Synthesis and characterization of biomimetic materials inspired by sandcastle worm

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NANYANG
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Synthesis and Characterization of Biomimetic Materials

Inspired by Sandcastle Worm

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School of Materials Science and Engineering

2016

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Table of Contents

Acknowledgements	v
List of Figures	xiii
List of Tables	xxi
List of Abbreviations	xxiii
Chapter 1: Introduction	1
1.1 Introduction to biomimetics.....	1
1.2 Marine adhesive materials	2
1.2.1 Current adhesive glues.....	3
1.2.2 Applications of under-water adhesives	3
1.3 Hypothesis and scope of this project	5
1.4 Organization of this thesis	7
Chapter 2: Literature review	9
2.1 Inspiration glues from sandcastle worm	9
2.2 Glue cement composition	11
2.3 Mechanism of formation and curing for the sandcastle worm adhesive	14
2.4 Coacervation from phase separation.....	16
2.4.1 Self - coacervation	16
2.4.2 Complex coacervation	17
2.4.3 Theory of complex coacervation	19
2.5 The role of Dopa.....	21
2.6 The role of phosphoserine	23
2.7 Synthetic mimicking	25
Chapter 3: Materials and methods	29
3.1 Protection of L-3,4-dihydroxyphenylalanine (Dopa)	29
3.2 General synthesis of N-Carboxyanhydride (NCA) monomers.....	29
3.3 ^1H , ^{13}C and ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy	30
3.4 Amino Acid Analyzer (AAA)	31
3.5 Size, zeta potential and molecular weight measurements	31
3.6 Uv-Vis spectrometry	33
3.7 Optical microscopy of coacervates	33
3.8 Rheology.....	33
3.9 Contact angle measurements	34
3.10 Quartz crystal microbalance with dissipation (QCM-D).....	35
3.11 Surface Plasmon Resonance (SPR)	36
Chapter 4: Synthesis and characterization of negatively charged co-polypeptides with tunable degrees of phosphorylation	37
4.1 Introduction	37
4.2 Experiment design	38
4.2.1 Ring opening polymerization (ROP).....	38
4.2.2 Phosphorylation	38
4.2.3 Deprotection	39
4.3 Results and discussion	41
4.3.1 Molecular weight measurements by light scattering	42
4.3.2 Amino acid composition studies by NMR	43
4.3.3 Co-polypeptide phosphorylation degree by ^{31}P NMR and AAA	46
4.3.4 Zeta potential results of the co-polypeptides	48
4.4 Conclusions	53

Chapter 5: Synthesis and characterization of positively charged co-polypeptides.....	55
5.1 Introduction	55
5.2 Experiment design	55
5.2.1 Polymerization.....	55
5.2.2 Deprotection	56
5.3 Results and discussion	58
5.3.1 Molecular weight measurements by light scattering	59
5.3.2 Amino acid composition studies	60
5.3.3 Zeta potential and size measurements	63
5.3 Conclusions	64
Chapter 6: Interaction studies of oppositely charged polypeptides	67
6.1 Introduction	67
6.2 Experimental design	67
6.2.1 Binding affinity of negatively charged co-polypeptides with Ca^{2+}	67
6.2.2 Interaction of oppositely charged polypeptides A2 and B3 by QCM-D	69
6.2.3 Interaction of oppositely charged polypeptides with metal ions and gel formation	70
6.2.3.1 Interaction of oppositely charged polypeptides A2 and B3 with FeCl_3	70
6.2.3.2 Interaction of oppositely charged polypeptides A2 and B3 with MgCl_2	71
6.2.4 Interaction of negatively charged co-polypeptide with Lanthanide ions.....	72
6.3 Results and discussion	72
6.3.1 Binding affinity of negatively charged co-polypeptides with Ca^{2+}	72
6.3.2 Interaction of oppositely charged polypeptides A2 and B3 by QCM-D.....	79
6.3.3 Oppositely charged polypeptide mixture solutions with metal ions in the gel formation	82
6.3.3.1 Interaction of oppositely charged polypeptides A2 and B3 with FeCl_3	82
6.3.3.2 Interaction of oppositely charged polypeptides A2 and B3 with MgCl_2	86
6.3.4 Luminescence resulted from the interaction of negatively charged co-polypeptide with lanthanide ions.....	89
6.4 Conclusions	91
Chapter 7: Complex coacervation studies.....	93
7.1 Introduction	93
7.2 Experiment design	93
7.2.1 Condition optimization for coacervation.....	93
7.2.2 Preparation and characterization of coacervates	94
7.2.3 Phase diagram of co-polypeptide mixtures: pH and polypeptides ratio as two leverages for tunable coacervation	95
7.2.4 PBS buffer as solvent	96
7.3 Results and discussion	97
7.3.1 Condition optimization of coacervates	97
7.3.2 Observation of coacervate and solution phase	100
7.3.3 Phase diagram of co-polypeptide mixtures: pH and polypeptides ratio as two leverage for tunable coacervation.....	102
7.3.4 PBS buffer as solvent	105

7.3.5 Characterization of concentrated complex coacervates.....	106
7.4 Conclusions	113
Chapter 8: Conclusions and outlooks	115
8.1 Conclusions	115
8.2 Future recommendations	116
References	119
Publications.....	131
Conferences.....	131
Appendix I	133

Abstract

The intertidal zone abounds of organisms who critically depend on their ability to produce water-resistant adhesives for their survival. Sandcastle worm (*Phragmatopoma californica*, *Pc*) is one of the marine organisms whose molecular-scale adhesive strategy has been revealed in the past decade. Sandcastle worm are polychaetes, which construct protective, honeycomb-like tubular structures by gathering sub-millimeter sediment particles from their surroundings that they glue together using a proteinaceous adhesive. The glue secreted from sandcastle worm can adhere to different materials under seawater environment. The major components of the secreted glue are oppositely charged proteins with highly repetitive sequences, and a key characteristic of the glue is related to its processing by the formation of complex coacervates. These characteristics make the native glue cement an intriguing mimicking model for the development of adhesive materials that could be used in wet environment. This research was aimed at developing wet-adhesive materials, with the long-term goal to be used in biomedical applications.

With a strong understanding of protein structures from native glue cement of sandcastle worm, we started with a co-polypeptide synthesis that mimicked both the side-chain of the sandcastle worm glue, with an amino acid composition that matches *Pc* glue proteins. A series of negatively charged polypeptides containing Tyr and Ser with tunable degrees of phosphorylation were developed through ring opening polymerization (ROP) of N-Carboxyanhydrides (NCAs). Co-polypeptides with variable physico-chemical properties could be prepared, including zeta potential, hydrodynamic radii, divalent ion affinity, or charge density of the colloidal suspension. Synthetic route for positively charged polypeptide containing Gly, Lys,

Di-hydroxyphenylalanine (Dopa) and Tyr were also developed and their physico-chemical properties characterized.

The metal-ligand coordination ability of our synthetic co-polypeptides was assessed, as metal coordination is increasingly recognized to play a critical role in many biological materials including in adhesion, cohesion and self-assembly. Next, the coacervation capability of our oppositely charged co-polypeptides was demonstrated, as reports have shown that the adhesive of sandcastle worms is delivered through the mixture of oppositely charged proteins into a so-called complex coacervate phase. The role of different parameters on coacervation was investigated, including pH, ionic strength, polypeptide concentrations, and polypeptide molar ratios. Tunable coacervation was developed by mixing different ratios of oppositely charged co-polypeptides at different pHs. The concentrated coacervate was subsequently characterized by rheology and contact angle measurements. The successful synthesis of oppositely charged co-polypeptides that closely mimic the composition of Pc-1, Pc-2 and Pc-3 and their subsequent processing into complex coacervates represent a first key step towards the engineering of biomimetic water-resistant adhesive materials that mimick the natural glue from sandcastle worm.

List of Figures

- Figure1-1.** Examples of living organisms living in underwater environment and that secrete bioadhesives for their survival. **(A)** Caddishfly larva. **(B)** Sandcastle worm. **(C)** Mussel displaying an extensive byssus attached to a mica surface. **(D)** Acorn barnacles living in their thatched.
- Figure2-1.** Pictures of sandcastle worm and their reef-buildings. **(A)** Reef-building with honeycomb-like outward appurtenance constructed by sandcastle worm. **(B)** Close look of sandcastle worm colony. **(C)** Sandcastle worms in and out of their worm tube. The longer white color arrow indicates the sandcastle worm's building organ, while its thoracic part is pointed out by a shorter arrow.
- Figure2-2.** Sandcastle glues with different materials: **(A)** Scanning electron micrograph (SEM) of the tube, showing particles bonded together and the glue bridges between neighboring particles. **(B)** A tube made with 0.5 mm glued glass beads. **(C)** A tube made with glued egg shells. **(D)** Silicon glued by the sandcastle worm cement. **(E)** Cortical bones glued by the sandcastle worm cement.
- Figure2-3.** Pc-1, Pc-2, and Pc-3 sequences deducted from *P. californica* cement gland cDNA with internal repeats. The underlined initial sequence denotes the signal peptide. About 64 pct. of S residues are post-translationally modified into pSer.
- Figure2-4.** Complex coacervation mechanism model for *P. californica* glue cement. **(a)** Secretion and separation processes: oppositely charged Pc proteins are secreted from their glands and quickly into phase separate through complex coacervation. **(b)** Gelation and hardening processes ensures when the coacervates enter in contact with divalent ions later with seawater. At pH 8 corresponding to seawater, Dopa residues quickly oxidize into quinone, and covalently react with His or Cys to cure the glue.
- Figure2-5.** Principle of complex coacervation. **(a)** Photograph of two phases after centrifuge with the white arrow pointing to the coacervate phase. **(b)**

- Illustration of the denser coacervate droplet observed under an optical microscope. (c) Schematic illustration of a ternary complex coacervate.
- Figure2-6.** Pathways of oxidation, adhesion and cross-linking reactions for Dopa and Dopa ortho-quinone residues. (a) Chelation of catechol side chain to a metal to form mono, bis, or tris complexes. (b) Coupling of Dopa-quinone with amine group to form an imine crosslink. (c) Crosslinking of Dopa-quinone with amine group in Michael addition reaction. (d) Formation of free-radical- species containing Dopa or Dopa-quinone. (e) Oxidation of Dopa to Dopa-quinone. (f) Dimeric cross-linked products (biaryl) from Dopa-based radicals. (g) Adsorption of Dopa to metal- or metal-oxide-bearing surfaces through hydrogen bonding or other means.
- Figure3-1.** a. Schematic strategy for NCA synthesis. b. The structures of synthesized amino acid NCAs.
- Figure4-1.** Overall synthetic route for phosphorylated co-polypeptides. Phosphorylation degree is controlled by the initial ratio of Bzl-protected Ser-NCA vs. unprotected Ser-NCA.
- Figure4-2.** ^{13}C NMR spectra of the co-polypeptides **A2** after ROP. The three peaks from left to right correspond to the signal of carbonyl groups of Tyr-O-Bzl, Ser-OH, Ser-O-Cbz, respectively.
- Figure4-3.** Composition of co-polypeptides after ring-opening polymerization of NCAs, as measured by ^{13}C NMR: (a) Target vs. measured composition. (b) Measured relative amount of unprotected Ser group vs. targeted amount of unprotected Ser (dotted is a guide line).
- Figure4-4.** (a) Degree of phosphorylation in the co-polypeptides as quantified by ^{31}P NMR after post- phosphorylation (red) and after final deprotection (blue). Degree of phosphorylation in co-polypeptide **A4** as quantified by Ninhydrin-based amino acid analysis (AAA) (see also b-c) after deprotection (magenta). (b) Phosphorylation degree of serine in polypeptide **A4** as quantified by Ninhydrin-based amino acid analysis (AAA) with different hydrolysis time. pSer content in polypeptide **A4** decreases with increasing the hydrolysis time and the pSer content in

the polypeptide is obtained by extrapolation at time zero. (c) Amino acid analyzer profiles of phosphorylated polypeptide **A4** at various hydrolysis times, showing the decay in pSer and concomitant increase in Ser with hydrolysis time.

- Figure4-5.** Zeta potential (ζ) values of phosphorylated co-polypeptides. (a) ζ values pre- and post-phosphorylation and after final de-protection. (b) Comparison of ζ values of the phosphorylated co-polypeptides after deprotection with the calculated values, assuming full de-ionization of side-chains.
- Figure4-6.** Plot of zeta potentials vs. pH for polypeptide **A2** indicating a pI of 1.62.
- Figure5-1.** Synthetic route for the positively charged polypeptide (composition: Dopa, Gly, Tyr and Lys).
- Figure5-2.** Scheme for the hydrolysis of HMDS.
- Figure5-3.** ^{13}C NMR spectra of the co-polypeptide **B3** after ROP. The four peaks from left to right correspond to the carbonyl groups of Lys-Cbz, Tyr-O-Bzl, Dopa-(Cbz)₂, and Gly-H, respectively.
- Figure5-4.** Feed ratios (mol. %) and actual amino acid composition as measured by ^{13}C NMR and amino acid analysis (AAA) mol. % of the positively charged co-polypeptide after deprotection. (a) Co-polypeptide **B1**. (b) Co-polypeptide **B2**. (c) Co-polypeptide **B3**.
- Figure5-5.** (a) Zeta potential (ζ) values of positively charged co-polypeptides after deprotection. (b) Plot of ζ vs. pH for polypeptide **B3** indicating a pI of ~ 8.9 .
- Figure6-1.** Surface plasmon resonance measurements. (a) Sensograms of the fully phosphorylated co-polypeptide **A4** (5 mg/ml). Residual signal after CaCl_2 and buffer rinse indicates affinity between the polypeptides and Ca^{2+} ions. (b) SPR sensograms of the same polypeptide (25 mg/ml), showing the influence of Ca^{2+} concentrations on the affinity. (c) SPR sensograms of the polypeptides **A4**, showing the influence of EDTA on the affinity between the polypeptides and Ca^{2+} ions. Note that in comparison to (a) and (b), the Δ signal returns to its value before the polypeptide (low concentration) injection.

- Figure6-2.** Quartz microbalance measurements of polypeptide **A4** (10 mg/mL) and CaCl_2 solution **(a)** Sensogram ($\Delta f_3/3$ vs. time) of polypeptides with loading of Ca^{2+} at various concentrations and rinsing cycles conducted with in deionized water or low pH buffer. **(b)** Change in resonance frequency Δf as a function of Ca^{2+} concentration.
- Figure6-3.** Affinity of phosphorylated co-polypeptides with Ca^{2+} ions as probed by DLS. **(a)** Initial hydrodynamic radius (black curve) of the co-polypeptides as a function of the phosphorylation degree and relative change in hydrodynamic radius upon incubation with CaCl_2 (blue curve). **(b)** Changes of hydrodynamic radius of polypeptide **A4** with addition of Ca^{2+} (0.5M). Following addition of EDTA (0.1M), the hydrodynamic radius returns to its original value, strongly suggesting that aggregation is Ca^{2+} -induced. **(c)** Schematic illustration of Ca^{2+} -induced aggregation (fully phosphorylated co-polypeptide) and recovery after adding EDTA.
- Figure6-4.** Changes of frequency ($\Delta f_3/3$) with time during quartz microbalance measurements of **(a)** polypeptide **B3** (40 mg/mL); **(b)** polypeptide **A2** (40 mg/mL); **(c)** mixture solution of polypeptides **A2** and **B3** at relative ratio of 5:5 (40 mg/mL); **(d)** mixture solution of polypeptides **A2** and **B3** at relative ratio of 9:1 (40 mg/mL). (1) = Milli-Q water rinsing; (2) = loading of polypeptide solution.
- Figure6-5.** Frequency changes ($\Delta f_3/3$) for thiol buffer modified crystal surfaces after loading of different samples followed by Milli-Q rinsing.
- Figure6-6.** **(a)** Plot of storage modulus (G') as a function of frequency for polypeptide **B3** with different Dopa to Fe^{3+} molar ratios. **(b)** Storage modulus (G') at angular frequency 50 rad/s for polypeptide **B3** with different Dopa to Fe^{3+} molar ratios. **(c)** Plot of storage (G') and loss moduli (G'') of polypeptide **B3** (100 mg/mL) with a Dopa to Fe^{3+} molar ratio of 2.5:1. **(d)** Plot of storage modulus (G') at 50 rad/s for samples with different concentrations, showing that higher polypeptide concentration increased gel stiffness.
- Figure6-7.** Rheology measurements of the mixture solutions at different **A2** to **B3** polypeptides ratios, after addition of FeCl_3 at alkali pH. **(a)** G' vs.

angular frequency. **(b)** G'' vs. angular frequency. **(c)** Damping factor vs. angular frequency. **(d)** Storage modulus (G') at angular frequency 20 rad/s vs. percentage of **B3** in mixture solutions.

Figure6-8. **(a)** Plot of storage modulus (G') as a function of angular frequency for polypeptide mixture solutions for different amounts of $MgCl_2$. **(b)** Plot of storage modulus (G') for mixture solutions ($\omega = 50$ rad/s) as a function of Mg^{2+} concentration in the mixture solutions.

Figure6-9. Rheology measurements for the mixture solutions of different ratios of oppositely charged polypeptide **A2** and **B3** after addition of $MgCl_2$ under alkali pH. **(a)** G' vs. angular frequency. **(b)** G'' vs. angular frequency. **(c)** G' at angular frequency 20 rad/s vs. percentage of polypeptide **B3** in mixture solutions.

Figure6-10. Solution of phosphorylated anionic polypeptide **A2** with 50% of Ser phosphorylated (10mg/mL) mixed with Ln^{3+} at pH 3, 7 and 10 (from left to right) under UV light of 254nm. **(a)** With addition of $TbCl_3$. **(b)** With addition of $EuCl_3$. **(c)** Fluorescence spectrum of **A2** + $TbCl_3$ at excitation wavelength 365nm. **(d)** Fluorescence spectrum of **A2** + $EuCl_3$ at excitation wavelength 390 nm.

Figure7-1. **(a)** Turbidity measurements as a function of pH for a mixture solution with 50 wt. % co-polypeptide **A2** and 50 wt. % **B3** (total polypeptide concentration: 10 mg/mL, without NaCl addition). **(b)** Turbidity measurements as a function of mixture ratios between oppositely charged polypeptide **B3** and **A2** at pH 6.8 (total polymer concentration: 5mg/mL, without NaCl addition).

Figure7-2. Turbidity measurements as a function of NaCl concentration for polypeptide mixtures at three different concentrations (polypeptide **B3** : **A2** = 60:40 wt. %, at pH 6.8).

Figure7-3. Photographs of **(a)** oppositely charged polypeptides **A2** (left) and **B3** (right) showing clear solutions. **(b-c)** Photographs of vials before and after centrifugal separation respectively, illustrating the formation of a coacervate phase at the bottom of the tube. **(d)** Optical micrograph of the mixture solution with coacervate on the glass slide. Blue arrow lines point out to coacervate droplets. **(e)** Mixture solution of

coacervate on a glass slide (with cover slide). The micrograph was taken immediately after adding NaCl to the mixture. **(f)** The same coacervate droplets as in (e) several minutes after formation, showing that the size of the coacervate droplets increased over time.

Figure7-4. Optical micrographs of: **(a)** coacervate concentrate phase on glass slide with scale bar 100 μm ; **(b)** Higher magnification view of the blue circle shown in (a) with scale bar 20 μm .

Figure7-5. Zeta potential for mixtures of positively and negatively charged polypeptides **B3** and **A2** taken at different ratio and at different pH (total polymer concentration is 40mg/mL): **(a)** Without salt. **(b)** With addition of 4.25M NaCl.

Figure7-6. Two-dimensional projection of Figure 7-5 (obtained using the “Table Curve 3D” software). Green regions correspond to domains where the overall charge is negative, whereas blue regions correspond to domains where the zeta potential is positive. The interface between green and blue represents possible conditions (mixture ratio and pH) at which zero zeta potential values are obtained, and thus where complex coacervation should be possible. **(a)** Without NaCl. **(b)** With NaCl addition (4.25 M). **(c)** Formation of complex coacervates for three mixture solutions at the conditions indicated in (b). Coacervates were obtained at pH 7, 6.8 and 4.8 at polypeptides ratio (**B3** : **A2**) of 8:2, 6:4, and 2:8, respectively (from left to right).

Figure7-7. Photographs of coacervate droplets formed after adding NaCl to a mixture of oppositely charged polypeptide **A2** and **B3** in PBS buffer solutions. **(a)** 10s; **(b)** 2 mins; **(c)** 5mins; **(d)** 15 mins. The scale bar for all images is 0.1 cm. **(e)** Coacervation solution with two separated phases.

Figure7-8. **(a)** Static contact angle on a glass and hydrophobic surfaces of the coacervate concentrate phase and comparison with the equilibrium dilute phase, the polypeptide mixture solution in non-coacervate formation conditions (**B3** + **A2**), polypeptide **B3**, polypeptide **A2**, and DI water. **(b)** Tilting experiment of the coacervate concentrate phase on hydrophobic surface, where the sample was tilted with 90 °.

- Figure7-9.** Plot of viscosity as a function of shear rate: **(a)** for coacervate concentrate phase. **(b)** for other samples: dilute phase, mixture without coacervate formation (**B3** + **A2**), polypeptide **B3**, polypeptide **A2** and DI water.
- Figure7-10.** Average viscosity for the concentrated coacervate at shear rate range 30 - 100 1/s and for the other samples: dilute phase, mixture without coacervate formation (**B3** + **A2**), polypeptide **B3**, polypeptide **A2** and Milli-Q water at shear rate range 150 - 1000 1/s.
- Figure7-11.** Frequency sweeps of concentrated coacervate phase showing storage and loss modulus as a function of angular frequency.

List of Tables

- Table 2-1.** Amino acid composition in mol. % (residues per 100 residues) determined by amino acid analysis of *P.californica* cement and deducted from cDNA for the precursor proteins, Pc-1, Pc-2 and Pc-3.
- Table 4-1.** Characteristics of synthesized polypeptides before phosphorylation.
- Figure5-1.** Characteristics of synthesized polypeptides after de-protetction.
- Table 7-1.** Phosphate buffered saline (PBS) solutions for coacervate formation.
- Table 7-2.** Surface tension of concentrated coacervates measured using the Pendant drop method and the Neumann's equation two methods.

