2013년 8월

박사학위 논문

Biomimetic Adhesive Materials Containing Cyanoacryl Group for Orthopaedic Application

조 선 대 학 교 대 학 원

의 학 과

조 승 환

Biomimetic Adhesive Materials Containing Cyanoacryl Group for Orthopaedic Application

시아노아크릴 그룹을 포함하는 정형외과용 생체모방 접착재료

2013년 8월 23일

조 선 대 학 교 대 학 원

의 학 과

조 승 환

Biomimetic Adhesive Materials Containing Cyanoacryl Group for Orthopaedic Application

지도교수 하 상 호

이 논문을 의학 박사학위신청 논문으로 제출함

2013년 4월

조 선 대 학 교 대 학 원

의 학 과

조 승 환

조승환의 박사학위 논문을 인준함

위원장　조선대학교 교수　이 상 홍 (인)

위　원　조선대학교 교수　유 재 원 (인)

위　원　전북대학교 교수　박 명 식 (인)

위　원　조선대학교 교수　이 인 화 (인)

위　원　조선대학교 교수　하 상 호 (인)

2013년　06월

조 선 대 학 교　대 학 원

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

시아노아크릴 그룹을 포함하는 정형외과용 생체모방 접착재료

조 승 환

지도교수 : 하 상 호

조선대학교 대학원 의학과

서론: 최근까지 오랜 기간에 걸쳐 의학적 접착제에 대한 연구가 있었으나 아직까지 충분한 접착강도, 생체적합성, 젖은 환경에서 부착성 등의 조건을 만족하는 접착체는 실용화되지 못한 상태로 이러한 접착체가 개발될 경우 정형외과 영역에서 매우 유용하게 사용될 수 있다. 본 연구는 해양생물 유래의 자연모방 수중접착제를 개발하고 이를 소에서 채취된 대퇴골의 절단면에 젖은 환경에서 부착한 후 이에 대한 부착 정도를 측정하여 실제 정형외과 영역에서 골절 치유시 적용이 가능할지에 대해 알아보고자 하였다.

실험 및 방법: 접착 물질을 만들기 위해 우선 3,4-dihydroxyphenylalanine (DOPA), methoxyethyl acrylate (MEA) 및 시아노아크릴 (CEA) 그룹을 포함하는 트리코폴리머 형의 고분자를 합성하여 주요 접착 기능을 부여하였다. 이를 접착력을 강화시킬수 있는 일급아민 측쇄의 폴리카티온과 2가 양이온인 Ca^{++}, Mg^{++}와 친수성 및 소수성을 조절할수 있는 삼중블럭고분자인 PEO (polyethylene oxide)-PPO (polypropylene oxide)-PEO (polyethylene oxide) 존재 하에서

트리코폴리머와 복합 코아세르베이트 (complex coacervate)를 형성하였다. 이들의 조성비를 달리하여 5종류의 접착 물질 (Complex coacervate - 1,2,3,4,5)을 만들어 각각에 대한 생체모방 수중 접착능력을 시험하였다. 인장력 및 전단력을 측정하기 위하여 소 대퇴골 간부를 절단한 후 인산완충식염수로 단면을 젖게 한후 생체 모방 접착물질을 적용하였다. 제조된 5 종류의 접착 물질 및 대조군으로 사용한 Super Glue에 대하여 Universal testing machine (UTM) 을 이용하여 인장력 및 전단 모듈러스를 6회 반복 측정한 후 이에 대한 평균치를 구하여 비교하였다. 또한 접촉각 측정기를 이용하여 각각의 접착물질에 대한 유리기판에서의 접촉각을 측정하였다.

결과: 접합체 검사에서 CC-4 가 165kPa 의 강한 인장력을 보여주었고 전단 모듈러스는 33 MPa 로서 이는 각각 Super Glue 인장력의 60 % 및 전단 모듈러스의 79 % 에 해당하는 값이며 기존의 생체모방 접착제보다 월등히 높은 값이다. 전단 모듈러스가 가장 높았던 CC-2의 경우 인장력이 96 kPa로 비교적 낮게 측정되었다. 접착 각도는 CC-4 와 CC-5가 110도로 기존의 Super Glue 와 가장 유사한 수치를 나타냈다.

결론: 본 연구에서 만들어진 물질은 인체 독성을 최소화하도록 해양생물 유래 접착 물질을 이용하였으며 접착체 합성에 사용된 시아노독성 유도 물질인 시아노아크릴은 화학적으로 안정한 고분자의 곁사슬로 결합되어 있고 사슬 얽힘 속에 묻혀있게 됨으로 인체독성을 일으킬 가능성이 낮다. 또한 본 연구에서 만들어진 5가지 종류의 접착제를 소대퇴부 절단면에 적용후 실험한 결과 CC-4에서 젖은 환경에도 불구하고 기존에 보고된 자연모방 접착물질 중 가장 높은 접착력을 보여 주었다. 그러나 향후 실용화를 위하여 독성 실험 및 동물 실험 등의 후속연구가 지속적으로 이루어져야 할 것이다.

Ⅰ. INTRODUCTION

Since the last century, synthetic polymeric adhesives were given more and more attention and have increasingly replaced mechanical fasteners in many fields. In medical sense, adhesion is an attractive technique to link divided materials as it is relatively easier and more rapid way to perform compared to conventional methods. Currently, although treatment of choice for most long bone shaft fracture is minimal invasive fixation encouraging secondary healing of the bone, there are often cases where bone bonding agent may seem attractive as in comminuted intra-articular fractures. Compare to using nails, screws or pins to connect fractured bones, using adhesives are advantageous as it can provide optimal load transfer from one fracture surface to another. In specific, a connection is made over the whole surface rather than just a spotted contact as is the case when using conventional methods.

However, up to recently, adhesives are used in limited ways in the field of orthopedic surgery. At present, orthopedic surgery has a striking reliance on internal and external fixation to hold fractured bones in place.

Lately, new generations of bioadhesives have been considered for tendon and bone fixation, thus anatomically fixating small fragments and avoiding further surgical operations to remove the mechanical devices.[1-4] Despite the longstanding history of research in this field, a clinically applicable alternative in the field of bone adhesion has not yet been found.[5,6] The

applications that tried to develop a bone adhesive system include epoxy resin, cyanoacrylates, polyurethanes, and the fibrin adhesives (Table 1). These premature developments failed because they did not meet the medical requirements such as biocompatibility, nontoxicity, ease of application and adhesion in wet environments.[7-10)]

Out of this materials, cyanoacrylates shows tremendous bonding strength and ability to bond in wet environments and due to its rapid adhesion effect, the usage of medical adhesives containing cyanoacrylate as a replacement for the classical suture has been reported.[11-13)] Yet, the cyanoacrylate adhesives still have some problems. The cyanoacrylate family form brittle adhesive layers in vivo which may cause biodegradation of polymer by adverse tissue response. They also have been known to generate toxic formaldehide by the biodegradation. Therefore, there is a need to get high quality medical adhesives based on cyanoacrylates at an affordable condition.

Biocompatible and more flexible bonds might be formed using biomimetic adhesive groups and other peptides with cyanoacryl group: thus, biomimetic adhesive molecules could be modified with cyanoacrylates to obtain different repeating units and chain length copolymers. Hence, the polymers having appropriate moiety of cyanoacryl group would give a strong adhesion effect and lower toxicity with lower degradation rate.

In search of such biomimetic adhesives, we came to focus on marine mussels firmly adhere to rock in the ocean. The underwater mussel adhesive has been

Table 1. Previous reports in medical adhesives.

Adhesive Materials	Test Specimens	Adehesion Force (MPa)	Reference
GRF	Porcine cortical	0.2 (dry)	Chivers et al[14]
	Sheep aorta	0.17 (dry) 0.048 (wet)	Albes et al[15]
GRG	Porcine cartilage	0.021 (dry	Chivers et al[14]
n-butyl cyanoacrylate	Bovine cortical	2.9 (dry)	Brauer et al[16]
	Porcine cortical	1.4 (dry)	Chivers et al[14]
Fibrin	Bovine cancellous	0.0005 - 0.017 (dry)	Weber et al[17]
	Porcine cortical	0.011 (wet)	Chivers et al[14]
	Porcine cortical	1.4 (dry)	Maurer et al[18]
Sandcastle Glue	Bovine cortical	0.1 (dry)	Shao et al[19]
PMMA bone cement	Bovine cortical	0.35 (dry)	Vanio et al[20]
	Human cortical	1.1 (dry)	Ishihara et al[21]
	Bovine cancellous	1.0 (dry)	Weber et al[17]

a worthwhile model for the design of synthetic and blending adhesives. Synthetic polyelectrolyte analogs with the same side chain such as catechols and/or cyano acrylate and side chain molar ratios as the underwater adhesive protein (UAP) could be mixed in similar proportions as the natural glue, including divalent cations, formed complex coacervates that qualitatively mimicked the entire range of natural glue properties.

Mussels adhere tightly to surfaces underwater using the byssus secreted from their foot, which consists of a bundle of threads, where dehydrophenylalanine (DOPA) is discharged as under water bioadhesives (UWB) (Figure 1).

UWBs are not particularly strong in initial stage of adhesion.[22] In controlled laboratory tests, byssal thread and plaque assemblies created by mussels (*Mytilus edulis*) on glass or Al substrates had tensile bond strengths of 0.2-0.3 MPa.[23] Bond strengths of barnacle and sandcastle worm (*Phragmatopoma californica*) glues were also in the range of 0.2-0.3 MPa.[24] While these comparative estimates must be interpreted cautiously because of the differences in test methods, the natural bond strengths are a fraction of the 20 MPa bond strengths of contemporary dental adhesives.[18] Clearly, the underwater bond strengths of bioadhesives are not miraculous. Because of its various usages, mimetic adhesives for human technology must achieve higher underwater bond strengths than that of the natural adhesives. It is clear that a new generation of bioadhesive is needed in orthopedic surgery. Moreover, the importance of this issue will be emphasized more in the future

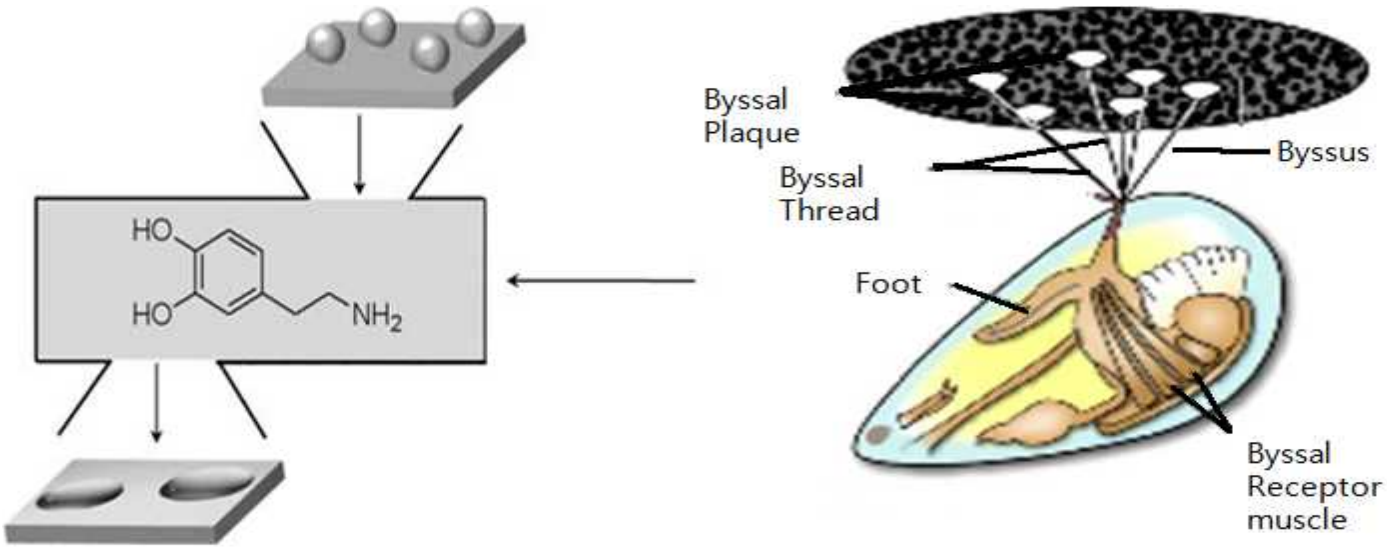


Figure 1. Mussel-inspired surface modification by DOPA.

and more studies on biocompatibility and bond strength of new bone adhesives will follow.

The goal of the current study is to follow the mechanisms of wet bonding to create synthetic water-borne underwater medical adhesives having cyanoacrylate group to be used in orthopedic field. We will create biomimetic bone adhesives using DOPA and other synthetic materials and test them in wet environment using bovine femur specimens.

