitated polymer.

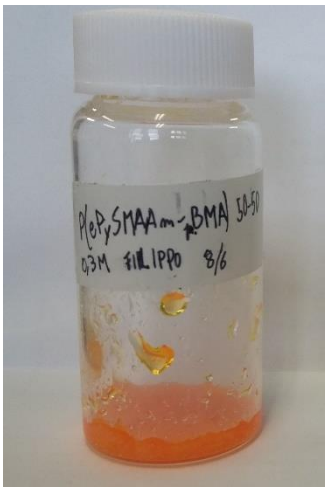


Figure 6.3: Sample of P(PySMAAm-BMA) 50-50.

The molecular weight of the samples could be evaluated in an eluent different from 0.4M $NaNO_3$, however it wasn't possible at the time because of instrumentation issues.

Solubility of the copolymers was tested in some solvents; again the stability in PBS was checked to have a first idea of the stability of the sample in aqueous environment.

From the ^{1}H-NMR spectra the copolymerization was confirmed and the final composition of the copolymers was evaluated. The copolymers showed more stability compared to the P(PySMAAm-HEMA) samples when it comes to final composition: the 80-20 sample kept the same composition after polymerization (Figure 6.4), 50-50 showed still some instability since at the end of the process composition was 35-65 and 20-80's composition was almost unchanged when the ^{1}H-NMR was evaluated: 17-83. Again, 1M NaCl in D_2O was used for the samples with higher PySMAAm content, meanwhile TFE d3 was the solvent used for high BMA content.

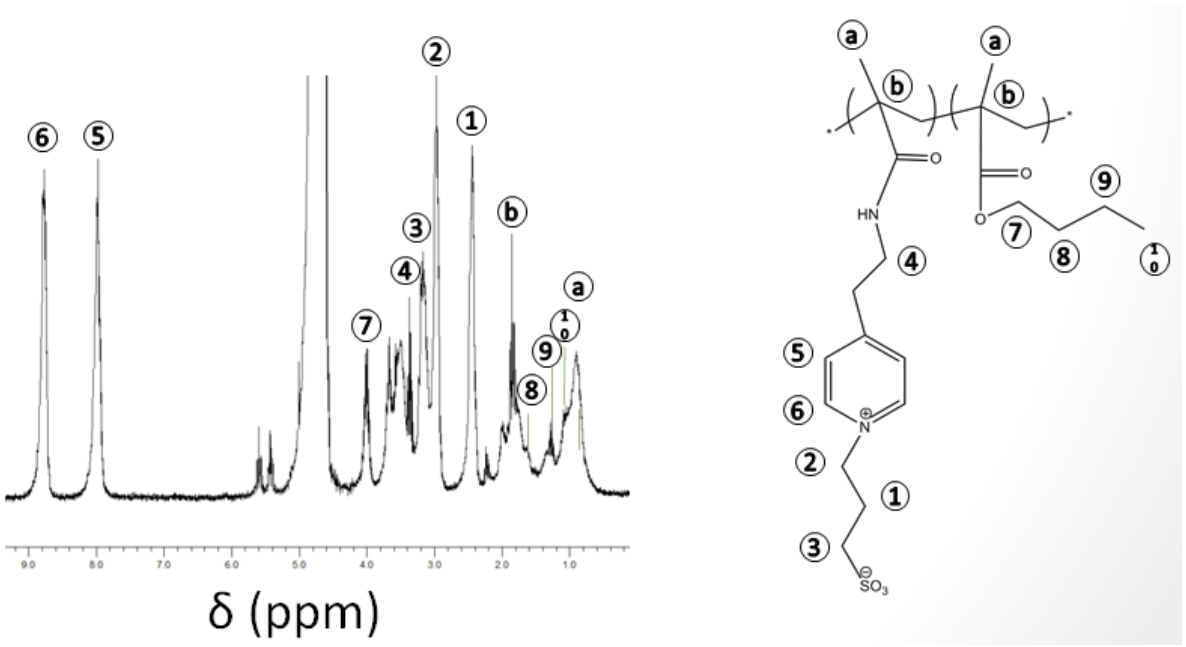


Figure 6.4: P(PyMAAm-BMA)80-20 ^{1}H-NMR spectrum with peaks assigned.

6.3 SEM MEASUREMENTS

The purpose of this analysis was to observe the surface of the coating and to evaluate its thickness. 3 samples were observed: P(PySMAAm-HEMA) 50-50, P(PySMAAm-BMA) 50-50 and P(PySMAAm-BMA)20-80.

Even though the surface of the coatings was clear, it was not possible to evaluate the thickness of the coating, probably because it was too thin. The SEM images provide however a good view of the polymer coated on the silicon surface.

Two images are shown in Figure 6.5 as an example: the surface of the coating appears to be granular and uniform, which are signs of a good coating.