List of Abbreviations

R	Arbitrary substituent
Cbz (Z)	Carboxybenzyl
Bzl	Benzyl
Pc	<i>Phragmatopoma californica cement</i>
ROP	Ring-opening polymerization
NCA	N-Carboxyanhydride
pSer	Phosphor-serine
Ser	Serine
Tyr	Tyrosine
Lys	Lysine
Gly	Glycine
Dopa	3,4-dihydroxyphenyl-L-alanine
pI	Isoelectric point
POCl₃	Phosphoryl Chloride
THF	Tetrahydrofuran
DI water	Deionized water
MW	Molecular weight
Ln³⁺	Trivalent lanthanide
QCM-D	Quartz Crystal Microbalance with Dissipation
GPC	Gel Permeation Chromatography
SLS	Static Light Scattering
DLS	Dynamic Light Scattering
AAA	Amino Acid Analyzer
NMR	Nuclear Magnetic Resonance
SPR	Surface plasmon Resonance
HMDS	Hexamethyldisilazane
MALDI-ToF	Matrix-Assited Laser Desorption/Ionization Time-of-Flight
DMSO	Dimethyl sulfoxide
CDCl₃	Deuterated chloroform
Pd/C	Palladium carbon
MWCO	Molecular weight cut off
PBS	Phosphate buffered saline

D₂O	Deuterium oxid
AA	Amino acid
EDTA	Ethylenediaminetetraacetic acid
PDI	Polydispersity index
% wt	Percentage in weight
Mol. %	Percentage in mole
Pct.	Percentage

Chapter 1: Introduction

1.1 Introduction to biomimetics

In recent years, terms such as “biomimicry”, “biomimetics”, “bionics”, or “bio-inspiration” have all been used to describe the concept of learning from Nature. Nature is a wonderful design partner, and provides great inspiration for many engineering development. It lights up the frontier researches in biology, physics, medicine, architecture and chemistry. Biomimetic science studies biological materials including their formation, structure and functions for the purpose of producing similar products artificially.¹⁻⁴ Biomimetics can be found in numerous examples of our daily life. For example, aircraft fuselages initially inspired by bird flights could be one of the early biomimetic design examples, which later evolved into important concepts for research and engineering applications.^{1, 5} At the molecular scale, elastin is a classical example which has been very well studied as an ideal mechanical energy storage material with long lifetime.^{6, 7} Once elastin primary sequences had been discovered, various elastin-like polypeptides have been synthesized, either by recombinant DNA expression⁸ or chemical synthesis.⁹ As another example, lotus leaf has taught us the development of waterproof or self-cleaning materials to be used in our daily life.¹⁰ Moreover, materials that are light, but still stiff and strong,^{11, 12} have been fabricated with the inspiration coming from the cellular architecture from various biological materials, including bone or wood.^{13, 14}

Biomimetics can provide sustainable solutions that can be applied in a wide range of technological fields. It is an interdisciplinary scientific subject. For example, many case studies¹⁵ have illustrated that biomimetics combines biology with

architecture for innovative design to improve the quality of our building environment. Biomimetics thus has the opportunity to provide solutions to problems for which there are currently technological limitations.

There are different approaches to biomimetics. It can be top-down, which means biomimetics by analogy, and it can also be a bottom-up approach, which can be referred to “molecular biomimetics”. Through millions of years of evolution, Nature has developed powerful peptide-based polymeric materials using the 20 standard amino acids as monomeric building blocks.¹ The discovery of such proteins has of course been a central interest of biochemists in the past decades, and has accelerated in the Genomic era,¹⁶⁻¹⁸ providing many interesting strategies for molecular biomimicry. To understand such solutions that evolved over time, it is important to fundamentally understand the multi-scale structure/properties relationships all the way down to the molecular scale.

1.2 Marine adhesive materials

In the past decades, biomimetic materials science has gained increasing interest from a dynamic community of chemists, materials scientists, and physicists, as well as life scientists. It maintains a large potential and opportunities for further development. One extensive sub-area of bio-inspired research has been the outstanding mechanical properties of many natural biological structures.^{15, 19} In this project, we are specifically interested in the synthesis of novel biocompatible protein-based adhesive materials inspired by marine organisms,²⁰ which is an area that has intrigued Material Scientists in the past two decades or so.^{21, 22}

1.2.1 Current adhesive glues

Glues or adhesives have been widely used in our daily life for a very long time. Many complex products are assembled using adhesive technology, which have been used in the manufacturing industry for many daily-usage products such as books or packaging. The initial adhesives come from nature, such as plant resin, casein, or gelatin. Later, with the development of chemical industries, synthetic adhesive materials have become the major fraction of the adhesive market. However, due to their specific properties, some biologically-based adhesive products have persisted in the marketplace. Adhesives have been diversified and currently there exists a large range of adhesives that have been developed for specific substrates and applications. However, there still remains needs for better and improved products in special applications.²³ Adhesive in medical application is one of such area where adhesion still remains a challenge. With current adhesive technologies, water or moisture acts as a surface contaminant and as weak boundary layer. The usage of medical adhesives inside of the body or in wet environment is still very limited.

1.2.2 Applications of underwater adhesives

Over the past decade a wide range of marine organisms that secrete adhesive materials have been studied, including freshwater caddisfly larva,²⁴ blue mussels,²⁵⁻²⁷ acorn barnacles,²⁸ and sandcastle worms,²⁹ (Fig. 1-1). These marine species can produce remarkable moisture resistant adhesives that effectively stick to almost any hydrated underwater surfaces. The increasing attention on underwater adhesive materials is largely motivated by clinical applications demand in areas such as orthopedics, plastic surgery, cardiology and wet living tissue repairing.³⁰ Wet-

resistant adhesives are also attractive as an alternative method for internal organs, and bone fragments or fixation of implants in the body.³¹

Figure 1-1. Examples of living organisms living in underwater environment and that secrete bioadhesives for their survival. **(A)** Caddishfly larva.³² **(B)** Sandcastle worm.³³ **(C)** Mussel displaying an extensive byssus attached to a mica surface.²⁶ **(D)** Acorn barnacles³⁴ living in their thatched.

In general, adhesives used in medical applications should have the following characteristics: (1) good adhesion on wet surface in moist environment; (2) sufficient adhesion strength, especially for bone repair applications; (3) rapid curing of the adhesive after application; (4) biocompatibility and non-poisonous reactants. Since adhesives produced by marine animals –mussels, sandcastle worms, and barnacles– are secreted and cured in a moist environment, they have a high potential as

biomimetic models for the development of adhesives in biomedical applications. Moreover, these adhesives are all protein-based, which are usually highly biocompatible. Reports have shown that the adhesives of sandcastle worm are delivered through the mixture of oppositely charged proteins into a so-called complex coacervate phase.^{22, 35} Coacervates exhibit physico-chemical characteristics that make them particularly suitable as underwater adhesives, notably their fluid-like, yet water-insoluble properties³⁶ and their low surface tension.^{37, 38}

1.3 Hypothesis and scope of this project

This study was motivated by the need for adhesives in biomedical applications, and the model system we set out to mimic is the glue secreted by the sandcastle worm. The utility of natural bioadhesive proteins extracted from marine animals is intrinsically limited by the amount that can be extracted and purified. Towards their usage in commercial applications, it will thus be important to produce them in large quantities and at reasonable cost. Recombinant technologies are limited, notably due to limitations in incorporating post-translationally modified residues, *e.g.*, 3,4-dihydroxyphenyl-L-alanine (Dopa), which is a key amino acid residue of bioadhesive formulations.³⁹ Consequently, synthetic co-polypeptides inspired from natural bioadhesive proteins have been the focus of a significant research, particularly with regard to their potential use as surgical tissue adhesives. In this report, oppositely charged co-polyelectrolytes inspired from the sandcastle worm adhesive proteins were synthesized and studied. The goal was then to achieve complex coacervation from these oppositely-charged co-polypeptides, owing that coacervation has been recognized as a key attribute of the sandcastle glue cement, as described in the Literature Review (Section 2.3).

We aim to achieve the following in this project:

Aim 1: To synthesize oppositely charged co-polypeptides inspired by adhesive Pc proteins and characterize these synthetic co-polypeptides.

The amino acid sequences of adhesive Pc proteins are known. Based on the main components of Pc-1 and Pc-2 proteins, positively charged polypeptides containing Dopa, glycine (Gly), lysine (Lys), and tyrosine (Tyr) with different ratios will be synthesized by ring-opening polymerization (ROP) of N-Carboxyanhydrides (NCAs). Similarly, negatively charged co-polypeptides containing Tyr and serine (Ser) will be synthesized and Ser residues will be phosphorylated to various degrees in order to mimic the natural glue system. These co-polypeptides will be characterized using techniques such as Nuclear Magnetic Resonance (NMR), Dynamic Light Scattering (DLS) and Static Light Scattering (SLS).

Aims 2: To study the interaction of synthetic co-polypeptides with metal cations.

The interaction of polypeptides or proteins with cations play critical role in adhesion, cohesion and self-assembly.^{40, 41} Thus, interactions of the synthesized co-polypeptides with divalent and trivalent ions such as Ca^{2+} and Fe^{3+} will be studied using various physico-chemical methods.

Aim 3: To study the coacervation behaviors of the synthetic co-polypeptides.

The coacervation behaviors of oppositely charged co-polypeptides will be studied as a function of pH, concentration, ionic strength, and co-polypeptide ratio. Coacervates will be prepared from the identified optimized conditions, following which their physical, mechanical and chemical properties will be characterized. The final goal is

to mimick the adhesive properties of the native protein cement from the sandcastle worm.

1.4 Organization of this thesis

This thesis contains 8 chapters and is organized as follows:

Chapter 1 provides brief background information to biomimetic material science and a general introduction of marine adhesive materials. This chapter also includes brief theoretical information of complex coacervation, hypothesis and scope of this thesis, and this organization.

Chapter 2 gives a thorough background literature review including the primary sequence and compositions of the sandcastle worm Pc proteins, their adhesive mechanisms, and the functionalities of some critical amino acids from the glue. This chapter also summarizes previous work aimed at mimicking the sandcastle worm glue. The novelty of this project is also emphasized in this chapter.

Chapter 3 describes the materials and techniques used in this project.

Chapter 4-7 report the major experimental results and discussions generated in this project.

Chapter 8 summarizes the significant conclusions from the experimental data and provides recommendations for possible future studies.

Chapter 2: Literature review

2.1 Inspiration glues from sandcastle worm

Phragmatopoma californica is commonly known as the “sandcastle worm”, or the “honeycomb tube worm”. The name “sandcastle” and “honeycomb” are due to the fact that they live in colonies, building tube reefs with honeycomb-like outward appearance as shown in Figure 2-1A. The colonies can cover an area of up to 2 x 2 meters.^{42, 43} Sandcastle worms reside in the intertidal zone along the coast of California. As shown in Figure 2-1C, sandcastle worms possess a crown of lavender tentacles and a dark brown body with a length of up to about 7.5 cm. Within tube reefs, sandcastle worms can protect themselves from the harsh environment along the coast. The worms remain in their tubes and are almost never seen. When the tide is low and the colonies are above the water level, sandcastle worms close the entrance to their tubes with a shield-like operculum made of dark setae. When submerged, they extend their tentacles out of the tube to catch food particles and sand grains.⁴²

Figure 2-1. Pictures of sandcastle worm and their reef-buildings. (A) Reef-building with honeycomb-like outward appurtenance constructed by sandcastle worm.⁴³ (B) Close look of sandcastle worm colony.⁴³ (C) Sandcastle worms in and out of their

worm tube.⁴² The longer white color arrow indicates the sandcastle worm's building organ, while its thoracic part is pointed out by a shorter arrow.

This intriguing creature builds its castle home by secreting a unique glue and by gathering the mineral components as sand grains and shells.²⁹ The glue is secreted from their thoracic, the first three parathoracic segments (Fig. 2-1C). The secreted glue adheres strongly, rapidly, and irreversibly to a variety of wet materials, (Fig. 2-2B-E) in seawater and the harden time takes about 30 sec. Therefore, this special glue cement has been an intriguing model for biomimetic adhesives.

Figure 2-2. Sandcastle glues with different materials:³⁵ (A) Scanning electron micrograph (SEM) of the tube, showing particles bonded together and the glue bridges between neighboring particles. (B) A tube made with 0.5 mm glued glass beads. (C) A tube made with glued egg shells. (D) Silicon glued by the sandcastle worm cement. (E) Cortical bones glued by the sandcastle worm cement.

2.2 Glue cement composition

The structure and composition of glue cement from sandcastle worm has been extensively studied. The glue is mostly proteinaceous and also contains significant levels of phosphate, calcium, and magnesium.^{44, 45} The precursor proteins of the glue cement have been isolated from the worm's thoracic glands and sequenced by Zhao and colleagues.⁴⁶ They demonstrated that the adhesive cement comprises two types of proteins with widely different electrostatic charges. The sequences of Pc proteins are shown in Figure 2-3, and the amino acid compositions determined by amino acid analysis of Pc cement is summarized in Table 2-1.

The major proteins of the first protein types are Pc-1, and Pc-2. Both Pc-1 and Pc-2 are positively charged as a result of the high amount of basic Lys amino acid residues. The iso-electric point (pI) values for Pc-1 and Pc-2 are 9.7 and 9.9, respectively, and the complete sequences of Pc-1 and Pc-2 have been sequenced by Waite *et al.*⁴⁷ Three variants have been sequenced for Pc-1. All the three variants have a molecular weight around 18 kDa, and their sequences are high repetitive, containing 15 repeats of a consensus GGYGYGG unit as shown in Figure 2-3. They consist mostly of three amino acids, Lys, Gly and Tyr, where Tyr is extensively post-translated into 3,4-dihydroxyphenyl-L-alanine (Dopa).⁴⁷ For Pc-2, currently, only one variant has been found with a mass of 21 kDa. The sequence of Pc-2 is also highly repetitive which contains a consensus HPAVHKALGGYG, but with considerable variety in connecting and flanking sequences.⁴⁶ Similar to Pc-1, most of the Tyr residues in Pc-2 exist as Dopa in the mature protein.^{46, 47}

Pc-1; M = 18,240; pI = 9.74 <u>MKFVIVLALVSAA</u> YGCGVGIGC- AGGRCGGACG GK GGYGG -K LYGAYGKGG IGGYGKG C VGGYGG LG AGK----- LGGYGG GSK CGGYGG GQK LGGYGG GKK LGGYGA AKK VGGYGG AKK VGGYGG AKK VGGYGG AKK VGGYGG AKK VGGYGG AKK VGGYGG AKK VGGYGG VKK VGGYGG ---	+
Pc-2; M = 21,226; pI = 9.91 <u>MKVLIFLATVAVYG</u> CGGAGG WRS G SCGG RWG HPAV --- HKALGGY G-G YGAHPAVHAAV HKALGGY GAGAYGAG AWG HPAV --- HKALGGY GAGA WGHPAV--- HKALGGY G-G YGAHPAV HVAV HKALGGY GAGACGHKTGG YGG YGAHP -VAV---KA--AY-NHGFNYGANN AIKSTKR FGG YGAHP -V-VK--KA FSRGL SHGAY-AGSKAATGYGYGSGKAAG YGY	+
Pc-3A; M = 13,979 <u>MKLLSVFAIVLAVYITHVEA</u> DSSSSSYSSSSSYSSSSSSSSSSSYSSSSSYSSSSSSSY SSSSSSYSSSSSYSSSSSYSSSSSYSSSSSYSSSSSILTSTSSSDW KRKVPARRVLRTTRFLKCVTRCTLR CILFR SAKTCARKCSR RCLKR VF	—
Pc-3B; M = 30,525; pI = 2.5 <u>MKSFTIFAAILVALCYIQISEAG</u> CCKRY SSSSSYSSSSSSSSSSSYSSSSSSSSSSSYSSSSSYSSSSSSSYSSSSSYSSSSSSSY SSSSSSSSSSSYSSSSSYSSSSSSSSSSSYSSSSSSSSSSSYSSSSSYSSSSSSSYSSSSSYSS SSSSSSSSSYSSSSSYSSSSSSSSSYSSSSSSSSSYSSSSSSSYSSSSSYSSSSSSSYSSSSSYSS SSSSSSSYSSSSSYSSSSSSSSSYSSSSSSSSSYSSSSSSSYSSSSSYSSSSSSSYSSSSSYSS SSSSSYSSSSSYSSSSSSSSSYSSSSSSSSSYSSSSSSSYSSSSSYSSSSSSSYSSSSSYSS	—

Figure 2-3. Pc-1, Pc-2, and Pc-3 sequences deduced from *P. californica* cement gland cDNA with internal repeats.⁴⁶ The underlined initial sequence denotes the signal peptide. About 64 pct. of S residues are post-translationally modified into pSer.

Positive charged Pc proteins show some similarities with other characterized marine adhesive proteins. One of the major similarities is the high level of Dopa and Lys in Pc-1 and Pc-2 like almost all mussel byssal adhesive proteins. Like a few mussel adhesive proteins, Pc-1 is also Gly-rich.⁴⁶ The consensus repeat of

VGGYGYGGK in Pc-1 shares many functionalities of AGYGGVK⁴⁸ repeat in fp-1 from *Aulacomya ater*, which is a species of mussels containing adhesive polyphenolic proteins.

Table 2-1. Amino acid composition in mol. % (residues per 100 residues) determined by amino acid analysis of *P.californica* cement and deducted from cDNA for the precursor proteins, Pc-1, Pc-2 and Pc-3.⁴⁶

Amino acid	Cement	Pc-1 ^a	Pc-2	Pc-3 ^a
Asp + Asn	2.8	0	2.1	0.8
Thr	2.2	0	1.6	2.2
Ser	28.5	0.6	3.7	72.9
pSer^b	(>25)			(>64)
Glu + Gln	1.4	0.6	0	0
Pro	2.7	0	3.7	0.3
Gly	26.2	45.0	27.5	0.3
Ala	9.8	6.8	19.0	0.9
Cys/2 or Cys ^c	0.4	3.6	1.6	1.0
Val	3.4	6.0	6.4	1.2
Met	0	0	0	0
Ile	0.6	1.2	0.5	0.6
Leu	3.4	3.0	3.2	2.2
Dopa^b	2.1	(9.8)	(7.3)	(?)
Tyr	4.0	18.7	9.0	10.0
Phe	1.1	0	1.6	0.9
His	3.5	0	9.0	0
Lys	4.4	13.9	6.9	2.2
Trp	ND ^d	0	2.1	0.3
Arg	2.9	0.6	2.1	4.1
Total	99.9	100	100	100

a. Pc-1 and Pc-3 compositions are calculated from the average composition of three variants for Pc-1 and seven variants for Pc-3.

b. Parenthetical levels of pSer and Dopa represent the repeats of initial Ser and Tyr modified to pSer and Dopa, respectively.^{49, 50}

c. Detected as cysteine (Cys/2) post-hydrolysis in cement but as cysteine for the deducted compositions of the three Pc proteins.

d. Not determined.

The second type of protein, Pc-3, is negatively charged. Two major variants, Pc-3A and Pc-3B, have been found together with several minor variants for the sequence of Pc-3. They are comprised of mostly Tyr and Ser, in the form of long (4 to 13) runs of Ser 4-13 punctuated with single tyrosine residues. Pc-3A variants contain 50-60 mol. % Ser with a MW of about 14 kDa. The carboxyl terminus for Pc-3A variant is highly basic.⁴⁶ Pc-3B variant has no basic amino acids at its C-terminus and contains about 90 mol. % of Ser. A salient feature is that a large fraction of serine residues is post-translationally modified into phosphoserine (pSer). The average amount of Ser (including pSer) for all Pc-3 variants is 72.9 mol. %, as shown in Table 2-1. Moreover, more than 80% of the Ser exists as pSer, resulting in the highly negatively charged Pc-3 with an extremely low pI of 2.5.

2.3 Mechanisms of formation and curing for the sandcastle worm adhesive

To explain the amazing masonry ability of sandcastle worm on wet surfaces, a mechanism model based on complex coacervation has been proposed by Stewart and co-workers in 2004.⁴⁴ As shown in Figure 2-4, the complex coacervation model has been summarized as a four-step process. The first step is the secretion of Pc proteins. Precursor proteins, Pc-1, Pc-2, and Pc-3 are water soluble polyelectrolytes in acidic environment. Upon secretion from the worm's glands into seawater, oppositely charged polyelectrolytes separate from the initial homogeneous solution into two distinct liquid phases, called the coacervate phase and the equilibrium phase (Fig. 2-4a). The phase separation process is pH-triggered and driven by charge neutralization as the proteins travel through the secretory pathway (pH 5.5) to seawater (pH 8.2). The coacervate phase containing concentrated proteins has a higher density and is not

dispersible in aqueous media. These characteristics make the coacervate spread ideally over most surfaces (Fig. 2-4b). The third step is gelation. After spreading over the surface, divalent ions, mostly Mg^{2+} and Ca^{2+} help to balance the charge and regulate solubility of the coacervate phase.⁵¹ During the gelation, phosphate groups from Pc-3 interact with Mg^{2+} or Ca^{2+} and the viscosity of coacervate increases. Following gelation, the hardening (curing) step occurs and is based on the intermolecular cross-linking, making use of the Dopa side-chains from Pc-1 and Pc-2. The catecholic Dopa side chain can form stable interactions with oxide surfaces to stabilize coacervates.⁵² Direct evidence of coupling between Dopa and the nucleophilic thiol side chain of Cys has been obtained by mass spectrometry of glue hydrolysates.⁴⁶ Furthermore, triggered by the pH shift, Dopa is oxidized into *o*-quinone, which can quickly react to form covalent cross-links with Cys and histidine (His) side-chains, hence “curing” the glue (Fig. 2-4b). Moreover, the interaction of phosphoserines with minerals such as apatite and iron oxides can also form insoluble ionic bonds.⁵³

Figure 2-4. Complex coacervation mechanism model for *P. californica* glue cement.

(a) Secretion and separation processes: oppositely charged Pc proteins are secreted

from their glands and quickly into phase separate through complex coacervation. **(b)** Gelation and hardening processes ensures when the coacervates enter in contact with divalent ions later with seawater. At pH 8 corresponding to seawater, Dopa residues quickly oxidize into quinone, and covalently react with His or Cys to cure the glue.⁵⁴

2.4 Coacervation from phase separation

Coacervation is a liquid-liquid phase separation process. During this process two immiscible liquid phases are formed from a homogeneous aqueous solution of charged macromolecules. The denser phase is the coacervate phase, which is polyelectrolyte-rich. The other phase is the dilute equilibrium phase. The major difference between coacervates and aggregation or precipitation is their physical status. The coacervate is a fluidic phase due to the water entrapped between empty sites of associated polyelectrolytes, whereas the aggregation or precipitation results in a solid phase. Coacervation was firstly observed by Gungengerg de Jong in the system of gum arabic-gelatin.⁵⁵ In the past decades, extensive research has been carried out in the area of coacervation and has attracted a wide range of applications.⁵⁶⁻⁵⁸ Due to their high fluidity and processing in aqueous environment, coacervation is extensively used for the production of pharmaceutical⁵⁹ like microcapsules for drug delivery.^{60, 61} Coacervation can also be used for film and fiber coatings,⁶² for isolation of proteins,⁶² for food processing,⁶³ and for cosmetics.⁶⁴ Coacervation can be classified into self-coacervation and complex coacervation.⁶⁵

2.4.1 Self - coacervation

Self-coacervation is a phase separation resulted from the molecular dehydration of polyelectrolytes under certain conditions.⁶⁵ The dehydration process can be

achieved by a temperature change or by addition of a dehydrating agent, like micro-ions which can promote polymer-polymer interactions over polymer-solvent interactions. The formation of simple coacervation is highly dependent on the structure of the macromolecules and on the pH environment. For example, studies⁶⁶⁻⁶⁸ have shown that gelatin molecules with a compact coil structure can give simple coacervation, and the compact coil structure is only obtained at pH value which is close to gelatin's pI value. Once the pH is far away from the protein pI, the balance of charge interactions on the molecules is distorted and the coil structure becomes unfolded due to repulsive forces. Therefore, the change of pH prevents coacervation formation. Furthermore, addition of salt can also prevent the simple coacervation formation by screening of the charged functional groups and decreasing the intramolecular attractive force. Usually, excess of salt will result in precipitation instead of coacervation.