II. MATERIALS AND METHODS

A. Materials for adhesive

To develop an adhesive, following materials were obtained. Triblock poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO), Pluronic F-127 (Molecular Probe, Eugene, USA) as a trade name, with an average molecular weight of 12,600 g/mol(oxyethylene content, 71.5-74.9%, content of PEO 80%) and an inhibitor-removing column, Sephadex LH-20 (GE healthcare, Uppsala, Sweden) were purchased and used without further treatment. Methoxyethyl acrylate (MEA), 2-ethyl cyanoacrylate (ECA, 99 %, Tm 84-86°C) and azobisisobutyronitrile (AIBN) 97% were purchased from Aldrich Chemical Co. (St Louis, USA) and used as received. Super Glue, Loctite 401 (Henkel, Dusseldorf, Germany), was used as a control. All other chemicals and organic solvents of the highest purity available were obtained from commercial sources.

B. Syntheses of Adhesives

Synthesis of dopamine methacrylamide (DMA), DOPA analog monomer, poly(dopamine methacrylamide-co-Methoxyethyl acrylate), [poly (DMA-co-MEA)], poly(dopamine methacrylamide-co-Methoxyethyl acrylate-co-2-ethyl cyano acrylate), [poly(DMA-co-MEA-co-ECA), PDMC].

For synthesis of dopamine methacrylamide (DMA), DOPA analog monomer, Messersmith's method was employed with slight modifications as follows.[25]

1) Sodium borate (20 g) and sodium bicarbonate (8 g) were dissolved in deionized water and bubbled with aragon (Ar). 10 g of dopamine-HCl (52.8 mmol) was then added, followed by the dropwise addition of 9.4 ml of methacrylate anhydride (58.1 mmol) in 100 ml of tetrahydrofuran (THF) to keep the pH above 8 with addition of 1M NaOH.
2) The reaction mixture was stirred overnight at room temperature with Ar bubbling. The aqueous mixture was washed several times with 300 ml of ethyl acetate and then the pH of the aqueous solution was reduced to less than pH 2.0 and extracted with 100 ml of ethyl acetate three times.
3) The final three ethyl acetate layers were combined and dried over MgSO4 to reduce the volume to around 50 ml. 800 ml of hexane was added with vigorous stirring and the suspension was kept in a refrigerator overnight.
4) The product was recrystallized from cold n-hexane and dried to yield 8.8 g of grey solid.

Polymer, p(DMA-co-MEA) was synthesized by free radical copolymerization of the DMA and MEA monomers by slight modification of published procedure[25] as shown from the scheme of Figure 2 and its molecular weight was analyzed by the size exclusion chromatography (Wyatt Technology, Santa Barbara, USA).

The synthesis procedure of p(DMA-co-MEA) is as follows.

1) MEA was passed through a column packed with Al203 to remove inhibitor.

2) 7.5 g of purified MEA (60 mmol), 17 g of DMA (74 mmol), and 120 mg of AIBN (0.71 mmol) were added to 70 ml of DMF in an airtight flask. The solution mixture was degassed through pump-freeze-thaw cycles three times.

3) While sealed under vacuum, the solution was heated to 65 ℃ and stirred overnight.

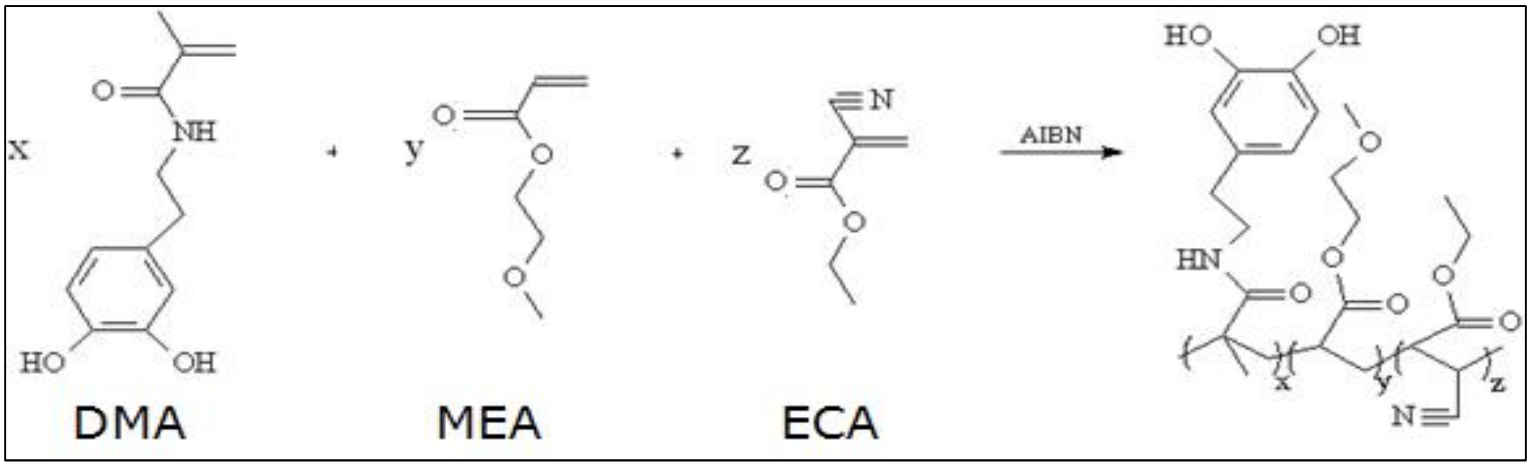
4) The reaction mixture was diluted with 250 ml of methanol and added to 500 ml of methyl ethyl ketone to precipitate the polymer.

5) After precipitating in dichloromethane three times and drying in a vacuum desiccator, 11.2 g of brownish, sticky solid was obtained.

6) Gel permeation chromatography was done in concert with multi-angle laser light scattering (Wyatt Technology), with mobile phase of 20 mM LiBr in DMF and Shodex-OH Pak columns: weight-average molecular mass 420 kDa, polydispersity 25.2.

For control experiments, a catechol-free p(MEA) homopolymer (molecular mass (average)5100 kDa, Scientific Polymer Products) was used.

ECA, DMA and MEA were copolymerized in the monomer feed ratio 2:1:1 by free radical polymerization on the same way for poly DMA-co-MEA preparation



Polymers

DMA; Dopamine Methacrylamide

MEA; 2-methoxyethyl acrylate

ECA; Ethyl 2-cyanoacrylate

PDM; poly(DMA-MEA), x=y= 0.5, z= 0.0

PDMC; poly(DMA-MEA-ECA), x=y=0.5, z= 1.0

Figure 2. Synthetic scheme of poly(DMA-co-MEA) and poly(DMA-co-MEA-co-ECA) from DMA + MEA + ECA.

which is as follows.

1) Cyanoacrylate was passed through an inhibitor-removing column to remove the polymerization inhibitor (monomethyl ether hydroquinone).
2) In a typical synthesis procedure, a mixture of 6.5 g of purified MEA (50.0 mmol), 11.1 g of DMA (50.0 mmol) and 12.5 g of ECA (100.0 mmol) were added to 100 ml of DMF in an airtight flask, and 300 mg of AIBN was placed into a borosilicate glass vial covered with a sleeve rubber stopper and equipped with a gas inlet/outlet.
3) The mixture was deoxygenated by nitrogen flow overnight at 10°C and immersed in oil bath. The bath was heated from 10 to 60°C and then was kept at 60°C for 48 hrs.
4) Then the vial was allowed to equilibrate at a room temperature and the polymer was precipitated in chloroform. The polymer sample produced was dried in vacuum condition to constant weight.

The polymers synthesized were characterized by FTIR, H^1-NMR and C^{13}-NMR spectroscopy analyses. NMR spectra of the polymers were obtained with a Varian NMR 300 model at a proton resonance frequency of 300MHz on using $CDCl_3$ as a solvent and tetramethylsilane (TMS) as an internal reference. Nicholet FTIR spectrometer model 6700 (Thermo Scientific, St Waltham, USA) was used to evaluate the extent of functional groups. For the purpose of this study, 1mg of polymer sample was mixed with 100mg of KBr containing 1 wt percentage

of KSCN.

The main polymeric fraction, washed with chloroform, was dried in vacuum condition to constant weight and analyzed by gel-permeation chromatography (GPC) to determine average molecular weight of the prepared polymer. GPC analyses were run on a GPC Waters model 600 E in tetrahydrofuran (THF) on two Waters Ultrastyragel linear columns and in water on two Waters Ultrahydrogel linear columns by using a RI detector.

C. Polymer Characterization Procedure and Preparation of Blends and Complex Coacervates

Blends of poly (DMA-co-MEA)/PEO-PPO-PEO, PDM/EPE or poly (DMA-co-MEA-co-ECA)/PEO-PPO-PEO, PDMC/EPE and were prepared in 1:1 ratio each by dissolving the components in ethanol (50 vol. %) at their required compositions.

The mixtures were stirred for 1 day at a room temperature and finally for 10 minutes at 60 °C, after which they were transparent. Calcium sulfate and Magnesium sulfate (0.5 wt % each) in DI water were added into the each solution to get a complex coacervate. The compositions of complex coacervates are shown in Table 2.

Table 2. Compositions of the complex coacervates in weight portion.

	PDM	PDMC	EPE	Ca^{+2}/Mg^{+2}
CC-1	1	0	1	0.1
CC-2	0	1	1	0.1
CC-3	1	1	1	0.1
CC-4	1	2	1	0.1
CC-5	1	2	1	0.0

PDM; poly(DMA-MEA), x=y= 0.5, z= 0.0
PDMC; poly(DMA-MEA-ECA), x=y=0.5, z= 1.0

D. Bone Adhesion Property Test - Measuring stress at break, shear modulus and contact angle.

Thirty six bovine femurs from freshly sacrificed Korean native kettle were purchased from the local grocery store. Using a band saw, bovine femurs were sawed perpendicular to long axis so that transverse section could be exposed for later adhesive application (Figure 3). The size of the transverse section after the cutting was 26 ㎠ in average (range 23-29 ㎠). The 36 specimens were divided in random into six experimental groups; each assigned to one of five complex coacervates and Super Glue. Super Glue was used as a control because there are no hard tissue medical adhesives available for comparison.

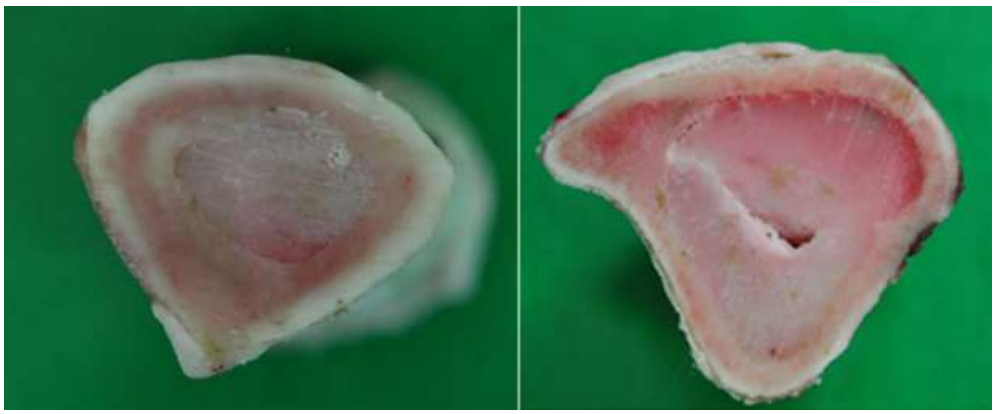


Figure 3. Femur from Korean native kettle cut to transverse sections.

The adhesives (CC-1, 2, 3, 4 and 5) were prepared at pH 7. Ascorbic acid was added at ratio of 1:5 to the polymers to prevent premature DOPA

oxidation in the specimens. Bone samples were soaked in phosphate buffered saline (20 mM Phosphate, 150 mM NaCl, pH 7.4) for 5 minutes and NaIO4 at a 1:2 molar ratio to DOPA side chains was evenly applied onto each faces of wet bone specimens. A volume sufficient to fill the space completely between the bone interfaces of the test specimens was applied with a homemade spray-gun. The two pieces of bone specimens were then pressed together, clamped for 10 seconds and were immediately wrapped with PBS soaked gauze. All the bonded specimens were incubated in a sealed container containing soaked sponges to maintain sufficient humidity at 37° C for at least 24 hours. Because of the problems in determining the exact contact area, the values in modulus (shear) were taken from the tests in stainless-steel adhesion, on the other hand the values in stress at break were measured from the bone specimen tests.

Universal Testing Machine (Autograph, Shimadzu Co., Tokyo, Japan) was used to test the stress at break. For this test, one piece of bone from a bonded pair was clamped laterally 1 mm from the bond interface. The other piece was pressed against a dull blade positioned 1 mm lateral to bond interface with a crosshead speed of 0.02 mm/s until break (Figure 4a). Mechanical bond tests were performed in the water jacket at 37°C. The tests were repeated 6 times for each adhesive using different specimen.

To test shear modulus, stainless steel plate (Height : 10mm, Width : 10mm. thickness : 5mm), commercially acquired, was used. The stainless steel plate was fully submerged in a temperature controlled water bath at 37°C during the

test (Figure 4b). The test for shear modulus was also repeated 6 times for each specimen.

