Proceedings of the 28th IEEE
EMBS Annual International Conference
New York City, USA, Aug 30-Sept 3, 2006

Synthesis and Characterization of Novel Injectable, Biodegradable and *In situ* Crosslinkable Poly(hexamethylene-carbonate-fumarate), Poly(hexamethylene carbonate) Diacrylate and Poly(ethylene glycol fumarate-co-hexamethylene carbonate-fumarate) Scaffolds for Bone Tissue Engineering

S. Sharifi[*], H. Mirzadeh[†*], M. Imani[†], M. Atai[†], R. Bakhshi[†] and F. Ziaee[†]
[*]Biomedical Eng. Dept., Amirkabir University of Technology, Tehran, Iran
Email: sh.sharif@gmail.com
[†]Drug Delivery and Biomaterials Dept., Iran Polymer and Petrochemical Institute, Tehran, Iran

Abstract- **A series of novel self-crosslinkable and biodegradable polymers; poly(hexamethylene carbonate-fumarate), poly(hexamethylene carbonate) diacrylate and their amphiphilic copolymers with polyethylene glycol, poly(ethylene glycol fumarate-co- hexamethylene carbonate-fumarate) (PEGF-co-PHMCF) were developed for tissue engineering using novel synthesis approach. These novel polymers were fully characterized using nuclear magnetic resonance spectroscopy, Fourier transform infrared spectroscopy, gel permeation chromatography, differential scanning calorimetry, rheometry and shrinkage strain measurement. The cytocompatibility of macromers and their networks were evaluated by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay. The synthetic macromers were light colored with self-crosslinking ability via both photocrosslinking and chemical crosslinking. These polymers can be used as precursors to prepare polymer networks and scaffolds with controlled hydrophilicity, biodegradability and mechanical characteristics for application in cell delivery, tissue engineering and controlled release of biologically active agents.**

.

I. INTRODUCTION

Attempts to find tissue engineering solutions to cure orthopedic injuries/diseases such as spinal arthrodesis, total joint arthroplasty, osteoprotic insufficiency fractures and bone loss after skeletal trauma have made necessary the development of new polymers that meet a number of demanding requirements including ability of scaffold to provide mechanical support during tissue growth and gradually degrade to biocompatible products [1]. *In situ* polymerizable compositions that can function as cell delivery systems and /or biodegradable scaffolds in the form of an injectable liquid/paste offer attractive routes to existing methods. A major advantage would be the possibility of administrating the gel arthoroscopically avoiding surgery in many cases. However, many of the currently available degradable polymers require significant chemical changes to their structure if they are to be formulated for such applications.

For the last two decades aliphatic polycarbonates have been widely investigated because of their biodegradability, biocompatibility and non-toxicity [2, 3]. However, most of these polymers are hydrophobic and undergo slow degradation. In order to improve their hydrophilicity, biodegradability and rendering them to self-crosslinkable and injectable materials we developed a series of novel, self-crosslinkable and biodegradable copolymers consisting poly(hexamethylene carbonate-co-fumarate) (PHMCF), poly(hexamethylene carbonate) diacrylate (PHMCA) and poly(ethylene glycol fumarate-co-hexamethylene carbonate fumarate) (PEGF-co-PHMCF). By incorporation of double bond containing fumarate group in the backbone, the copolymers may crosslink without presence of any potential toxic crosslinking agent such as N-vinyl pyrollidone (NVP) or methacrylic acid due to chain flexibility of the polycarbonate. The polymer networks were prepared through both chemical and photocrosslinking method and injectability, hardening and crosslinking characteristics were evaluated by rheometry and polymerization contraction strain measurement.

II. METHODOLOGY

A. Synthesis of PHMCF, PHMCA and PEGF-co-PHMCF macromers

PHMCF macromer was synthesized by condensation polymerization of polyhexamethylene carbonate diol (PHMC diol) with fumaryl chloride (FUCl) in presence of propylene oxide (PO) as a novel replacement catalyst for common triethylamine, because polycondensation in the presence of the tertiary amines usually leads to dark colored products. All chemicals were obtained from Aldrich (Milwaukee, WI, USA). Fumaryl chloride was distilled at 161ºC and methylene chloride was dried over calcium hydride. Two PHMC diols with number average molecular weight of 860 and 2000 $g.mol^{-1}$ were used. The purified FUCl and PHMC diol were reacted in 0.99:1 molar ratio. PHMC diol was dissolved in 100 mL anhydrous methylene chloride and an appropriate amount of PO was added to this solution. The FUCl was dissolved in 25 mL anhydrous methylene chloride and added dropwise to stirred solution under nitrogen atmosphere and using reflux condenser at ambient temperature. After adding all the FuCl-methylene chloride solution, the reaction mixture was allowed to stir for an additional 24 hrs. Upon completion of the reaction, the solution was washed with brine (0.1 M) to remove chlorinated propanol and the solvent was removed by rotavaporation. The residue was dissolved in small amounts of acetone/methanol (90% V/V) and appropriate amount of water (non-solvent) was added to precipitate the polymer.