2.4.2 Complex coacervation

Complex coacervation refers to a phase separation driven by electrostatic interactions between two or more macromolecules (Fig 2-5c). Oppositely charged polyelectrolytes form stoichiometric colloidal complexes in aqueous solutions.⁶⁹ When the net charge is adjusted towards neutrality, the complexes separate into a denser coacervate phase and a dilute equilibrium phase.^{16,18} Figure 2-5a shows an example of complex coacervation, which was prepared by mixing oppositely-charged polyelectrolytes aqueous solutions.⁷⁰ Figure 2-5b illustrates polymer-rich droplets observed by optical microscopy, depicting the coalescence of smaller droplets into larger coacervates.

Many factors including pH, ionic strength, molecular weight, concentration and mixing ratio affect the formation of complex coacervate.^{71, 72} Charge is a most critical factor. Coacervation may be suppressed at very low charge densities. At very high charge densities, precipitation or gelation may be obtained. Usually, samples with equal and opposite charges give the best coacervate yield. The mixing ratio of polyelectrolytes and pH also affect the coacervation formation, as they can alter the amount of each polyelectrolyte available for electrostatic interaction. The molecular weight of the macromolecules must fall within a critical range for complex coacervation formation.^{73, 74} If the molecular weight is too high, gels or precipitates may occur. If the molecular weight is too low, the interaction is ion pairing rather than coacervate. Low temperature ($< +20\text{ }^{\circ}\text{C}$) is favored by complex coacervation as solvent-solvent, solvent-solute, and solute-solute interactions are stronger at high temperature.⁷⁵⁻⁷⁸ Complex coacervation can either be “self-suppressed” or “salt-suppressed”. The term “self-suppressed” refers to the suppression of coacervation at high macromolecule concentrations. In concentrated mixtures, oppositely charged polyelectrolytes are in close proximity with one another and the molecular skeins may overlap, which is unfavorable for the formation of a separated coacervate phase. Self-suppression is also observed for simple coacervation. Salt suppression refers to the effect of ionic strength, which can either happen at high salt concentration or low salt concentration. At high salt concentration, the large charge density of ions around the polyelectrolytes can prevent electrostatic interaction between the polyelectrolytes.⁷² At low salt concentration, on the other hand, highly charged macromolecules will be an extended molecular conformation,^{58, 79, 80} which is again unfavorable for coacervation.

Figure 2-5. Principle of complex coacervation. (a) Photograph of two phases after centrifuge with the white arrow pointing to the coacervate phase.⁷⁰ (b) Illustration of the denser coacervate droplet observed under an optical microscope.⁷⁰ (c) Schematic illustration of a ternary complex coacervate.⁸¹

2.4.3 Theory of complex coacervation

There are several different theories of complex coacervation. The Voorn-Overbeek theory⁸²⁻⁸⁷ is the first one. This theory is developed based on the data of de Jong⁷² for gelatin/acacia complex coacervation. It has the following assumptions: (1) in both phases, polyelectrolytes are distributed in random coil chain; (2) the Huggins (solvent-solute) interactions are negligible; (3) electrostatic interactive forces

distribute uniformly in both phases and the charges are free to move. Voorn and Overbeek interpret complex coacervation as a competition between electrostatic forces and entropy effect.⁸⁸ Electrostatic forces tend to accumulate the charged macromolecules whereas entropy effect tends to disperse them.⁶⁵ Complex coacervation is deemed to be a spontaneous process driven by electrostatic interaction on mixing the oppositely charged polyelectrolytes. The water entrapped in the coacervate phase contributes to the entropy of the system, allowing a number of possible arrangements of the macromolecules⁵⁸ and resulting in the fluidity of the coacervate. The Voorn-Overbeek theory is limited to a system of polyelectrolytes with low charge densities due to the random distribution assumption.

The Veis and Aranyi theory⁷⁵⁻⁷⁷ using the “dilute phase aggregate model”, explains a practical case of coacervation between two oppositely charged gelatins, where the Voorn-Overbeek theory does not apply. The Veis and Aranyi theory assumes Huggins (solvent-solute) interaction is non-negligible. Based on the Veis and Aranyi theory, the complex coacervation process of oppositely charged gelatins has two steps. The gelatins firstly spontaneously aggregate by specific ion pairing to form neutral aggregates, following with the rearrangement of the aggregates coacervate to give phase separation. The rearrangement occurs slowly, over hours or even days.⁵⁸ It is driven by the configurationally entropy gain resulting from the formation of a randomly mixed coacervate phase.⁸⁸ Studies show that temperature reduction facilitates the coacervation occurrence in this gelatin system. The Veis and Aranyi theory is also limited to systems with low charge densities⁸⁸ since it is proposed based on the coacervation data from gelatin/gelatin with low charge densities.

The third complex coacervation theory has been developed by Nakajima and Sato as a modification of the Voorn Overbeek theory.^{73, 74} Nakajima and Sato have studied the coacervation behavior of oppositely charged poly(vinyl alcohol) macromolecules system with high charge densities. They agree with some assumptions of the Voorn-Overbeek theory in that the electrostatic interaction forces should be treated as distributed uniformly⁵⁸ in nature and charges are free to move. However, they modify the Voorn-Overbeek theory by including the Huggins interaction parameter.

The fourth complex coacervation theory has been developed by Tainaka, which is an adaptation of the Veis and Aranyi theory.^{89, 90} Tainaka and co-workers agree with the two steps mode for coacervation. However, Tainaka suggest that the aggregate pairs formed between oppositely charged polyelectrolytes are not specific charge pairings. The aggregates can be symmetrical or asymmetrical with respect to the charge and molecular size of the polyelectrolytes. In the second step, the dilute aggregates condense to form coacervates.⁹¹ In the coacervate phase, the aggregates overlap with each other resulting in electrostatic energy gain.⁵⁸ Phase separation is driven by attractive forces between aggregates.⁵⁸ The driving forces are stronger with the greater charge densities and higher molecular weights of polyelectrolytes. The Tainaka theory is more broadly used than other theories, as it is applicable to both low and high charge density systems.^{90, 92}

2.5 The role of Dopa

Among the several amino acid functionalities at the adhesive interface, Dopa (a catechol side chain) has received considerable interest.^{22, 52, 93, 94} The role of Dopa in adhesive glues is better known in Mussel-inspired proteins and peptides.^{95, 96} The

adhesive precursor proteins from mussels have been isolated and sequenced. The one common feature of all these adhesive proteins is the presence of high levels of Dopa, which is post-translationally modified from Tyr.^{22, 26} Analyses indicate that the unique bonding and adhering properties of mussel adhesive proteins should be attributed to the presence of Dopa.⁹⁷⁻⁹⁹ As shown in Figure 2-6, numerous reactions have been proposed for Dopa and its derivatives,¹⁰⁰ which illustrate the wide range role of Dopa in the cross-linking process. Intense researches have been conducted on the adsorption mechanism of catechol and catechol derivatives on metal oxide surfaces.¹⁰¹⁻¹⁰⁴ The strong chelating power of catechol for metal ions and metal oxides has been proposed to explain the extraordinary adhesives capability of the adhesive proteins (Fig. 2-6a, g).¹⁰⁵ Dopa is able to displace water and bind to hydrophobic and hydrophilic surfaces.²⁶ The contribution through Fe(III)-L-Dopa metal chelation has also been established¹⁰⁶ and shown to provide cohesive integrity of the mussel adhesive plaque.²² In addition, di-Dopa¹⁰⁷ or cysteinyl-dopa¹⁰⁸ crosslinks also contribute the cohesive integrity of mussel adhesive plaques. After secretion, the sandcastle worm glue changes from progressively more reddish to a brownish color, which suggests a Dopa-mediated covalent crosslinking. In addition, a series of adsorption studies have shown that the ortho-dihydroxy configuration gives stronger absorption comparing to meta- or para-dihydroxybenzenes.^{109, 110} Clearly, Dopa with catechol side chains is believed to be primarily responsible for covalent cross-linking and chemisorption of the polymers to substrates.

Figure 2-6. Pathways of oxidation, adhesion and cross-linking reactions for Dopa and Dopa ortho-quinone residues. **(a)** Chelation of catechol side chain to a metal to form mono, bis, or tris complexes.¹¹¹ **(b)** Coupling of Dopa-quinone with amine group to form an imine crosslink. **(c)** Crosslinking of Dopa-quinone with amine group in Michael addition reaction. **(d)** Formation of free-radical- species containing Dopa or Dopa-quinone. **(e)** Oxidation of Dopa to Dopa-quinone. **(f)** Dimeric cross-linked products (biaryl) from Dopa-based radicals. **(g)** Adsorption of Dopa to metal- or metal-oxide-bearing surfaces through hydrogen bonding or other means.¹⁰⁰

2.6 The role of phosphoserine

Besides Dopa, another post-translational amino acid in sandcastle worm cement that plays a critical role is pSer. Phosphoserine containing proteins have also been detected in other underwater bioadhesives, such as mussels¹¹² and caddisfly silks.³² It

also exists in the sticky defensive secretion of sea cucumbers¹¹² and kelp spore adhesive.¹¹³ However, there are few known adhesive precursors like Pc-3 proteins with such high amount of Ser/pSer. In the sandcastle worm glue, the high amount of pSer results in the extremely low pI value for Pc-3 proteins below 3, leading to protein chain that are able to electrostatically interact with divalent ions and to drive the initial rapid solidification⁴⁶. The coacervate phase becomes less fluid due to the strong interaction of phosphate residues from pSer with divalent ions. As a result of these interactions, the coacervate phase becomes less soluble in seawater and can start spreading over the surface.

Phosphorylated polypeptides and proteins are well-known to play critical roles in controlling and regulating biomineralization process in living organisms.¹¹⁴ Notably, the low negative charge of proteins modified by post-translation phosphorylation at Ser residues plays an important role in the crystallization of calcium phosphates (apatite) in vertebrates, where nucleation, inhibition, and growth of apatite crystals are intimately associated with specific phosphorylated proteins. For instance the high content of Ser¹¹⁵ and the extent of Ser phosphorylation in the bone matrix protein osteopontin are key in controlling apatite mineralization in bone.¹¹⁶ Dentin phosphophoryn, another protein controlling dentin mineralization that is secreted by odontoblasts, contains 75% of aspartic and serine residues where 85-95% of the serine residues are phosphorylated.¹¹⁷ Phosphoserine-rich domain from chicken phosvitin is another example like Pc-3 that contain several runs of serine as long as 14 residues.¹¹⁸ These proteins are usually involved in binding with Ca^{2+} ions and amorphous calcium phosphate and /or hydroxyapatite. The phosphate moiety has also been extensively applied in surfaces modification due to its strong and water-resistant adsorption. One example is the phosphates-containing dental materials that

adhere to dentin and enamel.^{101, 102} Another example is the use of phosphates to inhibit corrosion of iron, steel, and aluminum.¹¹⁹ Studies on competitive adsorption¹⁰¹ have shown that phosphate can compete effectively with equal concentration of catechol for adsorption onto Al_2O_3 surface and has a similar affinity.²²

2.7 Synthetic mimicking

Due to its critical role in adhesion, the amino acid Dopa has been used extensively to synthesize natural underwater glues as well as non-fouling surfaces. Messersmith *et al.* were one of the early pioneers who modified surfaces with Dopa as an anchoring group. In 2003, this group modified biomaterials surfaces with poly(ethylene glycol) (PEG) where mPEG was conjugated to Dopa –contained peptides.^{93, 120, 121} They have shown that these conjugates adsorb on the TiO_2 surfaces when at least two catechol residues are used, and resist cell attachment. Later, another class of synthetic antifouling macromolecules containing Dopa and Lys was reported.¹²² The general design of these antifouling polymers consists of a functional peptide domain for robust adsorption to surfaces, coupled to an N-substituted glycine (peptoid) oligomer of variable length that provides resistance to protein and cell fouling. Dopa has also been used to oxidatively crosslink PEG into hydrogels for tissue engineering^{123, 124} and to form a sticky layer on a structured surface to create wet/dry adhesive.^{22, 125} Later on, the catechol functionalities of Dopa was put into the backbone of polystyrene through cross-linking for adhesive applications.¹²⁶ Inspired by the pH jump experienced by adhesive proteins, soft-gels formed through catechol- Fe^{3+} inter-polymer cross-linking that are pH-dependent have been reported.¹¹¹

Inspired from the sandcastle worm glue, side chains with phosphate and amine group, in addition to Dopa, have also incorporated into water soluble polyelectrolytes

for mimicking underwater adhesion.^{35, 69, 127} Stewart and co-workers first synthesized polyelectrolyte-based adhesives inspired by the biomolecular design of the sandcastle worm glue⁶⁹. In their formulation, they employed acrylic-based co-polymers and used amine and phosphate side groups in order to confer to the polymers positively and negatively charges, respectively. Catechol was also introduced in the formulation of the negatively-charged co-polymers. In further development, amine-modified gelatin was used as positively-charged polymer analog¹²⁷. They later demonstrated the functionality of the glue in cranofacial reconstructive surgery on rat models and established that the glue was non cyto-toxic¹²⁸. In addition, coacervates prepared with 2-(methacryloyloxy)ethyl phosphate dopamine methacrylamide and Ca^{2+} , following with cross-linking with polyethylene glycol diacrylate (PEG-dA) has been reported.¹²⁹ Co-polymers containing Dopa and Lys, analogous to the natural adhesive proteins, was reported for their moisture-resistant adhesive properties.¹³⁰

Above all, most of these efforts have focused on incorporating functionalities such as catechol, phosphate, and amines into macromolecule backbones, end groups, or side chains. In this project, we aimed at mimicking both the side-chain as well as the backbone chemistry of the sandcastle worm glue by synthesizing co-polypeptides with an amino acid composition that matches Pc proteins. We employed a ring-opening polymerization (ROP) strategy from N-carboxyanhydride (NCAs) precursors to polymerize negatively- and positively- charged co-polypeptides. The negatively-charged co-polypeptides closely mimicked Pc-3 composition, being comprised of Serine (Ser) and Tyrosine (Tyr), with Ser phosphorylated to various degrees using a post-polymerization phosphorylation method. The positively-charged polypeptides were composed of Gly, Lys, Tyr, and Di-Hydroxyphenylalanine (Dopa), with Lys providing positive charge and Dopa introduced in order to provide subsequent

adhesive functionality. The introduction of Gly and Tyr was added in order to mimic their content in Pc-1 and Pc-2 proteins. In the next step, complex coacervation behavior was studied using synthetic co-polypeptides in order to mimic the native sandcastle worm glue cement. As a valuable model, synthetic adhesive glues inspired by the sandcastle worm glue proteins have the following advantages: (1) They can be made inexpensively for scale-up in potential commercial application. (2) The adhesive working environment could be “wet”. The adhesive is coacervation-based, which is not dispersible in water. (3) Adhesives are polypeptide based; one advantage is that they are generally biocompatible, although it is necessary to carefully assess it. An adhesive modeled after the sandcastle worm glue has been used to fix rat skull bones both *in vitro* and *in vivo*, and showed no cell toxicity or inflammation.¹²⁸ They did not impede new bone growth, and did not induce persistent inflammation.¹²⁸ (4) Besides all of the above advantages, co-polypeptides with a tunable phosphorylation degree as a main goal of this project will provide a way to vary the glue physico-chemical properties and to possibly tune their adhesive strengths in various applications.

Chapter 3: Materials and methods

3.1 Protection of L-3,4-dihydroxyphenylalanine (Dopa)

L-Dopa (1 equiv, 5 g, 25.35 mmol) was added to a solution of 50 mL of NaOH (0.5 M) aqueous solution. The solution was stirred vigorously under nitrogen at -10 °C for 10 min. Then, benzyl chloroformate (3 equiv, 12.97 g, 76.05 mmol) in 70 mL of Et₂O and 85 mL of NaOH (1 M) were added simultaneously into the reaction solution drop-wise over a period of 75 min.¹³¹ The reaction solution was stirred for an additional 1 hour at 0 °C, following by 2 hours at room temperature. The crystalline sodium salt was removed by filtration. The filtered solution was adjusted by citric acid (1 M) up to pH 1-2, washed with Et₂O and H₂O. The organic layer was collected, dried over Na₂SO₄, following by solvent removing by evaporation. The product was crystallized from CH₂Cl₂-petroleum ether and dried in a vacuum oven.

3.2 General synthesis of N-Carboxyanhydride (NCA) monomers

Here I described the synthesis of NCA of Z-Ser(Bzl) as an example: α -Pinene (2.4 equiv, 10.9 g, 80.16 mmol) was added to a solution of respective amino acid (1 equiv, 11 g, 33.4 mmol) in anhydrous THF (10 mL/g amino acid) under an argon atmosphere. To the reaction mixture was added triphosgene (0.4 equiv, 3.96 g, 13.36 mmol) in anhydrous THF (10 mL / 1.5 g triphosgene) and the solution stirred for 4 hours at 45 °C.¹³² The reaction mixture was concentrated *in vacuo* to 25% of initial volume, and anhydrous n-hexane (9:1) was added to the residue for crystallization of the crude product. The resulting crude product was re-crystallized twice from anhydrous ethyl acetate : n-hexane (1:9), following with drying under freeze dryer. NCAs of Z-Tyr(Bzl), Z-Ser(Bzl), Z-Ser-OH and Z-Gly, were prepared by this method.

NCA of Z-Lys(Z) and Z-Dopa(Z)₂ were prepared by this methods, except that CH₂Cl₂-petroleum ether was used for the precipitation or re-crystallization instead of hexane. All NCAs structures were verified with NMR. ¹H and ¹³C NMR spectra are provided in Appendix I.

Figure 3-1. **a.** Schematic strategy for NCA synthesis. **b.** The structures of synthesized amino acid NCAs.

3.3 ¹H, ¹³C and ³¹P Nuclear Magnetic Resonance (NMR) spectroscopy

¹H, ¹³C and ³¹P NMR spectra were obtained with a Bruker 400 NMR spectrometer. Chloroform-d or dimethyl sulfoxide-d₆ (DMSO) was used as solvent for ¹H and ¹³C NMR analyses of NCA monomers. Polypeptide samples (typically 10-20

mg/mL) in protected state were dissolved in DMSO, whereas samples after deprotection were prepared in deuterium oxide. For phosphorylated polypeptides, phospho-serine was used as an external standard for phosphorylation degree calculation.

3.4 Amino Acid Analyzer (AAA)

A Ninhydrin-based amino acid analyzer (Sykam 433, Sykam GmbH, Eresing, Germany) was used to quantify the amino acid content as well as the degree of phosphorylation of the co-polypeptides. Sample preparation: For phosphorylation degree study, deprotected co-polypeptide (5 mg) was hydrolyzed for 1 to 24 hours in a solution of 6M HCl (150 μ l) and phenol (5 μ l) under a vacuum at 110 °C. Following hydrolysis, the acid was washed using a ScanVan (Labogen) vacuum concentrator with adding 100 μ l of Milli-Q water (3x) followed by 70 μ l of methanol (2x). For the amino acid content study, the hydrolysis time was 24 hours. Sample analysis: all amino acid peak locations, including pSer and Dopa, were first calibrated using external standards and their amount quantified by peak area integration using calibrated extinction coefficients.

3.5 Size, zeta potential and molecular weight measurements

Particle size and zeta potential ζ of polypeptides were obtained using a Malvern Zeta sizer ZS (Malvern, UK) equipped with a He/Ne ion laser (633 nm). The zeta potential of initial non-phosphorylated polypeptides was measured in anhydrous ethanol at pH = 5.4 (1 mg/mL). For phosphorylated polypeptides, the zeta potential was measured in ethanol at pH = 12 (1 mg/mL), corrected by the addition of 2M NaOH. For deprotected positively charged polypeptides, the zeta potential was

measured in water at pH = 7. The pI values of polypeptide **A2** and **B3** were calculated by measuring zeta potentials of samples in water solutions (1 mg/mL) at different pH. Light scattering measurements were conducted at 25 °C with a detection angle of 173°. The molecular weight was determined by measuring the sample at different concentrations and applying the Rayleigh equation.

$$\frac{K \cdot C}{R_\theta} = \frac{1}{M_w} \cdot \left(1 + \frac{1}{3} \cdot q^2 \cdot R_g^2 \right) + 2 \cdot A_2 \cdot C \quad \text{Eq. 3-1}$$

where

$$q = \frac{4 \cdot \pi \cdot n_0 \cdot \sin(\theta/2)}{\lambda}$$

$$K = \frac{2 \cdot \pi^2}{\lambda_0^4 \cdot N_A} \cdot \left(n_0 \cdot \frac{dn}{dC} \right)^2$$

$$R_\theta = \frac{I_A \cdot n_0}{I_T \cdot n_T^2} \cdot R_T$$

$$R_T = \frac{I_0 \cdot r^2}{I \cdot f \cdot V}$$

where C : concentration of analyte, (mg/mL); M_w : molecular weight (g/mol); R_g : constant calculated at $C = 0$ slope; n_0 : solvent refractive index; dn/dC : the refractive index increment of the solution (ml/mg) which was calculated based on the measured refractive index for samples at different concentrations; A_2 : constant calculated at $\theta = 0$ slope; θ : measurement angle (degree); λ : laser wavelength (nm); N_A : Avogadro number; R_T : Rayleigh ratio of standard solution (toluene); r : distance from the point

of scattering (mm); f : compensation for polarization phenomena = 1; V : scattering volume, (L).

Six solutions in anhydrous ethanol (for protected samples) or Milli-Q water (for samples after deprotection) with concentration 0.25, 0.5, 0.75, 1, 1.5 and 2.0 mg/mL were used for molecular weight measurements of synthesized co-polypeptides.