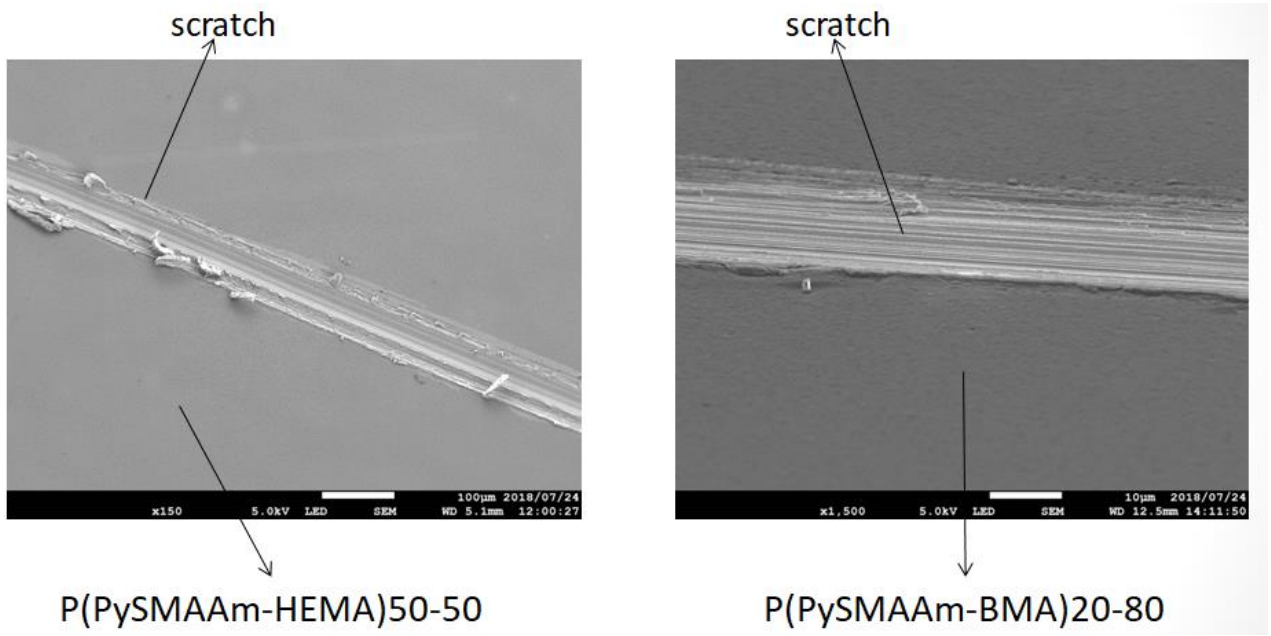


Figure 6.5: SEM images of the coated silicon substrates.

6.4 FTIR MEASUREMENTS

This measurement was performed in order to assess the stability of the coatings after immersion in PBS for one day. Initially the spectrum of a not-coated PET substrate was measured (Figure 6.6).

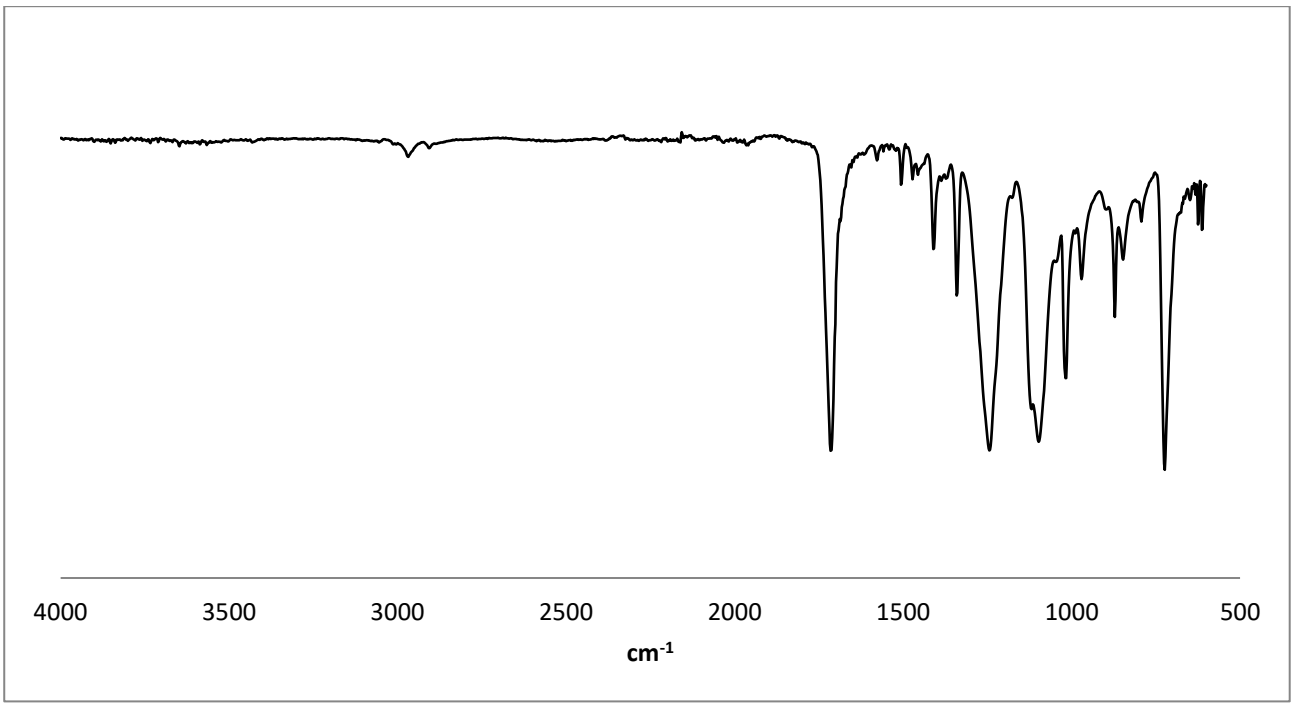


Figure 6.6: PET FTIR spectrum.

The idea was to detect the differences between the PET spectrum and the spectra of the coated samples, including those of control samples of P(HEMA) and P(BMA) homopolymers.

Unfortunately the analysis failed: the spectra of coated and not-coated PET are basically identical, no significant difference can be identified (Figure 6.7). Again it has been hypothesized that the reason is the thickness of the coating, too thin to be studied.