The polymer was dried in vacuum at 30ºC for 12 hr. and stored at -20ºC until used.

PHMCA was synthesized by acylation of PHMC diol 860 by acryloyl chloride in the presence of PO. Briefly, 15 mM of PHMC diol 860, 0.04M of PO and 300 mL of methylene chloride were charged into 500 mL four-necked flask equipped with mechanical stirrer and acryloyl chloride (0.04 M) added dropwise at ambient temperature and esertification proceeded at room temperature for 12 hrs. The work up procedure was the same as PHMCF.

For synthesis of PEGF-co-PHMCF, PEG 1K and PHMC diol 860 with an equal weight of 10 g were dried by an azeotropic distillation in toluene and then evacuated under reduced pressure to remove residual trace of water. Fumaryl chloride, the total amount of hydroxyl functional group in the mixture of PEG, PHMC diol and propylene oxide were measured out in 0.999, 1, 2 molar ratio. The mixture of PHMC diol and PEG 1k and PO formed earlier was dissolved into 100 mL methylene chloride and fumaryl chloride added dropwise to the stirred solutions at ambient temperature. The rest of the reaction procedure to synthesize PEGF-co-PHMCF was similar to PHMCF described above. The same procedure was also applied to the synthesis of PEGF-co-PHMCF from PEG 4k.

B. Network synthesis

Polymer networks were prepared by self-crosslinking of the fumarate carbon-carbon double bonds via free radical polymerization (Red-Ox) and visible photocrosslinking. In chemical crosslinking, benzoyl peroxide (BPO) and dimethyl toluidine (DMT) were used as the crosslinking initiator and accelerator. As a typical procedure, 20 mg BPO, dissolved in 40 µl NVP, was mixed thoroughly with 1 g PHMCF at 37ºC and the mixture was placed in cavity of a copper mold (D=10mm, h=3mm) and 5 µl DMT was added to this mixture, mixed rapidly and immediately transferred to oven at 37ºC and held for 1h. After crosslinking, scaffolds were removed from the mold. In the photocrosslinking process, crosslinking was initiated with visible light (blue region) using camphorquinone (CQ), and DMT. Briefly, 10 mg CQ and 5 µl DMT were mixed thoroughly with 1 g PHMCF in glass watch and the samples (cylindrical shape with 10 mm diameter and 3 mm height, mounted between lower part of apparatus) (Fig. 1) were cured for 220s using light source with intensity of 550 mW/cm^2 (Optilux 500, USA)

C. Polymerization shrinkage strain and shrinkage strain rate measurement

The bonded disk technique was used to measure the shrinkage of samples during light curing [4]. Briefly a disk shaped specimen was placed at center of a square cross-section brass ring adhesively bonded to a rigid glass plate and the top edge of the ring and the disk specimen were covered by a flexible diaphragm consisting of square microscope cover glass. A centrally aligned LVDT displacement transducer was positioned in contact with the center of cover slip. The light source was placed beneath the

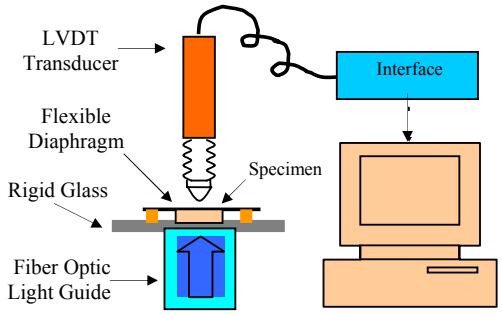

Fig1.Shrinkage strain test apparatus

rigid glass plate and upon initiation of the reaction, the cover slips deflected due to polymerization shrinkage and LVDT which was connected to signal conditioning unit, microcomputer transient recorder and data logging system, monitored the deflection of cover slip over time. The total shrinkage strain of the samples was measured 400 seconds after starting the light irradiation, at which time the contraction had plated-out. The schematic of apparatus is illustrated in Fig. 1.