3.6 Uv-Vis spectrometry

Turbidity during coacervate formation was measured by means of UV-Vis spectrophotometer (Nanodrop 2000, thermo scientific) at the wavelength of 600 nm and at the temperature 25 °C. The absorbance of the synthesized polypeptide solution (not in the mixture or coacervate form) was negligible at 600 nm. Turbidity was defined using the standard relation $-\ln(T/T_0)$, where T and T_0 are the light transmittance with and without sample, respectively.^{133, 134}

3.7 Optical microscopy of coacervates

Optical micrographs of the coacervate were obtained with a ZEISS, AxioCam MRc5 microscope before and after coalescence of coacervate droplets. Glass slides were used as substrates for the coacervate for all microscopic observations, which were bare or covered with another piece of glass slide.

3.8 Rheology

Rheological measurements were performed on a Physica MCR 501 rheometer (Anton Paar) fitted with parallel plate (10 mm in diameter) or cone plate (25 mm in diameter) geometry. Storage and loss modulus of oppositely charged polypeptide mixture solutions with addition of cations were measured at an amplitude τ of 0.5 Pa

in the frequency range 1-100 rad/s. Coacervate samples for the rheological measurements were centrifuged for 5 min at 10k rpm in a micro-centrifuge. After centrifugation, the dilute equilibrium phase was removed, whereas the coacervate phase was left at the bottom of the vial. Samples were loaded onto the plate and allowed to equilibrate for 2 min. Rotation test with a steady shear rate sweep was performed to measure the viscosity of the coacervate phase. Storage and loss modulus of concentrated coacervate were measured at an amplitude τ of 0.5 Pa in the frequency range 1-100 rad/s.

3.9 Contact angle measurements

Contact angle is a quantitative measurement of wetting of a solid by a liquid. Static contact angle measurements of the complex coacervate were obtained using a goniometer (Dataphysics OCA 15Pro). 3 μ L of each sample was loaded on two types of surfaces, namely hydrophilic and hydrophobic surfaces. The hydrophilic surface was bare glass, whereas the hydrophobic surface consisted of a gold-coated quartz crystal onto which a self-assembled monolayer (SAM) of HS(CH₂)₁₁CH₃ was grafted via thiol-gold attachment¹³⁵. Gold-coated quartz crystal was immersed in solution of HS(CH₂)₁₁CH₃ with a concentration of 100 μ M in 99.5% ethanol for 24 hours, in the dark, at room temperature. After incubation, the surfaces were rinsed with ethanol, ultrasonicated in ethanol for 3 min and then dried under nitrogen flow. Under sessile drop mode, three to five measurements were performed for each sample to give the average contact angle. Tilting experiments were done by tilting the sample on hydrophobic surface up to 90° following with measurements of receding and advancing contact angle measurements. Advancing contact angle (θ_a) and receding

contact angle (θ_i) are the contact angles formed by expanding and contracting the liquid, respectively.

Surface tension of the coacervate concentrate phase was measured under pendant drop model with 6 μL dispersion volume and following equation:¹³⁶

$$\gamma = \Delta\rho g d_s^2 \frac{1}{H} \quad \text{Eq. 3-2}$$

Where γ is the surface (interfacial) tension; g is the gravitational constant; ρ is liquid density; $1/H$ is correction factor depending on the results of d_e/d_s ; d_e (equatorial diameter) is the maximum diameter of droplet; d_s is the drop diameter measured horizontally at a distance d_e away from the bottom of the drop.

The density ρ of the coacervate concentrate phase was calculated by measuring the weight of certain volume of coacervates. Three measurements were done and an average density value 1.23 g/cm^3 was used for the surface tension measurement.

3.10 Quartz crystal microbalance with dissipation (QCM-D)

The QCM-D measurements were conducted using a Q-sense instrument (D-Sense), which allows for the simultaneous measurement of frequency change (Δf) and energy dissipation (ΔD) variations by periodically switching off the oscillation of the sensor crystal and by recording the decay of the damped oscillation. Changes in mass on the quartz surface are related to changes in frequency of the oscillating crystal through the Sauerbrey ($\Delta m = -C^* \Delta f$) relationship. Dissipation measurements enable qualitative analysis of the structural properties of adsorbed molecular layers. Five megahertz AT-cut quartz crystals (Q-Sense AB) were used for all experiments. The Sauerbrey sensitivity of these crystals is $1\text{Hz} = 17.7 \text{ ng/cm}^2$. The frequency and

dissipation responses were recorded at 15, 25 and 35 MHz, corresponding to the overtones, $n = 3, 5$ and 7 , respectively. Here, the normalized frequency shift ($\Delta f_{\text{normalized}} = \Delta f_3/3$) was used. All measurements were conducted at ambient temperature, and the noise level of the frequency and dissipation factor with liquid load were ~ 0.3 Hz and $\sim 2 \times 10^{-7}$, respectively. The liquid loading speed was 50 $\mu\text{L}/\text{min}$.

3.11 Surface Plasmon Resonance (SPR)

SPR is a label-free detection and surface-sensitive analytical technique based on the ability to detect dielectric constant changes induced by molecular adsorption at a noble metal film.¹³⁷ It is widely used in bio-molecular interaction studies, including determination of affinity constants and kinetic binding parameters. In this project, SPR is used to investigate the binding affinity of negatively charged co-polypeptides with Ca^{2+} . The light beam is generally directed on the opposite side of the metal film through an optical prism. The surface plasmas in the metallic film are excited if the incident angle is appropriate. The resonance angle is extremely sensitive to the polarizability and number density of the adsorbed molecules. For most biological molecules, the polarizabilities are similar, so the shift in the resonance angle is proportional to the mass of the adsorbed molecules.¹³⁸⁻¹⁴⁰

The SPR cell utilizes the Kretschmann setup for SPR measurements and consists of a holder for a 60° BK7 prism and a flow cell. The SPR surfaces consisted of 12×12 mm glass slides coated with a 45 nm thick film of gold (GE Healthcare-Biacore). Measurements were conducted at a wavelength of 741nm with a 70° angle of incidence. Sample preparation: gold substrates were prepared by refluxing for 5 min in (25%) NH_3 : (30%) H_2O_2 : Milli-Q water solution (1:1:5).

Chapter 4: Synthesis and characterization of negatively charged co-polypeptides with tunable degrees of phosphorylation

4.1 Introduction

This chapter mainly comes from my published paper entitled “Synthesis of biomimetic co-polypeptides with tunable degrees of phosphorylation”¹⁴¹ (co-first author paper).

This chapter describes the synthetic strategy towards negatively charged co-polypeptides based on the ring-opening polymerization (ROP) of N-Carboxyanhydrides (NCAs), followed by controlled phosphorylation of Ser residues. The molecular design, including amino acid composition and molecular weight of polypeptides, was mimicked after the phosphorylated protein Pc-3. The major components of negatively charged Pc-3 are Ser and Tyr, with up to 70% of Ser residues phosphorylated into phospho-serine (pSer) (Table 2-1 and Fig. 2-3). The high degree of phosphorylation shifts the isoelectric point (IEP) of Pc-3 below 3, leading to protein chains that are able to electrostatically interact with counter ions. Three NCA monomers were synthesized as described in Chapter 3, namely Ser with free -OH group, and Ser and Tyr with protected -OH groups, and subsequently polymerized with various feeding ratios in order to obtain a broad range of final amino acid composition. In the following steps, phosphorylation targeting free -OH group of Ser was conducted before deprotection. With this strategy, the degree of phosphorylation is governed by the initial amount of unprotected -OH groups of the precursor Ser-

NCA, and the final co-polypeptides contain Tyr and pSer amounts that can be tailored, yielding a composition and molecular weight that closely match Pc-3.

4.2 Experiment design

4.2.1 Ring opening polymerization (ROP)

All polypeptides were synthesized with constant molar ratios Ser : Tyr of 4:1 with targeted molecular weight of 50 kDa. Two different types of serine NCAs were used for the synthesis containing either protected or unprotected side chain -OH group. Four polypeptides with protected and unprotected -OH groups of Ser were prepared according to the following ratios: 0:1; 1:3; 1:1; 3:1. Stoichiometric amount of NCA monomers¹⁴²⁻¹⁴⁷ were dissolved in anhydrous THF. To this mixture, a calculated amount of HMDS was then added (chosen to reach a target amount of 10g) as an initiator to achieve molecular weight of 50 kDa. The reaction solution was stirred for 4 days at room temperature under argon atmosphere. Polypeptides were precipitated by hexane addition, and then dried in a vacuum oven at room temperature to give either a white powder or a viscous product depending on the polypeptide composition.

4.2.2 Phosphorylation

Polypeptides (1 g) were dissolved in 50 mL solution of THF and H₂O (4:1) under argon atmosphere. POCl₃ (10 equiv) in anhydrous THF (40 mL) and 2 M NaOH solution were added into the reaction simultaneously from two dropping funnels over 60 min. Temperature was maintained in the range 0-5 °C by means of an ice bath during one hour. Then the reaction was stirred for one more hour at room temperature. The pH was kept at 9-12 by adjusting the dropping speed of the two reagents. The organic solvent was then evaporated in a rotary evaporator at room

temperature. The concentrated solution was dialyzed against DI water for at least 24 hours. About 5 to 10 wt. % of the polypeptide was present in the form of precipitates, which was attributed to cross-linking with POCl_3 . The insoluble precipitates were removed by filtration and the final products were dried in vacuum oven for overnight at room temperature.

4.2.3 Deprotection

Polypeptide deprotection was done using a slightly modified procedure described by *Gowda et al.*¹⁴⁸ To a solution of respective polypeptide (1 equiv) in cyclohexene : ethanol (1:2; 10 mL / 1 g of polypeptide) under argon atmosphere was added 10% palladium/carbon (100% w/w) and the solution was heated to reflux for 45 min. The precipitate was collected, washed with ethanol (1 x 10 mL) and 0.1M HCl solution (3 x 10 mL). The liquid washings were collected and evaporated to give deprotected polypeptides. Schematically, the overall synthetic route of NCAs' copolymerization, phosphorylation and deprotection is presented in Figure 4-1.

Figure 4-1. Overall synthetic route for phosphorylated co-polypeptides. Phosphorylation degree is controlled by the initial ratio of Bzl-protected Ser-NCA vs. unprotected Ser-NCA.

With the above methods, the following negatively charged co-polypeptides were synthesized (the numbers in brackets represent the feed ratios of amino acid NCAs compositions in mol. %):

Co-polypeptide **A1**: Poly(Ser-co-pSer-co-Tyr) (60-20-20);

Co-polypeptide **A2**: Poly(Ser-co-pSer-co-Tyr) (40-40-20);

Co-polypeptide **A3**: Poly(Ser-co-pSer-co-Tyr) (20-60-20);

Co-polypeptide **A4**: Poly(pSer-co-Tyr) (80-20).

The amino acid composition and the degree of phosphorylation of the resulted polypeptides were characterized by NMR spectroscopy and ninhydrin-based Amino Acid Analysis. The molecular weights and zeta potential values were measured by

light scattering techniques. All these experimental procedures were described in Chapter 3.

4.3 Results and discussion

Sandcastle worm Pc-3 protein, which constitutes the mimic protein for our synthetic phosphorylated polypeptides, can be considered as a multiblock co-polymer of poly Ser intervened by regularly-spaced Tyr residues, with Ser and Tyr contents of 73 and 10 mol%, respectively.¹⁴⁹ The choice of Ser : Tyr ratios was further narrowed by considering two additional requirements: (i) the synthetic polypeptides need to be water-soluble, and (ii) we hypothesized that the polypeptides should exhibit a mostly random-coil structure with minimal crystallinity in order to be efficiently used as a precursor for complex coacervation. We performed preliminary co-polymerization synthesis with a Ser : Tyr ratio of 9:1 and discovered through DSC measurements that it led to a relatively high crystallinity close to pure poly-serine. We also prepared polypeptide with Tyr content of 30 mol. %, however this co-polypeptide exhibited low water solubility due to the high amount of hydrophobic Tyr residues. Therefore, we targeted a composition of synthetic polypeptide with a Ser : Tyr ratio of 80:20 resembling similar ratios to that of the natural Pc-3 protein.

Various initiators were attempted during polymerization, including hexamethylamine, benzylamine, trimethylamine, ethanolamine, HMDS, sodium borohydride, and sodium ethoxide. HMDS gave the best match with the target polypeptides in terms of molecular weight and amino acid ratios and has previously been shown to be an efficient initiator for NCA ring opening polymerization.¹⁵⁰ The usage of HMDS also provided good yield, high stability of polymerization, and good

agreement between the polymer composition and the monomer feed ratio in ring opening polymerization.

4.3.1 Molecular weight measurements by light scattering

In order to assess molecular weight of the polymers, various methods were tried, including GPC, LS and MALDI-Tof. With MALDI-Tof, one broad peak was observed, which prevented accurate measurements. Similarly with GPC, one broad peak was noticed. It is likely that during the MALDI measurements, poor ionization of polypeptides occurred, which is attributed to the high affinity between the phosphorylated polypeptides and the plates. Thus the MW of phosphorylated co-polypeptides is intrinsically very challenging to be measured by MALDI-ToF.

Light scattering is an alternate tool to determine the molecular weight of polymers in solution, using the method described in Section 3.5. Light scattering measurements indicated a molecular weight ranging from 15 to 20 kDa for the polypeptides (Table 4-1). This is considered relatively high for co-polypeptides synthesized by NCAs ROP, in particular for co-polypeptides synthesized from three or more monomeric units. More critically for this study, it is close to the native Pc-3 protein. It is important to note that light scattering is more suitable for polymers with very high molecular weight and it may be insensitive for low molecular weights (< 50 kDa) which do not scatter as much. By using light scattering measurement, the molecular weight may be overestimated and the PDI underestimated slightly. Based on the polydispersity value, a fraction of the polypeptides will indeed exhibit a lower molecular weight. However, the average value is still close to the target.

We note a significant decrease of MWs for polypeptides with higher feed ratios of unprotected –OH groups. . The obtained PDI for our samples by DLS technique is ranging from 0.4 to 0.85. The PDI values increase with the augmentation of serine with unprotected OH groups in ROP. This points on the possible participation of small fraction of unprotected –OH groups in ROP. By DLS instruction, samples with PDI in the range of 0.08 - 0.7 are considered as middle range polydispersity particles. Polydispersity index above 0.7 is considered as very high. We have one sample **A4** with polydispersity which is higher than 0.7. Polydispersities for other samples are in the middle range. It is also worth mentioning that at 50:50 ratio of protected : unprotected –OH groups of Ser gave the lowest crystallinity with a viscous texture of the products, whereas at high and low amount of –OH groups the polypeptides were more crystalline.

Table 4-1. Characteristics of synthesized polypeptides before phosphorylation.

Polypeptides	Polypeptides with Ser unprotected – OH groups (target), [%]	Molecular weight by SLS, [kDa]	Yield of polypeptide, [%]	Melting enthalpy (serine), [J/g]	PDI*
A1	25	22.8±2.2	85	83.9	0.476
A2	50	19.3±2.0	52	23.2	0.605
A3	75	19.2±5.7	64	38.8	0.647
A4	100	15.7±4.5	50	67.3	0.845

*PDI values were obtained by Dynamic Light Scattering measurements.

4.3.2 Amino acid composition studies by NMR

The amino acid composition of polypeptides after ROP was measured by ^{13}C NMR, using the signals from the carbonyl groups of Ser protected (Ser-O-Cbz – 172.2 ppm) and unprotected OH (Ser-OH – 172.6 ppm) groups, as well as the shift from the Tyr residue (Tyr-O-Bzl – 173.9 ppm) (Fig. 4-2) . The intensities of these distinct peaks were used to calculate the ratio of amino acids in the synthesized polypeptides and the results are summarized in Figure 4-3.

^{13}C NMR compositional measurements were verified by measuring selected co-polypeptides by Ninhydrin-based quantitative amino acid analysis. The relative differences between the two techniques were less than 5% (Fig. 4-4a). Given its ease and speed of operation, all subsequent measurements were done only by ^{13}C NMR. All four co-polypeptides exhibited a composition that closely matches the target ratio, and also resulted in a fraction of unprotected -OH group of Ser that is close to the target level (Fig. 4-3a). The amount of free -OH groups of synthesized polypeptides was slightly higher compared to the desired target, which was attributed to (i) increased reactivity during ROP of NCA with free -OH compared to its protected variant, and (ii) the probability that initial protected amino acid Cbz-protected Ser contains a small fraction (<2%) of the unprotected variant as the purity for the purchased Cbz-protected Ser amino acid is 98%. The appearance of final product obtained from dialysis bag depended on the degree of phosphorylation, ranging from solid dense agglomerate to fluffy flakes with low bulk density.

Figure 4-2. ^{13}C NMR spectra of the co-polypeptides **A2** after ROP. The three peaks from left to right correspond to the signal of carbonyl groups of Tyr-O-Bzl, Ser-OH, Ser-O-Cbz, respectively.

Figure 4-3. Composition of co-polypeptides after ring-opening polymerization of NCAs, as measured by ^{13}C NMR: **(a)** Target vs. measured composition. **(b)** Measured

relative amount of unprotected Ser group vs. targeted amount of unprotected Ser (dotted is a guide line).

4.3.3 Co-polypeptide phosphorylation degree by ^{31}P NMR and AAA

The amounts of phosphate groups after phosphorylation and final deprotection were measured by ^{31}P NMR. Ninhydrin-based AAA was also utilized after deprotection to measure the pSer content, thus providing an additional method to verify the phosphorylation content. All data are presented in Figure 4-4a. The degree of phosphorylation was slightly lower than the degree of unprotected $-\text{OH}$ groups, indicating that not all available groups were functionalized, with deprotection resulting in additional loss of phosphate groups. However, we were able to obtain phosphorylation degrees that exceed those obtained by protein recombination methods followed by kinase incubation. More critically, a wide range of polypeptide phosphorylation from $\sim 10\%$ to nearly 100% was achieved as also verified by AAA (Fig. 4-4b-c). Thus the synthetic strategy enables to tailor the phosphorylation content to a high accuracy.

Figure 4-4. (a) Degree of phosphorylation in the co-polypeptides as quantified by ^{31}P NMR after post- phosphorylation (red) and after final deprotection (blue). Degree of phosphorylation in co-polypeptide **A4** as quantified by Ninhydrin-based amino acid analysis (AAA) (see also b-c) after deprotection (magenta). (b) Phosphorylation degree of serine in polypeptide **A4** as quantified by Ninhydrin-based amino acid analysis (AAA) with different hydrolysis time. pSer content in polypeptide **A4** decreases with increasing the hydrolysis time and the pSer content in the polypeptide is obtained by extrapolation at time zero. (c) Amino acid analyzer profiles of phosphorylated polypeptide **A4** at various hydrolysis times, showing the decay in pSer and concomitant increase in Ser with hydrolysis time.

4.3.4 Zeta potential results of the co-polypeptides

The zeta potential of all polypeptides is presented in Figure 4-5. In order to clearly distinguish the effect of phosphorylation, the measurements were done at pH well-above the pK_as of all phosphate groups, thus ensuring full dissociation of –OH groups. As expected the absolute values of zeta potential increased with the degree of phosphorylation. The zeta potential ζ values were also compared to the theoretical values calculated from Eq. 4-1:¹⁵²

$$\zeta = \frac{4 \cdot \pi \cdot \eta \cdot \mu}{\varepsilon} \quad \text{Eq. 4-1}$$

where η is the viscosity of the solution, ε the dielectric constant of the media, and μ is the electrophoretic mobility, given by:¹⁵³

$$\mu = \frac{z \cdot D}{K \cdot T} \quad \text{Eq. 4-2}$$

where z is the net charge of polypeptide, K is the Boltzmann constant, T is the temperature and D is the diffusion coefficient given by:¹⁵⁴

$$D = \frac{K \cdot T}{6 \cdot \pi \cdot \eta \cdot R} \quad \text{Eq. 4-3}$$

where R is the hydrodynamic radius of the macromolecules.

We assumed full dissociation of all phosphorylated Ser residues as well as Tyr residues since the measurements were all done at pHs above the pK_as of these residues. As shown in Figure 4-5b, a close correlation is obtained between the calculated and measured values, although the measured values are systematically lower than the calculated values at high degrees of phosphorylation. We attribute

these differences to the assumption of constant solution viscosity in all cases, which is likely not the case when the phosphorylation content increases. Regardless of this small discrepancy, the measurements indicate that our phosphorylated polypeptides can exhibit a wide range of zeta potential values, thus in principle allowing to vary their colloidal stability.

Figure 4-5. Zeta potential (ζ) values of phosphorylated co-polypeptides. **(a)** ζ values pre- and post-phosphorylation and after final de-protection. **(b)** Comparison of ζ values of the phosphorylated co-polypeptides after deprotection with the calculated values, assuming full de-ionization of side-chains.

Figure 4-6. Plot of zeta potentials vs. pH for polypeptide A2 indicating a pI of 1.62.

The isoelectric point (pI) of polypeptide **A2** was obtained out by means of zeta potential measurements at different pH. As shown in Figure 4-6, the calculated pI value for polypeptide **A2** was 1.62, which is close to the target Pc-3 (pI = 2.5).

Given the well-established role of phosphorylated proteins in binding inorganic ions and regulating biomineralization processes, there is a strong interest in phosphorylated proteins and peptides for use in regenerative medicine applications. However key limitations have hindered broader usage of these biomacromolecules in such investigations. First, it is challenging to synthesize large-scale quantities of phosphorylated peptides and proteins. Extraction of *native* phosphorylated proteins is possible –although deemed difficult– for biochemical studies,¹¹⁴ though the obtained yield in the micro-gram range drastically limits their usage in the context of bio-inspired synthetic materials. Second the degree of phosphorylation is difficult to control. In fact, even when producing recombinant proteins in bacteria, studies of *in vitro* post-translation modification using kinases have shown that only 10% of the targeted Ser are phosphorylated.^{155, 156} Thus there has been a strong interest in producing artificial phosphorylated proteins and peptides, with the first synthetic phosphor-peptide reported as early as 1948.¹⁵⁷ However, most synthetic phosphorylated peptides have been limited to low MWs in the range of 2–5 kDa.