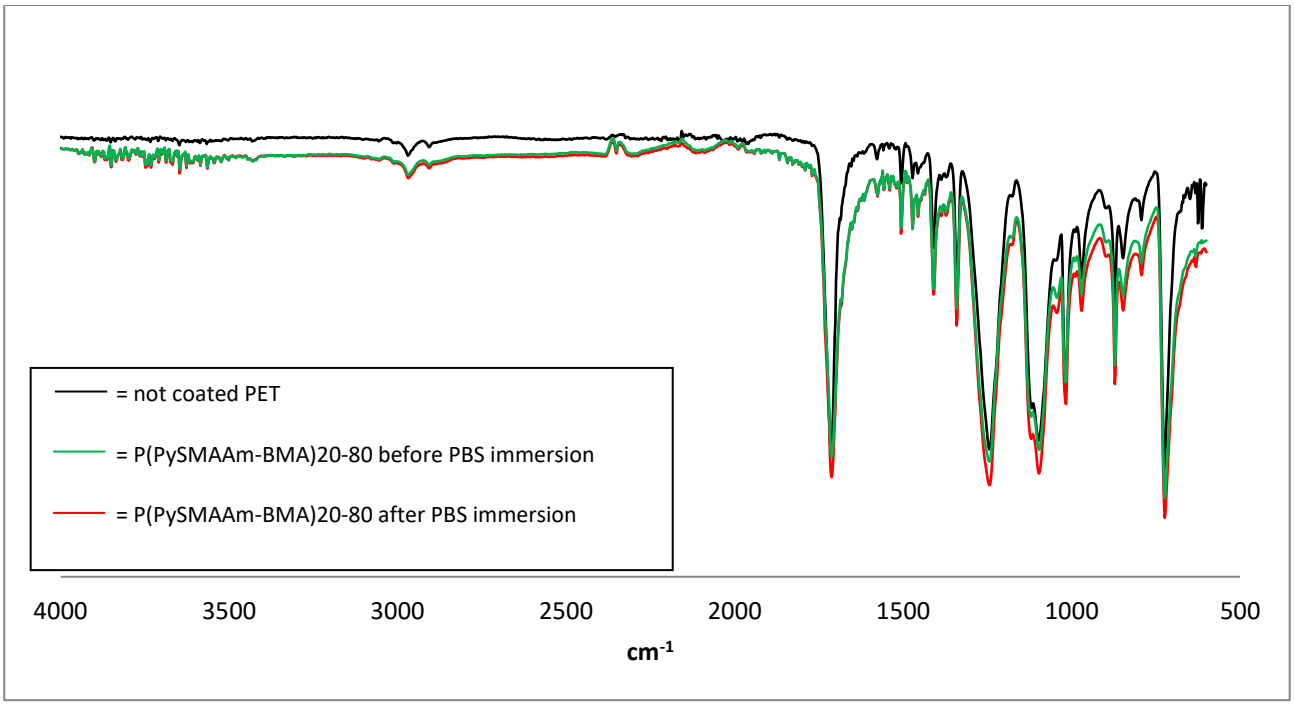


Figure 6.7: overlapped FTIR spectra of PET and P(PySMAAm-BMA)20-80 coated substrates before and after PBS immersion.

It was then decided to verify, like it was done by ^{1}H-NMR, the success of the copolymerization of the samples. Copolymer powder or slime was placed on the detector and the spectra were analyzed, assigning all the peaks to the corresponding molecule groups.

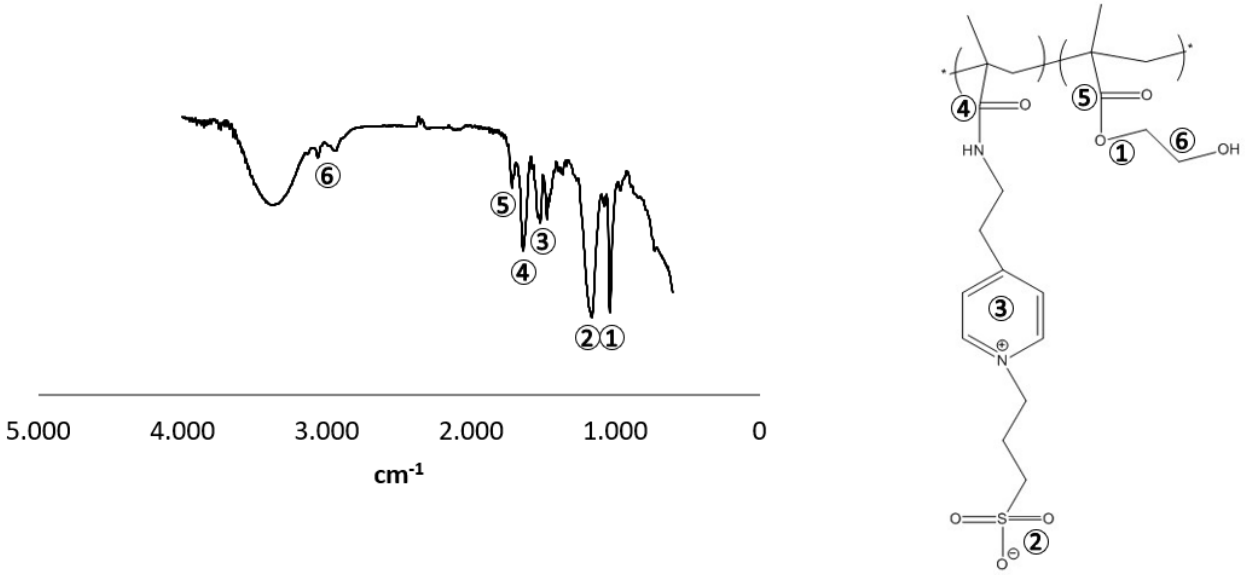


Figure 6.8: FTIR of P(PySMAAm-HEMA)80-20 with peaks assigned.

