ABSTRACT

Title of Document: A NOVEL CYCLIC ACETAL BIOMATERIAL AND ITS USE IN CLEFT PALATE REPAIR

Jennifer Lynn Moreau, Master of Science, 2006

Directed By: Dr. John P. Fisher, Assistant Professor
Department of Chemical and Biomolecular Engineering and Bioengineering Graduate Program

Cleft lip and/or palate are the most prevalent congenital craniofacial birth defect in humans. While myriad surgical techniques have been described to repair orofacial clefts, several complications have been associated with the repair techniques. To overcome these complications, a tissue engineering strategy may be employed. In particular, we are investigating strategies for regenerating the alveolar bone that is often missing as a result of cleft palates. Numerous materials have been explored as biomaterials for bone tissue engineering, however there are disadvantages to these, including compromised mechanical properties and harmful degradation products. To overcome this issue, a novel class of biomaterials has been created. These materials are crosslinked networks of monomers of 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate. The study presented here was designed to determine the effects of the material's formulation scheme on several of its physical properties, so as to develop a novel bone tissue engineering material suitable for cleft palate repair.

A NOVEL CYCLIC ACETAL BIOMATERIAL AND ITS USE IN CLEFT PALATE REPAIR

by

Jennifer Lynn Moreau

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Advisory Committee:
Professor John P. Fisher, Chair
Professor William E. Bentley
Professor Jose Helim Aranda-Espinoza
Professor John F. Caccamese

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Chapter 1: Introduction

There are several types of orofacial clefts, including cleft palate and cleft lip with or without cleft palate[1]. Cleft lip with or without cleft palate is the most prevalent congenital craniofacial birth defect in humans and is seen in about 1 in 700 live births[2, 3]. In addition, cleft lip and/or cleft palate comprise the second most common congenital anomaly after clubfoot[4]. The incidence of facial clefting varies among ethnic groups, with Native American and Asian populations having the highest rate at 3.6 per 1000 live births and 2.0 per 1000 live births, respectively. People of European ancestry and African populations have the lowest prevalence at 1.0 per 1000 live births and 0.3 per live births, respectively[5, 6]. This translates into approximately 15,000 newborns with clefts each year, with a total United States population with clefts at any one time of 330,000[7].

In general, about 30% of all clefts are those of the lip, about 20% are isolated cleft palates, and the remaining approximately 50% are combination cleft lip and palate[8]. Cleft lip with or without cleft palate is more frequently seen in males, whereas isolated cleft palate is twice as common among females[6]. Approximately 70% of cleft lip and/or cleft palate cases are non-syndromic, occurring as an isolated condition. The remaining 30% of cases are syndromic and are present in association with another disease or disorder[9].

Individuals with orofacial clefts often require interdisciplinary treatment into adulthood. These individuals, along with their families, may experience the financial burdens of extensive treatment, the morbidity of multiple procedures, and

a variety of psychosocial issues[5]. A multitude of surgical techniques have been described to repair orofacial clefts. The goals of cleft lip and palate surgery are directed toward achieving a normal facial appearance, as well as the ability to feed, speak, and hear without significantly affecting the facial development of the patient. However, several complications have been associated with the repair of cleft palate deformities. Patients are often subjected to multiple revision surgeries over their lifetime[10, 11]. Other complications resulting from surgical cleft palate repair include wound dehiscence, scarring of the palate, fistula formation, disturbances in facial growth, and mandible retrusion.

In order to combat these complications, a tissue engineering strategy may be employed in the repair of cleft palate. The field of tissue engineering aims to restore function to or replace damaged or diseased tissues through the application of engineering and biological principals. These principles include the selection and manipulation of cells, identification and use of pertinent biological signaling molecules, and the design of biomaterials used as scaffold matrices.

The biomaterial scaffold must provide a solid framework for cell growth allowing cell attachment and migration. Scaffolds for engineering bone, and palates in particular, should be osteoconductive, osteoinductive, biocompatible, and biodegradable[12]. Several materials have been explored as biomaterials for bone tissue engineering. However, there are disadvantages to these materials, including compromised mechanical properties and harmful degradation products. To overcome these issues, a novel class of biomaterials has been created. These new materials are based upon a cyclic acetal unit. Specifically, the material that has been

examined in this study is 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD). These monomers, when reacted together, crosslink to form a rigid plastic material.

The work presented in this study was designed to determine the effects of the EHD network formulation parameters on several properties of the networks. By optimizing these properties, the most appropriately functioning biomaterial can be created for use in a tissue engineering strategy for the repair of cleft palate, and specifically for the replacement of lost alveolar bone.

Chapter 2: Tissue Engineering Solutions for Cleft Palates

2.1. Functional palate anatomy

2.1.1. Anatomy

The human skull is comprised of 22 separate bones, as well as 20 deciduous and 32 permanent teeth. The bony skull is formed from two components – the neurocranium and the viscerocranium. The latter includes the bones of the face, such as the mandible, maxilla, zygoma, and nasal, as well as the palatal, pharyngeal, temporal, and auditory bones[13].

The mammalian palate consists of two components: the primary and the secondary palate (Figure 1). These structures together form the roof of the mouth and the floor of the nose, separating the oral cavity from the nasal cavities and the nasopharynx. The palate consists of two distinct regions, the bony and immobile hard palate, and the mobile posterior fibro-muscular part, known as the soft palate.

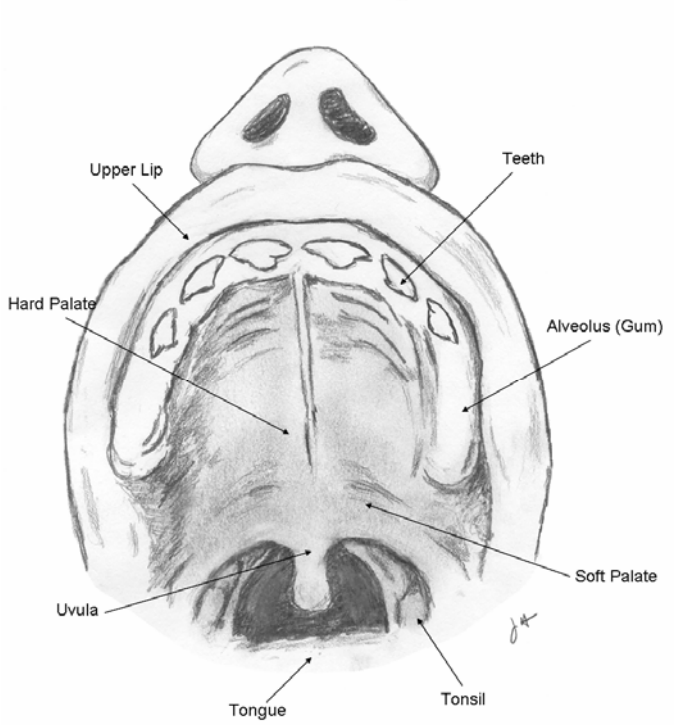


Figure 1. The anatomy of a normal palate. Adapted from [37].

The hard palate is formed by the palatine processes of the maxillae and the horizontal plates of the palatine bones. The primary palate is a small but significant contributor to the hard palate and is located anterior to the incisive foramen. The primary palate has particular relevance in the cleft deformity. Anteriorly and laterally, the hard palate is bounded by the alveolar processes and the gingivae. Posteriorly, it is continuous with the soft palate.

There are no muscles in the hard palate, but it is covered by keratinized oral mucosa which is continuous with the periosteum. There are numerous minor salivary glands in its posterior portion.

The soft palate is the posterior portion of the palate. It is a mobile muscular fold that is attached to the posterior border of the hard palate. It extends

posteroinferiorly to a curved free margin where the uvula is suspended from the midline. Laterally, the soft palate is continuous with the wall of the pharynx and the tongue and the by the palatoglossal and palatopharyngeal muscles identified externally as the anterior and posterior tonsillar pillars, respectively.