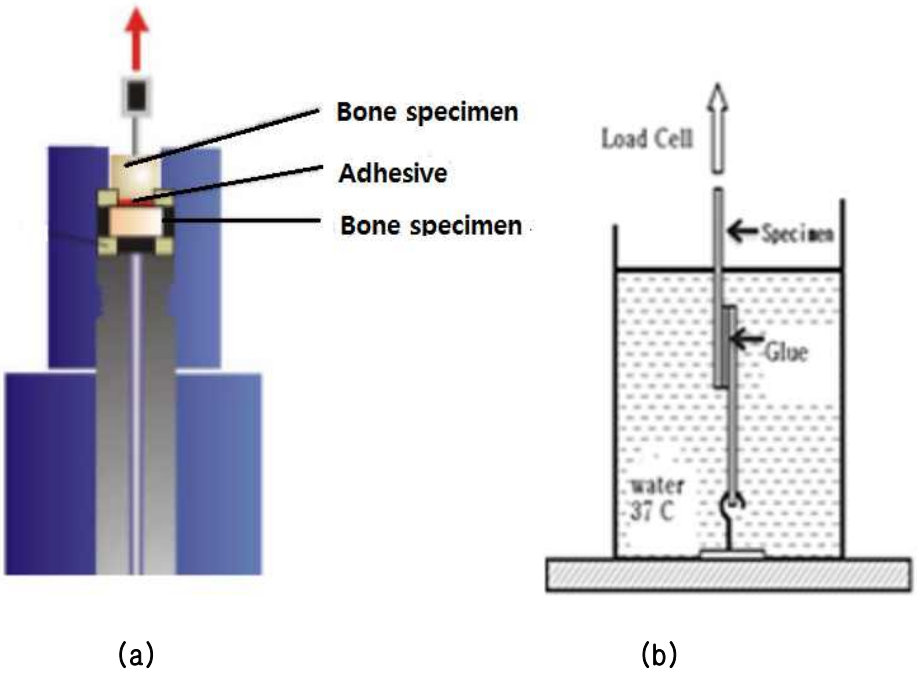


Figure 4. Test of stress at break (a) and for shear modulus (b).

Super Glue was again used as a control. Control specimens were bonded with sufficient amount of Super Glue in exactly the same manner. The shear mechanical tests were performed with UTM in a water jacket at 37°C and a 1 kg load cell.

Contact angle measurements of the complex coacervates and Super Glue were taken from six clean glass surfaces. A contact angle analyzer (Phoenix 300, SEO Co, Ansung, Korea) was used. Data were calculated using the static half-angle method with the Girifalco-Good-Fowkes-Young mode. Reported values are averaged from 10 measurements.

E. Sample Designation

DMA : dopamine methacrylamide

MEA : methoxyethyl acrylate

ECA : 2-ethyl cyano acrylate

PDM : poly(dopamine methacrylamide-co-Methoxyethyl acrylate), poly (DMA-co-MEA)

PDMC : poly(dopamine methacrylamide-co-Methoxyethyl acrylate-co-2-ethyl cyano acrylate), poly(DMA-co-MEA-co-ECA)

EPE : poly(ethyleneoxide)-poly(propyleneoxide)-poly(ethyleneoxide) triblock copolymer

CC : complex coacervates

III. RESULTS

A. Synthesis of dopamine methacrylamide (DMA), poly(dopamine methacrylamide -co-Methoxyethyl acrylate), PDM, and poly(dopamine methacrylamide-co-Methoxyethyl acrylate-co-2-ethyl cyano acrylate), PDMC.

Dopamine-HCl was reacted with methacrylate anhydride to produce DMA with 61% in yield. The prepared DMA was characterized by H^1 NMR and C^{13} NMR. DMSO-d6 and TMS were used as a deuterized solvent and an internal standard respectively for NMR measurements. Following tests were conducted to characterize newly created adhesives:

For 1H-NMR (300MHz, DMSO-d/TMS): δ 8.7-8.6 [2H, (OH)2-Ar-], 7.9[1H, -C(=O)-NH-], 6.5-6.6 [2H,C6H2(OH)2-], 6.42[1H, C6H2H(OH)2-], 5.61 [1H, -C=O)-C(-CH3)=CHH], 5.30 [1H, -C(=O)-C(-CH3)= CHH], 3.21[2H,C6H3(OH)2-CH2-CH2(NH)-C(=O)-], 2.55 [2H, C6H3(OH)2-CH2-CH2(NH)-C(=O)-], 1.84 [3H, -C(=O)-C(-CH3)=CH2]. (Italic letters indicate the atom taking the peak.)

For 13C-NMR (300MHz, DMSO-d/TMS): δ 167.3 (s, 1C, -NH-C(50)-C(CH3)5CH2), 145.0 (s, 1C, -NH-C(=O)-C(CH3)=CH2), 143.5--115.5 (6C, C6H3(O-C(=O)-CH3)2), 130.3 (s, 1C, -NH-C(=O)-C(CH3)=CH2), 41.0 (s, 1C, C6H3(OH)2- CH2-CH2(NH)-C(=O)-), 34.6 (s, 1C, C6H3(OH)2-CH2- CH2(NH)-C(=O)-), 18.7 (s, 1C, -C(=O)-C(-CH3)=CH2).

Figure 5 illustrates H^1-NMR spectra of DMA and Figure 6 shows C^{13}-NMR spectra of DMA. Figure 7 shows the Infrared (IR) absorption spectra of DMA.

The functional groups of DMA were identified at 1650 Cm^{-1}($\upsilon_{C=C}$), 1670Cm^{-1}(υ_{C6H5}), 2750 Cm^{-1} υ_{OH}, 3250Cm^{-1} υ_{-NH}, 1560Cm^{-1} $\upsilon_{C=O}$.

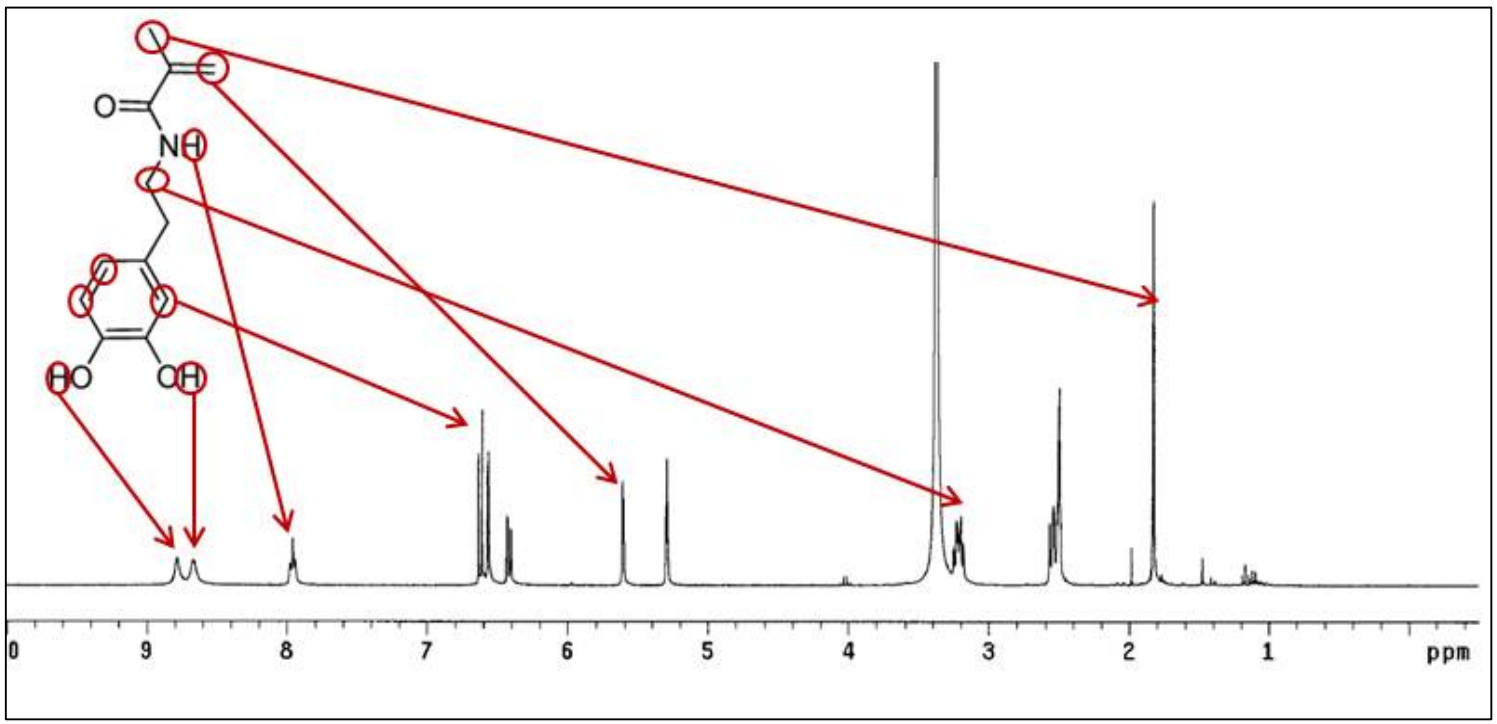


Figure 5. H^1-NMR spectra of DMA. Arrows indicate typical spectrum of H^1 in DMA.

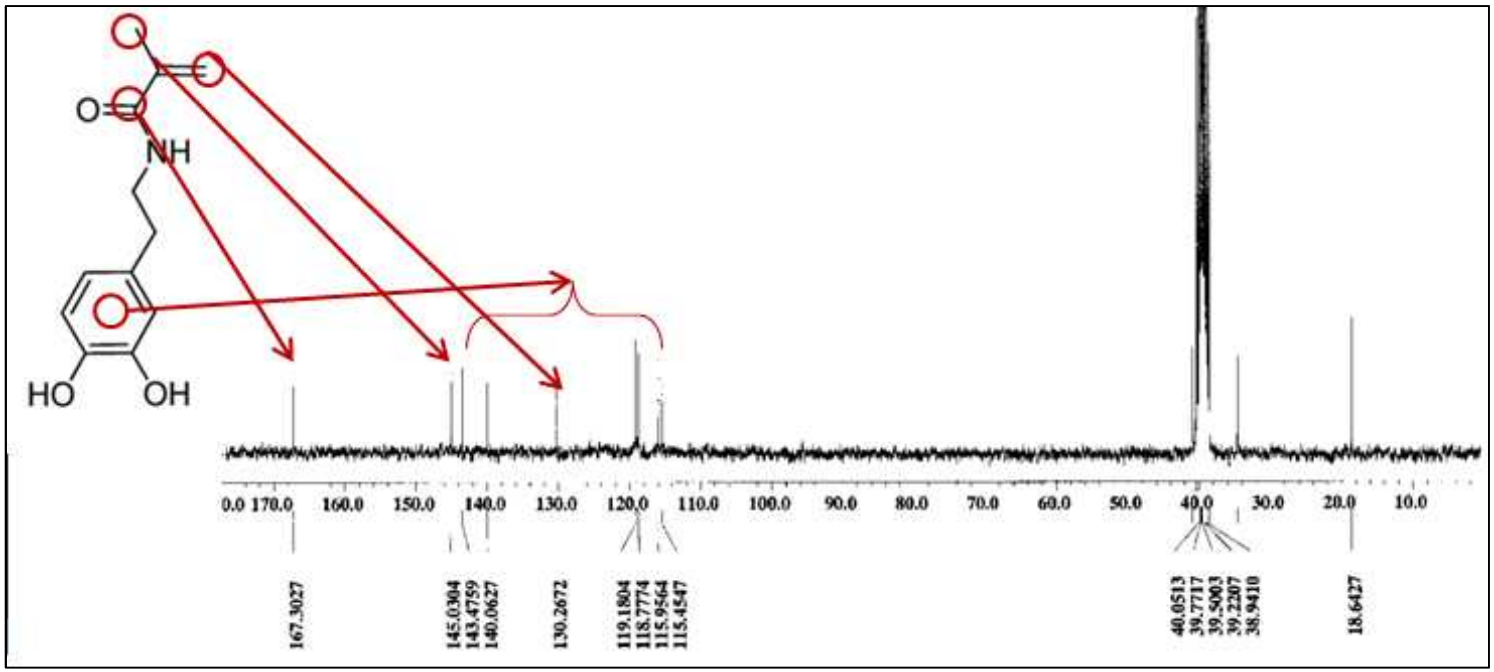


Figure 6. C^{13}-NMR spectra of DMA. Arrows indicate typical spectrum of C^{13} in DMA.