Peak 1 corresponded to the C-O bond in HEMA, peak 2 derived from the SO_2 group, peak 3 corresponded to the C=C bond in the pyridinium ring, as it often comes in the form of multiple peaks. Peak 4 was assigned

to the C=O bond in the PySMAAm monomer, peak 5 instead identified the same bond in the HEMA, because derived from a carboxylic group. Finally, peak 6 referred to the C-H bond in the HEMA (Figure 6.8). The P(PySMAAm-HEMA) copolymer spectrum presented the same peaks as the P(PySMAAm-BMA) one (Figure 6.9). The difference between the two of them and also between the spectra of the same copolymer but with different composition was the intensity of the peaks, that allowed the identification of the bonds.

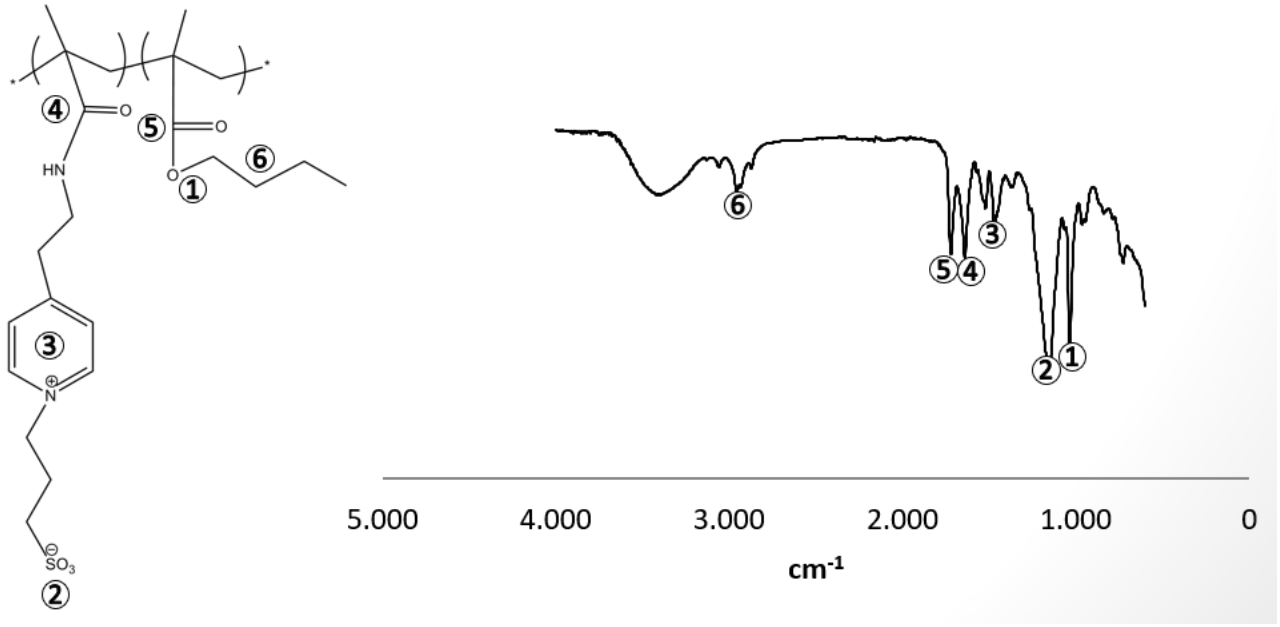


Figure 6.9: FTIR of P(PySMAAm-BMA)20-80 with peaks assigned.

6.5 CONTACT ANGLE MEASUREMENTS

The stability of the coatings was investigated by contact angle measurements. Coatings of both the copolymers were analyzed, in order to see the difference in the contact angle before and after PBS immersion for 20h. The influence of the surface ozone treatment was studied on all the samples for both PySMAAm-HEMA and PySMAAm-BMA copolymers.

Initially, as control samples, the contact angle of not-coated PET, of P(HEMA) coated PET and of P(BMA) coated PET was investigated (Figure 6.10). PET and P(BMA) are hydrophobic therefore they presented a high contact angle, bigger than 70°. P(HEMA) instead, provided a hydrophilic coating with low contact angle, around 25°. The coated control samples were evaluated after PBS immersion too, to assess their stability. Contact angle of the P(HEMA) coated sample was unchanged, whereas P(BMA) coated sample increased its wettability, supposedly by little hydration of the surface, and showed a decreased contact angle of 46°.

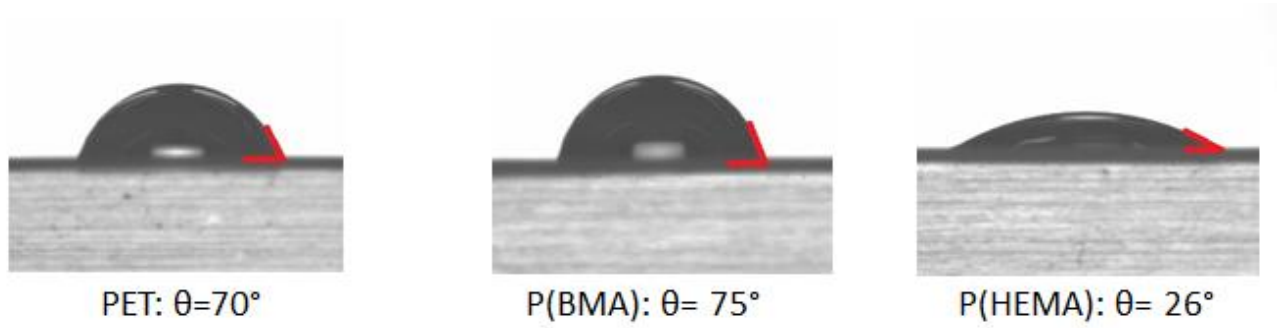


Figure 6.10: control samples contact angle before PBS equilibration.