The soft palate is covered on its nasal and oral surfaces by non-keratinized mucosa. Numerous mucous glands are present on both surfaces, and collections of lymphoid tissue are found in the submucosa. There are five muscles of the palate including the levator veli palatini, the tensor veli palatini, the palatoglossus muscle, the palatopharyngeus muscle, and the musculus uvulae. The soft palate is strengthened by the palatine aponeurosis, formed by the expanded tendon of the tensor veli palatini muscle. This aponeurosis is attached to the posterior margin of the hard palate and the muscles of the soft palate are attached to the palatine aponeurosis[14, 15].

2.1.2. Development of the palate

The entire viscerocranium and part of the neurocranium are formed from the neural crest – a pluripotent cell population derived from the lateral ridges of the neural plate during the early stages of embryogenesis[13, 16]. During craniofacial development, cranial neural crest cells migrate ventrolaterally as they populate the branchial arches. The formation of mesenchymal structures of the head and neck, including the palate, can be attributed to the migration of the cranial neural crest cells[16].

The human palate, as well as the palates of other mammals, is formed by the unification of three elements – the primary palate, the inferior most extension of the frontonasal process, and the two lateral maxillary palatal shelves that together will form the secondary palate[3]. The development of the palate starts at about the sixth week of gestation and is a multi-step process that involves palatal shelf growth, shelf elevation, midline fusion of palatal shelves, and the disappearance of the midline epithelial seam[2, 16].

The primary palate develops from the fusion of medial nasal prominences. This consists of the prolabium that forms the philtrum of the upper lip and the triangular-shaped premaxilla that houses the four maxillary incisor teeth. This segment represents a small but a critical portion of the hard palate. It is here where the failure to fuse during embryogenesis results in the commonly seen cleft lip and palate deformity.

Between the eighth and twelfth weeks of gestation, the development of the secondary palate begins with the formation of the palatal shelves from the maxillary processes[17]. The shelves grow through cell replication and matrix deposition[18]. As the palatal shelves grow, the first branchial arch is oriented vertically downwards and surrounded by a layer of undifferentiated epithelial cells. Then, the palatal shelves elevate from a vertical to a horizontal position about the dorsum of the tongue[2]. The final stage of secondary palate development occurs when the medial edge epithelium fuses the palatal shelves with each other to form a midline palatal seam. This then separates the oral and nasal cavities. Mesenchymal cells are able to

flow across the intact horizontal palate as the medial edge epithelium disintegrates[17].

By the ninth week of gestation, the developing palate begins to fuse at the nasal surface with the nasal septum, and this process is completed by the twelfth week. The hard palate is formed by ossification in the primary palate and the anterior portion of the secondary palate, while the soft palate and uvula are formed from the posterior portion of the secondary palate[4].

2.2. Cleft palates

2.2.1. Classifications of clefts

A cleft palate occurs when the palatal shelves fail to fuse properly. Numerous systems have been devised to classify clefts of the lip and palate, however, most simply, cleft palates can be categorized as complete or incomplete and described further according to which aspect of the palate they involve. In the authors' minds, accurate anatomic description more than classification, leaves less to the imagination and serves best to facilitate surgical planning.

Complete cleft palates involve both the primary and secondary palates in which no fusion between the palatal shelves or the primary palate has taken place. These types of clefts are usually associated with a cleft lip. Incomplete cleft palates can either involve the primary or the secondary palate in isolation or in combination, where fusion between the facial processes or palatal shelves has been initiated but not completed. Hence, the incomplete cleft may involve only the

posterior portion of the soft palate (Figure 2a), it may extend through the soft and hard palates to the incisive foramen (Figure 2b), or may be limited to the alveolus.

Clefts of the secondary palate can be further classified as unilateral or bilateral. When only one palatal shelf fuses to the nasal septum, the cleft is considered unilateral (Figure 2c), and the cleft defect is confined to one side of the midline. When neither palatal shelf fuses with the nasal septum, the cleft is classified as bilateral (Figure 2d)[19].

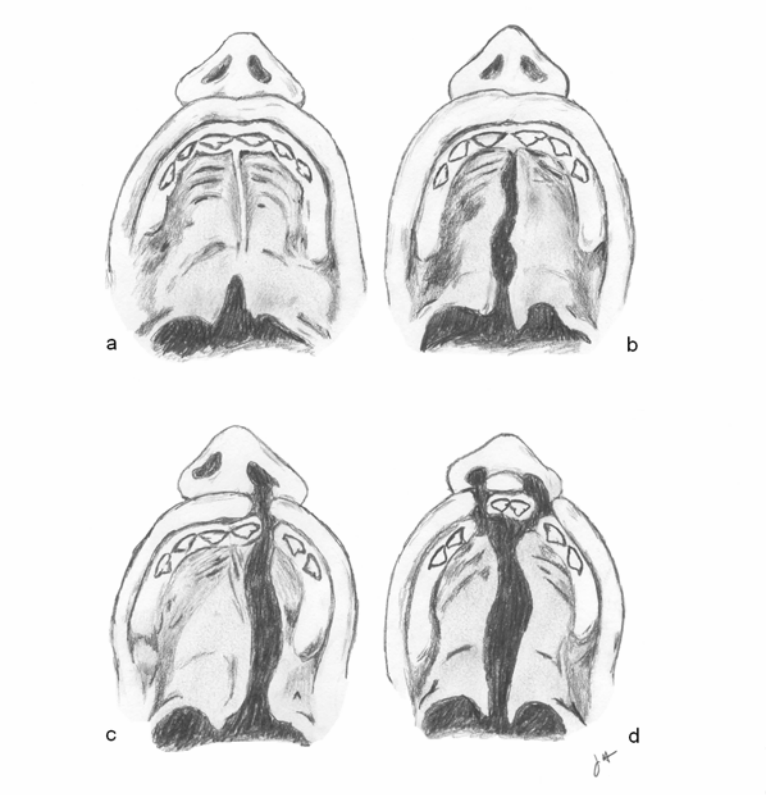


Figure 2. (a) A cleft of the soft palate; (b) A cleft of the soft and hard palate; (c) A complete unilateral cleft of the lip and palate; (d) A complete bilateral cleft of the palate and lip. Adapted from [37].

Lastly, the submucous cleft involves the separation of the intrinsic soft palate musculature while the overlying soft palatal mucosa remains intact. This common type of posterior palatal cleft is sometimes difficult to diagnose.

2.2.2. Causes of clefts

Both genetic and environmental factors contribute to the formation of orofacial clefts. Specific gene defects, maternal smoking, fetal hypoxia, and teratogenic drugs have all been linked to the typical cleft dysmorphology.

Clefting of the primary palate occurs when there is failure of the lateral nasal and maxillary processes to make contact with the medial nasal process. Alterations in the rate of cell proliferation and morphogenetic movements of the facial processes are the driving force for normal fusion. Abnormalities in cell signaling as well as alterations in the epithelium and peridermal cells lining these processes appear to be involved in the development of the defect[20].

Clefting of the secondary palate may result from a disturbance at any stage of palate development. Defective palatal shelf growth can result in a cleft palate. At the precise developmental stage when the palatal shelves have completed growing, the shelves begin to elevate. This movement occurs quickly, on the scale of minutes to hours, and any delay in the elevation of the palatal shelves may result in the failure of their fusion[2]. Cleft palate can also result from a disturbance in the fusion or mesenchymal penetration of the palatine shelves[6].