Synthetic methods to produce phosphorylated polypeptides can be classified into two main groups, which are differentiated by whether the phosphorylation reaction is achieved post- or pre-polymerization. In the first case, introduction of the phosphate groups can be obtained by targeting residues –OH group residues.¹⁵⁸ Phosphorus oxychloride (POCl₃) was perhaps the first reagent used for such a reaction and has been used in both aqueous as well as non-aqueous media.¹⁵⁸ The main

drawbacks of POCl_3 are the heat and low pH generated when POCl_3 is added to an aqueous solution, as well as side reactions such as polyphosphate formation. Phosphoric acid has also been used in conjunction with trichloroacetonitrile¹⁵⁹ and many other phosphorylating agents have been attempted.^{29, 157-169} Phosphorylation of polypeptides, however, is impeded by several obstacles. The use of simple reagents such as POCl_3 leads to complications in controlling the phosphorylation content with impeding intermolecular crosslinking occurring.¹⁵⁹ The uses of more complicated chemicals, such as dibenzyl phosphoryl chloride provide higher accuracy but do not protect the final product from undesirable secondary reactions, which are often accompanied by coloration of polypeptides. It should also be noted that conditions for phosphorylation of long macromolecule may be quite different from phosphorylation of small molecules and that the majority of previous work has been devoted to phosphorylation of small molecules and short synthetic polypeptides.^{162, 170}

The second approach to prepare phosphorylated polypeptides with targeted composition is by Fmoc-based synthesis¹⁷¹ or by polymerization of phosphorylated NCAs.^{171, 172} The former is limited to short polypeptides in the range of 10 AAs long, thus the latter method is deemed more suitable in order to prepare large MW co-polypeptides. In order to reach target degrees of phosphorylation, phosphorylated NCAs precursors are employed. However, NCA polymerization is further limited by the initial preparation of phosphorylated serine NCA, which is drastically complicated by the limited phosphoserine solubility in organic solvents,¹⁷² as also observed in our own initial attempts (data not shown). The method we have developed here combines both approaches and involved four steps: (i) synthesis of NCA (Ser(Z)-OH; Ser(Z)-OH(Bzl); Tyr(Z)-OH(Bzl); (ii) polypeptides synthesis by means of ring opening polymerization of NCAs; (iii) phosphorylation; and (iv) removal of protecting groups.

The relative straightforward approach of NCAs ring-opening polymerization was exploited to prepare large MW co-polypeptides (*i* and *ii*). Using various ratios of protected and unprotected Ser, post-polymerization phosphorylation using POCl₃ that targets free –OH groups was subsequently performed. Since the final amount of attached phosphates scales with the amount of unprotected –OH groups of Ser, this allows for the facile control of the final phosphorylation degree, as illustrated in Figure 4-1. It should be mentioned that phosphorylation was effective thanks to the use of the less conventional Z-protecting group. Other attempted protecting groups were much less efficient in protecting the polypeptides during the phosphorylation step.

As a way to illustrate the potential of controlled phosphorylation, we synthesized Ser-pSer-Tyr co-polypeptides with an amino acid composition that closely resembles that of sandcastle worm glue protein, Pc-3, meaning that similar biochemical properties as that from the native Pc-3 should be readily achievable with our synthetic co-polypeptide, including its ability to act as a polyanion precursor for complex coacervation. By varying the feed ratio and incorporating additional side-chain motifs during NCA polymerization, we can easily envision preparing co-polypeptides with higher levels of compositional complexity. The phosphate side-chain has been recognized as a critical element of most water-based adhesives³⁵ because its physico-chemical properties are deemed ideal in order to promote strong interfacial interactions in a wet environment. Likewise, the binding ability of phospho-proteins strongly depends on the level of phosphorylation. For instance phosphophoryn facilitates nucleation of apatite crystals, but non-phosphorylated recombinant phosphophoryn or de-phosphorylated native proteins are unable to do so.¹⁷³ Clearly, the ability to control the phosphate side-chain content offers an extra

level of tunability for further development of water-based adhesives and biomimetic templates.

In addition, complex coacervation often depends on commercially available polyelectrolytes.^{169, 174, 175} Consequently, whereas polypeptide concentrations, stoichiometry, and ionic strength can be varied, there is little room to maneuver with regard to changing the linear charge density of the polyelectrolytes, which is recognized as a key parameter in coacervation.¹⁶⁹ By controlling the degree of phosphorylation and by introducing specific amino acids within the polypeptide (here Tyr), charge density, zeta potential (Fig. 4-5), or peptide backbone flexibility can be tailored, in turn allowing for controlled charge-charge interactions and colloidal stability in a more systematic manner, which will be useful in future development.

4.4 Conclusions

We have described a synthetic approach in order to prepare gram-scale quantities of phosphorylated co-polypeptides with tunable degrees of phosphorylation. The synthesis was achieved by means of ring opening polymerization followed by phosphorylation of unprotected –OH groups from Ser residues, with the final amount of phosphate side groups controlled by the initial amount of –OH groups available for phosphorylation. The deprotection method was optimized such that protecting group removal occurred with minimum loss of the phosphate groups. This strategy allows tuning of the degree of phosphorylation by simple variation of the initial feed ratio of protected Ser vs. unprotected Ser NCAs. Our results show that phosphorylated polypeptides that closely mimic the composition of Pc-3 can be obtained, and with tunable degree of phosphorylation between 10 to 100%. With this method, tunable physico-chemical properties of the polypeptides can be prepared, including zeta

potential, hydrodynamic radii, or specific weight density of the colloidal suspension. Other amino acids can also be readily incorporated within the phosphorylated polypeptides. As an illustrative example, co-polypeptides were prepared by adding Tyr NCAs within the initial mixture, allowing closely mimic the molecular composition of the highly phosphorylated Pc-3 protein from the sandcastle worm glue. The ability to prepare quite high MW, phosphorylated co-polypeptides with tunable physico-chemical properties is highly desirable in order to control binding of inorganic ions and specific interactions involved in biomineralization or as a way to tailor complex coacervation processes used to prepare adhesives for hard tissue repair, which will be addressed in subsequent chapters.

Chapter 5: Synthesis and characterization of positively charged co-polypeptides

5.1 Introduction

In this chapter, the synthesis and characterization of positively charged co-polypeptides is described. Native positively charged polypeptides Pc-1 and Pc-2, which were chosen as models for our synthetic positively charge polypeptides, possess highly repetitive sequences with high amount of Gly and Tyr.¹⁴⁹ Pc-1 and Pc-2 also contain distinctive amount of 3,4-di-hydroxyphenylalanine (Dopa), which is a central theme in a wide range of marine adhesive proteins.^{96, 176} The high amount of Lys in Pc-1 and Pc-2 (13.9 mol. % in Pc-1 and 6.9 mol. % in Pc-2) may also be involved in covalent cross-linking and is responsible for the high positive zeta potential of Pc-1 and Pc-2.¹⁴⁹ Similar to the synthesis of negatively charged polypeptides in Chapter 4, positively charged co-polypeptides were synthesized by ring opening polymerization from initial NCAs followed by de-protection. The synthesis of NCA monomers has been described in Chapter 3. By feeding different ratios of NCAs, polypeptides with a broad range of final amino acid composition can be obtained.

5.2 Experiment design

5.2.1 Polymerization

Figure 5-1 summarizes the synthetic route and structure of positively charged polypeptides. Here details on the synthesis of co-polypeptide **B3** are provided as an example. Other co-polypeptides **B1** and **B2** were prepared in a similar way, with corresponding NCA monomer and initiator amounts. Positively charged polypeptide,

B3 with, a target of 30 mol. % Dopa, 50 mol. % Gly, 10 mol. % Lys and 10 mol. % Tyr, was synthesized by dissolving NCAs: Z-Tyr(Bzl)-NCA (1.1 g, 2.5 mmol), Z-Gly-NCA (3.0 g, 12.7 mmol), Z-Lys(Z) -NCA (1.2 g, 2.5 mmol), and Z-Dopa(Z)₂-NCA (4.78 g, 7.5 mmol) in distilled THF (20 mL) under argon atmosphere. To the mixture solution, HMDS (18.4 μ L) and Hexylamine (11.7 μ L) were added as initiator to achieve a molecular weight of 50 kDa. The reaction solution was stirred for 4 days at room temperature under argon atmosphere. Polypeptides were precipitated by hexane addition, and then dried in a vacuum oven at room temperature to give a viscous liquid product (6.1 g, 60%).

5.2.2 Deprotection

The polymerized polypeptide was de-protected by HBr in a TFA solution. To a solution of polypeptide (2 g) in TFA (~ 15 mL), 33% HBr in acetic acid (w/w) (4 equiv. 7.5 mL) was added with stirring. The mixture was stirred for 3 hours at room temperature under argon atmosphere. The product was quenched with Milli-Q water, and purified by dialysis with 1K MWCO for 24 hours. Water in solution was removed under freeze-drying to give de-protected positively charged polypeptides, **B**.

Figure 5-1. Synthetic route for the positively charged polypeptide (composition: Dopa, Gly, Tyr and Lys).

To mimicking Pc-1 and Pc-2 proteins, the following positively-charged co-polypeptides were designed and synthesized (the numbers in the bracket are the feed ratios of amino acid NCA compositions in mol. %):

Co-polypeptide **B1**: Poly(Dopa-co-Gly-co-Lys-co-Tyr) (10-50-30-10);

Co-polypeptide **B2**: Poly(Dopa-co-Gly-co-Lys-co-Tyr) (20-50-20-10);

Co-polypeptide **B3**: Poly(Dopa-co-Gly-co-Lys-co-Tyr) (30-50-10-10).

The amino acid compositions of synthetic positively-charged polypeptides **B1-3** were characterized by ^{13}C NMR spectroscopy and ninhydrin-based amino acid

analysis. Using light scattering techniques, the molecular weights and zeta potential were measured. Detailed experimental procedures can be found in Chapter 3.

5.3 Results and discussion

To mimick the major amino acid composition in natural polypeptides Pc-1 and Pc-2, three positively charged co-polypeptides **B1**, **B2** and **B3** were designed and synthesized. Gly and Tyr amount in co-polypeptides were fixed at 50 mol. % and 10 mol. %, respectively. From **B1** to **B3**, the Dopa amount increased from 10 mol. % to 30 mol. %. In contrast, the Lys amount decreased from 30 mol. % to 10 mol. %. Similar to negatively-charged co-polypeptide described in Chapter 4, the ratio design took into account the requirements of water solubility and of random-coil structures, the latter deemed important for bioadhesives.¹⁷⁷ Moreover, the ratio design of Dopa and Lys was narrowed down by considering the adhesion properties and charge of the resulted co-polypeptides: Dopa is well-established to be a primary moiety to promote adhesions, whereas Lys provides the positive charge to the polypeptide.

Typically, an initiator should maintain living controllable polymerization, provide low poly-dispersity, and prevents formation of side-products. Different initiators, NaBH₄, hexylamine, benzylamine, sodium ethoxide, hexamethyldisilazane and triethylamine, were used in the co-polymerization for initiator optimization. As a result of lower poly-dispersity and high molecular weight, mixture of hexamethyldisilazane and hexylamine (50:50) was used as initiator to process the ROP of amino acid-NCAs for positively charged co-polypeptides. We found that the 50:50 mixture was the best initiator. H₂O led to hydrolysis of Hexamethyldisilazane (Fig. 5-2) as evidenced by the presence of trimethylsilanol in the reaction product,

which protects ROP from the detrimental influence of moisture. In turn, hexylamine led to the lowest PDI for the co-polypeptides.

Figure 5-2. Scheme for the hydrolysis of HMDS.

Palladium/carbon in the mixture solution of ethanol and cyclohexene were initially used for the de-protection of positive charged co-polypeptides. Unfortunately, the de-protected sample was very difficult to purify as Dopa residues adsorbed on the carbon strongly, giving rise to a dark color of the product. Then, polypeptides were de-protected by HBr in TFA and product was obtained as a milky white flurry solid.

5.3.1 Molecular weight measurements by light scattering

Gel Permeation Chromatography (GPC) and Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-ToF) are effective tools to determine the molecular weight of polymers. Unfortunately, GPC did not work for our polymers, which may be due to the samples strongly adhering to the stainless steel parts of the equipment. Due to the high poly-dispersity and viscous behavior of the polypeptides, MALDI-ToF also gave unreliable results.

Light scattering is an alternate tool to that can be employed to characterize the molecular weight of polymers in solution, using the method described in Section 3.5. The results obtained for co-polypeptides **B1-3** are outlined in Table 5-1 and indicate

molecular weights ranging from 12 to 18 kDa. The molecular weight is considered relatively high for co-polypeptide synthesized by ROP of NCAs, especially for co-polypeptides with four different monomeric units. The molecular weight is also close to the molecular weight of Pc-1 (18K Da) but slightly less than Pc-2 proteins (21 kDa). The obtained PDI indicated middle range poly-dispersity particles for the synthetic polypeptides. The yield for the synthesis route ranged from 50% to 80%. These yields are considered acceptable for ROP with four monomeric units.

Table 5-1. Characteristics of synthesized polypeptides after de-protetction.

Polypeptides	Molecular weight by SLS (kDa)	Yield %	PDI [*]
B1	12.6 ± 2.4	79	0.624
B2	16.6 ± 2.9	50	0.751
B3	17.1 ± 2.1	61	0.609

* PDI values were obtained by Dynamic Light Scattering measurements.

5.3.2 Amino acid composition studies

The compositions of protected polypeptide after ROP were measured by ¹³C NMR, using the signals from the carbonyl group of Tyr (Tyr-O-Bzl – 173.8 ppm), Gly (Gly-H – 172.0 ppm), Dopa (Dopa-(Cbz)₂ – 173.5 ppm), and Lys (Lys-Cbz – 174.4 ppm) (Fig. 5-3). The intensities of these distinct peaks were used to calculate the ratio of amino acids. In parallel, ninhydrin-based Amino acid analysis (AAA) was also used to study the amino acid composition of the co-polypeptides after de-protection. The results are summarized in Figure 5-4.

Figure 5-3. ^{13}C NMR spectra of the co-polypeptide **B3** after ROP. The four peaks from left to right correspond to the carbonyl groups of Lys-Cbz, Tyr-O-Bzl, Dopa-(Cbz)₂, and Gly-H, respectively.

Figure 5-4. Feed ratios (mol. %) and actual amino acid composition as measured by ^{13}C NMR and amino acid analysis (AAA) mol. % of the positively charged co-

polypeptide after de-protection. (a) Co-polypeptide **B1**. (b) Co-polypeptide **B2**. (c) Co-polypeptide **B3**.

After ROP, the actual amount of Tyr was slightly higher than the targeted values, which may be due to its higher NCA reactivity. The actual amount of Dopa, Lys and Gly were higher than the targeted values in some polypeptide and lower in other polypeptides. This may be due to the existence of multi-monomeric NCA units, which result in the high competition of reactivity between different NCA monomers. After de-protection, the amino acid composition was generally close to their feeding amount. In particular, the amino acid composition for polypeptide **B1** and **B3** were close to the target ratios.

The multi-amino acid composition of our co-polypeptide led to challenges in precisely matching the target amino acid composition. However, synthesis of co-polypeptides with more than three amino acids and a high molecular weight has not been widely reported.^{178, 179} Furthermore, we managed to achieve co-polypeptides with distinct relative ratios of Gly, Tyr, Lys, and Dopa, thus providing us with a sufficient range of co-polypeptides for further experiments. Therefore the results obtained with our synthesis strategy are considered successful towards the next step of complex coacervation. Beside co-polypeptides containing Dopa, Lys, Gly and Tyr, we also attempted the synthesis of co-polypeptides with one more component, namely His or Cys. However, the resulted amino acid composition was far away from the target amount and these co-polypeptides were not considered further.

5.3.3 Zeta potential and size measurements

Results of zeta potentials and size measurements of co-polypeptides **B1-3** are shown in Figure 5-5 (a). The charges of the polypeptides are highly dependent on the pH. Samples were dissolved in water and pH was adjusted to 7 by using 6M NaOH. As expected, the zeta potential gradually decreased from polypeptide **B1** to **B3**, in line with the corresponding amount of Lys residues. Polypeptide **B1** with the highest Lys amount gave the highest zeta potential, whereas polypeptide **B3** with the least amount of Lys has the lowest value.

Figure 5-5. (a) Zeta potential (ζ) values of positively charged co-polypeptides after deprotection. (b) Plot of ζ vs. pH for polypeptide **B3** indicating a pI of ~ 8.9 .

The isoelectric point (pI) of polypeptide **B3** was obtained by means of zeta potential measurement of the sample solutions at different pHs. As shown in Figure 5-5 (b), the calculated pI value for polypeptide **B3** was 8.9, which is relatively close to native Pc-1 (pI = 9.7) and Pc-2 (pI = 9.9) proteins.

Due to the well-established role of Dopa residues in adhesion and cohesion of bioadhesives, there is a strong interest to prepare Dopa-containing proteins or

polypeptides for wet-resistant bioadhesives. However, extraction of native Dopa-containing proteins is highly limited in the yield and amount that can be extracted and purified. Another option is to produce recombinant proteins in bacteria, following by post-translation modification using the tyrosinase enzyme.¹⁸⁰⁻¹⁸² However, it is challenging to control the obtained amount of Dopa and the post-translational modification is time-consuming. With our method, we developed a synthetic route to prepare gram-scale quantities of co-polypeptide with a good control of Dopa content (Fig. 5.3). Our co-polypeptides exhibited an amino acid composition that is relatively close to that of the sandcastle worm glue proteins, Pc-1 and Pc-2. Therefore, our synthetic positively-charged co-polypeptides should exhibit similar biochemical properties as that from native Pc-1 and Pc-2. Furthermore, by varying the feed ratio of Dopa and Lys during NCA polymerization, co-polypeptides with varying iso-electric points could be prepared. In summary, we have successfully prepared co-polypeptides with tunable Dopa content and iso-electric point values. Dopa plays a critical role in many water-resistant adhesives (Section 2.5) whereas charge density, polypeptide concentration and stoichiometric ratios of oppositely-charged polyelectrolytes are key parameters during complex coacervation. The critical building blocks for subsequent biomimetic adhesives have thus been established.

5.3 Conclusions

In conclusion, a synthetic approach has been described for the preparation of gram-scale quantities of positively charged co-polypeptides which are inspired by the wet adhesive ability of the cement proteins from sandcastle worm *P. californica*. The synthesis was based on ROP of NCAs followed by de-protection. This method allows the synthesis of polypeptide to closely mimic the molecular composition of native Pc-

1 and Pc-2 proteins from the sandcastle worm and tuning the ratio of each amino acid composition by adding different initial feed amount of protected amino acid NCAs. With this method, we synthesized three positively charge polypeptides **B1-3** with different physico-chemical properties that will be used for complex coacervation studies in subsequent Chapters.

Chapter 6: Interaction studies of oppositely charged polypeptides

6.1 Introduction

Synthetic co-polypeptides are targeted as templates for biomineralization or as bioadhesives for hard tissues. In both cases, a key requirement is that the co-polypeptides are able to exhibit specific affinity towards divalent cations.^{35, 46, 114} Moreover, metal-ligand coordination plays critical roles in many biological materials including adhesion and self-assembly. In this chapter, the binding affinities of negatively charged co-polypeptides with Ca^{2+} were studied. Next, interactions between oppositely charged polypeptides **A2** and **B3** in the presence of different metal ions were carried out in order to establish whether metal-ligand coordination found in nature could also be achieved with our synthetic co-polypeptides. Finally, given the previously established affinity of phosphorylated peptides towards lanthanides ions,^{183, 184} we were also interested in exploring whether our phosphorylated co-polypeptides could specifically interact with such ions, thus providing the basis for future work.

The experiments, results and discussions for section of “Binding affinity of negatively charged co-polypeptides with Ca^{2+} ” are mainly from my published paper entitled “Synthesis of biomimetic co-polypeptides with tunable degrees of phosphorylation”.¹⁴¹

6.2 Experimental design

6.2.1 Binding affinity of negatively charged co-polypeptides with Ca^{2+}

Negatively charged polypeptide **A4** with 100% phosphorylation was used for the binding affinity study with Ca^{2+} . SPR and QCM-D measurements were initially conducted to assess their specific interactions, followed by DLS measurements. For SPR measurements, solution A was prepared by adding 250 μL HCl (37%) into 50 mL of Milli-Q water. 0.5M and 0.05M CaCl_2 solutions were prepared in solution A. Similarly, different amount of polypeptides **A4** were dissolved in solution A to give 5 mg/mL and 25 mg/mL solutions. Prior to measurement, all prepared polypeptide solutions were thoroughly filtered with 0.2 μm syringe filter.

SPR measurements: gold-coated surfaces were first rinsed with buffer solution A at a 30 $\mu\text{L}/\text{min}$ flow rate. Then, the polypeptide solution was loaded at the same rate onto the gold surface. After 10 mins, the coated surface was rinsed with the buffer solution A, followed by loading of the CaCl_2 solution and final rinsing with buffer solution A. The change of refractive index during the whole process was monitored in terms of Δ vs. time, where Δ is the phase shift of $-p$ and $-s$ components of polarized light.

For QCM-D measurements, polypeptide **A4** was dissolved in acid buffer solution A ($\text{pH} = 1.3$) with a concentration of 10 mg/mL. Different amounts of CaCl_2 were dissolved in acid buffer A to give 0.1 M, 0.2 M and 0.5 M solutions. The loading sequence was following as: (i) loading polypeptide; (ii) loading CaCl_2 with 0.1 M concentration; (iii) loading CaCl_2 with 0.2 M concentration; (iv) loading CaCl_2 with 0.5 M concentration. Before polypeptide sample solution and every CaCl_2 solutions loading, the gold-coated sensor crystal surface was rinsed with buffer A solutions. This QCM-D measurement was also repeated using Milli-Q water as solvent.

Upon addition of CaCl_2 , changes in particle size of polypeptides were detected. Solutions of de-protected co-polypeptide **A1-4** were dissolved in 1 mL Milli-Q water with 10 μL of HCl (37%) at concentration of 5 mg/mL and the particle size of polypeptide was measured by DLS before addition of CaCl_2 . To 1mL polypeptide solution, 0.5 mL of CaCl_2 solution (5 mg/mL) was added directly into the cuvette and three to five subsequent measurements were conducted with 60 sec intervals between measurements. To polypeptide **A4** solution with addition of CaCl_2 , EDTA (0.1M) solution was added following with its hydrodynamic radius measurement.

6.2.2 Interaction of oppositely charged polypeptides **A2** and **B3** by QCM-D

The surface of gold-coated AT-quartz crystal was firstly modified using thiol solution with positively charged ending groups. 100 μM thiol solution was prepared by dissolving 2.6 mg of (11-mercaptopoundecyl)-N,N,N-trimethylammonium bromide into 80 mL of anhydrous ethanol. The gold-coated quartz crystal was immersed in the thiol solution for 24 hours and covered with aluminum foil. The modified quartz crystal was washed twice with ethanol, following with sonication in ethanol for 5 mins. The crystals were dried with N_2 gas blowing.