6.5.1 P(PySMAAm-HEMA) copolymers

The investigation of the contact angle in P(PySMAAm-HEMA) copolymers coated samples showed that the copolymers had a positive effect on the substrate before PBS immersion. All of the samples had a contact angle comparable or smaller to the only P(HEMA) coated substrate, which was expected because of the hydrophilic properties of both HEMA and PySMAAm. After PBS immersion the coatings behaved differently:

- P(PySMAAm-HEMA)50-50 0.1M: this coating, that presented one of the smallest contact angles before PBS immersion, significantly decreased its wettability after the equilibration in aqueous solution: The contact angle increased from 14° to almost 60°. This sample, as expected, wasn't stable in aqueous environment. The low molecular weight caused instability in the coating and its dissolution.
- P(PySMAAm-HEMA)80-20: this coating, due to the small amount of HEMA in composition, had a contact angle higher than P(HEMA) coated samples, however it remained consistent after the immersion in PBS solution. Unfortunately, since the angle was high it can't be assumed that the coating is stable. The comparison with P(HEMA) only demonstrated that the hydrophilicity of the surface is lower.
- P(PySMAAm-HEMA)50-50: the behavior of this coating before and after PBS immersion is similar to the previous one, however the contact angle is slightly smaller.
- P(PySMAAm-HEMA)50-50 synthetized with 0.8% of initiator: this sample is the one with the higher measured Mw. Its contact angle was one of the smallest (15°) and, even though the angle increased after PBS immersion (30°), it was still comparable to the control sample. The results allow to believe that the coating is stable.
- P(mPySMAAm-HEMA)50-50: this coating was successful in the stability evaluation: the initial contact angle was smaller than P(HEMA) (15°) and the decrease of wettability after equilibration in PBS was limited (contact angle=30°). The results are really similar to the P(PySMAAm-HEMA)50-50 08% initiator.
- P(PySMAAm-HEMA)20-80: the sample with the higher content of HEMA in composition, as expected, gave the best results: the contact angle was, before and after PBS immersion, <20°, even lower than the control sample. This coating was confirmed to be stable thanks to the presence of HEMA (Figure 6.11).

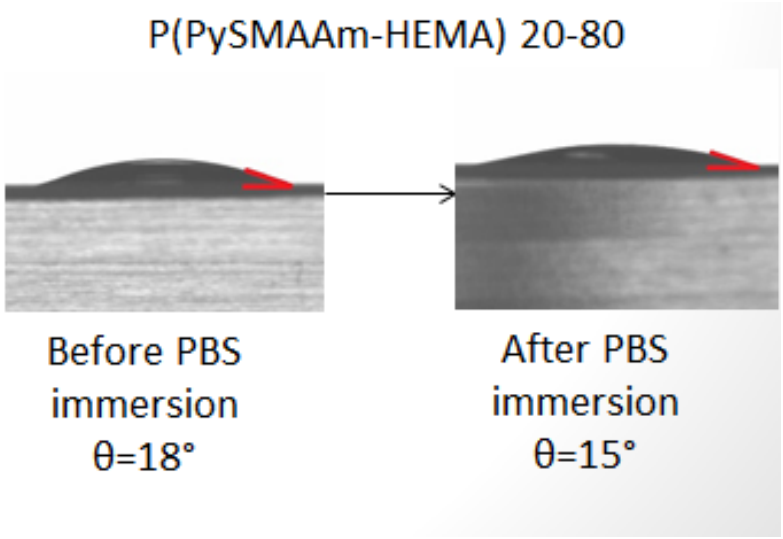


Figure 6.11: contact angle measurements of P(PySMAAm-HEMA)20-80.

For what it concerns the effect of the surface treatment on the substrates, it was verified that it didn't provide an improvement in the stability of the coatings: the adhesion of the copolymer to the surface of the substrate was improved but didn't have any positive effect on the stability of the coating after the immersion in the PBS solution. In most cases the stability was instead decreased compared to the not treated surface (Figure 6.12).