2.2.2.1. Implicated Genetic Factors

Several studies have been completed to test a number of genes that may be linked with cleft palate. The consensus from these studies is that there is not a straightforward single gene that is responsible for clefting. In fact, different genes may be acting in different populations[21]. Several genes may play a role in

predisposing an individual to cleft palate, including msh homeobox homolog-1 (*Msx1)*, transforming growth factor-β_3 (*TGF-*β_3), T-box transcription factor-22 gene (*TBX22*), poliovirus receptor like-1 (*PVRL1*), interferon regulatory factor-6 (*IRF6*), and patched (*PTCH*). The effects of the *Msx* family of proteins and *TGF-*β_3 in relation to cleft palate formation have mainly been studied in mice, but the conclusions can be applied to humans. There have been studies about the effects of *TBX22*, *PVRL1*, and *IRF6* in humans.

2.2.2.1.1. Msh homeobox homolog-1

The *Msx* family of proteins is an important modulator of craniofacial, limb, and nervous system development. It has been estimated that *Msx1* mutations may contribute to up to 2% of all non-syndromic cleft lip with or without cleft palate cases[22]. In *Msx1*-deficient mice, the bilateral primordial palatal shelves form and elevate normally, but fail to make contact and never fuse, which results in a cleft palate[3]. Similarly in humans, mutations in or loss of the *Msx1* gene result in non-syndromic clefts of the secondary palate. The homeodomain of *Msx1* is crucial in mediating several functions, including DNA binding, protein-protein interactions, protein stability, and transcription repression[23].

2.2.2.1.2. Transforming growth factor-β_3

TGF-β signaling plays a pivotal role in regulating palatogenesis. During mouse palatal development, *TGF-*β_3 is expressed in the medial edge epithelium of the palatal shelves. Upon fusion of the palatal shelves and disappearance of the

midline epithelial seam, the expression of *TGF-β_3* is lost, suggesting crucial functions of *TGF-β* signaling in regulating palatal fusion. Mutations in *TGF-β_3* results in 100% penetrance of cleft secondary palate[16]. The development of cleft palate in *TGF-β_3*-null mutant mice is apparently due to a failure of fusion of palatal shelves[24].

2.2.2.1.3. T-box transcription factor-22 gene

Cleft palate with X-linked inheritance has been considered a rare form of cleft palate and is characterized by isolated cleft palate and ankyloglossia[25]. Recent studies by Marçano et al. have found that mutations in *TBX22* may cause X-linked cleft palate. *TBX22* encodes a T box containing transcription factor that is expressed primarily in the palatal shelves and tongue during palatogenesis, indicating a specific role of *TBX22* in both palatal and tongue development[26].

2.2.2.1.4. Poliovirus receptor like-1

PVRL1 was shown to have a significant association with non-syndromic cleft lip with or without cleft palate[27]. *PVRL1* encodes a cell adhesion molecule called nectin-1, which is expressed at the medial edge epithelium of the developing palatal shelves and the skin surface epithelium[25]. Normal *PVRL1* function is important in mediating fusion of the palatal shelves during the later stages of palatogenesis[9].

2.2.2.1.5. Interferon regulatory factor-6

IRF6 encodes a transcription factor which regulates the expression of Interferon-α and Interferon-β following viral infection. The exact role of *IRF6* during development is unknown. However, in the developing mouse embryo, *IRF6* demonstrates high levels of expression in a variety of craniofacial structures, including the medial edges of the fusing palatal processes. This expression pattern implies an important role during craniofacial development, with some suggestion that it mediates interactions between members of the transforming growth factor-β superfamily[28].

2.2.2.1.6. Patched

PTCH is the human homolog of the *Drosophila* segment polarity gene patched; it encodes a 12-unit transmembrane protein and is the proposed receptor for the morphogen sonic hedgehog (SHH). By binding SHH, PTCH transduces a repressing signal through smoothened in a downstream pathway to the nucleus. Loss of SHH signaling in the chick embryonic face causes defects similar to cleft lip and palate in humans. Mutations in PTCH are associated with a variety of birth defects and are implicated in the development of nevoid basal cell carcinoma syndrome and cleft palate is found in 4% of these cases[29].

2.2.2.2. Syndromes

It is estimated that 300 syndromes include some form of cleft palate in their presentation and a syndrome is diagnosed in one third to one half of isolated cleft palate patients[19]. Van der Woude syndrome is the most common form of syndromic cleft lip with or without cleft palate and accounts for about 2% of all of these cases. This syndrome is characterized by cleft lip with or without cleft palate, pits or mucous cysts on the lower lip, and hypodontia. Van der Woude syndrome is caused by a mutation on the *IRF6* gene[25].

Treacher Collins syndrome is dominantly inherited. The principle features are underdeveloped facial bones, cleft palate, and conductive hearing loss. Treacher Collins syndrome is caused by mutations in the *TCOF1* gene[13]. Stickler syndrome is an autosomal dominant disorder of collagen connective tissue associated with ocular, auditory, articular, and craniofacial manifestations. Approximately 25% of those individuals with Stickler syndrome exhibit some form of midline clefting, including cleft palate[9].

Deletions at 22q11 cause DiGeorge syndrome, and cleft palate occurs in about 14% of people with this deletion. Another cause of cleft lip with or without cleft palate includes Opitz syndrome, which is caused by mutations in the *MID1* gene, and ectrodactyly, ectodermal dysplasia and cleft lip with or without cleft palate, which is caused by mutations in the *TP63* gene[13].

2.2.2.3. Environmental factors

Epidemiological studies on different populations have demonstrated links between several environmental factors active during pregnancy and a higher risk of having a child with a cleft. Some of these include heavy alcohol consumption, cigarette smoking, consumption of pharmaceuticals such as anticonvulsants, and accidental intake of pesticides[30, 31]. There has also been recent, preliminary data provided which suggests that early embryonic exposure to the cholesterol-lowering statin drugs may confer a risk for a wide range of birth defects, including cleft palate[32]. Many teratogens including steroids, ethanol, 13-cis retinoic acid, and ionizing radiation along with periods of hypoxia and hypothermia have been shown to cause clefting in animal models. Infections, such as rubella and toxoplasmosis during the first trimester, have also been associated with clefting[4].

For several years, it has recommended that pregnant women and those women planning on having a baby should take folic acid as a daily dietary supplement. Several studies have been completed to determine whether maternal consumption of folic acid reduces the risk for orofacial clefting in their children. These studies have produced mixed results on the effect of folic acid and the occurrence of clefts. However, several studies have proven a protective effect of maternal multivitamins containing folic acid on the incidence of clefts[33, 34].

Higher risk of orofacial clefts has also been associated with maternal obesity and diabetes mellitus. According to one study, the pregnancies of women who were both obese and diabetic were 3.1 times more likely to result in an offspring with a defect than were those of nonobese, nondiabetic women, which suggests that

obesity and diabetes mellitus may act together to increase the risk of congenital anomalies, including cleft palate[35].

2.3. Current treatments for cleft palate

The goals of cleft lip and palate surgery are directed toward achieving a normal facial appearance, as well as the ability to feed, speak, and hear without significantly affecting the ultimate facial and psychosocial development of the child. Cleft lip repair typically occurs at three months of age with palate surgery normally performed when a child is between the ages of nine and 18 months. This correlates with speech development and lessens the likelihood of interfering with ultimate facial growth[4]. According to a study performed by Weinfeld et al, single-staged cleft palate repair is used in 97% of cases[36]. Multiple surgical techniques are used worldwide, all with relative advantages and disadvantages. The most common palatoplasty techniques being used currently are the von Langenbeck technique[37], the Bardach two-flap palatoplasty[38], the Veau-Wardill-Kilner closure[4, 39], the Schweckendiek technique, and the Furlow palatoplasty[40].