Polypeptides **A2** and **B3** were prepared at concentrations of 40 mg/mL by dissolving the corresponding amount of polypeptide in 1mL of Milli-Q water. In addition, two mixture solutions were prepared by mixing oppositely charged polypeptides **A2** and **B3** solutions (40 mg/mL) with volume ratio of **A2** : **B3** at 5:5 or 9:1. The quartz crystal surface with an amine functionalized surface was first rinsed with Milli-Q water following with rinsing of polypeptide containing solutions. After loading polypeptide solution, the surface was left for 10 - 15 mins before water rinsing. Changes in frequency were recorded with time.

6.2.3 Interaction of oppositely charged polypeptides with metal ions and gel formation

Growing evidence supports a critical role of metal-ligand coordination in many attributes of biological materials including in self-assembly, adhesion, toughness, and strengthening without mineralization.^{111, 185} Here, Fe^{3+} and Mg^{2+} were used to investigate their metal-ligand coordination capability with our synthetic oppositely charged polypeptides **A2** and **B3**. The amount of metal ions was firstly optimized for the individual co-polypeptides using rheology measurements, followed by studies of the role of oppositely charged polypeptides ratio on gel formation.

6.2.3.1 Interaction of oppositely charged polypeptides A2 and B3 with FeCl_3

Influence of Dopa : Fe^{3+} ratio on gel formation for positively charged polypeptide B3.

Polypeptide **B3** was prepared at 40 mg/mL in Milli-Q water and FeCl_3 was prepared at 800 mM in Milli-Q water. During rheology measurements, corresponding volume of FeCl_3 was added into 70 μL polypeptide **B3** solutions to prepare different ratios of Dopa to Fe^{3+} ranging from 1:1 to 12:1. Dopa and Fe^{3+} ratios were calculated theoretically using the target amount of Dopa from polypeptide **B3**. 10 μL of NaOH (6M) was added into the mixture solution for pH adjustment above 10. The mixed samples were measured under a steady shear rate at an amplitude τ of 0.5 Pa in the frequency range 1-100 rad/s.

*Concentration influence of positively charged polypeptide **B3** in the formation of gel*

Several polypeptide **B3** solutions at different concentration, namely 4 mg/mL, 25 mg/mL, 40 mg/mL, 100 mg/mL and 200 mg/mL were prepared. Similar conditions were used to study the role of concentration at a fixed Dopa : Fe³⁺ ratio of 2.5:1.

Influence of ratio of oppositely charged polypeptides

Mixture solutions were prepared by mixing polypeptide **A2** and **B3** with ratios ranging from 1:9 to 9:1, with 0.2 increments. The total polypeptide concentration for all mixed solutions was 40 mg/mL. FeCl₃ at concentration of 800 mM was used and Dopa : Fe³⁺ ratio was fixed at 2.5:1. Rheology measurements for these mixture solutions were done similarly as described above. Results are shown in Figure 6-7.

6.2.3.2 Interaction of oppositely charged polypeptides A2 and B3 with MgCl₂

Interaction of oppositely charged polypeptide **A2** and **B3** with MgCl₂ was studied in a similar way as with FeCl₃. Polypeptide mixture solution (**A2** : **B3** = 3:7) was prepared at 40 mg/mL in Milli-Q water, and MgCl₂ was prepared in 800 mM in Milli-Q water. Different amount of MgCl₂ (5 µL, 7.8 µL, 10 µL, 15 µL, 18 µL) was added into 70 µL of the polypeptide mixture solutions, with addition of 10 µL NaOH (6M) for pH adjustment, followed by rheology measurements.

After optimization of MgCl₂ amount, the influence of ratio of oppositely charged polypeptides was studied. Polypeptide mixture solutions of oppositely charged polypeptides were prepared at **A2** : **B3** ratios ranging from 1:9 to 9:1 with 0.2 increments. The total polypeptide concentration for all mixed solutions was 40

mg/mL. A fixed volume of 15 μL of MgCl_2 (800 mM) was added into 70 μL of the polypeptide mixture solutions with addition of 10 μL NaOH (6M) for pH adjustment. Rheology measurements for these mixture solutions were done similarly as described above.

6.2.4 Interaction of negatively charged co-polypeptide with Lanthanide ions

Negatively charged polypeptide **A2** with 50% of Ser phosphorylated was prepared in Milli-Q water with 10mg/mL concentration. Lanthanide ions (LaCl_3 , TbCl_3 and EuCl_3) were dissolved in Milli-Q water at concentration of 13 mM. The polypeptide and lanthanide ions were mixed with a relative ratio of $\text{PO}_4^{2-} : \text{Ln}^{3+}$ of 3:2. The pH of mixed solution was adjusted from 3 to 10 with the addition of NaOH (4 M). Mixed solutions at three pH values (3, 7 and 10) were characterized. UV light at wavelength of 254 nm was used for the fluorescence study.

6.3 Results and discussion

6.3.1 Binding affinity of negatively charged co-polypeptides with Ca^{2+}

We first conducted SPR measurements as a means to assess specific interactions between the polypeptides and Ca^{2+} ions.¹⁸⁶ Measurements were all conducted on 100% phosphorylated polypeptide **A4** using concentrations of 5 mg/mL and 25 mg/mL, respectively. The polypeptides were first adsorbed onto the SPR gold-coated chip before CaCl_2 solution was flown over the chip. The SPR response of a complete sequence is shown in Figure 6-1 (5 mg/ml polypeptide concentration) and consists of (i) buffer rinse; (ii) loading polypeptide; (iii) buffer rinse; (iv) loading of CaCl_2 ; and (v) regeneration with EDTA. The change in SPR signal (Δ signal) after loading the polypeptide (Fig. 6-1a) indicates adsorption of polypeptide onto the gold

surface. After flowing of CaCl_2 , a clear interaction between the polypeptide and Ca^{2+} ions was observed, as indicated by the increase in SPR signal intensity. Higher polypeptide concentration (25 mg/mL) resulted in a higher adsorption on gold (Fig. 6-1b), but upon application of 0.05 M CaCl_2 the interaction of polypeptide and Ca^{2+} did not increase. However, increased binding was observed when the concentration of CaCl_2 was increased up to 0.5 M, which is consistent with the large amount of phosphate groups available for calcium binding at higher concentrations of polypeptide. It is also worth mentioning an unusual feature of our phosphorylated polypeptide: upon loading on the SPR chip, the phosphorylated polypeptide displayed a higher adsorption on the unmodified gold surface than on the modified surface with amine or carboxylate groups, which may be related to the high affinity of phosphate side chains to a wide variety of solid surfaces.³⁵

Figure 6-1. Surface plasmon resonance measurements. **(a)** Sensograms of the fully phosphorylated co-polypeptide **A4** (5 mg/ml). Residual signal after CaCl_2 and buffer rinse indicates affinity between the polypeptides and Ca^{2+} ions. **(b)** SPR sensograms of the same polypeptide (25 mg/ml), showing the influence of Ca^{2+} concentrations on the affinity. **(c)** SPR sensograms of the polypeptides **A4**, showing the influence of EDTA on the affinity between the polypeptides and Ca^{2+} ions. Note that in comparison to (a) and (b), the Δ signal returns to its value before the polypeptide (low concentration) injection.

As a control experiment, an SPR measurement was conducted in which an additional step of EDTA flow was conducted before the final wash (Fig. 6-1c) in order to chelate Ca^{2+} ions bound to the polypeptide. In contrast to washing without

EDTA treatment, the Δ signal intensity returned to its initial value before CaCl_2 loading, confirming that Ca^{2+} ions interact with the phosphorylated polypeptide.

SPR measurements were complemented with QCM-D experiments, which monitored the phosphorylated polypeptide/ Ca^{2+} ions affinity in terms of mass changes on the quartz micro-crystal during loading and rinsing of Ca^{2+} solutions. Different concentrations of Ca^{2+} were used to observe the influence of the polypeptide : Ca^{2+} ratio on the binding affinity, and the experiments were performed using either deionized water or pH = 1.3 solution for the rinsing steps (Fig. 6-2). The decrease of frequency after loading polypeptide solution indicated the adsorption of polypeptide on the gold-coated crystal surface. Overall, the QCM-D data are in agreement with SPR results, showing a clear interaction of polypeptide with Ca^{2+} ions as observed by the resonance frequency decrease during loading of the CaCl_2 solution onto the adsorbed polypeptide. The frequency change increased with the Ca^{2+} concentration (Fig. 6-2b), denoting the high affinity potential of highly phosphorylated polypeptides for Ca^{2+} . When deionized water was used as rinsing solution, the resonance frequency variation was more pronounced in comparison to rinsing with an acid buffer solution, which is consistent with the increased level of dissociation of hydroxyl and phosphate side-chains at lower pH. After loading of CaCl_2 and rinsing, the resonance frequency of the QCM decreased slowly, which may be due to time-dependent molecular re-arrangement of polypeptide when they dynamically interact with Ca^{2+} ions.

Figure 6-2. Quartz microbalance measurements of polypeptide **A4** (10 mg/mL) and CaCl_2 solution (a) Sensogram ($\Delta f_{3/3}$ vs. time) of polypeptides with loading of Ca^{2+} at various concentrations and rinsing cycles conducted with in deionized water or low pH buffer. (b) Change in resonance frequency Δf as a function of Ca^{2+} concentration.

To further corroborate any observed phosphorylated polypeptide/ Ca^{2+} interactions, we conducted Dynamic Light Scattering (DLS) experiments and monitored the variation of polypeptide hydrodynamic radius in water upon addition of Ca^{2+} ions. Polypeptide **A1-4** with phosphorylation degree ranging from 25% to 100% was used for the interaction study. DLS measurements are summarized in Figure 6-3(a) and indicate the following trends. First, for the 100% phosphorylated polypeptide **A4**, the initial particle size was monodispersed with an average size of 100 to 150 nm. These values are larger than would be expected from a 16 kDa MW polypeptide (as estimated by DLS), suggesting a substantial degree of hydration of the phosphorylated chains, which results in a higher hydrodynamic radius. Lower hydrodynamic radii were achieved when measurements were conducted in a solvent with a lower dielectric constant such as ethanol. Upon addition of Ca^{2+} ions (5 mg/mL at a ratio of 1:1) and stabilization for 60 sec, the average particle size quickly rose approximately 10-fold to values of 1 μm . Second, for lower degrees of phosphorylation (25 and 50%

nominal degrees), the initial size of the polypeptides was slightly higher, which is consistent with molecular weight measurements. After Ca^{2+} addition, the average particle size also increased but to a lesser extent in comparison to the 100% phosphorylated polypeptide **A4**. For all polypeptides, the average particle sizes stabilized to smaller values after an additional 60 sec delay, suggesting dynamic sedimentation and equilibration of large aggregates with time.

In one control experiment on polypeptide **A4**, the aggregate after Ca^{2+} incubation was treated with EDTA (Fig. 6-3c). The hydrodynamic radius returned almost instantaneously to its initial value before Ca^{2+} incubation, confirming the ability of Ca^{2+} ions to drive the formation of larger phosphorylated polypeptides/ Ca^{2+} aggregates.

Figure 6-3. Affinity of phosphorylated co-polypeptides with Ca^{2+} ions as probed by DLS. **(a)** Initial hydrodynamic radius (black curve) of the co-polypeptides as a function of the phosphorylation degree and relative change in hydrodynamic radius

upon incubation with CaCl_2 (blue curve). **(b)** Changes of hydrodynamic radius of polypeptide **A4** with addition of Ca^{2+} (0.5M). Following addition of EDTA (0.1M), the hydrodynamic radius returns to its original value, strongly suggesting that aggregation is Ca^{2+} -induced. **(c)** Schematic illustration of Ca^{2+} -induced aggregation (fully phosphorylated co-polypeptide) and recovery after adding EDTA.

For both targeted applications (hard tissues adhesives and biomineralization templates) a key requirement of the phospho-polypeptides is their ability to sequester and bind divalent ions such as Ca^{2+} or Mg^{2+} . The interaction measurements that were conducted corroborate this critical requirement of affinity between the polypeptides and counter ions. SPR measurements show that our phosphorylated co-polypeptides are able to bind Ca^{2+} ions in solution. These data indicate that the affinity between phosphorylated polypeptides and counter ions can be adjusted by tuning the relative molar concentration of polypeptides and Ca^{2+} (Fig. 6-1). Concomitant measurements with QCM-D confirmed that the phosphorylated co-polypeptide bound calcium ions. Here the frequency shift Δf increases monotonically with Ca^{2+} concentration and no saturation in Δf was observed up to 0.5M CaCl_2 (Fig. 6-2b), indicating the high binding capacity of the **A4** co-polypeptide. Moreover, the binding capacity is pH dependent with higher Ca^{2+} bound at higher pH (> 6), which is consistent with the increased level of phosphate side-chain ionization at higher pH. The interaction between the phosphorylated co-polypeptides and Ca^{2+} ions was further confirmed by the increase in co-polypeptides hydrodynamic radii when mixed with a CaCl_2 solution. The increase in radius suggests that phosphorylated co-polypeptides and Ca^{2+} ions interact to form large aggregates, with Ca^{2+} ions possibly playing the role of intra-chain bridges as illustrated in Figure 6-3c. The addition of even small concentration of Ca^{2+} has also been documented to affect the secondary structure of phosphorylated

proteins.^{173, 187} Such ionic-induced conformation changes are plausible in the present phosphorylated co-polypeptides, but they remain to be confirmed. Taken together, these interaction assay studies show that our phosphorylated polypeptides hold significant potential as a way to bind divalent ions. Since the degree of phosphorylation is tunable, we envisage that it will be possible in the future to control the binding capacity and strength of these interactions, which could prove critical in order to optimize complex coacervates or biomineralization templates.

6.3.2 Interaction of oppositely charged polypeptides A2 and B3 by QCM-D

As shown in Figure 6-4, changes of frequency with time were recorded during quartz microbalance measurements. Polypeptide **A2** {Poly(Ser-co-pSer-co-Tyr) (40-40-20)} and polypeptide **B3** {Poly(Dopa-co-Gly-co-Lys-co-Tyr) (30-50-10-10)} as well as the mixture solution of polypeptide **A2** and **B3** with relative ratios of 5:5 and 9:1 were studied by QCM-D. Polypeptide **A2** and **B3** were selected for their high water solubility. The high content of Dopa in polypeptide **B3** was selected because it was expected to lead to enhanced adhesion. The total polypeptide concentration for all solutions was 40 mg/mL. The crystal surface, which was modified with positively charged amine groups, was rinsed until the signal was stable before polypeptide loading. Polypeptide containing solutions were rinsed over the amine functionalized surface on a QCM crystal. It was expected that the higher negative charge, the stronger the interaction with the amine modified surface leading to an increase in the Δf signal. The loading volume for all polypeptide solutions was fixed at 1 mL, which led to saturation of the interaction as shown in Figure 6-4, followed with water rinsing off the un-adsorbed polypeptides. For all cases, the frequency decreased with polypeptide loading and increased with water rinsing.

Figure 6-4. Changes of frequency ($\Delta f_3/3$) with time during quartz microbalance measurements of (a) polypeptide **B3** (40 mg/mL); (b) polypeptide **A2** (40 mg/mL); (c) mixture solution of polypeptides **A2** and **B3** at relative ratio of 5:5 (40 mg/mL); (d) mixture solution of polypeptides **A2** and **B3** at relative ratio of 9:1 (40 mg/mL). (1) = Milli-Q water rinsing; (2) = loading of polypeptide solution.

The frequency decrease after polypeptide loading indicated molecular adsorbance to the crystal surface.¹⁸⁸ After water rinsing, the non-adsorbed polypeptide solutions in the upper layer were replaced by water and resulted in a mass decrease on the surface as indicated by the frequency increment.

Figure 6-5. Frequency changes ($\Delta f_3/3$) for thiol buffer modified crystal surfaces after loading of different samples followed by Milli-Q rinsing.

The changes of frequency after loading of polypeptide **A2**, **B3** or their mixture solutions following with water rinsing are summarized in Figure 6-5. Since the crystal surface was modified with positively charged amine groups, interactions between the surface and negatively charged polypeptide **A2** are likely electrostatically driven between the phosphate moieties from pSer residues and the substrate. The frequency change for the cationic polypeptide **B3** (13 Hz) was higher than polypeptide **A2** (6.6 Hz), indicating a slightly higher mass adsorbance of polypeptide **B3** on the modified surface. This counterintuitive result may be due to cation- π interactions between amine group and Dopa¹⁸⁹ or Tyr¹⁹⁰ as the positively charged polypeptide **B3** contains high amount of Dopa (~ 30 mol. %) and Tyr (~ 10 mol. %).

The frequency changes for two solutions with mixed oppositely charged polypeptides were more than 6-fold higher than that for either polypeptide **A2** or **B3**

solutions, indicating a higher mass adsorbed on the crystal surface and stronger interactions. The higher mass adsorbed and stronger interactions on the crystal surface for mixed solutions were due to molecular complexation between the oppositely charged polypeptides. This behavior is consistent with the formation of a denser complex coacervate formed during the mixing of oppositely charged polypeptides. Complex coacervation formation is presented in more details in Chapter 7.

6.3.3 Oppositely charged polypeptide mixture solutions with metal ions in the gel formation

6.3.3.1 Interaction of oppositely charged polypeptides A2 and B3 with FeCl_3

Influence of Dopa : Fe^{3+} ratio in the formation of gel for positively charged polypeptide B3.

Since Fe^{3+} is expected to form coordination complexes with Dopa and provides mechanical stiffening,^{111, 191} the ratio of Fe^{3+} and Dopa during gel formation was first studied. The solution was adjusted in alkali pH since previous studies have shown that high pH is needed to ensure cross-link stability.¹¹¹ The angular frequency dependence of storage modulus measured for samples with different ratios of Dopa and Fe^{3+} is shown in Figure 6-6 (a). By varying the Dopa : Fe^{3+} ratio from 12:1 to 1:1, the storage modulus increased from a few Pa to a few thousand Pa. Catecholic moieties can form tri-complexes with Fe^{3+} at basic pH, therefore the optimum molar ratio of Dopa to Fe^{3+} was expected to be 3:1. The strongest interaction was obtained with a Dopa to Fe^{3+} ratio of 2.5:1, with the highest storage modulus around 450 Pa (Fig. 6-6b). This small discrepancy may be due to the actual Dopa content, which is slightly different

than the target amount of Dopa in **B3** used to calculate the ratio. The ratio of Dopa to trivalent ions of 2.5:1 was used for subsequent characterizations.

Figure 6-6. (a) Plot of storage modulus (G') as a function of frequency for polypeptide **B3** with different Dopa to Fe^{3+} molar ratios. (b) Storage modulus (G') at angular frequency 50 rad/s for polypeptide **B3** with different Dopa to Fe^{3+} molar ratios. (c) Plot of storage (G') and loss moduli (G'') of polypeptide **B3** (100 mg/mL) with a Dopa to Fe^{3+} molar ratio of 2.5:1. (d) Plot of storage modulus (G') at 50 rad/s for samples with different concentrations, showing that higher polypeptide concentration increased gel stiffness.

At Dopa to Fe^{3+} molar ratios between 2.5:1 to 1:1, all samples exhibited gel behavior. Their respective loss modulus (G'') was significantly lower than the storage modulus (G') within the frequency regime used, which is indicative of a gel

material.¹⁹² As an example, Figure 6-6 (c) shows G' and G'' as a function of angular frequency for polypeptide **B3** at a concentration of 100 mg/mL, with a Dopa : Fe^{3+} of ratio of 2.5:1. With low amount of Dopa (Dopa : Fe^{3+} at 1.8:1 or 1:1), the storage modulus was lower at around a few hundred Pa. In this case, it is suggested that mono- or bis- catechol- Fe^{3+} complexes (Fig. 2-5), resulting in lower mechanical properties. For samples with Dopa : Fe^{3+} molar ratios higher than 2.5:1, the storage modulus decreased as the Dopa content increased, likely because excess Dopa residues are not coordinated by Fe^{3+} , resulting in liquid-like behavior. There is repulsion for Fe^{3+} with the other positively-charged residues (Lys) that are stronger than the attractive interactions with Dopa (coordination bonding)

Influence of polypeptide concentration

The storage modulus of hydrogels with different concentrations at 50 rad/s is shown in Figure 6-6 (d). By increasing the polypeptide concentration from 4 mg/mL to 200 mg/mL, the storage modulus increased from a few Pa to more than thousand Pa. Above 100 mg/mL, the storage modulus remained constant.

Influence of ratio of oppositely charged polypeptides

Using the optimized Dopa : Fe^{3+} molar ratio obtained above, polypeptide solutions with different ratios of oppositely charged polypeptides **A2** and **B3** were assessed for their gel formation in the presence of FeCl_3 (Fig. 6-7). A damping factor less than 1 ($G'' < G'$) is indicative of a gel material. As shown in Figure 6-7 (c), only samples with polypeptide ratios **A2** : **B3** from 5:5 to 9:1 displayed hydrogel properties, whereas other ratios exhibited liquid behavior. The storage modulus increased slightly with the increasing of angular frequency. This near covalent stiffness (G') of

tri-catechol- Fe^{3+} cross-linked networks at high angular frequency supports the hypothesis that catechol-to- Fe^{3+} coordination bonds can provide significant strength to bulk materials. The storage modulus increased as the content of negatively charged polypeptide **A2** increased, and the sample with the polypeptide ratio **A2** : **B3** 9:1 gave the highest storage modulus (Fig. 6-7d). A plausible explanation could be that, in addition to Dopa/ Fe^{3+} complexation, electrostatic interactions occur between excessive anionic polypeptides and Fe^{3+} , which further increases the cross-linking density.

Figure 6-7. Rheology measurements of the mixture solutions at different **A2** to **B3** polypeptides ratios, after addition of FeCl_3 at alkali pH. **(a)** G' vs. angular frequency. **(b)** G'' vs. angular frequency. **(c)** Damping factor vs. angular frequency. **(d)** Storage modulus (G') at angular frequency 20 rad/s vs. percentage of **B3** in mixture solutions.