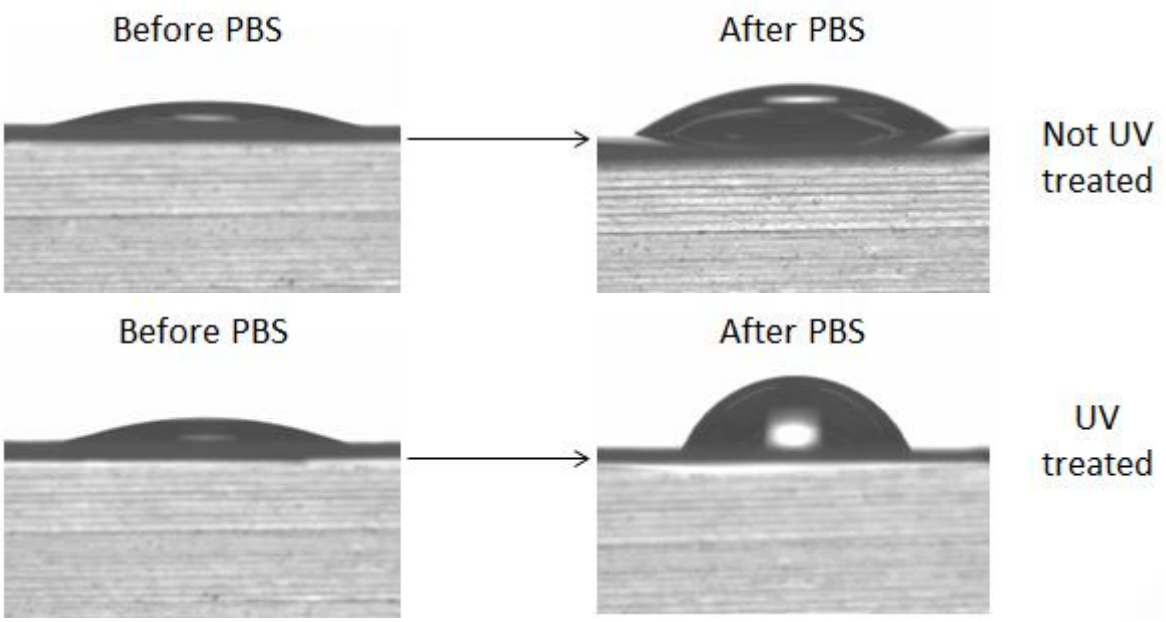


Figure 6.12: effect of UV surface treatment on the contact angle of P(PySMAAm-HEMA)50-50 coatings.

The treatment therefore didn't prove to be effective and was not repeated. The samples that showed stability were suitable for lower protein adsorption analysis.

6.5.2 P(PySMAAm-BMA) copolymers

Evaluation of the stability of these copolymer coatings was easier because of the hydrophobicity of BMA. If the contact angle after equilibration in an aqueous solution decrease, it's a sign of hydration of the polymeric coating and this can happen only if the coating is stable. Therefore the stability of P(PySMAAm-BMA) copolymers was assessed more easily compared to the other copolymer. The effect of the UV treatment on the surface of the substrates before coating was once again investigated.

- P(PySMAAm-BMA)80-20: this sample is a good example of the difference in wettability before and after the immersion in PBS: before, the sample had a relatively high contact angle (50°), which significantly decreased upon immersion in the aqueous solution (20°). The stability of the coating was confirmed.
- P(PySMAAm-BMA)50-50: the result of these samples, both the reprecipitated and the dissolved and freeze dried one, are unexpected. The contact angle both before and after the equilibration were extremely low (12° and 20° respectively). The stability of the coating was however confirmed.
- P(PySMAAm-BMA)20-80: this sample is again a perfect example of the hydration of the polymeric coating. The contact angle before was 85°, while after the immersion in PBS the wettability was significantly enhanced and the contact angle decreased to 25°. The coating was stable (Figure 6.13).
- P(PySMAAm-BMA)15-85: the behavior was similar to the 20-80 sample: high contact angle before immersion (75°) and after the hydration of the surface good wettability (27°)

An interesting fact that was noted is that the shape of the drop on the surface after PBS immersion was often not circular but ellipsoidal, a fact attributed again to the aggregation tendency of this copolymer.

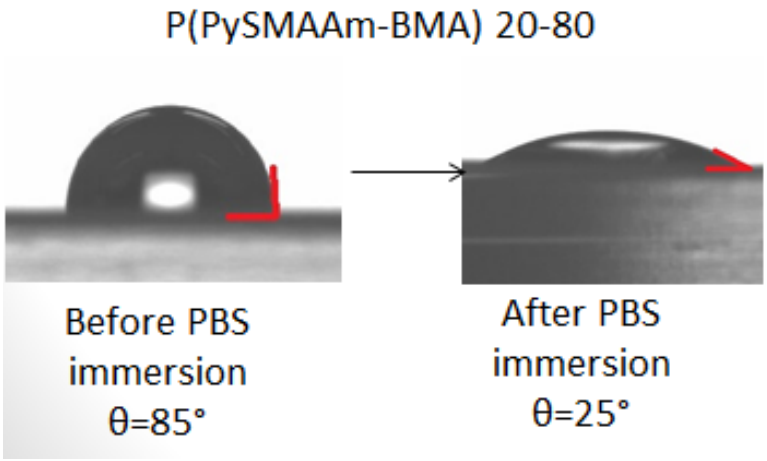


Figure 6.13: contact angle measurements of P(PySMAAm-BMA)20-80.

The effect of the UV treatment was the same as on the P(PySMAAm-HEMA) copolymer coatings: there was an increase in the adhesion between the substrate and the copolymer, but this improvement wasn't kept after the immersion in an aqueous environment.

All of the samples showed good stability to the aqueous media, therefore all of the compositions were suitable for lower protein adsorption analysis.

6.6 PROTEIN ADSORPTION EVALUATION

This analysis was the point of arrival of the present research project. In order to obtain an antibiofouling surface, adsorption of proteins has to be reduced as much as possible.

Micro BCA evaluation provided the absorbance of the coated samples and of standard solutions. There were 9 standard solution, with an increasing concentration of BSA in PBS of 0 (Blank)-0.5-1-2.5-5-10-20-40-200 µg/mL.

The average 562 nm absorbance reading of the Blank standard replicates was subtracted from the 562nm reading of all other individual standard and unknown sample replicates. With these values, it was calculated the slope in the absorbance/concentration graph and the equation of the slope was used to calculate the concentration of proteins adsorbed for each sample.

Each of the 4 coatings prepared for every composition of the copolymers occupied 2 wells of the microplate and the analysis was made with a density of three measurements per well. Therefore in the end there were 24 values of absorbance for each composition. Average and standard deviation were calculated for all the samples. These values were subsequently corrected by subtracting the Blank standard absorbance and finally put in the slope equation. The result was in [µg/mL], however it was preferred to change it to [µg/cm^2] because in papers it is usually found with this unit of measure. Since the substrates were 1X1 cm^2 and the adsorption was evaluated on both sides, dividing the result by 2 was enough to obtain the amount of BSA protein adsorbed per cm^2.

P(PySMAAm-HEMA) and P(PySMAAm-BMA) copolymer samples were compared to their respective homopolymers (P(HEMA) and P(BMA)).