In brief, the nasal side closure for most techniques involves the creation of a septal flap combined with a lateral nasal flap sutured to one another to create the nasal floor. For purposes of palatal closure, the von Langenbeck technique involves medial mobilization of full thickness bipedicled hard palate flaps with palatal alveolar crest releasing incisions[37]. The Bardach two-flap palatoplasty uses two large full-thickness hard palate flaps that are mobilized and closed anteriorly and medially without pushback[38]. The Veau-Wardill-Kilner palatoplasty, also known as

the three-flap/V-Y technique, is used to increase palatal length to improve function by means of a pushback maneuver[4, 39]. A substantial portion of the hard palate is left exposed to heal by secondary intention. The Schweckendiek method is a two-staged procedure. The soft palate is first repaired when the patient is three or four months old at the time of lip repair. The hard palate is then closed when the patient is about 18 months old. The Furlow technique is a double opposing Z-plasty procedure for the soft palate only. The purpose of the Furlow technique is to reorient the muscle of the palate into a functional transverse direction. For hard palate clefts, it is usually combined with the von Langenbeck technique. While this method is difficult to perform in wide clefts, it may have more utility when the cleft is narrow or if a submucous cleft exists[36].

No benefit has been convincingly demonstrated with any particular repair technique when one looks at dental arch form, speech outcome, feeding, or any other functional variable. At this point in our understanding, surgeons often consider their own experiences and training when repairing clefts, since definitive data suggesting that one repair is preferable over another are lacking.

2.3.1. Grafts for hard palate repair

Alveolar defects create serious disruption of the dentition and collapse of the alveolar segments, and are found in 75 percent of cleft patients[41]. Bony reconstruction of the maxillary-alveolar defect improves facial appearance and dental function. This surgical procedure decreases the need for removable prosthetic appliances and obturators by consolidating the dental arches and

eliminating the nasolabial fistula. Additionally, it establishes a bony environment to allow eruption of the permanent cleft-adjacent dentition with subsequent orthodontic, fixed prosthetic, or implant based habilitation of the cleft dental gap. Other advantages of alveolar bone grafting include provision of bone to support the upper lip as well as the cartilaginous skeleton and soft tissues of the nose resulting in a global improvement in facial symmetry and aesthetics[42].

2.3.1.1. Types of bone grafts

Bone grafting is categorized as primary, secondary, or tertiary based on the timing of the procedure. Primary bone grafting is performed during infancy, at the same time as the primary lip or palate repair. Secondary bone grafting is generally performed at the end of the mixed dentition driven by the patients' dental development. It is performed at the developmental stage when either the permanent cleft-adjacent lateral incisor or canine is near eruption. So-called tertiary grafting or late secondary grafting is performed following the eruption of the permanent canine tooth[43].

Primary bone grafting was used mainly in the 1950s and 1960s. The main objectives in primary bone grafting are elimination of bone deficiency, stabilization of the premaxilla to resist scar contracture from the lip and palate repair, creation of new bone matrix for eruption of the deciduous teeth in the cleft area, and augmentation of the alar base. There are also expectations of normalization and stimulation of maxillary growth[44]. However, many authors have demonstrated that

primary bone grafting causes significant growth disturbances of the middle third of the facial skeleton, and this procedure has been widely abandoned[45].

Secondary bone grafting, introduced in 1972 by Boyne and Sands[46], represents the mainstay of treatment in the habilitation of the alveolar cleft deformity. Secondary grafting is performed any time after the primary lip and palate repair and can be divided into early (two to five years of age), intermediate (six to 11 years of age), and late (after eruption of the permanent canine)[42]. Early secondary grafting, however, results in midface growth restriction similar to that seen in cases of primary bone grafting. For this reason, it has little utility on the management of the cleft alveolus. Intermediate secondary bone grafting is performed with the intentions of allowing tooth eruption through the grafted bone, stabilizing the dental maxillary arch, supporting the nasal soft tissue and cartilage, and improving the periodontal health of the dentition.

The purpose of late grafting is to optimize conditions for prosthetic dental treatment such as crowns, bridges, and implants[47]. Late secondary and tertiary bone grafts are performed to enable prosthodontic and periodontal rehabilitation and to assist in the closure of persistent bucconasal fistulae. However, this type of graft cannot repair bone loss to teeth adjacent to the cleft[45, 47].

Another technique to be considered is the gingivoperiosteoplasty (GPP). This technique attempts to avoid the need for future bone grafting by repairing the soft tissue component of the alveolar cleft at a very young age. Approximation of the gingival and periosteal tissues across the cleft usually takes place at the time of lip or palate repair. Surgeons who favor this technique hope to take advantage of the

osteogenic potential of the infant's periosteum, such that bone will grow *de novo* at the site of the repaired cleft. If successful, the GPP obviates the need for an additional surgical procedure and donor site morbidity. Outcome data regarding the effect of this procedure on the subsequent growth of the maxillofacial skeleton are insufficient at present to either advocate for its widespread use or complete abandonment.

2.3.1.2. Source of bone grafts

Various donor sites for harvesting bone grafts have been used. Autologous cancellous bone from the iliac crest is most often utilized, but may also be harvested from the tibia and the mandiblar symphysis. Bone from the cranial vault and rib have also been used, but has been shown by some to not perform as well as iliac and tibial bone[47]. Autologous grafts are advantageous because the entire cleft can be filled with viable bone and teeth that erupt through grafted bone respond well to orthodontic or orthopedic forces and to maxillary growth. The adjacent teeth around the former cleft have sufficient bony support, adequate closing of the oronasal fistulae is facilitated, and reliable support is added to the nasal framework[48, 49].

Success of an autologous bone graft depends on the ability of the graft to become revascularized. Cancellous bone is particularly advantageous because it is quickly revascularized due to its trabecular nature[49]. Additionally, a small number of transplanted cells survive providing a stimulus for osteogenic precursor cell invasion. Cancellous bone is more readily incorporated into the recipient site[50].

Cortical bone is another option for cleft grafting. While this type of bone provides initial form and strength for reconstruction, it lacks cellularity and revascularization is slow because of its compact structure with fewer surface openings, resulting in a lengthy transformation and weaker implant[49].

Disadvantages to autologous bone grafts include donor site pain and morbidity, a varied rate of success, and the limited volume of bone that is available at the donor site[51]. Moreover, there is blood loss at both the harvest site and the implantation site. Other donor site complications include wound dehiscence, hematoma, seroma, and paraesthesia.

Allogenic bone grafts have been utilized for repair of alveolar clefts[52]. These types of grafts reduce morbidity by eliminating the need for a second surgical site. Allogenic bone banks have been established, which make demineralized and freeze-dried cortical and cancellous bone available for immediate use "off the shelf." This bone has undergone extensive screening and processing to diminish the risk of disease transmission, reduce immunogenicity, and host rejection. Nevertheless, allogenic banked bone has disadvantages as well, which include eliciting a localized cellular immunologic reaction and retarding revascularization and osteoinduction. There may also be delayed incorporation at the recipient site and increased risk of dehiscence and sequestration[49].

Bone graft substitutes, referred to as alloplasts, have also been considered in cleft grafting. These materials, including β-tricalcium phosphate and hydroxylapatite, are not recommended in growing patients with unerupted teeth adjacent to the cleft site[53]. Histological evidence of fibrous incorporation was

found when hydroxylapatite was placed at a surgically created cleft site in an animal model by El-Deeb and Horswell[54]. Alloplasts, however, have been used for alveolar ridge contour augmentation in the adult patient where placement of endosteal implants is not anticipated[53, 55].

2.4. Limitations to current treatments

Several complications have been associated with the repair of cleft palate deformities. While these complications can often be minimized by meticulous technique and attention to detail, patients are often subjected to multiple revision surgeries over their lifetime. In a 1999 study of 374 patients with clefts completed by Mackay et al, it was found that patients underwent an average of 3.3 reconstructive procedures and 1.2 otolaryngologic procedures[11]. Cohen et al evaluated 67 patients aged 14 years or older with cleft lip and palate, finding that (1) patients with unilateral cleft lip and palate required an average of 6.12 procedures and, (2) patients with bilateral cleft lip and palate required an average of 8.04 additional procedures[10].