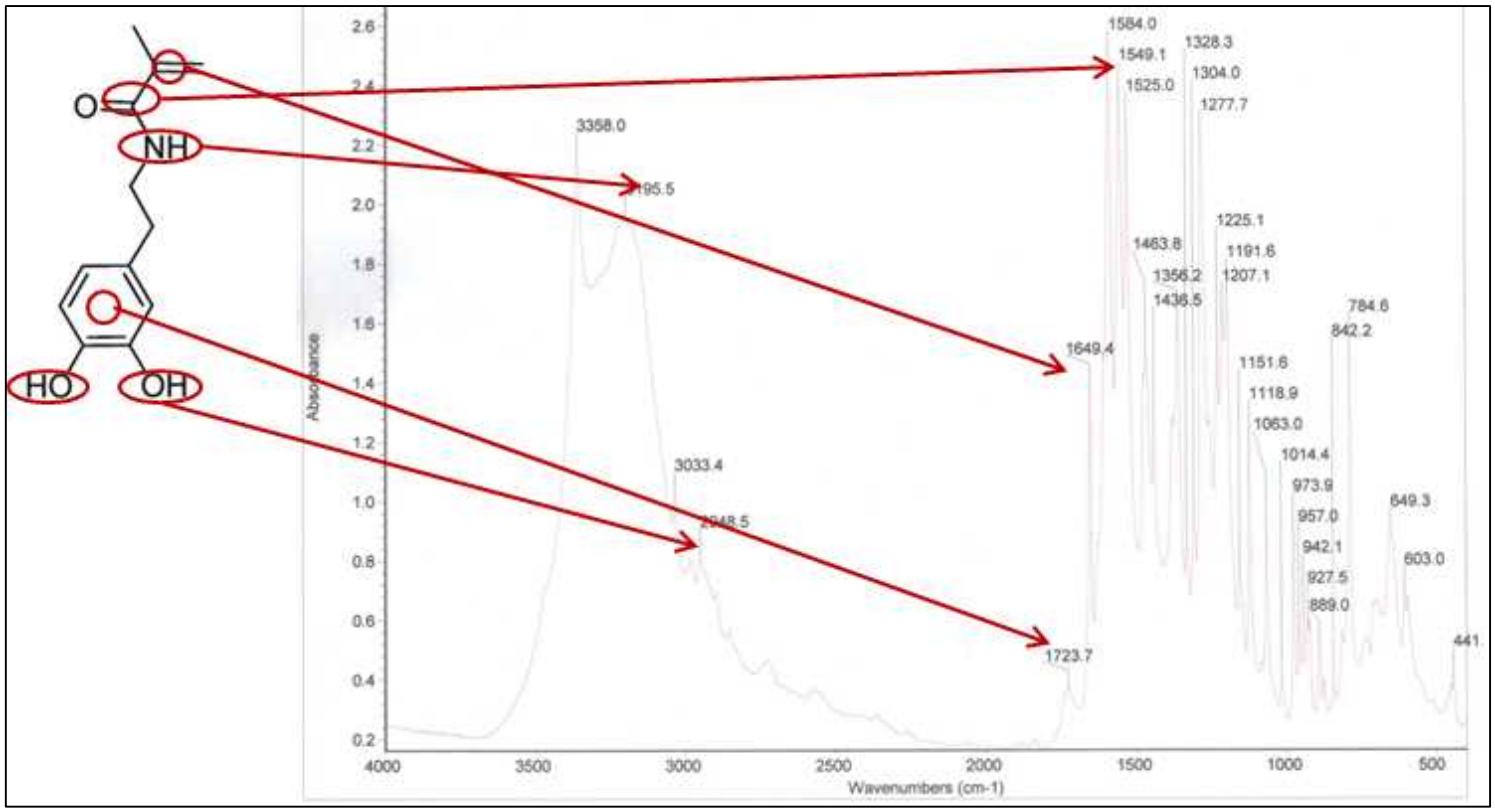


Figure 7. IR spectra of DMA. Arrows indicate typical spectrum of functional groups in DMA.

PDM was synthesized by free radical copolymerization of the DMA and MEA monomers with AIBN as a radical initiator. The monomer feed ratio of DMA and MEA was adjusted to 74 mmol (DMA) and 60 mmole respectively to produce 1 to 1 monomer unit contents in the final polymer composition after several prior trials.

1H-nuclear magnetic resonance spectra (300 MHz) were obtained with CDCl3 as a solvent and TMS as an internal standard (Figure 8).
1H : 6.81-6.70 (d, br, 2H, C6H*H2*(OH)2-), 6.58 (s, br, 1H, C6H2*H*(OH)2-), 4.20 (s, br, 2H, CH3-O-CH2-C*H2*-O-C(=O)-), 3.57 (s, br, 2H, CH3-O-C*H2*-CH2-O-C(=O)-), 3.36 (s, br, 3H, C*H3*-OCH2-CH2-O-C(=O)-), 2.69 (s, br, 2H, C6H3(OH)2-CH2-C*H2* (NH)-C(=O)-), 2.39 (s, br, 1H, -O-C(=O)-C*H*(CH2-)-CH2-), 2.14 (s, br, 2H, C6H3(OH)2-C*H2*-CH2(NH)-C(=O)-), 1.93 (s, 3H, -NHC(=O)-C(CH3)(CH2-)-CH2-), 1.68 (m, br, -O-C(=O)-CH(CH2-) -C*H2*-), 0.98 (m, br, -NH-C(=O)-C(CH3)(CH2-)-C*H2*-). This NMR analysis indicated a 1: 0.9 molar ratio of DMA to MEA in the copolymer composition.

IR spectrum of PDM gives a distinct hydroxhl group of cathecol assembly of DOPA unit at 2931cm^{-1} for cathecol OH and carbonyl C=O at 1732 cm^{-1} (Figure 9).

The yield of PDM was 65% and the average Mn was 12000 g/mol. The glass transition was observed at -15℃ on the 1st and 2nd heating runs of differential scanning calorimetry as shown in Figure 10.

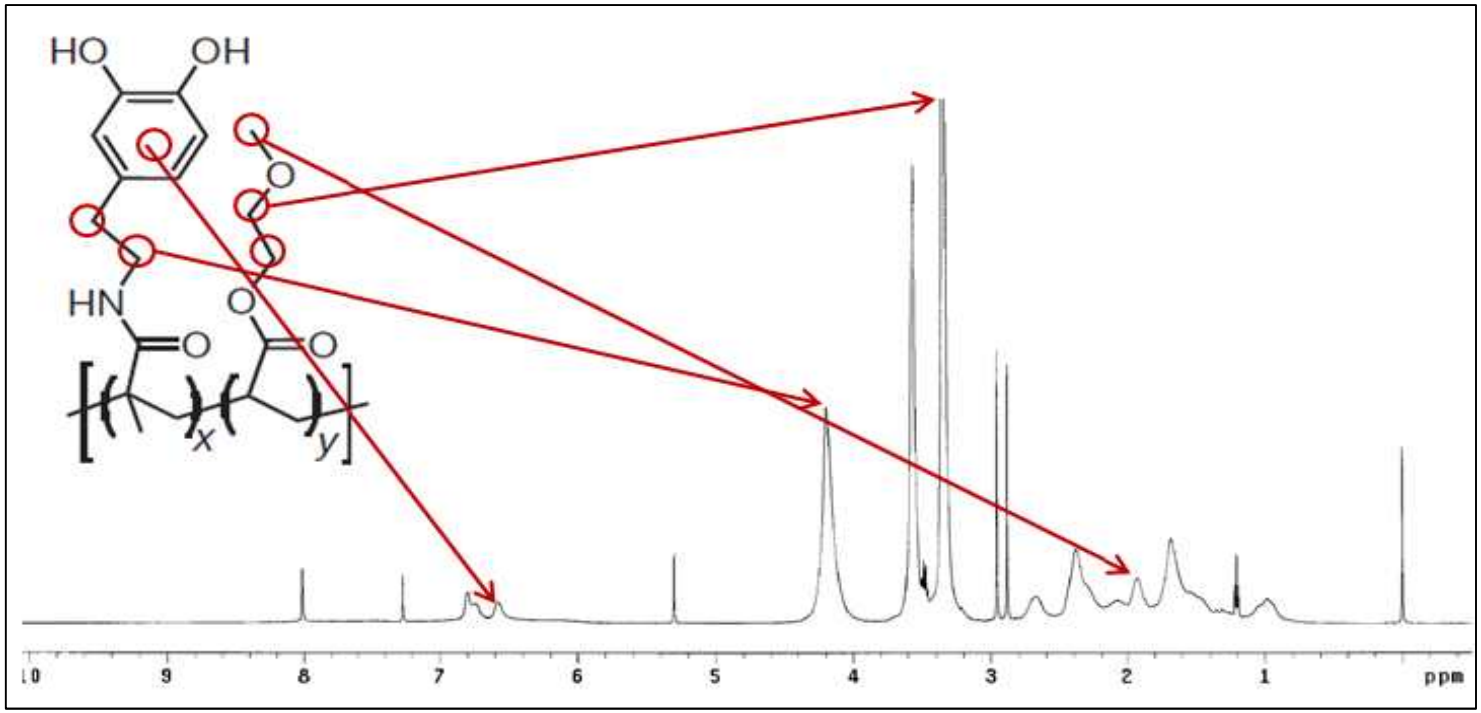


Figure 8. H^1-NMR spectrum of PDM. Arrows indicate typical spectrum of H^1 in PDM.

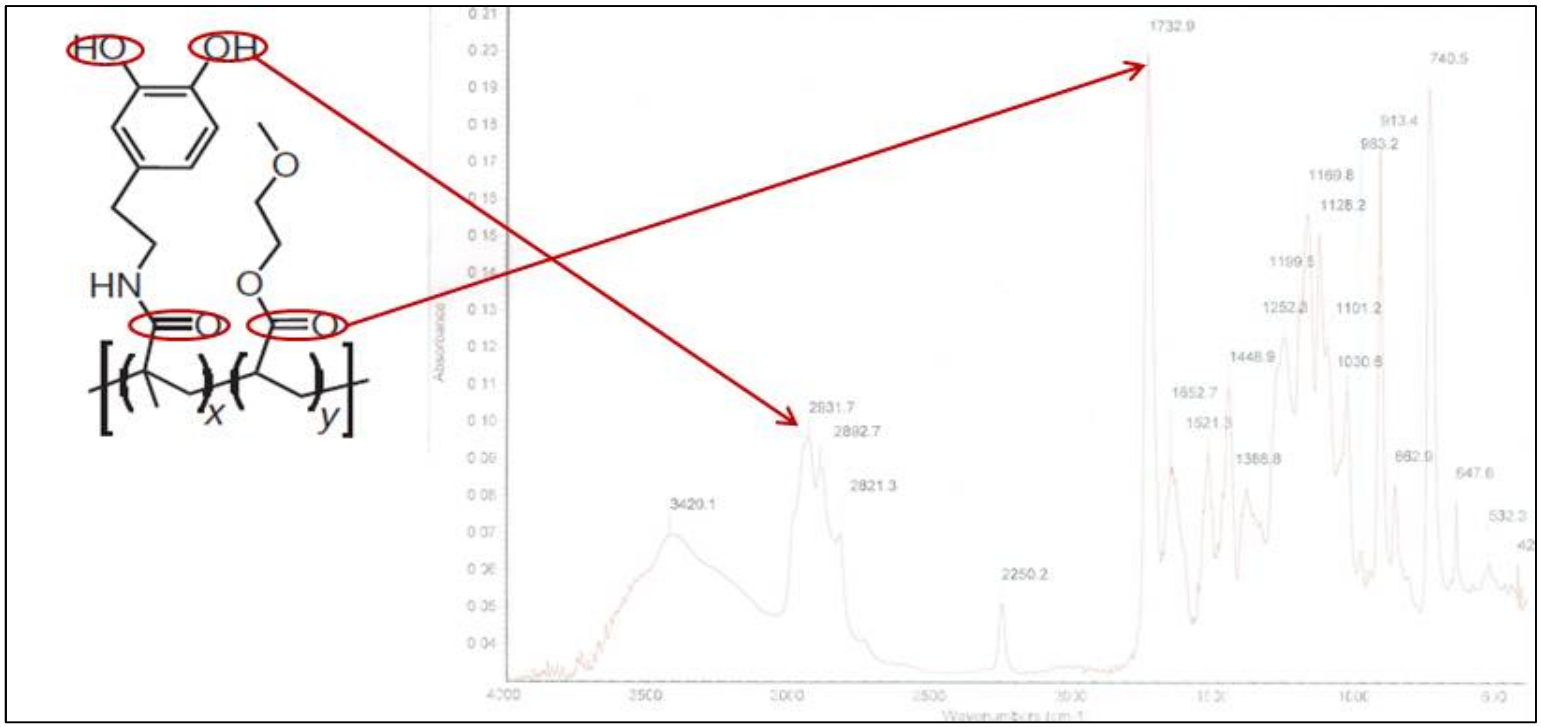


Figure 9. Infrared spectrum of PDM. Arrows indicate typical spectrum of functional groups in PDM.