Cross-linking of polypeptide network by metal-ligand coordination has several advantages:¹¹¹ (i) Metal-ligand coordination allows to form gels with comparable strength to covalent cross-linked gels. (ii) Cross-linking based on multiple ligand-metal complexes such as catechol-Fe offers a range of possible coordination valence from zero to three per metal ion.¹⁹³ (iii) Finally, the metal-ligand coordination cross-link offers easy control of viscoelastic properties over several orders of magnitude in modulus, from a low viscosity fluid to a stable gel material. Cross-linking with wide range viscoelastic properties will be very useful for the development of adhesives and coatings for applications in both dry and wet environment.^{125, 194}

6.3.3.2 Interaction of oppositely charged polypeptides A2 and B3 with MgCl₂

A control experiment was initially conducted to optimize the amount of MgCl₂ used for subsequent interaction studies with the polypeptide mixture solution. As shown in Figure 6-8, different amount of MgCl₂ (800 mM) was added to 70 μ L polypeptide mixture solutions (**A2** : **B3** = 3:7) at alkali pH, resulting in a final concentration of MgCl₂ ranging from 47 mM to 150 mM. The results of rheology measurements for these mixture solutions are shown in Figure 6-8 (a). The storage modulus initially increases with increasing the concentration of Mg²⁺ and reaches a maximum of 1800 Pa at a concentration of 126 mM. As the concentration further increased, G' decreased (Fig. 6-8 b). The initial increase of modulus may be related to coordination of Mg²⁺ with phosphate groups.¹²⁷ When Mg²⁺ concentration further increases, a screening effect may ensue, leading to repulsive interactions with the positively-charged polypeptides, which may result in network destabilization and to a decrease of the storage modulus.

Figure 6-8. (a) Plot of storage modulus (G') as a function of angular frequency for polypeptide mixture solutions for different amounts of $MgCl_2$. (b) Plot of storage modulus (G') for mixture solutions ($\omega = 50$ rad/s) as a function of Mg^{2+} concentration in the mixture solution.

Figure 6-9 shows the rheology measurements for the mixture solutions at different ratios of oppositely charged polypeptides **A2** and **B3** in the presence of $MgCl_2$ at alkali pH. Storage and loss moduli were monitored between angular frequencies of 0 to 100 rad/s. Mixtures with oppositely charged polypeptide ratios ranging between 9:1 to 1:9 were also studied, and all samples showed gel-like properties ($G'' < G'$). The storage moduli range from 1000 Pa to 4000 Pa as shown in Figure 6-8 (a) and the loss moduli range from 100 Pa to 1000 Pa. The storage moduli are more than 10 times higher than the loss moduli, which indicates gel formation for our oppositely charged polypeptides in the presence of $MgCl_2$. For some samples, the storage modulus decreases slightly in the high angular frequency (Fig. 6-9 a) as the cross-linking breaks partially. The mixture with polypeptide **A2** : **B3** ratio at 5:5 gave the highest moduli G' values with storage modulus 3300 Pa and loss modulus value 250 Pa. We noticed fluctuations of the storage modulus as a function of polypeptide **A2** to **B3** ratio (Fig. 6-9 c), which is however still unclear.

Figure 6-9. Rheology measurements for the mixture solutions of different ratios of oppositely charged polypeptide **A2** and **B3** after addition of MgCl₂ under alkali pH. (a) G' vs. angular frequency. (b) G'' vs. angular frequency. (c) G' at angular frequency 20 rad/s vs. percentage of polypeptide **B3** in mixture solutions.

These data indicate that our synthetic polypeptides are not limited to iron as the cross-linking ligand but that Mg²⁺ can also be used. The interactions are mostly the electrostatic between the phosphate group from polypeptide **A2** and Mg²⁺. As the storage modulus decreases at high angular frequency, this suggests that these electrostatic interactions are not as strong as catechol-Fe complex coordination.¹⁹⁵

6.3.4 Luminescence resulted from the interaction of negatively charged co-polypeptide with lanthanide ions

With their unique optical properties, usages of lanthanide ions are largely related to lighting and light conversion technologies,¹⁹⁶ such as television, lighting devices, and optical amplifiers. Most lanthanide ions are luminescent, but a few lanthanide ions exhibit luminescence only when forming ligands, a process called sensitization.¹⁹⁶ Furthermore, previous reports have demonstrated the strong affinity between phosphorylated peptides and proteins with lanthanide ions.¹⁸⁴ We thus hypothesized that our phosphorylated co-polypeptides would be able to form phosphate-lanthanide complexes with interesting luminescent properties.

To this hypothesis, TbCl₃, EuCl₃ and LaCl₃ were used for the luminescent study, by mixing them with the anionic polypeptide **A2**. After mixing TbCl₃ or EuCl₃ solutions with **A2**, precipitates ensued, especially in acidic environment (Fig. 6-10 a-b). The precipitate dissolved partly with increasing pH. As shown in Figure 6-10 (a-b), when illuminated with a UV light at 254 nm, the solution of polypeptide **A2** with Tb³⁺ and Eu³⁺ gave green and red luminescent light, respectively. Polypeptide **A2** also strongly interacted with LaCl₃, resulting in precipitation. However the precipitation did not yield luminescent light.

Lanthanides are unique in their chemical properties, particularly their oxidation states. This is usually explained by their electronic configuration and their derived ions, which essentially exist in the trivalent state Ln^{III} ([Xe]4fⁿ, n = 0-14) in aqueous solutions, in view of the various degrees of stabilization experienced by 4f, 5d, and 6s orbitals upon ionization.¹⁹⁶

Figure 6-10 (c) shows the emission spectrum of precipitates resulted from interaction of polypeptide **A2** with Tb^{3+} . The spectrum gave three emission peaks, 545 nm ($^5\text{D}_4\text{-}^7\text{F}_5$), 585 nm ($^5\text{D}_4\text{-}^7\text{F}_4$), and 620 nm ($^5\text{D}_4\text{-}^7\text{F}_3$).¹⁹⁷ The most intense transition, $^5\text{D}_4\text{-}^7\text{F}_5$ resulted in a green luminescent light. The emission spectrum shown in Figure 6-10 (d) gave two emission peaks for the interaction of polypeptide **A2** with Eu^{3+} , which are 592 nm ($^5\text{D}_0\text{-}^7\text{F}_1$) and 614 nm ($^5\text{D}_0\text{-}^7\text{F}_2$).¹⁹⁸ The emission peak at 614 nm corresponds to a red luminescent light.

Figure 6-10. Solution of phosphorylated anionic polypeptide **A2** with 50% of Ser phosphorylated (10mg/mL) mixed with Ln^{3+} at pH 3, 7 and 10 (from left to right) under UV light of 254nm. (a) With addition of TbCl_3 . (b) With addition of EuCl_3 . (c) Fluorescence spectrum of **A2** + TbCl_3 at excitation wavelength 365nm. (d) Fluorescence spectrum of **A2** + EuCl_3 at excitation wavelength 390 nm.

Precipitation together with luminescent light emission is a clear indication of the presence of coordination ligands between Ln^{3+} and PO_4^{2-} . Indeed, a strategy for phosphor-peptide enrichment has been reported using Ln^{3+} via the formation of a nine-coordinate complex between trivalent lanthanide ions and phosphorylated peptides¹⁸³. The luminescent system using co-precipitation of our synthetic polypeptide **A2** and Ln^{3+} was pH sensitive. In acidic pH, the co-precipitation yield was higher and the luminescence was stronger. This indicates a higher specificity for monoanionic phosphate.¹⁸⁴ This luminescent complexes based on our synthetic polypeptide **A2** could be useful for the further development of luminescent signaling systems. The coordination complexes with luminescence also have high potential to be used in future for molecular recognition. The significance of the detection and quantification of anions in the field of biochemistry and environmental science has attracted increasing attention for the development of luminescence probes.^{199, 200} Report has shown that different anions play critical role in the inner coordination sphere of central Eu^{3+} complexes due to their coordination capacity and radius of anions, which in turn affects the luminescence intensity.¹⁹⁸

6.4 Conclusions

The interactions of negatively charged polypeptides **A1-4** with Ca^{2+} were studied. Different techniques were used for the characterization of binding capacity and strength, suggesting the possibility to control the binding capacity and strength of the interaction since the degree of phosphorylation is tunable. Furthermore, interactions of oppositely charged polypeptide **A2** and **B3** were characterized by QCM-D and suggested that complexation between oppositely charged polypeptides resulted in molecular structural rearrangement and formation of viscoelastic complexes. Interactions of oppositely charged polypeptides with trivalent or divalent

cations were studied by adding FeCl_3 or MgCl_2 to the polypeptide solutions. Hydrogel materials were obtained that exhibited relatively higher storage moduli with the addition of metal ions. The influences of different parameters on the storage and loss moduli were screened. Samples that contained both oppositely charged polypeptides and metal ions gave the highest hydrogel mechanical property. The interaction of polypeptides with trivalent ions was higher than with divalent ion.

Furthermore, exploratory studies were conducted to assess whether our synthetic negatively charged polypeptide **A2** could interact with rare earth ions Ln^{3+} . The complexes resulted in strong fluorescence, which could be useful for further development of luminescent signaling systems. This topic could open new research avenues for phosphorylated co-polypeptides in the near future.

Chapter 7: Complex coacervation studies

7.1 Introduction

In the last Chapter, interactions between oppositely charged polypeptides **A2** and **B3** with metal ions were described. In the presence of metal ions, hydrogels could be obtained by mixing the synthetic oppositely charged polypeptides. In this chapter, I present the development of conditions to allow the mixtures of the oppositely charged co-polypeptides **A2** and **B3** to reach charge neutrality, and to form complex coacervates. Both contact angle measurements and rheological response confirmed the coacervate-like properties of our oppositely charged co-polypeptide mixtures. The results provide a key step towards the preparation of biocompatible adhesives formulated from polyelectrolytes directly inspired by the sandcastle worm glue, including the usage of a peptide backbone and the presence of the adhesive side-chains Dopa and phospho-serine (pSer). The work also expands the range of *de novo* designed complex coacervates with precise control over critical parameters such as charge density and side-chain chemistry.

7.2 Experiment design

7.2.1 Condition optimization for coacervation

Formation of complex coacervation with oppositely charged polyelectrolytes is directly dependent on various parameters of the solution, including pH, ionic strength, and polyelectrolytes concentrations. Turbidity is the most common technique for the study of complex coacervation due to its simplicity and reliability.^{70, 134} First, the pH effect on the turbidity of oppositely charged mixture solutions was studied. Mixture solutions of polypeptide co-polypeptides **A2** (50 wt%) and **B3** (50 wt%) was prepared

in Milli-Q water at total co-polypeptide concentration of 10 mg/mL. pH of the mixture solution was gradually adjusted from 3 to 10, and the turbidity of the solution at different pH values was measured using Nanodrop.

Second, relative ratios for polyanion **A2** and polycation **B3** were varied, while the pH was fixed at 6.8. This value was chosen because it is close to physiological conditions, while at the same rapid oxidation of Dopa residues can be avoided. At pH 6.8, mixture solution of polyanion **A2** and polycation **B3** was obtained by combining various relative amounts of negatively charged polypeptide **A2** with positively charged polypeptide **B3**. The total volume in all case was 1 ml and the relative ration **A2 : B3** was varied from 1:0 to 0:1 with 0.1 ml increments.

Third, the ionic strength of the solution also plays a critical role for the formation of coacervates, as described in early Chapters. Here, sodium chloride was used for the ionic strength adjustment. Turbidities of the mixture solution of polyanion **A2** and polycation **B3** at relative ratio 4:6 at pH 6.8 with different sodium chloride concentrations were measured. To study the effects of polypeptide concentrations on the coacervate formation, mixture solutions with three different concentrations, 10 mg/L, 20 mg/mL and 40 mg/mL, were prepared.

7.2.2 Preparation and characterization of coacervates

After screening various conditions coacervate formation, the latter were obtained by mixing aqueous solutions of oppositely charged polypeptides in the presence of sodium chloride. Oppositely charged polypeptides were dissolved separately in Milli-Q water with individual concentration of 40 mg/mL. The solution was prepared with relative ratios **A2 : B3** of 4:6 and the total volume in all case was

1 ml. The pH of the mixture solution was then adjusted to pH 6.8 and gradually increased by titrating with 1 M NaOH solution. The zeta potential was regularly measured in order to identify conditions of charge neutrality. To the 1 mL mixture solutions, 250 mg of sodium chloride was added to obtain the targeted level of ionic strength (4.25 M). After dissolving NaCl, the solution was centrifuged at 10k rpm for 5 min, resulting in two distinct phases, namely the coacervate phase and the dilute phase. All complex coacervates were prepared immediately before analysis and studied at room temperature (25 °C).

Both the non-centrifuged solution and the coacervate phase obtained after centrifuge were characterized. Contact angle measurements on both hydrophilic and hydrophobic surfaces were performed. Surface tension of the coacervate was measured by pendant drop method. The detailed experimental procedures for these measurements can be found in Chapter 3.

7.2.3 Phase diagram of co-polypeptide mixtures: pH and polypeptides ratio as two leverages for tunable coacervation

Zeta potential plays a critical role in the formation of complex coacervation. We noticed that zeta potential of the mixture of two polypeptides depends on polypeptides ratio, pH and salt concentration. We studied zeta potential of the mixture of positively and negatively charged polypeptides at different ratio and pH values. Mixture solutions of polyanion **A2** and polycation **B3** were obtained by combining various relative amounts of negatively charged polypeptide **A2** with positively charged polypeptide **B3** at a total polypeptide concentration of 40 mg/mL. The total volume in all case was 1 ml and the relative ratios **A2 : B3** was varied from 1:0 to 0:1 with

0.1 or 0.2 incremental units. The pH of each mixture varied from 3 to 12. Zeta potentials for all these solutions at different pH were measured.

7.2.4 PBS buffer as solvent

In addition to using Milli-Q water, phosphate buffered saline (PBS) solutions were used for complex coacervation formation of oppositely charged polypeptide **A2** and **B3**. Different PBS buffers were prepared as shown in Table 7-1. Initially, PBS 1 and PBS 2 were prepared. Unfortunately, these buffers either gave coacervate in very low yield or resulted in low solubility of polypeptide **A2** and **B3**. Later, boric acid or ascorbic acid²⁰¹ was added in order to prevent Dopa oxidation. With addition of ascorbic acid, buffer 3 and 4 were prepared, but the solubility of **B3** in both buffers with ascorbic acid was low. The highest concentration of polypeptide **B3** in buffer 3 or 4 was about 10 mg/mL. Eventually, considering the polypeptide solubility and coacervate yield, buffer 5 with 0.1 M Na₂HPO₄ and 0.1 M boric acid were chosen for further preparation of coacervates.

The amount of sodium chloride and relative polypeptide ratio needed for coacervation for oppositely charged polypeptide **A2** and **B3** in PBS 5 (pH 7.8) system was screened in a similar fashion as coacervation in water. With optimized condition, coacervation was prepared as follow: solutions of polypeptide **A2** and **B3** at concentration of 40 mg/mL were prepared by dissolving corresponding amount of polypeptide in PBS buffer 5. The solution of polypeptide **A2** and **B3** was mixed at a relative volume ratio of 5:5. To 1 mL mixture solution, 220 mg sodium chloride was added. After dissolving NaCl, the sample was configured at 5-10 k rpm for 5 mins and two phases were obtained. The surface tension of the concentrated coacervate phase was measured using the pendant drop method. Rheology measurements were

done to assess the viscosity and shear behavior of the obtained coacervate. The measurement methods are described in further detail in Chapter 3.

Table 7-1. Phosphate buffered saline (PBS) solutions for coacervate formation.

Buffer name	Buffer gradients	pH
PBS 1	Na_2HPO_4 (0.02M) + KH_2PO_4 (0.0014M) + NaCl (0.14M)	7.7
PBS 2	Na_2HPO_4 (0.44M) + citric acid (0.015M)	7.4
PBS 3	Na_2HPO_4 (0.2M) + ascorbic acid (0.1M)	6.3
PBS 4	Na_2HPO_4 (0.1M) + ascorbic acid (0.1M)	4.7
PBS 5	Na_2HPO_4 (0.1M) + boric acid (0.1M)	7.8

7.3 Results and discussion

7.3.1 Condition optimization of coacervates

Coacervation of oppositely charged polypeptides depends on the total final charge of the system, which is controlled by (i) the polypeptides ratio and concentration, (ii) the pH values since the ionization of the functional groups in the polypeptides is strongly pH dependent, and (iii) the ionic strength.

Influence of polypeptides ratio on coacervation

For our complex coacervation system, the effect of pH was first studied with oppositely charged polypeptide ratio (1:1). As shown in Figure 7-1 (a), the turbidity increased slightly between pH 6.5 to 7.5, followed by a dramatic increase at high pH above 8. The increase after pH 8 could be due to the oxidation of Dopa residues from the positively charged polypeptide at high pH as the solution first became pink and eventually dark particles were observed. Considering the target applications that will

required physiological environments, subsequent measurements were done at a fixed pH value of around 6.8. The oppositely charged polypeptides ratio was then optimized as shown in Figure 7-1b.

At pH 6.8, a 6:4 ratio between positively and negatively charged polypeptides (**B3** : **A2**) gave the highest turbidity and was accompanied by a zeta potential value close to zero (Fig. 7-1b).

Figure 7-1. (a) Turbidity measurements as a function of pH for a mixture solution with 50 wt. % co-polypeptide **A2** and 50 wt. % **B3** (total polypeptide concentration: 10 mg/mL, without NaCl addition). (b) Turbidity measurements as a function of mixture ratios between oppositely charged polypeptide **B3** and **A2** at pH 6.8 (total polymer concentration: 5mg/mL, without NaCl addition).

Ionic strength and coacervation.

Ionic strength also plays critical role during coacervation, and was studied using a solution of oppositely charged polypeptides with a fixed **B3** : **A2** ratio of 6:4. As shown in Figure 7-2, the turbidity was very low with low concentration of NaCl. When the NaCl concentration increased above to 4M, the turbidity of the solution

increased dramatically, followed by coacervate droplet formation. However, further addition of NaCl caused a decrease in the turbidity. When the NaCl concentration increased above to 4.7M, precipitation was obtained due to “salt suppression” as explained in Chapter 1.

Several studies of complex coacervate with low salt concentration have been reported.^{202, 203} For our complex system, the separation of phases occurs at high ionic strengths (high salt concentration), which may be due to the high charge density of our polypeptides in comparison to the natural polypeptides. Higher salt concentration is needed to provide the screening effect to densely-charged polyelectrolytes. This leads to an overall high charge density of the complexes and in turn to smaller hydration radii, which facilitates coacervation.^{204, 205}

Effect of polypeptide concentration on coacervation

Turbidity measurements for our synthetic oppositely charged polyelectrolytes with three different concentrations (10mg/mL, 20 mg/mL and 40 mg/mL) were conducted to investigate the effect of salt on the coacervate formation. As shown in Figure 7-2a, at all salt concentration, the turbidity of the mixture at higher polypeptide concentration was much higher than that at lower concentrations. Additionally, the optimal salt concentration shifts to higher values as the polypeptide concentration decreases. This may due to the fact that for more dilute polypeptide solutions, higher ionic forces are required to the reduce hydration radii and induce coacervation. Mixtures with a higher polypeptide concentrations, on the other hand, have a higher density of oppositely charged sites available to interact. Thus a higher density of complexes are formed, and the salt concentration needed to provide the screening effect to facilitate coacervation is lower.¹³⁴ For subsequent viscosity measurements

with polypeptide of concentration of 40 mg/mL, the optimal salt concentration was around 4.25 M which is also lower than for the sample with a polypeptide concentration of 20 mg/mL (where the optimal salt concentration is 4.5 M).

Figure 7-2. Turbidity measurements as a function of NaCl concentration for polypeptide mixtures at three different concentrations (polypeptide **B3** : **A2** = 60:40 wt. %, at pH 6.8).

7.3.2 Observation of coacervate and solution phase

The coacervate micro-droplets were detectable by optical microscopy. Figure 7-3 shows images on the morphology of the coacervate droplets at different magnifications. With addition of NaCl, the turbidity of the solution increased (Fig. 7-3b) dramatically. When the NaCl concentration reached to the optimum value,

coacervate droplets were formed as a result of liquid-liquid phase separation (Fig. 7-3c). After adding NaCl, the coacervation from the mixture solution (Fig. 7-3b) was immediately observed under the optical microscopy. Observations clearly showed the presence of coacervate droplets and the initial size for the droplet was around 2 μm in diameter. After several minutes, the diameter of these droplets increased, and reached 5 to 25 μm (Fig. 7-3f). Figure 7-3 (d) shows a micrograph of the coacervation on a glass slide without cover slip. During live observations, the droplets were highly mobile. Over time, the droplets coalesced into a larger coacervate phase. After centrifugation at 10k rpm for 5 mins, the solution separated into two distinguishable phases with the coacervate phase at the bottom.

Figure 7-3. Photographs of (a) oppositely charged polypeptides A2 (left) and B3 (right) showing clear solutions. (b-c) Photographs of vials before and after centrifugal separation respectively, illustrating the formation of a coacervate phase at the bottom of the tube. (d) Optical micrograph of the mixture solution with coacervate on the glass slide. Blue arrow lines point out to coacervate droplets. (e) Mixture solution of

coacervate on a glass slide (with cover slide). The micrograph was taken immediately after adding NaCl to the mixture. **(f)** The same coacervate droplets as in (e) several minutes after formation, showing that the size of the coacervate droplets increased over time.

Figure 7-4 (a) shows an optical micrograph of the coacervate phase. Figure 7-4 (b) is enlarged view of the blue circle from Figure 7-4 (a) at increasing magnifications. Comparing all these optical micrographs, we can conclude that the coacervate phase results from the coalescence of multiple smaller droplets.

Figure 7-4. Optical micrographs of: **(a)** coacervate concentrate phase on glass slide with scale bar 100 μm ; **(b)** Higher magnification view of the blue circle shown in (a) with scale bar 20 μm .

7.3.3 Phase diagram of co-polypeptide mixtures: pH and polypeptides ratio as two leverage for tunable coacervation

Figure 7-5 summarizes the zeta potential of mixtures of oppositely charged polypeptides **A2** and **B3** at different ratios and pH. As shown in Figure 7-5 (a), zeta potential increases with higher amount of positively charged polypeptide **B3**. As the

solution shifts from an acidic to an alkaline environment, zeta potential decreases. Figure 7-5 illustrates the strong influence of salt concentration on zeta potential. Salt decreases the zeta potential for the whole system by 5 to 10-fold. Without salt (Fig. 7-5a), the zeta potential varies from +20 to -30 mV for different polypeptides ratio, whereas when salt is added the zeta potential decreases in the range +2 to -6 mV (Fig. 7-5b).

Figure 7-5. Zeta potential for mixtures of positively and negatively charged polypeptides **B3** and **A2** taken at different ratio and at different pH (total polymer concentration is 40mg/mL): **(a)** Without salt. **(b)** With addition of 4.25M NaCl. Dots are real measured zeta potential values for mixture solutions at different conditions.