The most obvious complications resulting from palatoplasties include wound dehiscence resulting in persistent oral-nasal fistulae as well as scarred and immobile palates resulting in hypernasal speech and midface growth restriction. Scarring could be caused by the poor handling of the tissues, wound closure under tension, the use of large, tightly tied sutures, or sutures being left in place for too long[56].

Factors which contribute to dehiscence and fistula formation include the extent of clefting, errors in technique that include inadequate mobilization of the palatal flaps and closure of the incisions under tension, as well as patient and parent compliance with postoperative wound care and dietary instructions. Maxillary-alveolar (primary palate) fistulae, on the other hand, may be intentionally left to be repaired in the mixed dentition to minimized further growth retardation. Fistulae may simply present as asymptomatic conduits or may contribute to velopharyngeal incompetence, difficulties with oral hygiene, and nasal regurgitation of liquids and food.

Disturbances of facial growth are often the result of cleft palate surgery. Transverse maxillary hypoplasia and the resulting cross bite are common abnormalities in clefts and need to be managed by orthodontic maxillary expansion with fixed appliances. Once expansion is complete, bone grafting is performed to consolidate the dental arch correlated with the stage of canine development and closely coordinated with planned orthodontic therapy[4]. Midface retrusion is another common sequela of cleft palate repair. This results in class III skeletal and dental malocclusion. Orthognathic surgery in conjunction with orthodontic therapy is performed the time of skeletal maturity to establish a normal occlusion and improved facial form.

Donor site (tibia, mandible, ilium, cranium, rib) morbidity following bone graft harvest is still a recognized limitation in the reconstruction of the cleft primary palate. As with any grafting procedure, complications can be expected in rare instances from both the recipient and the donor sites. Early complications are seen

as in other grafting procedures and include pain, wound infection, bleeding, paresthesia, local tissue injury, poor mobility, and fracture of the donor bone. Late complications at the donor site include chronic pain, unaesthetic scarring, gait disturbance, and paresthesia. Growth disturbance has not been reported in the pediatric grafting population despite the immaturity of the ilium at the time of harvest.

Despite the widely reported successes and low complication rates of multiple bone donor sites utilized in the habilitation of cleft patients, the ideal method of reconstruction has not yet been realized. The number of complications associated with cleft palate repair is large. While these surgeries aim to restore proper function of the palate and provide the patient with normal aesthetics, these goals are sometimes not fulfilled. Therefore, other strategies may need to be employed to overcome the limitations associated with some surgical techniques.

2.5. Tissue Engineering Strategies for Cleft Palate

Tissue engineering strategies promise to deliver improvements in the way that bone defects, such as cleft palate, are repaired. Autologous bone is presently the preferred material for secondary and tertiary grafting procedures to repair a cleft palate. Grafts of this type provide a scaffold on which bone cells can proliferate, induce proliferation of undifferentiated cells and their differentiation into osteoblasts, and provide a reservoir of skeletal stem and progenitor cells that can form new bone[57]. However, autologous bone supplies are limited, and procurement

procedures can induce donor site pain and morbidity, and can lead to anatomical and structural complications[58].

The field of tissue engineering aims to restore function to or replace damaged or diseased tissues through the application of engineering and biological principles[59]. These principles include the selection and manipulation of cells, design of scaffold matrices, and identification and use of pertinent biological signaling molecules. The intended outcome of an implanted tissue engineered construct is a new tissue that is structurally and functionally integrated into the surrounding host tissue[59]. Grafting of the hard palate has been abandoned because it has been demonstrated that this impedes the maxillary growth in the cleft patient. Therefore, we propose a tissue engineering approach for the grafting of the alveolus and/or soft tissues in the palate.

The following sections provide a basic explanation of the scaffolds, cells, and signaling molecules that have been studied for their role in bone tissue engineering. These general principles are then further explored as they specifically relate to tissue engineering strategies for the repair of cleft palate. Specifically, tissue engineering applications for the repair of alveolar clefts and soft tissue augmentation are discussed.

2.5.1. Scaffolds

The scaffold provides a solid framework for cell growth and differentiation at a local site, allowing cell attachment and migration. The scaffold may be implanted alone to induce host cell migration to the wound site and initiate tissue

regeneration, or it may serve as a carrier for cells for the purpose of cell replacement therapy[60]. Scaffolds for engineering bone should be biocompatible, be absorbable with rates of resorption comparable to rate of formation of new bone, provide a platform on which bone cells can proliferate, have sufficient mechanical stability, and be easy to manufacture, sterilize, and handle in the operating room[57]. The scaffold should also have sufficient porosity to accommodate osteoblasts or osteoprogenitor cells, to support cell proliferation and differentiation, and to enhance bone tissue formation. High interconnectivities between pores are also desirable for uniform cell seeding and distribution and the diffusion of nutrients to and metabolites out from the cell/scaffold constructs[61].

Naturally derived or synthetic polymers are commonly used materials for bone tissue engineering scaffolds. Polymers have great design flexibility because the composition and structure can be tailored to specific needs[61]. Natural polymers are derived from plant or animal sources and are biocompatible, biodegradable, and substrates onto which cells can adhere and proliferate. Collagen, chitosan, and hyaluronic acid are some natural polymers that have been employed in bone tissue engineering applications[62-64]. Synthetic polymers that have been used in bone tissue engineering applications include poly(lactic acid), poly(glycolic acid), and poly(lactic-co-glycolic acid). Scaffolds for bone formation may contain inorganic compounds to enhance proliferation. Examples include hydroxylapatite, calcium phosphate cements, metals and calcium sulfate[60].

Scaffolds may be in a variety of forms including porous solid meshes, foams, and injectable gel networks. Injectable scaffolds are favorable because they

can be used to fill irregularly shaped defects, can be administered in a noninvasive manner, and can easily incorporate therapeutic agents or cells by simple mixing[65].

Surface properties of the scaffold, both chemical and topographical, can control and affect cellular adhesion and proliferation[66]. When a scaffold surface does not favor cell-matrix interactions, various cell-binding peptides can be anchored to the surface of the scaffold to induce cell adhesion and migration. These peptides mimic and induce cell-extracellular matrix protein interactions to the biomaterial surface through physical or chemical modification methods[57]. A commonly used peptide is the arginine-glycine-aspartic acid (RGD) sequence of amino acids[67].

2.5.2. Cells

The characteristics of an optimal cell source include no immunorejection, no graft-versus-host disease, no tumorigenicity, immediate availability, availability in large quantities, controlled cell proliferation rate, predictable and consistent osteogenic potential, and controlled integration into the surrounding tissues[57].

Autologous bone marrow is a source of mesenchymal stem cells that has these characteristics. The isolation of mesenchymal stem cells is easy as it relies primarily on the ability of these cells to adhere to tissue-culture plastic, they have a high proliferative potential, their default pathway of differentiation is into osteoblastic cells, and bone formation is not correlated to the number of cell passages as long as the cells retain their ability to proliferate[68, 69]. Mesenchymal

stem cells are thought to be recruited in the body for repair of injured tissues, and therefore are good candidates for cell-based tissue engineering therapies[70].

Embryonic stem cells are another source of cells that may be used in tissue engineering. These cells exhibit perpetual proliferation *in vitro* and the ability to differentiate into any cell type in the human body[60]. However, embryonic stem cells are a rare resource and for the foreseeable future will remain embroiled in ethical and political debates[71].

2.5.3. Signaling molecules

Often, tissue regeneration requires or is enhanced by the introduction of biochemical signals, in the form of growth factors, cytokines, or genes which direct cellular responses including proliferation, differentiation, and matrix synthesis[60]. These signals may lead to promotion or prevention of cell adhesion, proliferation, migration, and differentiation by up- or down-regulating the synthesis of proteins, other growth factors, and/or receptors[66].