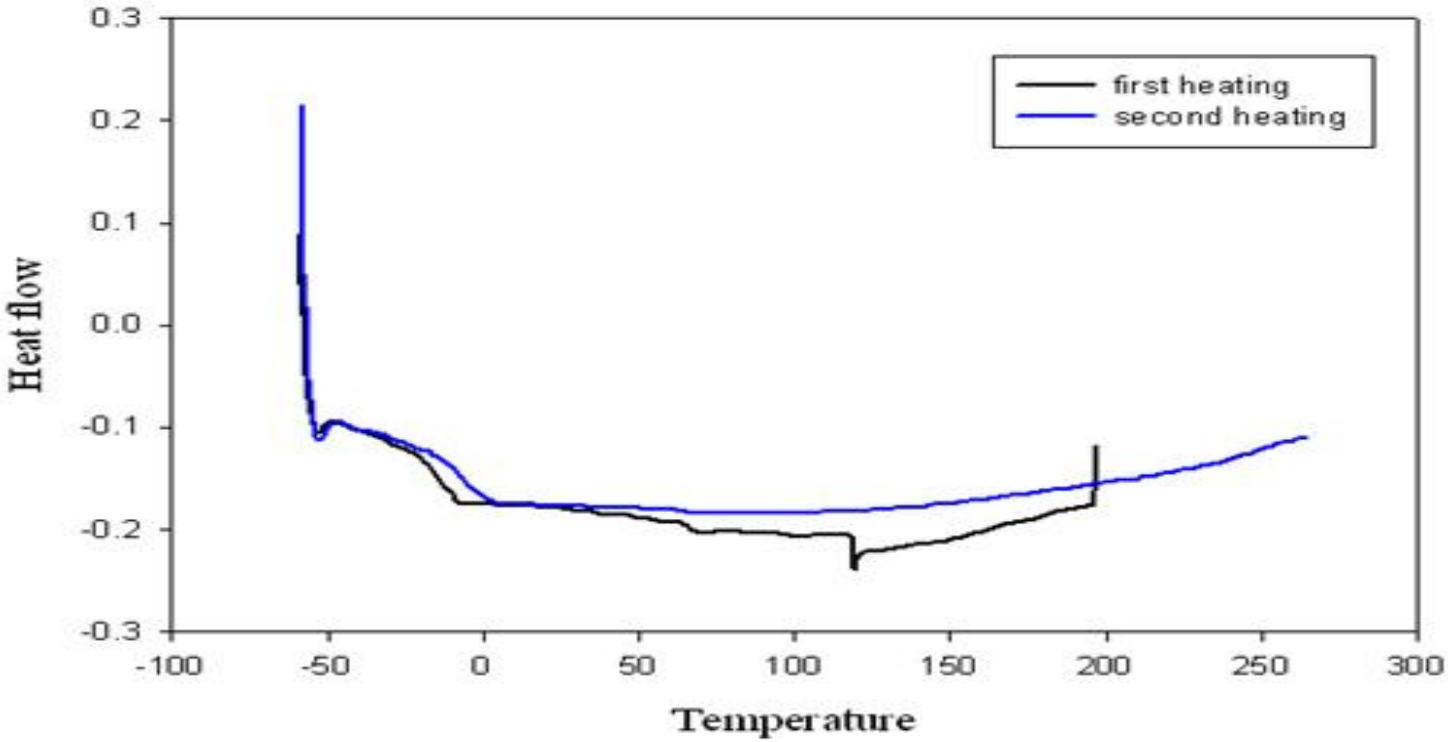


Figure 10. Differential scanning calorimetry diagram of PDM

PDMC was synthesized by a thermally initiated free radical polymerization of DMA, MEA, and ECA. The prepared polymer was characterized by 1H NMR to calculate composition ratios of each repeating unit in the final poly (DMA-co-MEA-co-ECA). 1H NMR and chemical structure assignments are shown in Figure 11. After testing samples which were prepared with various ratios of three repeating units, a tricopolymer which has 25% of DMA, 25% of MEA, and 50% of ECA was verified as the most suitable properties for use as an adhesive. Therefore, this study focused on an adhesive of this composition. According to 1H NMR characterization the proton of CH-CN showed at 3.4 ppm, the final composition reflects the initial feeds of monomers. 13 C NMR gives a distinct carbon of CN group in the PDMC at 162.6 ppm (Figure 12). IR spectrum of PDMC showed CN stretching vibration at 2040~2060 cm^{-1} (Figure 13).

The yield of the PDMC was 71% and the average Mn was 7500 g/mol. The melting transition was observed at 198 ^{o}C with quite crystalline morphology on the heating run of differential scanning calorimetry as shown in Figure 14.

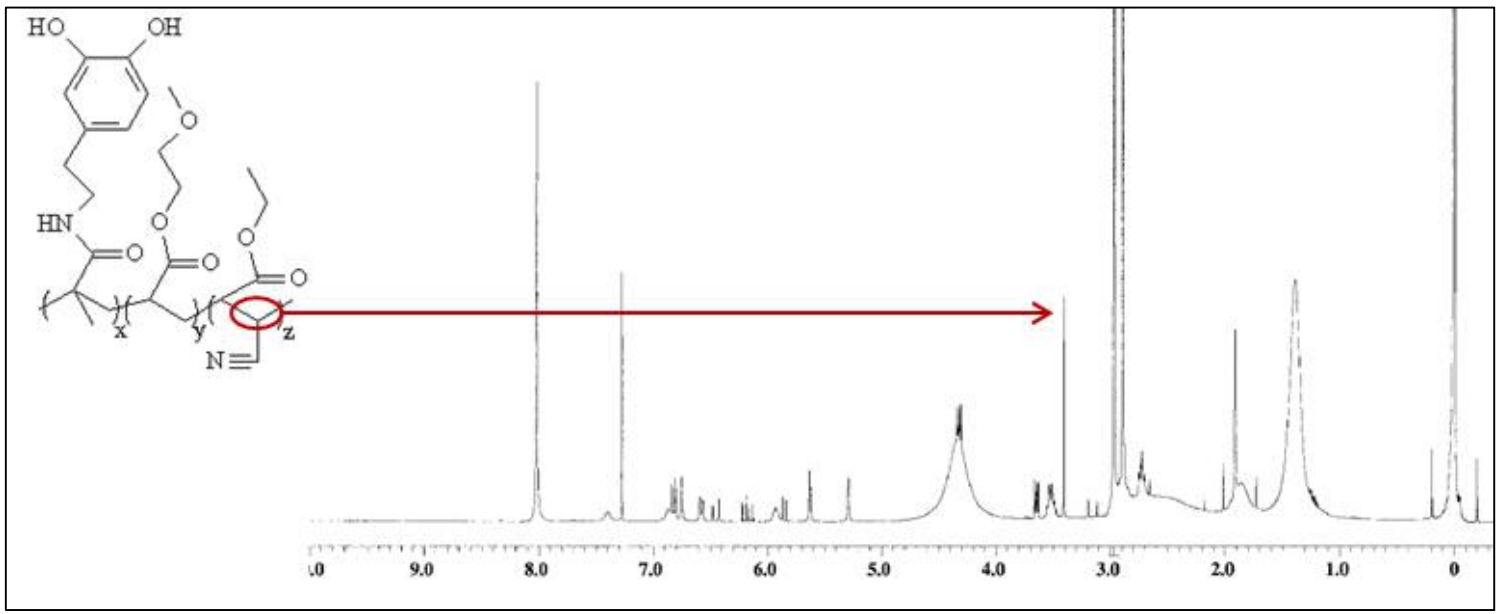


Figure 11. H^1-NMR spectrum of PDMC. Arrow indicates typical spectrum of H^1 in PDMC.

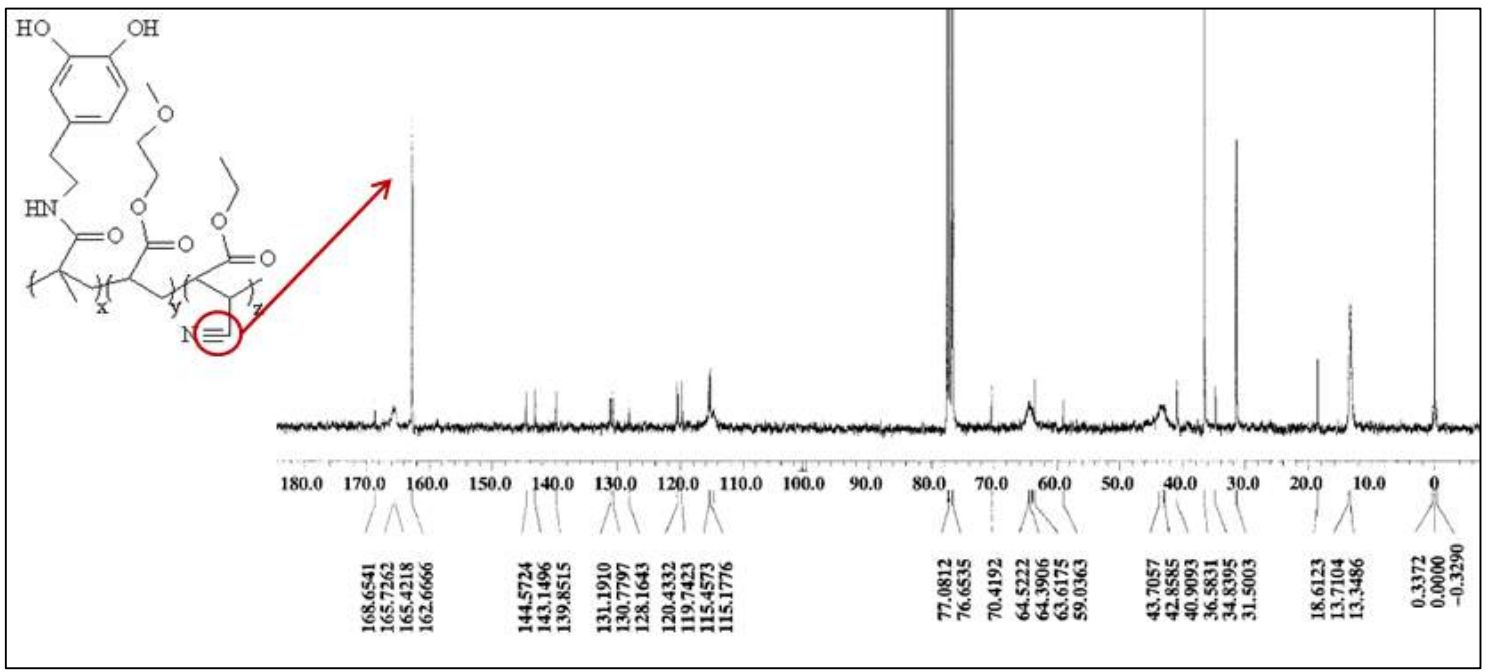


Figure 12. C^{13}-NMR spectrum of PDMC. Arrow indicates typical spectrum of C^{13} in PDMC.

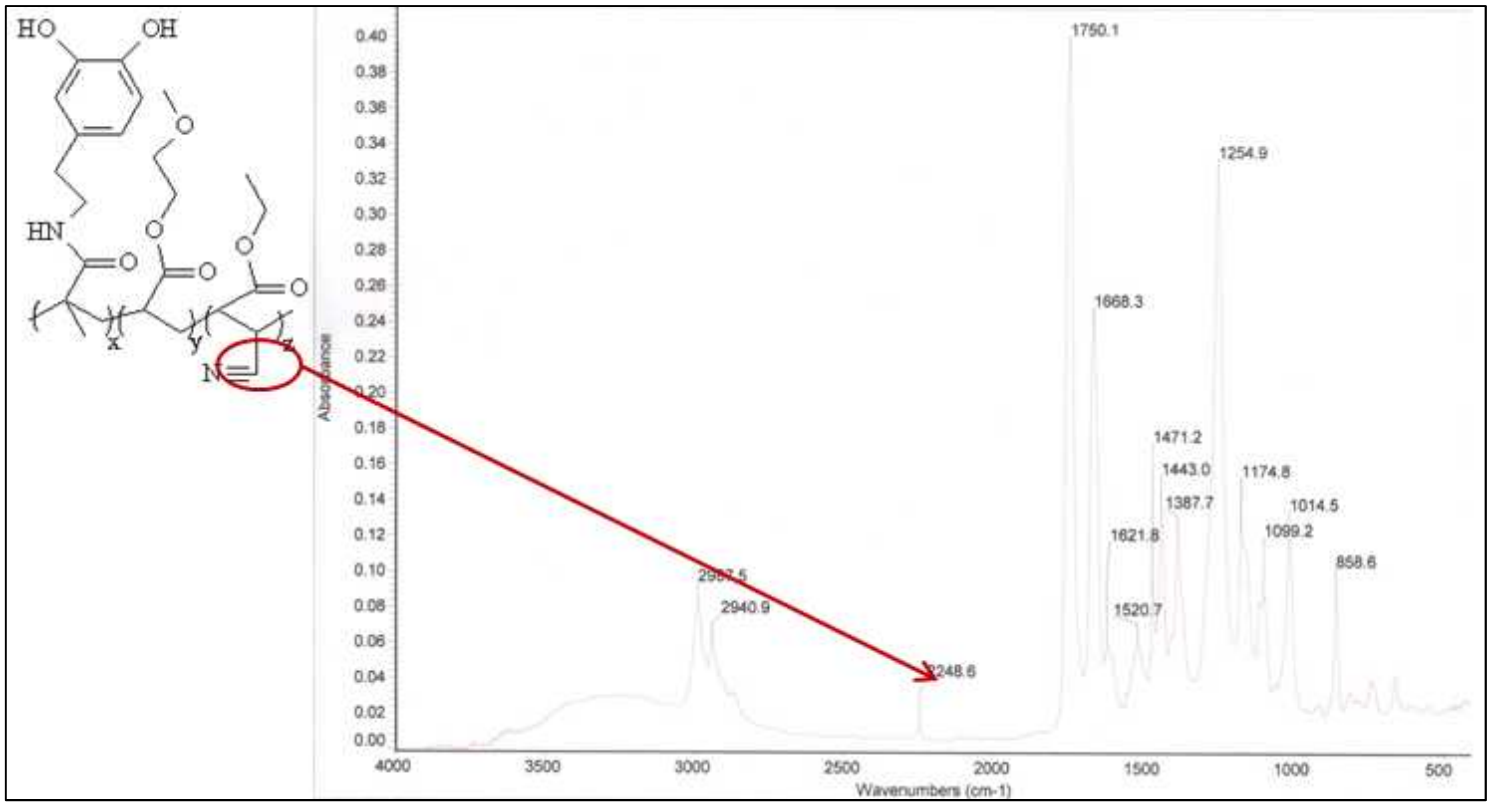


Figure 13. IR spectrum of PDMC. Arrow indicates typical spectrum of functional groups in PDMC.