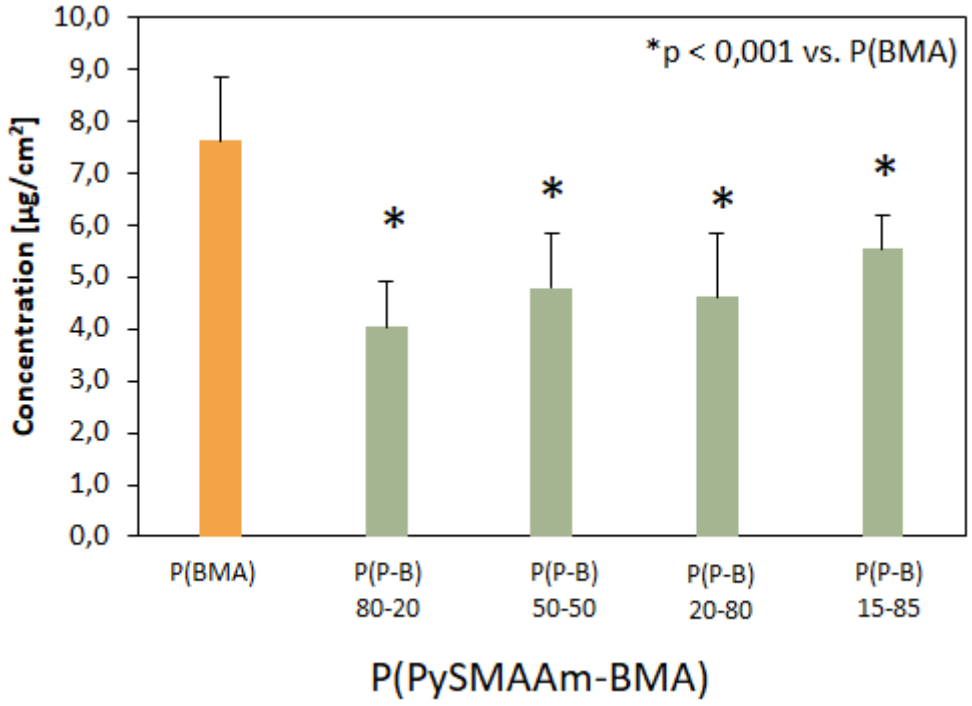


Figure 6.14: Protein adsorption of P(PySMAAm-BMA) copolymers.

P(BMA) is not an antibiofouling surface, therefore its protein resistance is not notably strong. It was observed in all of the samples a significant decrease in the protein adsorption. The effect was higher for higher content of PySMAAm, as it could be expected. The results were statistically evaluated by T-test, showing a big difference between P(BMA) and the others P(PySMAAm-BMA) copolymers and confirming the validity of the analysis (Figure 6.14).

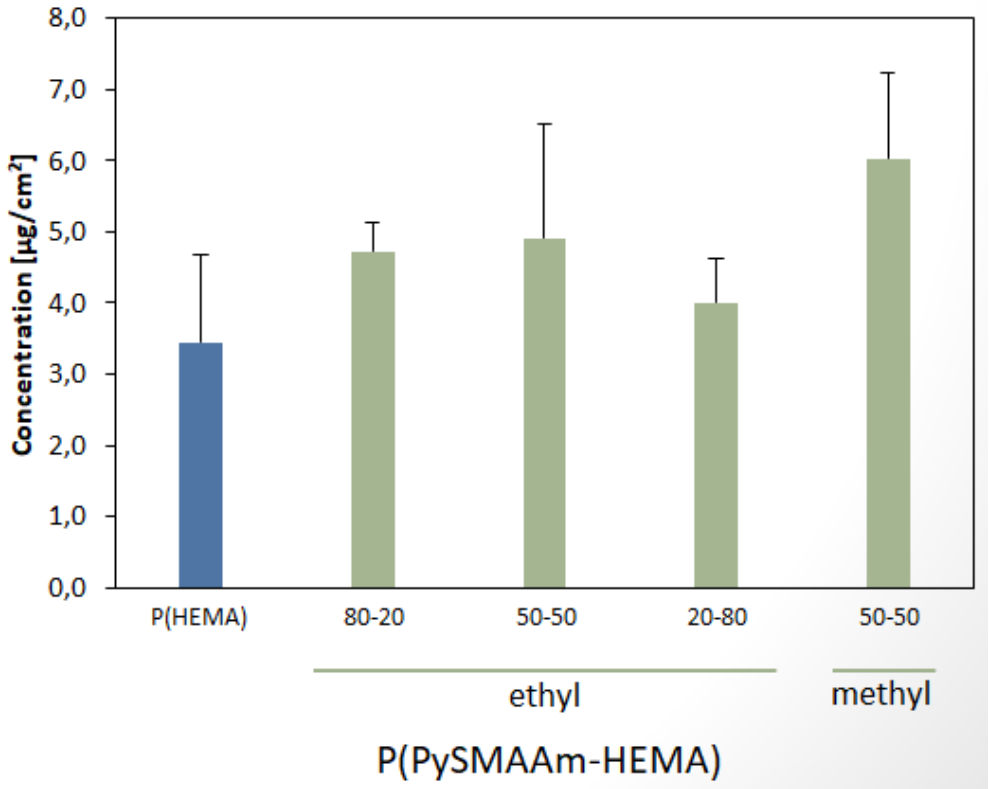


Figure 6.15: Protein adsorption of P(PySMAAm-HEMA) copolymers.