Bone morphogenetic proteins (BMPs) are categorized as a part of the transforming growth factor β (TGF-β) superfamily because they are similar in protein structure and sequence homology with TGF-β. While their main role is to recruit mesenchymal stem cells to the healing site, and then differentiate them into the osteogenic lineage, bone morphogenetic proteins also direct the progression of cells and their organizational format to tissues and organs in the embryo, influence body patterning, limb development, size and number of bones, and modulate post-fetal chondro-osteogenetic maintenance[72]. BMPs 2, 4, 6, and 7 are generally

considered to be the most osteoconductive of the bone morphogenetic proteins[66]. BMP-2, specifically, promotes undifferentiated mesenchymal cells into osteoblasts, leading to bone formation.

Other signaling molecules include TGF-β, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), and platelet derived growth factor (PDGF)[66]. TGF-β has been shown to stimulate cellular proliferation *in vitro* and to promote cellular differentiation. IGF genes are expressed by skeletal cells and have been found to stimulate type I collage synthesis and increase matrix apposition rate[73]. VEGF is commonly found in bone fracture healing sites and the plate growth, and regulates vascularization through the recruitment of endothelial cells to the healing sites[74]. FGF-2 is involved in the bone remodeling process. It is believed that it is involved in the regulation of the maintenance of the delicate balance between bone forming cells and bone resorbing cells and it promotes the development of new blood vessels. PDGF is produced by osteoblasts, platelets, monocytes, and macrophages, and is believed to have a role in the migration of mesenchymal stem cells to the wound healing site[66].

2.5.4. Alveolar bone engineering

Secondary bone grafting is routinely practiced as the alveolar cleft treatment in patients with cleft lip and cleft palate. However, harvesting a sufficient amount of bone is often difficult, and the procedure imposes a heavy burden on the patient. Tissue engineering strategies could be employed to lessen these implications. Several approaches have been studied.

A study by Shimakura and colleagues examined the possibility of achieving an autologous transplantation using cryopreserved human bone marrow cells[75]. Xiao et al used human osteoblasts that were isolated from alveolar bone and human gingival fibroblasts that were obtained from gingival tissue for bone regeneration[76].

The three-dimensional structures used today as scaffolds for cells include collagen matrices, synthetic organic polymers, and porous forms of hydroxyapatite. Hydroxyapatite is highly biocompatible and serves as the scaffold for the osteogenic precursor cells, promoting their differentiation into osteoblasts. From an examination of bone formation using porous hydroxyapatite with different pore sizes, Tsuruga et al concluded that hydroxyapatite with a pore size of 300 to 400 μm is best suited for the differentiation of osteoblasts and ingrowth of blood vessels[77]. Hydroxyapatite has also been combined with collagen for use as a bone-equivalent composite that can be used for the filling of irregular defects in maxillo-facial surgery[78]. Collagen type I matrices have been seeded with cells and implanted into sites where osseous damage has occurred[76]. Synthetic copolymers, such as poly-L-lactic acid/polyglycolic acid, have served as an osteoconductive material in bony defects[79, 80].

However, there are some disadvantages with these materials. Particulate hydroxyapatite lacks shape and cohesive strength; therefore, it tends to be dislodged and to migrate under externally applied forces during the healing period[78]. Most synthetic degradable polymers are based upon an ester backbone. These types of materials degrade when water is added to the ester linkage of the polymer backbone. The disadvantage of these materials is that their degradation products are

acidic. As the scaffold degrades, the local acidity of the native tissue increases, and this leads to an increase inflammatory response, and further premature degradation of the scaffold[81, 82].

Signaling molecules in the bone morphogenetic protein (BMP) family have been used to induce cell differentiation into osteoblasts. In 1988, Wozney et al sequenced recombinant human BMP-2 (rhBMP-2) and cloned it. Recombinant technology now allows production of large, pure quantities of rhBMP-2 that can be used clinically and in the laboratory. When placed in the proper environment, rhBMP-2 can cause bone formation. Bone forming cells are not necessary to initiate this process; rhBMP-2 acts to concentrate host stem cells at the site and then to influence their differentiation into bone forming cells. To achieve this effect, administration of super-physiologic doses is needed[83].

Chin and colleagues investigated the use of rhBMP-2 as a substitute for autologous iliac crest bone for repair of congenital facial clefts in humans. In this study, 49 of the 50 alveolar and facial clefts that were repaired had successful consolidation demonstrated by radiographs. These radiographs showed no evidence of root resorption in any patient and there was ossification across the alveolar cleft site only. No case exhibited bone formation outside of the site prepared for construction. There was no sign of adverse affects of the rhBMP-2 in any patient or on adjacent tissue[83]. Several other research groups have also used rhBMP-2 with positive results in terms of bone augmentation, some specifically for cleft palate repair[75, 84-86].

2.5.5. Soft tissue engineering

Although the underlying bone determines to the position of the soft tissue, in some cases, the soft tissue may be too thin, of poor vascularity, or of poor quality. In these cases, soft tissue augmentation may be required[87]. Oral reconstructions for cleft palate repair are often complicated by a shortage of mucosal tissue. Generally, no autologous grafts are used in cleft palate repair; therefore a tissue engineering approach could be employed to develop a substitute which could be used to limit the iatrogenic effects of cleft palate surgery.

Several techniques for the reconstruction of mucosa have been developed using keratinocytes cultured on a dermal substrate. Keratinocytes could be obtained from both the skin and palatal mucosa. Some researchers believe that the ideal mucosal graft should be constructed of autologous oral keratinocytes, grown in a serum-free or defined medium without a xenogeneic feeder layer[88]. A study by Ophof et al showed that canine oral keratinocytes cultured on a skin-derived substrate at the air-liquid interface form an approximately ten cell layers thick parakeratinized epithelium closely resembling normal palatal epithelium of the beagle dog[89].

The most widely used types of dermal substrates are skin-derived or collagen-based materials. Skin-derived materials are prepared from cadaveric donor skin. Human oral keratinocytes have been successfully grown on a decullularized human allograft skin[90, 91]. Type I collagen, sometimes supplemented with extracellular matrix components such as collagen type-IV, elastin, or glycosaminoglycans, is the basis for another type of dermal substrate[89].

2.6. Tissue engineering in children

Cleft lip with or without cleft palate is the most prevalent congenital craniofacial birth defect in humans and as such, cleft palate is primarily present in children. There are several considerations that must be taken into account when performing tissue engineering for eventual use in children.

Children have been shown to have a better reparative potential than adults perhaps due to the increased proliferative capacity of younger cells. For example, it has been widely observed that young children and juvenile animals are capable of re-ossifying large calvarial defects, while adult animals lack this endogenous tissue engineering capacity[92, 93]. It has also been demonstrated that juvenile osteoblasts derived from 2-day-old rats have significantly greater ability to proliferate and form bone nodules *in vitro* compared with adult osteoblasts derived from 60-day-old rats[94]. A study by Haynesworth et al proved that the number of mesenchymal stem cells produced in humans decreases as the donor age increases[95]. The ratio of stem cells is 1:10,000 bone marrow cells in newborns, 1:100,000 bone marrow cells in teenagers, 1:400,000 cells in persons in their fifties, and 1:2,000,000 cells in persons in their eighties[96]. Furthermore, another study demonstrated that bone marrow stromal cells obtained from elderly donors exhibited decreased proliferation potential and accelerated senescence compared with cells obtained from younger donors[97]. Similar results, showing an inverse relationship between donor age and proliferative potential of the cells, were found for arterial smooth muscle cells[98] and fibroblasts[99].

One significant limitation to tissue engineering in children is that the engineered construct must be able to account for ongoing growth in the child and the distortions that take place in the body following reconstruction. In a study by Farkas et. al., normal growth of the nasolabial features were studied in Caucasians aged 1 to 18 years and it was found that fast-growing features, such as the upper lip and nose, attained more than two-thirds of adult size by the age of five years[100, 101]. Prosthetic materials currently in use do not accommodate dynamic craniofacial development and therefore must be repeatedly replaced in pediatric patients as they grow[102]. The ideal tissue engineered construct will need to be designed to encourage unimpeded growth as well as hard and soft tissue healing in the short term, allowing its resorption and replacement by the body to occur with minimal scarring and local distortion.