D. ^{1}HNMR, ^{13}CNMR and Fourier transform infrared spectroscopy

Nuclear magnetic resonance was used to confirm the presence of fumarate and acrylate group in PHMCF, PHMCA and PEGF-co-PHMCF macromers. The chemical structure of their diol precursors was also determined by NMR spectroscopy. NMR spectra were recorded at ambient temperature employing NMR apparatus (Bruker ^{1}H400 MHz ^{13}C 100 MHz). $CDCl_3$ was used as a solvent. The chemical shifts were given in ppm from the signal of tetramethylsilane (TMS).

FTIR spectra (4000-400 cm^{-1}) were acquired on a Bruker, Equinox 55 spectrometer at 4 cm^{-1} resolution and 16 scans. The samples were examined as KBR disks at room temperature.

E. Rheology experiments

Linear viscoelastic properties of the synthesized copolymers were measured by dynamic mechanical spectrometer (rheometer,Rheolyst MCR300, TA Instruments, New Castle, DE) at frequencies (ω) ranging from 0 to 600 rad/s at 40ºC. The oscillatory shear measurements were carried out using a 20 and 50 mm diameter parallel plate flow cell and a geometry gap setting of 1 and 0.5 mm. Depending on the macromers, different initial strains (γ) ranging from 10% to 100% were applied. *In situ* rheology experiments were used to investigate the crosslinking kinetics. Modulus of the complex viscosity (η*), storage modulus (G') and loss modulus (G") of these compositions were monitored via a dynamic oscillatory test at 37°C. Typically, the composition (macromer 1g, BPO 0.02 g dissolved in 5µl NVP and 5 µl DMPT) was placed on a lower geometry and the parallel plate geometry (20mm diameter) was used and a gap was set to 1 mm. The frequency was constant during the experiment

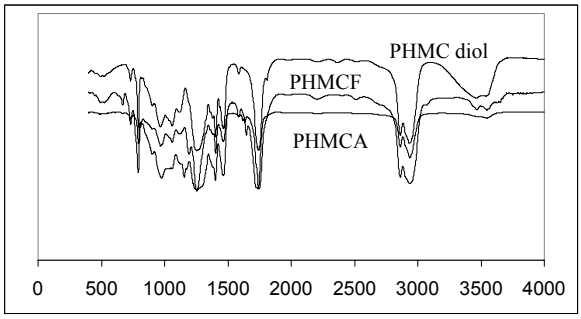

Fig.2. FTIR spectra of PHMCF, PHMCA and PHMC diol

(1 rad/s) and changes in G', G" and η* was monitored as a function of crosslinking time.

F. Cytotoxicity evaluations

For the cytotoxicity evaluation, MCF7 cells were grown in DMEM/F12 media containing 10% FBS in a humified CO_2 incubator. Pieces of sterilized photocrosslinked networks and uncured macromers were added to the media (5 mg/mL) and remained in the incubator for 15 days. Supernatant of this media after centrifugation at 15000 rpm for 3 hrs. was added in the concentrations of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 percent (V/V) to the cells. MTT assays were performed on these samples after 24 and 48 hrs. exposure. Morphology and growth rate of cells in the presence of photocrosslinked disks and macromers were also studied.

G. Differential scanning calorimetry analysis

Melting temperature, T_m, glass transition temperature T_g, and heat of fusion (ΔH_m) were measured by DSC with a PL instrument (PL, UK) at a heating rate of 10ºC/min via heating from -100ºC to 100ºC and nitrogen gas flow rate of 50 mL/min. The specimens were first heated from -80ºC to 100ºC at heating rate of 10ºC/min, quenched rapidly to -80 ºC and after a 2 minutes a second scan was recorded. The glass transition temperature T_g was taken as a midpoint of the heat capacity change. T_m and heat of fusion (ΔH_m) were determined from the maximum endothermic peaks position and integrating the endothermic area.