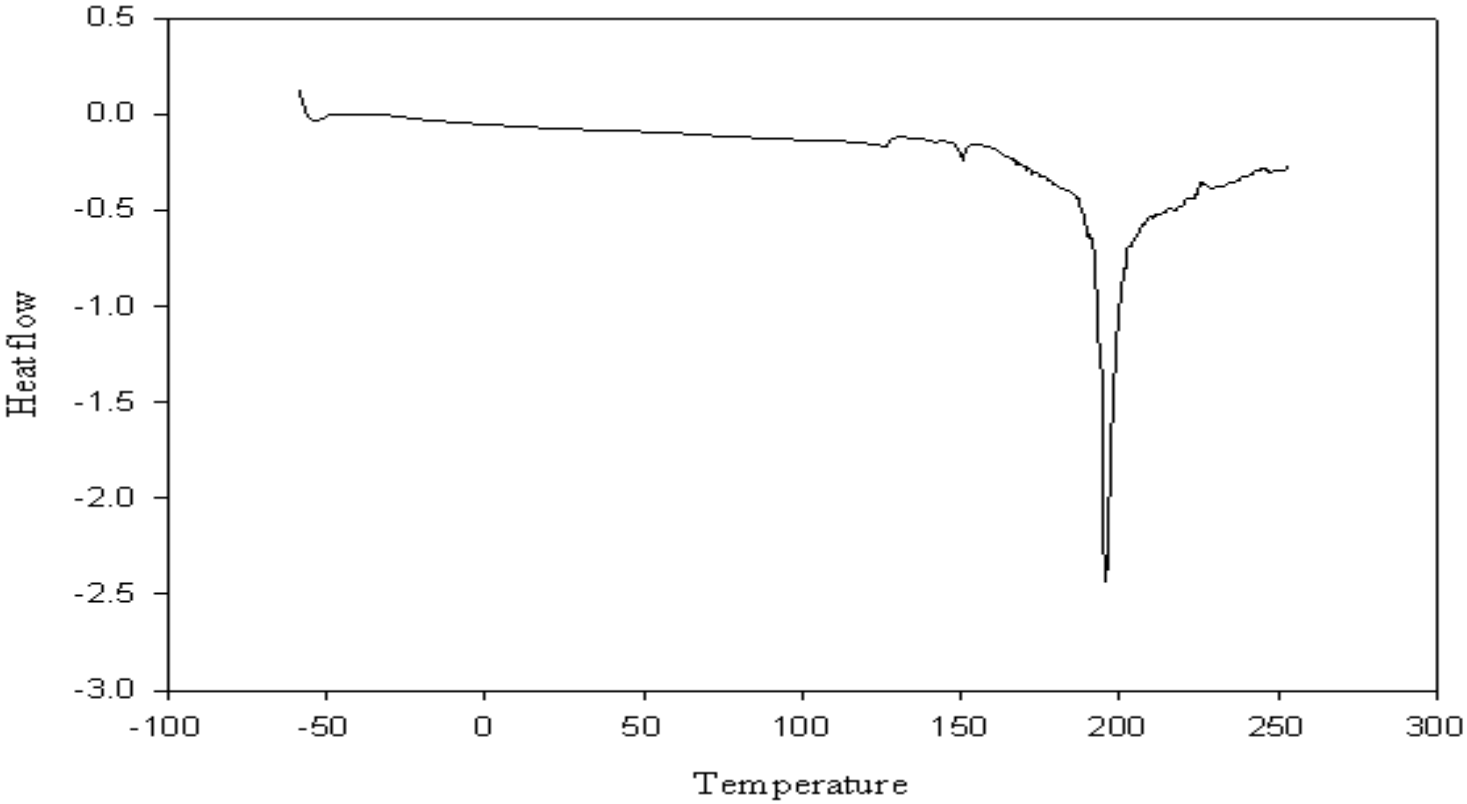


Figure 14. Differential scanning calorimetry diagram of PDMC.

B. Adhesion Properties

Tests for stress at break and shear modulus were performed 6 times for each of the 5 different compositions as described in Table 1. In specific, CC-1; PDM with EPE in 1:1 ratio, CC-2; PDMC with EPO in 1:1 ratio, CC-3; PDM and PDC with EPE in 1:1:1 ratio, CC-4; PDM and PDC with EPE in 1:2:1 ratio and CC-5; same as CC-5 but no Ca^{++}/Mg^{++} content, and for Super Glue which was used as a reference. All surfaces were handled according to the same procedure.

Data from 6 trials are described in table 3. The mean value and standard deviations of stress at break, shear modulus and contact angle are shown in Figure 15, Figure 16 and Figure 17 respectively. For adhesion in general, force is related to overlap area, with greater area yielding higher binding forces.[26] The average adhesion, however, varies dramatically depending upon the amount of PDMC content.

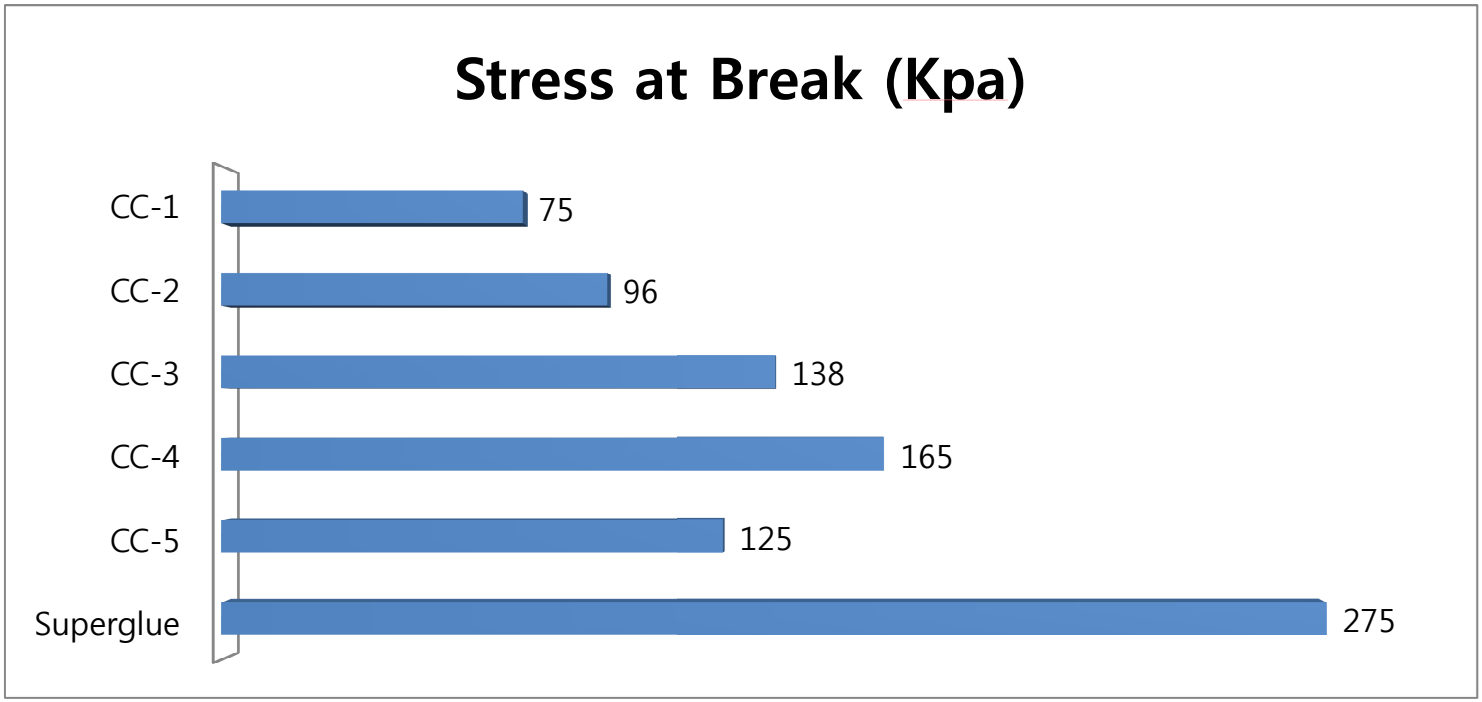


Figure 15. Stress at break of complex coacervates using UTM with Super Glue as a reference.

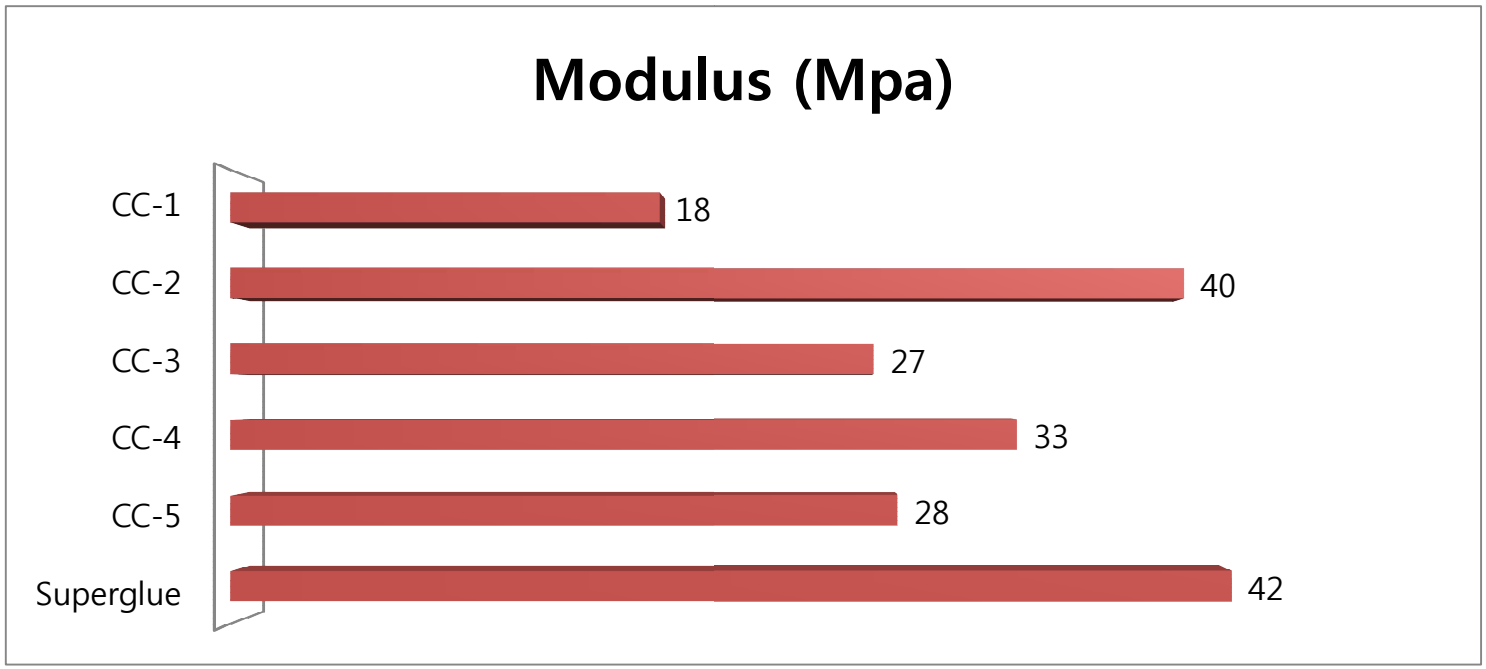


Figure 16. Shear modulus properties of complex coacervates on stainless steel plaques with Super Glue as a reference.