Another consideration with tissue engineering in children is the limitation in donor size. Bone marrow contains mesenchymal stem cells that are able to differentiate into several cell lineages, including osteoblastic cells. It is generally acceptable to obtain 1-5 milliliters of bone marrow per kilogram of patient weight[102, 103]. As children obviously weigh much less than adults, the amount of bone marrow available for harvest from children is limited. To overcome this, a much greater amplification of stem cells would be required to avoid the use of allogenic cells, which carry the risk of immunologic rejection and disease transmission.

Chapter 3: A Cyclic Acetal Biomaterial for Tissue Engineering

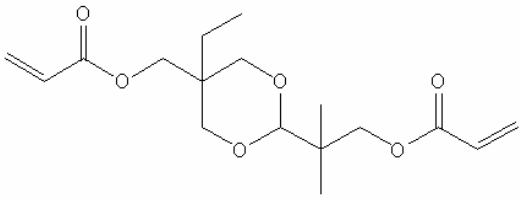
3.1. Introduction

Anatomical defects resulting from cleft palate are treated in several ways. These include a variety of surgical procedures and bone grafting of the alveolus. However, there are several disadvantages of surgical cleft palate repair including disturbances of facial growth, scarring, and mandible retrusion. Complications associated with bone grafting include donor site pain and morbidity, local tissue injury and fracture of the donor bone. In order to overcome these disadvantages, a tissue engineering strategy may be used in the repair of cleft palate. Specifically, we are investigating strategies for regenerating the alveolar bone that is often missing as a result of cleft palate. Tissue engineering combines cells, signaling molecules, and biomaterial scaffolds into a construct that is structurally and functionally integrated into the surrounding host tissue. The work presented in this thesis focuses on developing a biomaterial for use a tissue engineering scaffold.

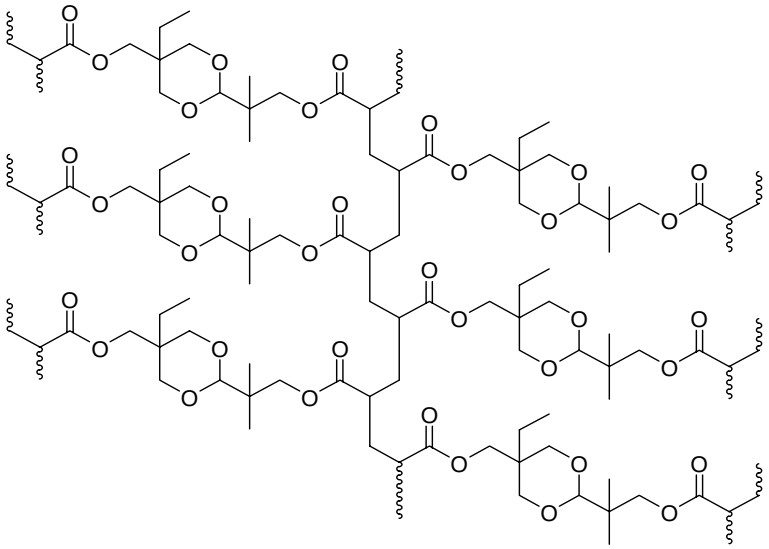
Several materials have already been studied for their role as a bone tissue engineering scaffold. These materials fall into the categories of ceramics, natural polymers, and synthetic polymers[57]. Ceramic materials include hydroxyapatite and hydroxyapatite-tricalcium phosphate, natural polymers include collagen and alginate, and synthetic polymers include polylactic acid, polyglycolic acid, and polycaprolactone[104]. However, there are disadvantages to all of these materials. Particulate hydroxyapatite lacks shape and cohesive strength and tends to be

dislodged from the implant site[78]. Natural polymers, such as collagen, have been reported to have mechanical properties significantly less than normal values[105]. Most synthetic degradable polymers are based upon an ester backbone. These types of materials degrade when water is added to the ester linkage of the polymer backbone. The disadvantage of these materials is that their degradation products are acidic. As the scaffold degrades, the local acidity of the native tissue increases, and this leads to an increased inflammatory response, and further premature degradation of the scaffold[81, 82].

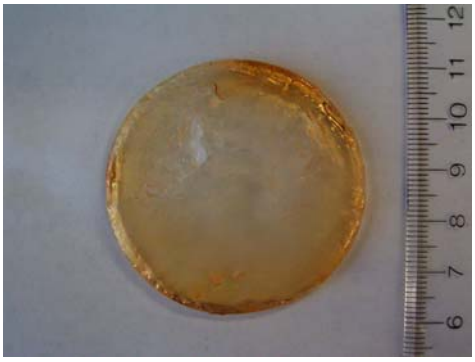
To overcome this issue, a novel class of biomaterials has been created. These novel materials are based upon a hydrolytically degradable cyclic acetal unit. These biomaterials degrade by hydrolysis of the cyclic acetal groups, forming diol and aldehyde degradation products. These types of compounds will not significantly affect the local acidity of the native tissues. Specifically, the material that has been examined in the study is 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD), which contains a cyclic acetal unit (Figure 3a). These monomers, when reacted with the initiator benzoyl peroxide (BP) crosslink to form a rigid plastic material (Figures 3b and 3c). BP is a polymerization initiator commonly used with acrylates because of its ability to form free radicals. The formation of EHD networks can be accelerated using heat or by the addition of N,N-dimethyl-p-toluidine (DMT).



(a)



(b)



(c)

Figure 3. (a) Chemical structure of 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD); (b) Chemical structure of EHD networks; (c) An EHD network.

These novel biomaterials can be used in one of two ways. They can be prefabricated *in vitro*, seeded with cells, and then implanted into a bone defect in the cleft palate patient. The polymer could also be designed to be an injectable biomaterial, which is introduced to the bone defect as a liquid, and then cured *in vivo* to form a solid material. We propose that the optimal method of use for the EHD networks is for them to be utilized as injectable materials.

Therefore, this study was designed to determine the effects of the EHD network formulation scheme on several properties of the networks. Specifically, the initiator content, volume of diluent, and amount of accelerator were varied according to a factorial design. The effect of these parameters on the EHD network gelation time, maximum reaction temperature, sol fraction, degree of swelling, and cell viability properties were measured and evaluated.

3.2. Materials and Methods

3.2.1. Materials

Benzoyl peroxide (BP), N,N-dimethyl-p-toluidine (DMT), 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD) were used as received from Sigma-Aldrich (Milwaukee, WI, USA). Reagent grade acetone was used as received from Fisher Scientific (Pittsburgh, PA, USA).

3.2.2. Experimental design

A three factor factorial design was employed. The three factors investigated were (1) weight percent of initiator, benzoyl peroxide (BP), (2) amount of diluent,

acetone, and (3) amount of accelerator, N, N-dimethyl-p-toluidine (DMT). BP content was examined at two levels, 2 weight percent and 5 weight percent. The amount of diluent was studied at two levels, 0.5 mL/g EHD and 1.0 mL/g EHD. Finally, the amount of DMT was examined at three levels, 1 μL/g EHD, 4 μL/g EHD, and 8 μL/g EHD. Therefore, a 2 x 2 x 3 design with 12 formulations was used. Table 1 presents the compositions of all formulations. For the BP and diluent contents, 0 represents the low level of the factor and 1 represents the high level of the factor. For DMT, 0 represents the lowest level of the factor, 1 represents the medium level of the factor, and 2 represents the highest level of the factor.

Table 1. Outline of the three factor 2 x 2 x 3 factorial design. The three factors that were investigated were benzoyl peroxide content (wt %), volume of diluent (mL/g EHD), and volume of N,N-dimethyl-p-toluidine (μL/g EHD). The first two factors were investigated at two levels (0 and 1), while the third factor was investigated at three levels (0, 1, and 2). Therefore, 12 formulations were tested.