III. RESULTS AND DISCUSSION

FTIR spectra of PHMCF, PHMCA and their precursor diol are shown in Fig. 2 (for simplicity spectrum of PEGF-co-PHMCF is not shown). The adsorption at 3466 cm^{-1} is due to hydroxyl end groups of PHMC diols. As the molecular weight of the macromers increases the hydroxyl bond is weakened or even omitted (in the case of PHMCA). The absorption band with peak position of 2937 and 2861 cm^{-1} are due to stretching of methylene group of PHMC diols. The absorption band with peak position at 1746 cm^{-1} is due to the carbonyl vibration of carbonate group in PHMC diol, fumarate groups and the formed ester group after reaction with fumaryl chloride. Bands with peak position at 1463 and 1403 cm^{-1} are due to bending and stretching vibration of methylene group. The weak absorption at 1646 cm^{-1} which is absent in PHMC diols is due to fumarate double bonds

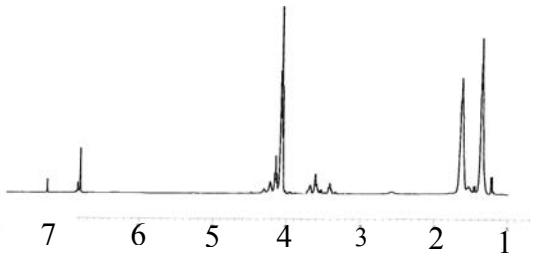

Fig.3. ^{1}HNMR of PHMCF

incorporated in PHMC backbone. The peak at 1059 cm^{-1} which is absent in synthetic macromers is due to C-O of terminal hydroxyl group. This band is replaced by C-O ester at 1155 cm^{-1} in macromers.

The ^{1}HNMR spectra of PHMCF are shown in Fig. 3. Peak assignments are as follows: ^{1}HNMR in $CDCl_3$ δ(ppm)= 4.11($OC(O)OCH_2$), 3.64 ($HOCH_2CH_2$), 2.2 (OH), 1.69 ($OC(O)OCH_2CH_2$), 1.58 ($HOCH_2CH_2$)

Signals in addition to those expected for a simple PHMC diol are due to incorporation of ethylene carbonate and ethylene oxide units in backbone since PHMC diol is prepared by condensation polymerization of 1,6-hexane diol and ethylene carbonate and ethylene carbonate is occasionally incorporated in backbone and partial decarboxylation of an added ethylene carbonate results in incorporation of ethylene oxide units [6]. The chemical shift with peak position at 6.8 ppm which is not present in PHMC diols is due to incorporated fumarate group. ^{13}CNMR in $CDCl_3$ δ(ppm)= 25.4-25.5 ($C(O)OCH_2CH_2CH_2$), 28.5-28.6 ($C(O)OCH_2CH_2$), 32.5 ($HOCH_2CH_2$), 62.7 ($HOCH_2$), 67.8 ($C(O)OCH_2$), 155.3 (C=O). Two additional peaks at 133.5 and 165 ppm which are not present in PHMC diols clearly indicate that fumarate group is incorporated into PHMC backbone.

Based on the MTT assays, the photocrosslinked networks and uncured macromers were not toxic to the cells and cells were growing neighboring to crosslinked networks and uncured macromers in the same rate and shape as the controls. As a conclusion, the solid photocrosslinked polymer and uncured macromers were not toxic to the cells at all.

T_m, T_g and ΔH_m of macromers and their precursors as determined by DSC are given in Table 1. As expected the T_m of PEG and PHMC diols increased with their degree of polymerization. The presence of fumarate groups in the backbone of PHMC decreased the melting temperature of the corresponding PHMC. All copolymers exhibited single glass transition suggesting that there is no microphase separation in amorphous phase. As indicated by heat of fusion of copolymers, the crystallinity and melting point of PHMCF and PEGF-co-PHMCF dropped when fumarate groups were introduced to copolymer backbone since fumarate group caused the crystalline structure to become imperfect.

The completion of crosslinking reaction was evaluated by rheometry as shown in Fig. 4, which shows the G', G" and η* as a function of crosslinking time at 37ºC. According to Fig. 3, the sample had reached above 90% of the final

Table 1: Thermal properties of synthetics macromers

Samples	T_g(°C)	T_m(°C)	ΔH(mcal/mg)
PHMC di acrylate	-75.37	33.49	3.8
PHMC diol 860	-60.67	39.58	3.22
PHMC diol 2000	-51.91	43.61	6.19
PHMCF 860	-47.69	21.79	2.56
PHMCF2000	-45.77	29.7	1.56
PEGF-co-PHMCF(PEG 0.4k)	-52.05	22.4	2.73
PEGF-co-PHMCF (PEG 1k)	-52.19	33.67	6.32
PEGF-co-PHMCF(PEG 4K)	-43.92	65.2	17.2
PEG 1k	-----	47.77	21.1
PEG 4k	-----	68.49	25.37

complex viscosity after 5 min crosslinking at 37ºC. The onset of gelation (gel point) which is the crossover point of G' and G" was identified to be at 30 seconds after initiation of crosslinking reaction.