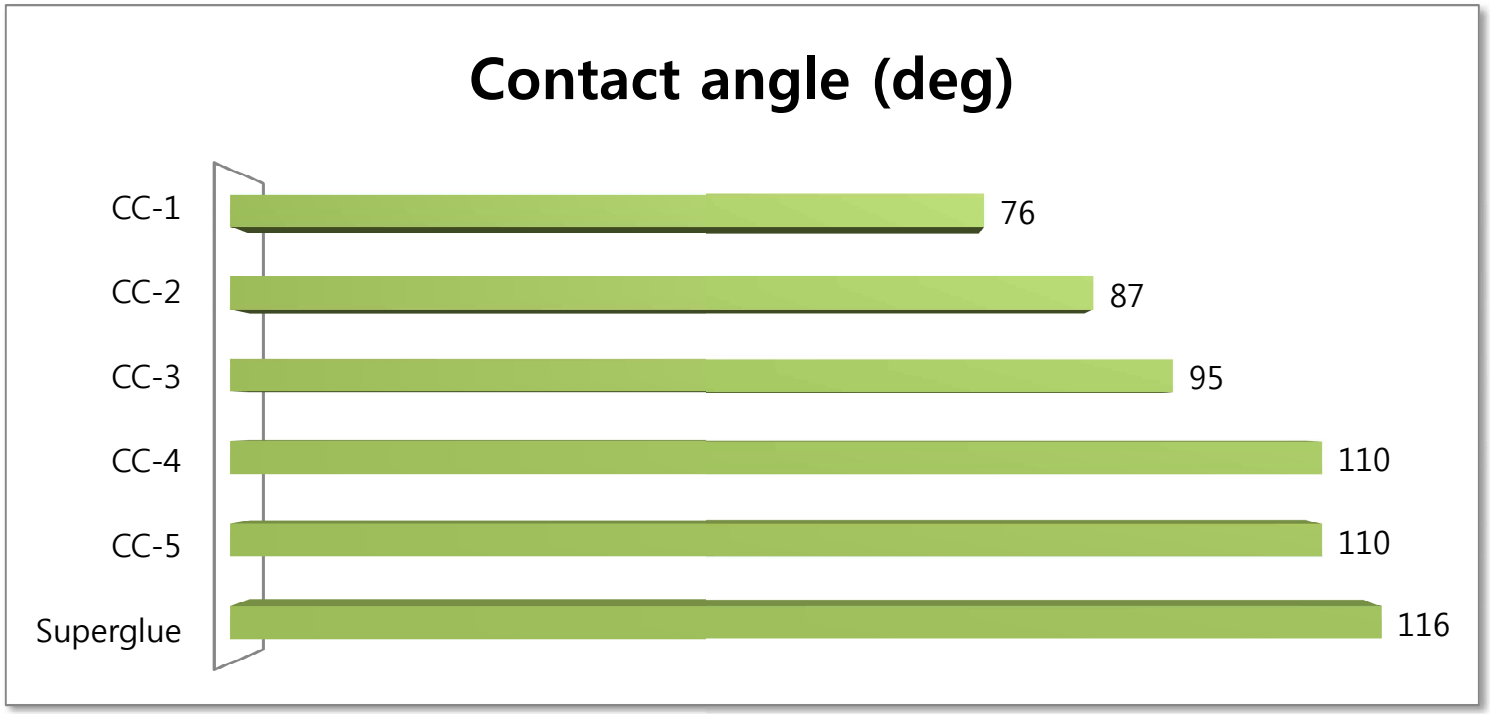


Figure 17. Contact angle of Complex coacervates on stainless steel plaques with Super Glue as a reference.

Table 3. Adhesive property data of complex coacervates from 6 reapeated trials respectively. Super Glue was used as a reference.

	Trials	Stress at Break	Shear Modulus
CC -1	1	77.4	17.2
	2	71.2	17.9
	3	73.8	18.3
	4	78.9	19.2
	5	70.0	18.4
	6	77.4	16.9
CC - 2	1	94.2	41.3
	2	96.4	38.8
	3	97.3	41.5
	4	98.1	39.5
	5	93.1	38.9
	6	97.0	40.2
CC - 3	1	137.1	26.9
	2	135.4	27.2
	3	132.8	26.2
	4	142.2	28.0
	5	141.9	27.8
	6	137.6	26.1
CC - 4	1	159.2	32.4
	2	165.8	33.7
	3	169.3	34.2
	4	166.4	33.8
	5	161.2	32.1
	6	168.3	31.9
CC - 5	1	128.3	27.8
	2	125.9	27.3
	3	121.3	28.4
	4	124.9	27.1
	5	120.8	28.3
	6	129.3	29.2
Super Glue	1	289.2	41.6
	2	269.2	41.2
	3	273.9	42.1
	4	266.3	42.3
	5	270.8	41.8
	6	281.3	43.2

For stress at break, the highest values was found for CC-4 with mean of 165 KPa while the lowest were observed in CC-1 with the mean of 75 KPa. The tensile force shown in CC-4 is about 65% of the strength of wet bones bonded with commercial cyanoacrylate adhesive (Super Glue).

Shear modulus of CC-2 showed 40 MPa which nearly approaches to that of Super Glue (42MPa) but 96 KPa, the value in stress at break, was quite low compared to that of Super Glue (273 KPa). CC-4 had shear modulus of 33 which is less than that of Super Glue but still considerably high compared to other complex coacervates.

The water contact angle was measured for each surface, prior to placement in the tanks. The data provide insight on the relative surface energy of each substrate. CC-1 having PDM and EPE with Ca^{++} and Mg^{++} cations showed 76^{o} in contact angle, on the other hand, CC-4 containing PDM, EPE and higher PDMC showed 116^{o} which is more hydrophobic due to higher ECA content.

Overall, we believe CC-4 has the optimal quality to be applied to actual in-vivo practice.

IV. Discussion

Bioadhesive materials have many potential applications in bone and surgical adhesion, wound closure, drug delivery carrier/vehicle, when they can function in wet environments. A notable biological model for wet adhesion is the mussel, which is well known for its ability to cling to wet surfaces. Marine mussels are known to anchor themselves to underwater surfaces. They secrete adhesive proteins that can rapidly cure to form adhesive plaques. The adhesion begins to set in several seconds under cold sea water and hardens to a tough leathery consistency over several hours. Structurally the adhesive appears to be an aggregation of 50-80 nm spheres that form a microporous water-filled foam with a steep gradient in porosity, from nearly solid at the outside edges to nearly empty foam at the center. The adhesive is comprised of several highly acidic and basic proteins and a good measure of Mg^{2+} and Ca^{2+} ions.[27] Also, mussels secrete specialized adhesive proteins containing a high content of the catecholic amino acid, 3,4-dihydroxy-L-phenylalanine (DOPA) (Figure 18), which is responsible for both strong interfacial binding and curing of these proteins.

DOPA is an amino acid that is believed to be responsible for the adhesive characteristics of underwater adhesive proteins. DOPA occurs in the adhesive plaque of mussels and has been proposed to play dual roles in interfacial adhesion and cohesive crosslinking. The biomimetic approach based on DOPA is to improve adhesive deliverability and performance in the presence of water.

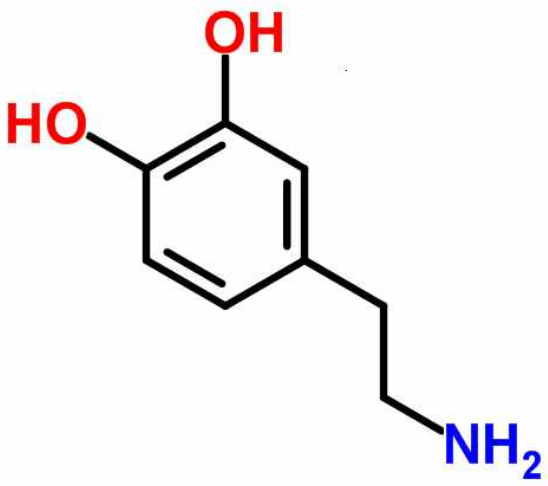


Figure 18. Chemical structure of DOPA

Recently, the Messersmith group has reported that the catechol form of DOPA bonds to wet titanium oxide surfaces with dissociation energies of 22 kcal/mol, the strongest noncovalent bond yet measured, providing support for DOPA's main role in interfacial adhesion.[28] UAPs are remarkable underwater bioadhesive (UWB) materials initially secreted as sticky fluids that harden in situ to form an adhesive plaque that anchors marine and freshwater mussels to the substrates upon which they reside. In addition to exhibiting good adhesion to metal, metal oxide, and polymer surfaces, DOPA-containing molecules have been found to strongly interact with a pig gastric mucin glycoprotein in dilute solution, suggesting that DOPA-containing proteins and biomimetic polymers may have useful mucoadhesive properties which can be exploited for medical applications.[29-32]

Despite these advantages, UWBs that are currently available rarely meet all the requirements for practical in-vivo applications due to extremely limited manufacturing, poor adhesion to wet tissues and toxicity concerns. The challenge is to develop effective adhesives for the repair of wet living tissues and bones with reasonable production methods.

A. Requirements for Bone Adhesion

There are several advantages of using the adhesives compared to mechanical fixation which is as follows[14,17,33]:

1) Enabling good fixation of small fragments of the articular surface replacing the numerous small diameter wires, especially for highly comminuted bone fracture,

2) Avoiding follow up surgical operations to remove the mechanical devices,

3) Leading to more homogeneous weight bearing distribution within the fracture site,

4) Allowing fixation of osteoporosis or low density bone tissues,

5) Avoiding the high capital equipment costs,

6) Being free from metal hardware interruption in radiologic evaluations,

7) Compensating joint surface displacement and minimizing interference with healing of the surrounding tissue in articular fracture.

There are many requirements for a medical bone adhesive to clinical

application in trauma and orthopedic surgery[34,35]:

1) Biocompatible to bone and surrounding tissue (no tissue irritation or tissue necrosis) with minimal exothermic during polymerization,
2) Non-toxic and non-teratogenic,
3) Biodegradable in a prefixed time, by degradation and cellular resorption without acting as a barrier to osteogenesis,
4) The degradation should act inversely proportional to healing, to ensure mechanical stability,
5) The adhesive must bond in moist environment (high bonding strength in situ) to allow early weight bearing,
6) Needs sufficient elasticity to ensure stability to tensile strength,
7) Easy preparation, practicability and applicability, minimal compression of volume, stability during storage.

However it's difficult to meet completely with the whole requirements. The applicable study in this field is just in its early stage. It would be highly desirable to apply biomimic chemical approach to match up the requirements.[19]

B. Complex Coacervates

A coacervate is a tiny spherical droplet of assorted organic molecules

such as lipid molecules which is held together by hydrophobic forces from a surrounding liquid. Complex coacervates are an intriguing state of matter. It refers to the phase separation of a liquid precipitate, or phase, when solution of two hydrophilic colloids is mixed under suitable conditions.

The general outline of the processes consists of three steps carried under continuous agitation. When the pH or other solution conditions are adjusted to change the net charge of the complexes, generally described as toward net neutrality, the complexes condense into a liquid phase of concentrated polymers (the coacervate phase) and a dilute equilibrium phase. The coacervate phase is an associative liquid with a dynamic structure in which the individual polymer components diffuse throughout the entire phase. Complex coacervates behave rheologically like a viscous particle dispersion rather than a viscoelastic polymer solution. In the case of *P. californica*, these properties explain how worms can secrete its adhesive under seawater as a watery liquid that readily spreads over the surface of wet mineral substrates, fills gaps, but does not disperse into the ocean. The trigger for the quick set and slower DOPA-mediated hardening reaction that occurs after secretion may be the pH jump experienced by the adhesive as it travels through the secretary pathway (pH 5) to seawater (pH 8.2).

In this context, poly(ethyleneoxide)-poly(propyleneoxide)-poly (ethylene eoxide), PEO-PPO-PEO triblock copolymers, are of a significant interest because they exist in different states of aggregation in aqueous solution depending on relative block sizes and on concentration and temperature.

Their self-association behavior in water has attracted great attention in the literature.[36-38] At moderately high temperatures and concentrations, the PEO-PPO-PEO copolymers self-assemble to form micelles because of the limited and temperature dependent solubility of the PPO block.[39,40] For these copolymers, owing to the polydispersity of the PPO and PEO blocks, the monomer-to-micelle transition occurs over a range in temperature and concentration, that is, with a much less sharp critical micelle temperature and critical micelle concentration, compared to ordinary surfactants. In this study, we described a UAP adhesive created by mimicking the composition and curing mechanisms of mussel adhesive with a composition of synthetic DOPA-cyanoacrylate containing copolymers and divalent cations with PEO-PPO-PEO triblock copolymer. Figure 19 gives a model of coacervate structure and adhesive mechanisms.

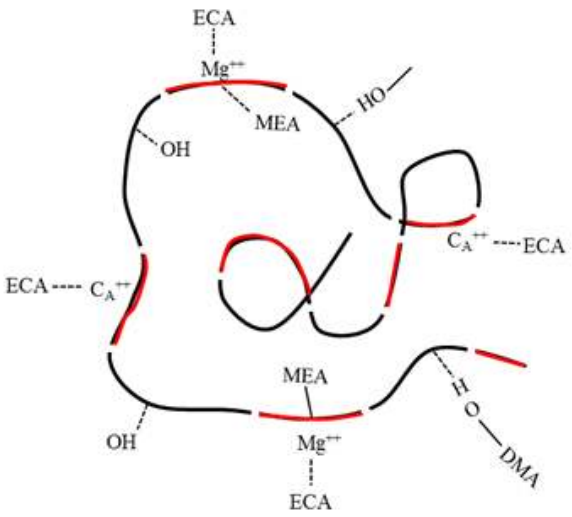


Figure 19. Model of coacervate structure and adhesive mechanisms.