In order to discuss the results for P(PySMAAm-HEMA) copolymers it must be noted that P(HEMA) is a widespread protein resistant polymer and its values of adsorption are generally low. From the histogram it can be seen that the values of protein adsorption for the copolymers synthetized from ePySMAAm are comparable to P(HEMA) homopolymer values and show therefore good protein resistance (Figure 6.15).
The P(mPySMAAm-HEMA)50-50 sample is the only one with values of adsorbance considerably higher compared to P(HEMA), therefore it is not suitable for the creation of an antibiofouling surface. P(mPySMAAm) was also the polymer that had higher cytotoxicity, so its properties are confirmed worse than P(ePySMAAm) in the biomedical field.

The mechanism of protein resistance for sulfobetaines copolymers is ascribed to their high hydrophilicity property and net neutral charge. The copolymers are strongly hydrated, which build up a stable defensive layer to resist protein invasion.

The results were compared with protein adsorption of MPC copolymers, since those have already been widely studied and it was found out that are really good antibiofouling polymers. In one of their works, Su et al. [20] blended a MPC/BMA (3/7 mol/mol) copolymer with polyethersulfone (PES) to obtain and ultrafiltration membrane. They subsequently studied the changes in protein adsorption by varying the content of zwitterionic copolymer in the membrane as reported in Figure 6.16.

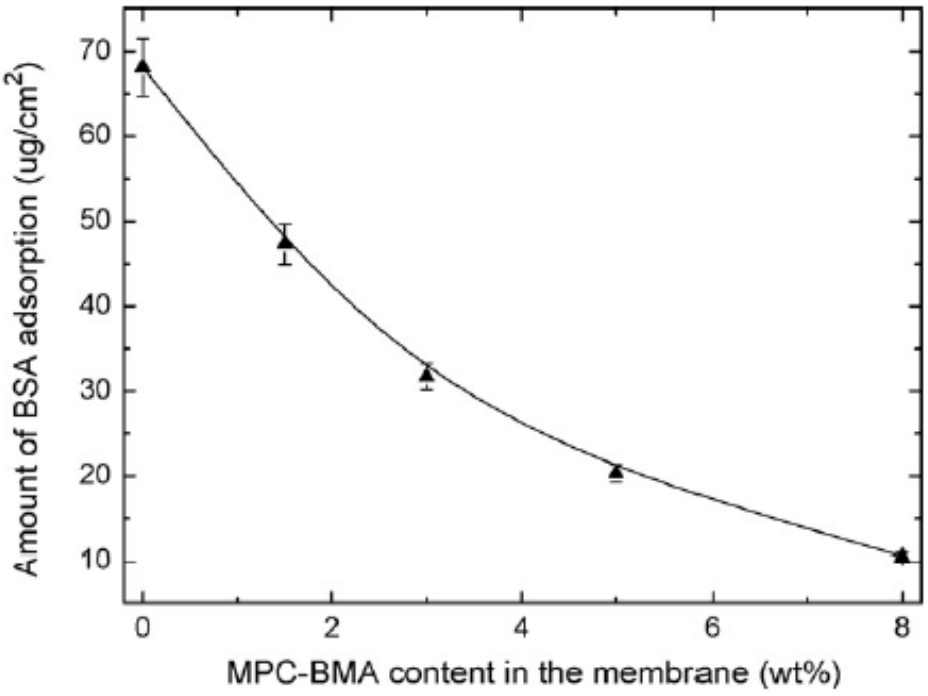


Figure 6.16: Protein adsorption amount on the MPC-modified PES membranes as a function of the MPC–BMA content in the casting solution.

It must be noted that the analysis was made after incubation of the samples at 25°C for 24 h in the 1.0mg/mL BSA solution at pH 7.0 (0.1M phosphate buffer solution (PBS)), so with different settings compared to the present analysis. After this clarification, the results can be compared: the amount of BSA adsorption on the membrane decreased drastically from 70 μg/cm^2 to 10 μg/cm^2 with an increase in the copolymer content from 0 to 8 %wt. The minimum amount of protein adsorbed is 10.6 μg/cm^2. This is higher than the adsorption of any of the copolymer tested. To have a proper comparison the analysis should be made in the same way, nevertheless the results seem promising and the antibiofouling properties of these copolymers should be further investigated.

7. CONCLUSIONS AND FURTHER DEVELOPMENT

This research project had the objective of the synthesis and characterization of a novel sulfobetaine copolymer for antibiofouling surfaces.

The synthesis process was optimized, molecular weight of the copolymers was increased almost 10 times and the yield was improved too.

The success of the copolymerization was confirmed by ^{1}H-NMR measurements, that allowed to measure the final composition of the copolymers too, showing that the P(PySMAAm-HEMA) copolymers tend to increase the quantity of HEMA in composition during the polymerization process and that P(PySMAAm-BMA) copolymers, instead, are more stable.

The stability in aqueous environment was assessed by contact angle measurements and most of the samples showed a good stability, which was necessary for the evaluation of the protein adsorption properties of the surfaces. PySMAAm copolymers exhibited low protein adsorption on the surface, therefore they could be utilized for antibiofouling surfaces.

Considering the comparison with P(HEMA), an already well-known protein resistant polymer, PySMAAm copolymers have the possibility to make one step further: even though the resistance to proteins is good for P(HEMA), its resistance to cell adhesion is not as much good. Instead, it is known that betaine surfaces can interact with phospholipids, which are found on the cell membrane, therefore PySMAAm copolymers have the potential to make for good cell resistant surfaces too.
The cell antiadhesion properties of these copolymers will have to be investigated.

Another aspect to be improved is the polydispersity of the P(PySMAAm-HEMA) copolymers, which has to be reduced; furthermore, it is necessary that the molecular weight and polydispersity of P(PySMAAm-BMA) copolymers are evaluated too, since the polymerization process of these copolymers could be further improved.

Finally, the stability and protein adsorption properties of the coatings must be further investigated, in order to identify the best composition of the copolymers for the antibiofouling surfaces.

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