Fig. 5 shows the shrinkage strain and their time-dependency during photocrosslinking. Because the polymerization shrinkage of the macromers is due to the conversion of the intermolecular van der Walls forces to the covalent single bonds during the polymerization, the shrinkage kinetics could be representative of polymerization reaction which follows the polymerization reaction kinetics [5]. The shrinkage strain rate calculated from derivative of shrinkage strain data with respect to the time using numerical differentiation is also shown, representing auto-acceleration and deceleration stages of polymerization reaction. The molecular weight of the starting PHMC diols as well as the molecular weight of the resulting PHMCF and PEGF-co-PHMCF determined by GPC showed that up to 6 or 3 repeating units of PHMC diol or PEG precursors could be connected together with fumarate segments.

IV. CONCLUSION.

A series of novel self-crosslinkable, biocompatible and biodegradable copolymers, PHMCF, PHMCA and (PEGF-co-PHMCF) have been synthesized and characterized for tissue engineering and drug delivery applications using propylene oxide as a replacement proton scavenger for triethylamine. Since each hexamethylene carbonate unit provides five free rotating C-C bonds, the PHMCF chains are flexible above their melting points and can be cured chemically or via photocrosslinking without use of any crosslinking agent. Additionally, the general properties of the copolymers can be controlled by modulating the composition and block length of its components. These new materials can be used as scaffold for tissue engineering and/or as an injectable material or prefabricated scaffolds for bone tissue substitution or drug delivery applications.

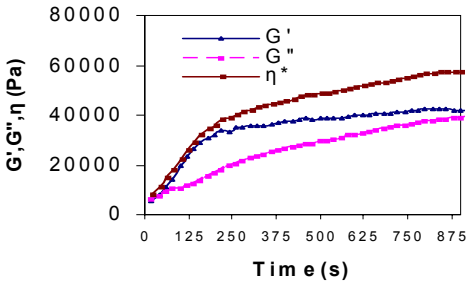

Fig 4. Profile of G', G" and η* changes during crosslinking reaction of PHMCF

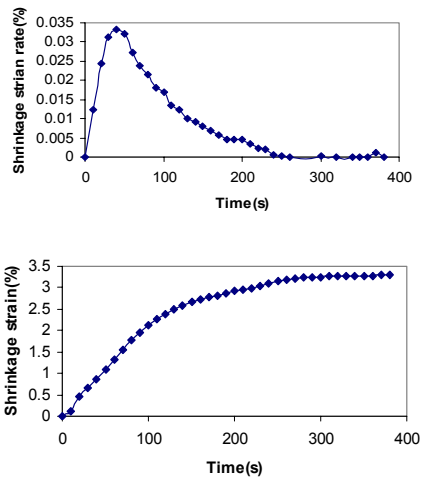

Fig 5. Shrinkage strain rate and shrinkage strain during photocrosslinking reaction of PHMCF

REFERENCES

[1] M.J. Yaszemski, J.B. Oldham, L. Lu and B.L. Currier, "Clinical needs for bone tissue engineering technology", in Bone Engineering, J.E Davis, Ed. Toronto: Em squared 2000; pp 541-547.

[2] Y. Zhou, R. Zhou and Z. Liu, Synthesis and characterization of novel aliphatic poly(carbonate-ester)s with functional pendent groups, Macromolecular Rapid Communication 2005; 26, 1309-1314.

[3] K.J. Zhu, R.W. Hendren, K. Jensen and C.G. Pitt, Synthesis, properties, and biodegradation of poly (1,3-trimethylene carbonate), Macromolecules 1991; 24, 1736-1740.

[4] D.C. Watts and J. Cash, Kinetic measurements of photo-polymerization contraction in resins and composites. Meas. Sci. Technol. 1991; 2: 788-794.

[5] M. Atai, D.C. Watts and Z. Atai, Shrinkage strain-rates of dental resin-monomer and composite systems. Biomaterials 2005; 26: 5015-5020.

[6] G. Rokicki and T .Kowalczyk, Synthesis of oligocarbonate diols and their characterization by MALDI-TOF spectrometry; polymer 2000, 41 9013-9031