DMA:PDM/ PDMC and PEO (black)-PPO (red)-PEO block with Ca^{++} and Mg^{++} form nm-scale complex domain in water. The coacervate can adhere to the bone surface through electrostatic interactions, 3, 4-dihydroxyphenol side chains, and quinone-mediated covalent coupling to matrix proteins. In the complex coacervates we created, anion-cation interactions could contribute to enhanced adhesive bonding between the plaques and surface. For example, the CC-5 containing the same polymer content with CC-4 but no Ca^{++} and Mg^{++} cations showed 125 KPa in stress at break and 28 MPa in modulus. These values are quite low compared with CC-4 which means that anion-cation interactions gives higher effect especially on the adhesion strength and modulus. Both the modulus and stress at break of the fully hydrated specimens increased with increasing divalent cation concentration. The coacervate with no Mg2+/Ca2+ resulted in the weaker bond. Other chemical contributions to strong adhesion on stainless-steel may include hydrogen bonding between the coacervate and the metal oxide surface as well as chelation of surface metal ions by the 3,4-dihydroxyphenylalanine (DOPA) of the polymers, PDM and PDMC. The bonds were dimensionally stable, neither shrinking nor swelling appreciably after complete submersion in PBS (10mM PO4, 120 mM NaCl, pH 7.2) for several weeks. Dimensional stability during cure and long exposure to physiological ionic strength and pH was an important requirement for a useful bone adhesive.

The liquid coacervate has low initial viscosity, specific gravity greater

than 1, and being mostly water by weight, it has low interfacial tension in an aqueous environment, all of which contribute to its ability to wet the bone surface and refill the requirement of effective underwater adhesion. The liquid coacervate adheres to wet bone fragments and other minerals through multiple mechanisms. The surface of the bone's hydroxyapatite mineral phase [Ca5(P04)3(0H)] displays an array of both positive and negative charges. The negative polyphosphate can interact directly with the positively surface charges or it can be bridged to the negative surface charges through the positive polyamine and/or divalent cations. Likewise, direct interaction of the polyamine with the negative surface charges bridged to the negative surface charges through the positive polyamine and/or divalent cations thereby contribute to adhesion.[19] Molecules containing catechol moieties have been shown to have strong absorptive properties and to readily wet hydroxyapatite. Correspondingly, its adhesive appears to be simpler in composition, to be less structurally organized, likely requires less sophisticated biological processing, and therefore, may be an excellent model for clinically practical adhesives.

C. Comparisons with Literature Data

To date, literature data for the stress at break of mussel plaques on various surfaces have focused on adhesion measurements. Although there could be some unsuitable test methods for comparison, the data obtained in this

study could provide correlations with foregone literature data. A variety of units have been used to report these data. Ackerman et al. performed whole animal studies with a spring scale and showed the detachment force of mussels on aluminum to be 0.46 ± 0.05 N.[41)]

The results from the current study, on bone and stainless steel plate, show a very high value of 165 KPa (= 16.5 N/Cm^2) which is almost 40 times higher than Ackerman et al's finding. Price group used a spring scale and reported the required detachment force of *Mytilus edulis* from rocks to vary between 1.2-2.4 kg over the course of a year in their natural habitat.[42)] With 1 kg = 9.8 N and mussels producing between 10-40 plaques, a rough conversion of these data to Newtons provides a force range of 0.5-1.2 N per plaque. On glass substrates, Dolmer and Svane reported detachment forces of individual threads to be just under 300 g, measured using a spring scale.[43)] This detachment force converts to 2.9 N. Crisp *et al*. described adhesion of mussels on glass, among other substrates, to be in a range of 320-750 kPa.[44)] However, the methods used for determining force and area were not described in detail.[44)] Presumably these authors used the same techniques outlined in a separate paper in which adhesion to glass was reported at 310 kPa.[45)] The latest study by Chung et al reported on three-component bio-inspired adhesive which includes terpolymer composed of a water-soluble segment, an interfacial adhesion segment, and a cross-linking segment but the test result only reached bond strength of approximately 5 kPa.[30)]

These experiments started by using a commercial cyanoacrylate adhesive for

bonding a metal wire to the thread.[45] Consequently, these prior data are not directly comparable with this current study in which we examined directly stress at break and shear modulus of the bone using stainless steel plates.

D. Application in the Field of Orthopedics

Biomimetic adhesive materials we have created definitely showed superior underwater adhesive capability compared to the other materials that have been manufactured. Also, as cyanoacryl group in our adhesives are confined in a chemically stable polymer, it is likely that this will have low toxicity compare to other adhesives that contains cyanoacryl group. Because of this quality, our adhesives can be applied to various orthopedic situations including articular fractures, the fractures in osteoporotic bone and in the arthroscopic managements of fractures. Although further in-depth study is required, we also believe these materials can be applied to bone to tendon attachment such as in arthroscopic rotator cuff tear repairs.

V. Conclusion

Complex coacervates are a state of matter with ideal but so far unexploited material and rheological properties for medical adhesives. Guided by a natural adhesive produced by a marine mussel, we have taken an alternative approach to developing adhesives for orthopedic surgery and other medical applications through biomimetic chemical syntheses and their blending for coacervate complexes. The adhesive we have created is less likely to be toxic yet has great adhesion force underwater. With in-vivo study, it could be used in many areas in the field of orthopedics.

REFERENCES

1. Kamino K. Biological adhesives. Springer; 2006. 145-66.

2. Waite J. Nature's underwater adhesive specialist. International Journal of Adhesion and Adhesives. 1987;7:9-14.

3. Tay FR, Pashley DH. Dental adhesives of the future. The journal of adhesive dentistry. 2002;4:91.

4. Kamino K, Inoue K, Maruyama T, Takamatsu N, Harayama S, Shizuri Y. Barnacle cement proteins importance of disulfide bones in their insolubity. Journal of Biological Chemistry. 2000;275:27360-5.

5. Heiss C, Schnettler R. Bioresorbable bone adhesives. Historical perspective and current status. Unfallchirurg. 2005;108:348-55.

6. Stewart RJ, Ransom TC, Hlady V. Natural underwater adhesives. J Polym Sci B Polym Phys. 2011;49:757-71.

7. Wang R, Liang Z, Hall M, Söderhäll K. A transglutaminase involved in the coagulation system of the freshwater crayfish, pacifastacus leniusculus. Tissue localisation and cDNA cloning. Fish & shellfish immunology. 2001;11:623-37.

8. Barlow D, Dickinson G, Orihuela B, Rittschof D, Wahl K. In situ ATR-FTIR characterization of primary cement interfaces of the barnacle Balanus amphitrite. Biofouling. 2009;25:359-66.

9. Dickinson GH, Vega IE, Wahl KJ, et al. Barnacle cement: a polymerization model based on evolutionary concepts. Journal of Experimental Biology. 2009;212:3499-510.

10. Monahan J, Wilker JJ. Cross-linking the protein precursor of marine mussel adhesives: bulk measurements and reagents for curing. Langmuir. 2004;20:3724-9.

11. Dougherty WJ. Carboxypeptidase activity of the zinc metalloprotease in the cement precursor secretion of the barnacle, chthamalus fragilis darwin. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 1997;117:565-70.

12. Rittschof D, Branscomb ES, Costlow JD. Settlement and behavior in relation to flow and surface in larval barnacles, balanus amphitrite darwin. Journal of Experimental Marine Biology and Ecology. 1984;82:131-46.

13. Wiegemann M, Watermann B. Peculiarities of barnacle adhesive cured on non-stick surfaces. Journal of adhesion science and technology. 2003;17:1957-77.

14. Chivers R, Wolowacz R. The strength of adhesive-bonded tissue joints. International journal of adhesion and adhesives. 1997;17:127-32.

15. Albes JM, Krettek C, Hausen B, Rohde R, Haverich A, Borst H-G. Biophysical properties of the gelatin-resorcinformaldehyde/glutaraldehyde adhesive. The Annals of thoracic surgery. 1993;56:910-5.

16. Brauer G, Kumpula J, Termini D, Davidson K. Durability of the bond between bone and various 2-cyanoacrylates in an aqueous environment. Journal of biomedical materials research. 1979;13:593-606.

17. Weber SC, Chapman MW. Adhesives in orthopaedic surgery. A review of the literature and in vitro bonding strengths of bone-bonding agents. Clin

Orthop Relat Res. 1984;249-61.

18. Maurer P, Bekes K, Gernhardt C, Schaller H-G, Schubert J. Comparison of the bond strength of selected adhesive dental systems to cortical bone under in vitro conditions. International journal of oral and maxillofacial surgery. 2004;33:377-81.

19. Shao H, Bachus KN, Stewart RJ. A water-borne adhesive modeled after the sandcastle glue of P. californica. Macromol Biosci. 2009;9:464-71.

20. Vainio J, Kilpikari J, Törmälä P, Rokkanen P. Experimental fixation of bone cement and composite resins to bone. Archives of orthopaedic and traumatic surgery. 1979;94:191-5.

21. Ishihara K, Nakabayashi N. Adhesive bone cement both to bone and metals: 4-META in MMA initiated with tri-n-butyl borane. Journal of biomedical materials research. 1989;23:1475-82.

22. Cheung P, Ruggieri G, Nigrelli R. A new method for obtaining barnacle cement in the liquid state for polymerization studies. Marine Biology. 1977;43:157-63.

23. Burkett JR, Wojtas JL, Cloud JL, Wilker JJ. A method for measuring the adhesion strength of marine mussels. The Journal of Adhesion. 2009;85:601-15.

24. Conlan SL, Mutton RJ, Aldred N, Clare AS. Evaluation of a fully automated method to measure the critical removal stress of adult barnacles. Biofouling. 2008;24:471-81.

25. Lee H, Lee BP, Messersmith PB. A reversible wet/dry adhesive inspired by mussels and geckos. Nature. 2007;448:338-41.

26. Wang TT, Ryan F, Schonhorn H. Effect of bonding defects on shear strength in tension of lap joints having brittle adhesives. Journal of Applied Polymer Science. 1972;16:1901-9.

27. Stevens MJ, Steren RE, Hlady V, Stewart RJ. Multiscale structure of the underwater adhesive of Phragmatopoma californica: a nanostructured latex with a steep microporosity gradient. Langmuir. 2007;23:5045-9.

28. Lee H, Scherer NF, Messersmith PB. Single-molecule mechanics of mussel adhesion. Proc Natl Acad Sci U S A. 2006;103:12999-3003.

29. Sedó J, Saiz-Poseu J, Busqué F, Ruiz-Molina D. Catechol-based biomimetic functional materials. Advanced Materials. 2012;

30. Chung H, Grubbs RH. Rapidly cross-Linkable DOPA containing terpolymer adhesives and PEG-based cross-linkers for biomedical applications. Macromolecules. 2012;45:9666-73.

31. Lee BP, Huang K, Nunalee FN, Shull KR, Messersmith PB. Synthesis of 3, 4-dihydroxyphenylalanine (DOPA) containing monomers and their co-polymerization with PEG-diacrylate to form hydrogels. Journal of Biomaterials Science, Polymer Edition. 2004;15:449-64.

32. Wei W, Yu J, Broomell C, Israelachvili JN, Waite JH. Hydrophobic enhancement of Dopa-mediated adhesion in a mussel foot protein. Journal of the American Chemical Society. 2012;135:377-83.

33. Benthien J, Russlies M, Behrens P. Investigating the effects of bone cement, cyanoacrylate glue and marine mussel adhesive protein from Mytilus edulis on human osteoblasts and fibroblasts in vitro. Annals of Anatomy-

Anatomischer Anzeiger. 2004;186:561-6.

34. Heiss C, Kraus R, Schlukerbier D, Stiller AC, Wenisch S, Schnettler R. Bone adhesives in trauma and orthopedic surgery. Eur J Trauma. 2006;2:141-8.

35. Walker G. The biochemical composition of the cement of two barnacle species, Balanus hameri and Balanus crenatus. J Mar Biol Assoc UK. 1972;52:429-35.

36. Chu B, Zhou Z. Physical chemistry of polyoxyalkylene block copolymer surfactants in Nonionic Surfactant Nace. Vol. 0,Marcel Dekker: New York; 1996. 67.

37. Almgren M, Brown W, Hvidt S. Self-aggregation and phase behavior of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) block copolymers in aqueous solution. Colloid and polymer science. 1995;273:2-15.

38. Alexandridis P, Alan Hatton T. Poly (ethylene oxide) poly (propylene oxide) poly (ethylene oxide) block copolymer surfactants in aqueous solutions and at interfaces: thermodynamics, structure, dynamics, and modeling. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 1995;96:1-46.

39. Kabanov AV, Alakhov VY. Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers. Crit Rev Ther Drug Carrier Syst. 2002;19:1-72.

40. Alexandridis P, Holzwarth JF, Hatton TA. Micellization of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) triblock copolymers in aqueous solutions: thermodynamics of copolymer association. Macromolecules.

1994;27:2414-25.

41. Ackerman JD, Cottrell CM, Ethier CR, Allen DG, Spelt JK. Attachment strength of zebra mussels on natural, polymeric, and metallic materials. Journal of Environmental Engineering. 1996;122:141-8.

42. Price HA. Seasonal variation in the strength of byssal attachment of the common mussel Mytilus edulis L. Journal of the Marine Biological Association of the United Kingdom. 1980;60:1035-7.

43. Dolmer P, Svane I. Attachment and orientation of Mytilus edulis L. in flowing water. Ophelia. 1994;40:63-74.

44. Crisp D, Walker G, Young G, Yule A. Adhesion and substrate choice in mussels and barnacles. Journal of Colloid and Interface Science. 1985;104:40-50.

45. Young G, Crisp D. Marine animals and adhesion. Adhesion. 1982;6:19-39.