Coacervation can be viewed as an agglomeration of oppositely charged polypeptides.⁸⁴ To induce coacervation, conditions where the zeta potential is close to zero need to be identified. By projecting the 3D plot shown in Figure 7-5 into a 2D representation, the regions with positive and negative zeta potential values can be readily identified as shown in Figure 7-6 (a) (without salt) and Figure 7-6 (b) (with salt), with the green regions corresponding to negative zeta potential values and the blue regions to positive zeta potential values, respectively. The interface between these two regions corresponds to zero zeta potential values. Without salt, a higher

concentration of positively charged polypeptide is needed to obtain zero charge as the pH increases. In the presence of salt (250 mg/mL), the content of cationic polypeptide needed to induce charge neutrality decreases for each pH value. At pH 6 in the presence of salt, the plot suggests that mixtures leading to zero charge should be approximately 50% cationic polypeptide **B3** and 50% anionic polypeptide **A2**. Without salt, at pH 6, the mixture solution leading to zero charge is about 80% positive polypeptide **B3** and 20% negatively charged polypeptide **A2**.

Figure 7-6. Two-dimensional projection of Figure 7-5 (obtained using the “Table Curve 3D” software). Green regions correspond to domains where the overall charge is negative, whereas blue regions correspond to domains where the zeta potential is positive. The interface between green and blue represents possible conditions (mixture ratio and pH) at which zero zeta potential values are obtained, and thus where complex coacervation should be possible. **(a)** Without NaCl. **(b)** With NaCl addition (4.25 M). **(c)** Formation of complex coacervates for three mixture solutions

at the conditions indicated in (b). Coacervates were obtained at pH 7, 6.8 and 4.8 at polypeptides ratio (**B3** : **A2**) of 8:2, 6:4, and 2:8, respectively (from left to right).

We also noticed that the addition of salt did not change significantly the location of the area where the zeta potential values are close to zero. We may assume that salt and pH are independent variables influencing the coacervation process.

To confirm our phase-diagram, three different coacervates corresponding to the different points in Figure 7-6 (b) were prepared, *i.e.* mixtures were prepared with the predicted polypeptide ratios and pH values. Photos of coacervate obtained at different condition are presented on Figure 7-6 (c) and confirmed that the phase diagram is a predictive tool in order to prepare complex coacervates. The dark coloration of the coacervate is attributed to Dopa oxidation.

7.3.4 PBS buffer as solvent

The coacervates prepared from milli-Q water solutions were quickly oxidized as evidenced by the color of the solution becoming first brown and then dark. Coacervates in PBS solutions were more stable than in Milli-Q water. The oxidation kinetics of coacervates was delayed by using PBS in the presence of boric acid. These coacervate could be stored in 4 °C fridge for 12 hours without any color change.

By using PBS as solvent, the rate of coalescence was also slowed down. Figure 7-7 shows the coacervate formation of oppositely charged polypeptide **A2** and **B3** using PBS buffer in Eppendorf tubes. The images illustrate the size increase of the dilute droplets. 20 mg of negatively charged polypeptide **A2** and 20 mg of negatively charged polypeptide **B3** were dissolved in 1 mL of PBS 5 (pH 7.8) solution. 220 mg of sodium chloride was added into the polypeptide PBS solution. As shown in Figure

7-7, the turbidity of the solution increases with the formation of small droplets, and the size of the coacervate droplets increased with time.

Figure 7-7. Photographs of coacervate droplets formed after adding NaCl to a mixture of oppositely charged polypeptide **A2** and **B3** in PBS buffer solutions. **(a)** 10s; **(b)** 2 mins; **(c)** 5mins; **(d)** 15 mins. The scale bar for all images is 0.1 cm. **(e)** Coacervation solution with two separated phases.

7.3.5 Characterization of concentrated complex coacervates

Two surfaces were used to characterize the wetting properties of the coacervates, namely *(i)* a standard glass surface, and *(ii)* a hydrophobic surface prepared by gold coating a quartz crystal followed by growing a self-assembled monolayer of

dodecanethiol HS-(CH₂)₁₁-CH₃. The contact angles of these surfaces were measured for the individual cationic and anionic polypeptides, for the concentrated coacervate phase as well as for the dilute equilibrium phase after coacervation (Fig. 7-8a). For the hydrophobic surface, the coacervate gave the lowest contact angle value of around 60 °. The relatively low contact angle value (for a hydrophobic surface) indicates that the liquid spreads on the surface, indicating the high wettability of the coacervate. For the glass surface, the cationic polypeptide gave the lowest contact angle, which is attributed to electrostatic interactions between the positively charged residues and the negatively charged glass surface. In order to quantitatively measure the coacervates' surface tension, the quality of the substrate surface was first verified using a goniometer with surface tilting.^{206, 207} As shown in Figure 7-8 (b) the coacervate droplet remained immobile up to 90 ° tilt angle of the hydrophobic surface. Contact angle hysteresis, which represents the difference between the advancing contact angle and the receding contact angle, was only around 5 °. Contact angle hysteresis arises mostly from the chemical and topographical heterogeneity of the surface, solution impurities adsorbing on the surface. This low contact angle hysteresis indicated that the surface was homogeneous and without defects and the contact angle obtained was thus reliable for the following surface tension calculation.²⁰⁶

Figure 7-8. (a) Static contact angle on a glass and hydrophobic surfaces of the coacervate concentrate phase and comparison with the equilibrium dilute phase, the polypeptide mixture solution in non-coacervate formation conditions (**B3** + **A2**), polypeptide **B3**, polypeptide **A2**, and DI water. (b) Tilting experiment of the coacervate concentrate phase on hydrophobic surface, where the sample was tilted with 90 °.

The surface tension of coacervates was calculated using the contact angle previously measured into Neumann's equation:²⁰⁸⁻²¹⁰

$$\cos \theta = 2 \times \sqrt{\frac{G_S}{G_L}} \times e^{[-b \times (G_L - G_S)^2]} - 1 \quad \text{Eq. 7-1}$$

where θ is contact angle; b is coefficient which equals to 0.0001247 m²/mJ; G_L and G_S are the surface tension of liquid and solid accordingly (J/m²). Here G_S is the

surface tension of the modified hydrophobic gold surface and is equal to 0.0196 J/m^2 .

ref. 135

A value G_L of 31.8 mN/m was obtained as shown in Table 7-2. The surface tension of the coacervate phase was also measured by the pendant drop method and gave an average value of $34.4 \pm 3 \text{ mN/m}$ (Table 7-2). The results obtained by these two methods are comparable.

Table 7-2. Surface tension of concentrated coacervates measured using the Pendant drop method and the Neumann's equation two methods.

Solvent	Surface tension (mN/m)	
	Pendant drop method	Neumann's equation
DI water	34.4 ± 3	31.8
PBS 5	14.5 ± 1.2	NP

The surface tension for the coacervate prepared in PBS buffer solution was also measured using the pendant drop method. The surface tension decreased to $14.5 \pm 1.2 \text{ mN/m}$. The significant decrease of surface tension using PBS buffer is attributed to less Dopa oxidation, since boric acid present in the buffer plays can delay Dopa oxidation.^{211, 212} Here, the Neumann's equation cannot be applied since this method is only valid for surface tension above 20 mN/m .²⁰⁸ A key property of coacervates is their low surface tension combined with their immiscibility in water, resulting in fluidic micro-droplets that can permeate small interstices. These characteristics are important for water-resistant adhesives and were confirmed in our coacervates. Some other studies on complex coacervate have been reported with a surface tension lower than 1 mN/m .³⁷ The surface tension for our coacervate is not as low as $< 1 \text{ mN/m}$.

One reason could be that there is still some oxidation of Dopa in our complex coacervates. Another reason could be the measurement method. In these studies, nano-scale force methods such as the colloidal probe atomic force microscopy (CP-AFM) were required²¹³ in order to measure the ultra-low surface tension values of complex coacervates. Such methods may be necessary in the future to measure the surface tension of our coacervates in a more accurate fashion. Nevertheless, surface tensions values smaller than 20 mN/m are already considered as quite low, typically in the range of lubricants,²¹⁴ whose low surface tension is an important functional characteristic.

After centrifugation, the coacervate phase was collected and their rheological properties measured. The viscosity of the coacervate concentrate phase was determined by carrying out steady-state shear experiments. As a comparison, the viscosity of the following phases was also measured for: (i) the equilibrium dilute phase (post-coacervation formation), (ii) the individual solution of cationic and anionic polypeptides, (iii) the mixture of oppositely charged polypeptides without coacervation formation, and (iv) DI water. Figure 7-9 (a) shows a plot of viscosity as a function of the shear rate for the concentrated coacervate. The plot indicates a shear-thinning behavior, with the viscosity rapidly decreasing with the shear rate at low shear rates. Shear-thinning in viscous materials is characterized by a strong decay of viscosity at high shear rates,²¹⁵ which is for instance useful for certain types of hydrogels to be delivered via a syringe system.²¹⁶ At shear rate above 30 s^{-1} , the concentrated coacervate led to a steady-state viscosity. For the other samples, steady viscosity was only obtained with shear rate above 150 s^{-1} (Fig. 7-9b). Figure 7-10 summarizes the steady-state viscosity at high shear rate for all samples. The coacervate concentrate phase viscosity was around 600 mPa's, which is hundred times

higher than the dilute phase. The viscosities for the oppositely charged peptides **B3** and **A2**, for the mixture without coacervate formation, and for the dilute equilibrium phase during coacervation were all close to the viscosity of water at around 2 mPa's.

Figure 7-9. Plot of viscosity as a function of shear rate: **(a)** for coacervate concentrate phase. Inset depicts the shear-thinning behavior of the coacervate concentrate phase. **(b)** for other samples: dilute phase, mixture without coacervate formation (**B3** + **A2**), polypeptide **B3**, polypeptide **A2** and DI water.

Figure 7-10. Average viscosity for the concentrated coacervate at shear rate range 30 - 100 1/s and for the other samples: dilute phase, mixture without coacervate formation (**B3** + **A2**), polypeptide **B3**, polypeptide **A2** and Milli-Q water at shear rate range 150 - 1000 1/s.

Loss modulus and storage modulus were also measured to assess the properties of the coacervate (Fig. 7-11). These measurements confirm a typical behavior of viscous materials.²¹⁵ Both loss and storage moduli increased with the angular frequency. The cross-over point where point G'' becomes higher than G' , corresponding to a gel-like behaviour²¹⁵ was around 50 rad/s.

Figure 7-11. Frequency sweeps of concentrated coacervate phase showing storage and loss modulus as a function of angular frequency.

7.4 Conclusions

In early Chapters, a synthetic approach has been described for the preparation of gram-scale quantities of oppositely charged polypeptides that were inspired by the wet-resistant adhesive ability of the cement proteins from sandcastle worm *P. californica*. Mimicking the composition of the major components of the natural proteins of sandcastle worm glue, we synthesized two oppositely charge polypeptides with MWs and pI values which are close to the natural Pc proteins.

We studied influence of sodium chloride concentration, pH and polypeptide ratios on coacervation, which was obtained by mixing oppositely charged polypeptides at certain conditions. We have shown that at certain pH, coacervation

can be obtained by changing the polypeptides ratio. In acidic environment (pH = 4.8) we need about 80% of negatively charged polypeptide and 20% of positively charged polypeptide to induce coacervation, whereas in environment with pH=7 a reverse polypeptide ratio is required to obtain coacervates. Varying the ratio between positively and negatively charged polypeptides, we can form coacervate at different pH, thus in principle making our system tunable. We provide two phase diagrams for coacervation governing by pH and polypeptide ratio with and without NaCl. Later on, PBS buffer solution (pH 7.8) with 0.1 M Na_2HPO_4 and 0.1 M boric acid was demonstrated to be a better buffer since it provided a more stable environment for coacervate preparation as Dopa oxidation was mostly prevented.

Coacervate solutions were characterized by simple optical microscopy and illustrated the coalescence of small droplet into the concentrated coacervate phase. Concentrated coacervates exhibited a relatively low contact angle on both hydrophilic and hydrophobic surface. Drops of coacervates did not move when the surface was tilted up to 90°. Surface tension of the coacervate was found to be low around 14.5 mN/m. Rheological measurements at controlled shear rate were conducted for the coacervates, which were shown to exhibit a shear thinning behavior and a steady-state viscosity of 600 mPa's at higher shear rate. This complex coacervate prepared from our synthetic oppositely charged polypeptides are highly concentrated with an extremely low surface tension. It meets some of the more crucial requirements of wet-adhesive materials and thus maintain potential for further development to be used in biomedical applications.

Chapter 8: Conclusions and outlooks

8.1 Conclusions

In this PhD study, a series of oppositely charged polypeptides that represent close chemical analogs to the sandcastle worm glue protein were synthesized by ring opening polymerization of α -amino acid-NCAs. Gram-scale quantities of negatively charged polypeptides with tunable degrees of phosphorylation were prepared. Synthesized polypeptides were studied through ^1H NMR and ^{13}C NMR for their purity, which also yielded information about the amino acid composition. Amino acid analysis was used for the amino acid composition analysis of positively charged polypeptides. The following conclusions are drawn from the experimental results generated in this work:

1. Our synthetic polypeptides exhibit variable physico-chemical properties, including zeta potential, hydrodynamic radii, divalent ion affinity, or charge density of the colloidal suspension.
2. Phosphorylated polypeptides have strong binding affinity with different ions. Hydrogel materials and luminescence materials could be obtained by using synthetic oppositely charged polypeptides with addition of cations.
3. Tunable complex coacervation could be prepared from our synthetic copolypeptides.
4. PBS buffer with boric acid provides stable environment for coacervate formation. Our coacervation follows “the Tainaka theory”.
5. Coacervates were shown to exhibit a low surface tension and shear thinning behavior.

Thus in summary, this thesis demonstrated that synthetic co-polypeptides mimicking from the sandcastle worm glue are able to form complex coacervates, with the coacervate phase exhibiting a shear-thinning behavior with low surface tension that meet the requirements of wet-resistant adhesives materials, and therefore have potential to be used for biomedical applications.

8.2 Future recommendations

One critical step towards the fabrication of adhesives biomaterials mimicking the cement from sandcastle worm is the curing step. Oppositely charged co-polypeptides have been developed to closely mimic the amino acid content native sandcastle worm glue. Coacervate has been prepared by mixing synthetic co-polypeptide under certain conditions. However, the final curing via cross-linking condition has yet to be optimized. The cross-linking reactions in the native cement are not yet fully understood. Various hypothetical cross-linking pathways for Dopa have been proposed as stated in early Chapters. Some evidences have shown that oxidation of Dopa to Dopa-quinone can lead to cross-linking either with Dopa residues via radical mechanism²¹⁷ or with an amine by Michael addition^{218, 219}. Moreover, Yu and Deming reported the gel formation through the cross-linking of Lys and Dopa^{130, 220} in the presence of oxidizing agents, such as mushroom tyrosinase, H_2O_2 , or NaIO_4 . It has been suggested that cross-linking via the chelation of Dopa and iron ions could also be used for the curing stage.²²¹

A range of different polypeptides have been synthesized. Further in-depth studies on the effects of functional group composition on the adhesive and cross-linking behavior will help in molecular engineering of adhesive biomaterials. The amino acid composition can be adjusted once we have gained a better understanding

of the synthesized adhesives. Some specific amino acid residues may play significant role during curing and this remains to be elucidated. Besides the hydroxyl groups of Dopa, Ser, and Tyr, some other functional groups may also be able to participate in cross-linking, such as the thiol side-chain Cys, the amino group of Arg, or the imidazole side-chain of His. Negatively charged polypeptides that also include a few pct. of Arg (as seen in the native glue) together with Ser and Tyr could be prepared. Then, positively charged polypeptide with higher amount of Cys or His can be synthesized, followed by their possible role in cross-linking. Currently, however, a challenge is to synthesize co-polypeptides via ring-opening polymerization containing more than four different amino acids and with a relatively high molecular weight.

Tunable coacervation has been developed by mixing different ratios of oppositely charged co-polypeptides at different pH in Milli-Q water. However, the coacervation in water solution proved to be unstable. PBS buffer solution containing boric acid provides a more stable environment for coacervate formation. Tunable coacervation in PBS solutions can be studied in subsequent studies. For instance PBS buffers with a systematic variation of pH could be prepared to study the coacervation process in more details. Moreover, the size of the colloidal particles could be studied by means of light-scattering experiments coupled with GPC.

The long term goal of this research is to fabricate underwater adhesive biomaterials mimicking the native adhesive cement from sandcastle worm for medical applications, such as orthopedics, plastic surgery and living tissue repair. Bones can be used for adhesion testing. The adhesive characteristics of synthesized polypeptide based glues can be tested by apply the glue on a variety of materials,^{35, 69} *e.g.*, bones, glass, and ceramic. The adhesion mechanical performance including shear and tensile

testing should be measured on cured adhesives. Many marine animals invertebrates use adhesive proteins to anchor the organism's bodies to inorganic surfaces, but not the organic scaffolding organ to living tissues. Although mimicking underwater adhesives for wet living tissue adhere may be challenging, there has been promising work in that direction in the past few years using coacervate-based adhesives. Thus the development of underwater adhesives for soft-tissue repair has a lot of potential for further improvements, and coacervate-based adhesives such as those developed in this thesis may be part for such developments.

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- **Zhang, L. H.**; Lipik, V.; Miserez, A. *J. Mater. Chem. B*. Accepted. Complex coacervates of oppositely charged co-polypeptides inspired by the sandcastle worm glue.
- Lipik, V.; **Zhang, L. H.**; Miserez, A. *Polym Chem-Uk* **2014**, 5, 1351. Synthesis of biomimetic co-polypeptides with tunable degrees of phosphorylation. (**co-1st author**)
- Ding, D., Guerette, P. A., Fu, J., **Zhang, L.**, Irvine, S. A. and Miserez, A. *Adv. Mater.* **2015**. From Soft Self-Healing Gels to Stiff Films in Suckerin-Based Materials Through Modulation of Crosslink Density and β -Sheet Content.

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Appendix I

N,O,O'-Tricarbobenzoxy-L-DOPA (9.8g, 65%): ^1H NMR (400 MHz, CDCl_3): δ 7.52-6.92 (m, 18H), 5.31-4.95 (m, 6H), 4.67 (m, 1H), 3.20-2.95 (d, 2H). ^{13}C NMR (400 MHz): δ 174.7, 156.0, 152.8, 152.7, 142.2, 141.4, 136.0, 135.2, 134.6, 134.5, 128.8, 128.7, 128.6, 128.5, 128.2, 128.1, 127.8, 124.1, 123.1, 70.7, 67.3, 54.5, 36.9.

N,O,O'-Tricarbobenzoxy-L-DOPA NCAs (Z-DOPA(Z)₂-NCA) (7.1g, 70%): ^1H NMR (400 MHz, DMSO): δ 7.5-6.8 (m, 17H), 5.3-5.2 (m, 2H), 3.5-3.2 (m, 5H), 3.1 (m, 1H), 2.8-2.9 (m, 1H). ^{13}C NMR (400 MHz) 173.5, 156.5, 152.5, 142.0, 141.0, 138.0, 137.4, 135.3, 129.1, 129.0, 128.8, 128.7, 128.3, 128.2, 128.0, 124.1, 123.4, 70.6, 67.5, 65.8, 55.6, 25.6.

N-Carbobenzyloxy-L-serine NCAs (Z-Ser-NCA) (7.2g, 65%): ^1H NMR (400 MHz, DMSO): δ 7.36 (m, 5H), 5.05 (s, 2H), 4.07 (m, 1H), 3.65 (m, 2H). ^{13}C NMR (400 MHz): δ 172.6, 156.5, 137.4, 128.8, 128.3, 128.2, 65.9, 61.8, 57.1.

Benzyloxycarbonyl-O-benzyl-L-serine NCAs (Z-Ser(Bzl)-NCA) (9.2g, 85%): ^1H NMR (400 MHz, DMSO): δ 7.62-7.33 (m, 10H), 5.04 (s, 2H), 4.47 (m, 2H), 4.29 (m, 1H), 3.68 (s, 2H). ^{13}C NMR (400 MHz): δ 172.0, 156.5, 137.3, 128.8, 128.6, 128.5, 128.2, 128.0, 127.8, 72.5, 69.7, 65.9, 54.7.

Benzyloxycarbonyl-O-benzyl-L-tyrosine (Z-Tyr(Bzl)-NCA) (9.0g, 85%): ^1H NMR (400 MHz, CDCl_3): δ 7.42-6.92 (m, 14H), 5.13 (s, 2H), 5.02 (s, 2H), 4.67 (s, 1H), 3.10-2.95 (d, 2H). ^{13}C NMR (400 MHz): δ 176.4, 158.0, 155.9, 136.9, 136.1, 130.4, 128.6, 128.5, 128.3, 128.1, 128.0, 127.7, 127.5, 115.1, 70.0, 67.2, 54.7, 36.9.

N-carbobenzyloxy-L-glycine NCAs (Z-Gly-NCA) (9.3g, 88%): ^1H NMR (400 MHz, DMSO): δ 8.2 (m, 1H), 7.6-7.2 (m, 4H), 5.0 (s, 2H), 3.7 (s, 2H). ^{13}C NMR (400 MHz): δ 172.1, 157.0, 137.5, 128.6, 128.3, 128.2, 128.1, 65.9, 43.0.

N,N'-dibenzylloxycarbonyl-L-lysine NCAs (Z-Lys(Z)-NCA) (7.7g, 60%): ^1H NMR (400 MHz, CDCl_3): δ 7.5-7.0 (m, 10H), 5.1 (s, 3H), 5.0 (s, 1H), 4.3 (m, 1H), 3.2 (m, 2H), 2.0-1.3 (m, 6H). ^{13}C NMR (400 MHz) 174.4, 172.1, 156.6, 152.4, 137.7, 137.5, 129.2, 128.2, 65.8, 65.6, 57.5, 54.3, 31.1, 29.4, 23.3.

Appendix Figure 1. (a) ^1H NMR spectra of Z-Dopa(Z)₂. (b) ^{13}C NMR spectra of Z-Dopa(Z)₂.

NCA of protected DOPA 20141128
DMSO

NCA of protected DOPA 20141128
DMSO

Appendix Figure 2. (a) ¹H NMR spectra of Z-Dopa(Z)₂-NCA. (b) ¹³C NMR spectra of Z-Dopa(Z)₂-NCA.

Appendix Figure 3. (a) ^1H NMR spectra of Z-Ser-NCA. (b) ^{13}C NMR spectra of Z-Ser-NCA.

Appendix Figure 5. (a) ^1H NMR spectra of Z-Tyr(Bzl)-NCA. (b) ^{13}C NMR spectra of Z-Tyr(Bzl)-NCA.

Appendix Figure 6. (a) ^1H NMR spectra of Z-Gly-NCA. (b) ^{13}C NMR spectra of Z-Gly-NCA.

NCA lysine z

NCA of Z-Lys-Z20141128
DMSO

Appendix Figure 7. (a) ^1H NMR spectra of Z-Lys(Z)-NCA. (b) ^{13}C NMR spectra of Z-Lys(Z)-NCA.