	Initiator Content (wt %)		Diluent Content (mL/g EHD)		Accelerator Content (μL/g EHD)	
	Low (0):	2	Low (0):	0.5	Low (0):	1
					Medium (1):	4
	High (1):	5	High (1):	1	High (2):	8
Formulation Number	Initiator Content		Diluent Content		Accelerator Content	
1	0		0		0	
2	0		0		1	
3	0		0		2	
4	0		1		0	
5	0		1		1	
6	0		1		2	
7	1		0		0	
8	1		0		1	
9	1		0		2	
10	1		1		0	
11	1		1		1	
12	1		1		2	

3.2.3. EHD network synthesis

EHD networks were synthesized using two methods, depending on their eventual use. For all studies except the cell attachment studies, the networks were formed into disks approximately 22 mm in diameter and 4 mm thick. In order to make these disks, 1 g of EHD was measured out. The appropriate mass of BP, depending on formulation, was dissolved in the proper amount of diluent. This solution was added to the EHD and mixed vigorously. The appropriate volume of DMT was added to the EHD solution and mixed well. The solution was then poured into a cylindrical glass vial (25 mm in diameter), where it gelled.

For the cell viability studies, a flat sheet of the EHD networks were made. In order to make these sheets, 8 g of EHD was measured out. The appropriate mass of BP was dissolved in the proper amount of diluent. This solution was added to the EHD and mixed well. The appropriate volume of DMT was added to the EHD solution and mixed thoroughly. The solution was then poured into a mold 150 mm by 75 mm by 0.9 m and sandwiched between two glass plates, where it gelled. Once the networks formed, circles approximately 22 mm in diameter were cut out using a cork borer. These networks were washed for 10 minutes each in PBS, acetone, and PBS on a shaker table at 100 rpm.

3.2.4. Cell Viability

Bone marrow was harvested from the femurs and tibias of adult male Wistar Hannover GALAS rats (101-125 g). The cells were cultured in α-minimal essential medium (αMEM) + 10% fetal bovine serum (FBS) in a T-75 polystyrene flask.

Osteoprogenitor cells adhere to the polystyrene. Media is changed every two days, and non-adherent cells are washed away, isolating the osteoprogenitor cells. Cells were grown until confluent and passaged cells were used in the cell attachment studies.

Confluent flasks were rinsed with 2 mL of phosphate buffered saline (PBS). Cells were incubated at 37 ºC for 8 minutes with 2 mL of trypsin to release the cells from the flask. The trypsin was then neutralized with 4 mL of αMEM media + 10% FBS. The cell solution was then placed in a 50 mL Falcon tube and 100 μL of the solution was removed to count the cells, using a hemacytometer. The cells were centrifuged for 5 minutes at 1500 rpm. The cell pellet was resuspended in αMEM media + 10% FBS.

Cells were seeded onto the surface of EHD networks at a density of 400,000 cells / 22 mm diameter disc. Osteoprogenitor cells were cultured with αMEM media + 10% FBS incubated at 37°C at 5% CO2. Osteoprogenitor cells were allowed to attach and proliferate in these conditions for 2 days. The cells were then assayed for viability using 0.4% trypan blue stain. Both viable and nonviable osteoprogenitor cells were counted using a hemacytometer. The cell viability was calculated using the formula:

$$\text{Cell viability} = (\text{Number of alive cells} / \text{Total number of cells}) \times 100\%$$

Each sample was run in triplicate; the reported values are the mean values and the associated errors are the standard deviations.

3.2.5. Gelation time

The gelation time was determined using a rheometer (Model AR 2000, TA Instruments, New Castle, DE) equipped with a 20 mm diameter stainless steel flat plate geometry. The gelation point was defined as the time corresponding to the formation of an infinite polymer network in which all of the chains are bound together at a minimum of one site[106]. At the gelation point, the polymer viscosity change with time asymptotically approaches infinity. A representative gelation time plot is shown in Figure 4.

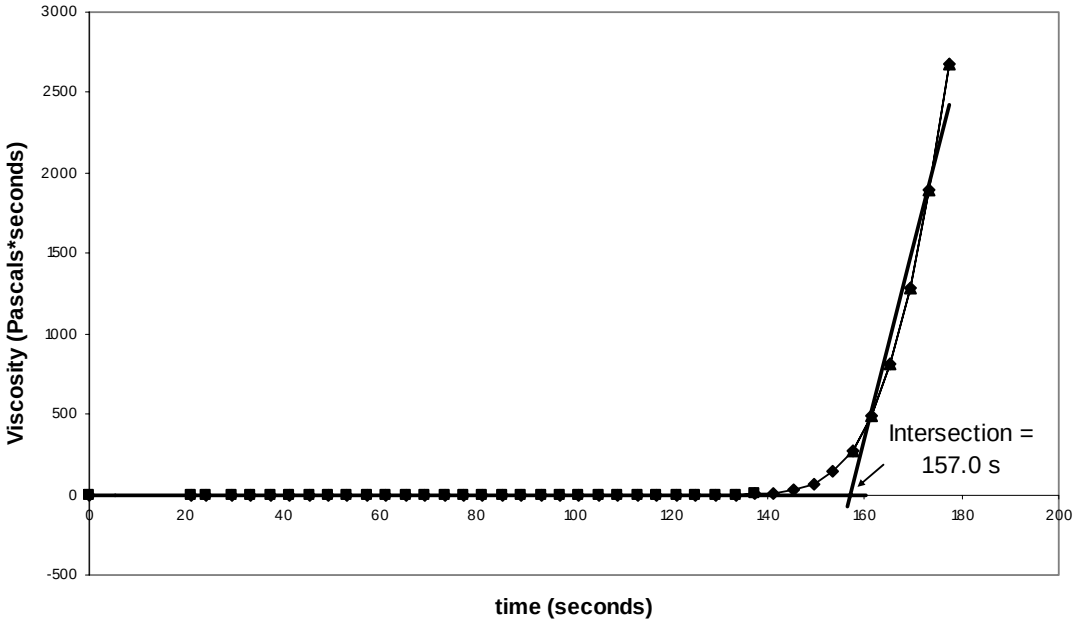


Figure 4. A representative gelation time plot. Gelation time was defined as the x value of the intersection between a line drawn through the initial viscosity region, and a line drawn through all points where the viscosity is greater than 100 times that of the initial viscosity.

The components of each formulation in Table 1 were weighed out, using 0.5 g EHD as a basis. The corresponding mass of BP was dissolved in the appropriate volume of diluent and mixed well. This solution was added to the EHD and mixed

well. Next, the proper amount of DMT was added to the EHD solution and mixed thoroughly. DMT was added at time zero and a stop watch was started at this point. The EHD solution was injected into the rheometer with the gap set to 500 μm. A time sweep was performed on each of the samples at a frequency of 20 rad/s and a constant 10% strain. Each sample type was run five times; the reported values are the mean values and the associated errors are the standard deviations.

3.2.6. Temperature profiles

The temperature of the EHD networks during the gelation time was measured using a thermocouple. One gram of EHD was measured out in a 25 mm diameter glass vial. The appropriate mass of BP was dissolved in the correct amount of diluent and this solution was added to the EHD and mixed well. The proper volume of DMT was added to the EHD solution and mixed thoroughly. A wire thermocouple (Control Company) was then inserted into the center of the sample. The thermocouple was kept away from the bottom and sides of the glass vial. Initial temperature of the samples was 23.2 ± 1.3 ºC. Temperature measurements were then taken and recorded using a Traceable Data Acquisition System (Control Company) once a second until the sample temperature returned to within 2 ºC of the initial temperature. The maximum temperature was then determined for each sample. A representative temperature profile is shown in Figure 5. Each sample type was run five times; the reported values are the mean values and the associated errors are the standard deviations.

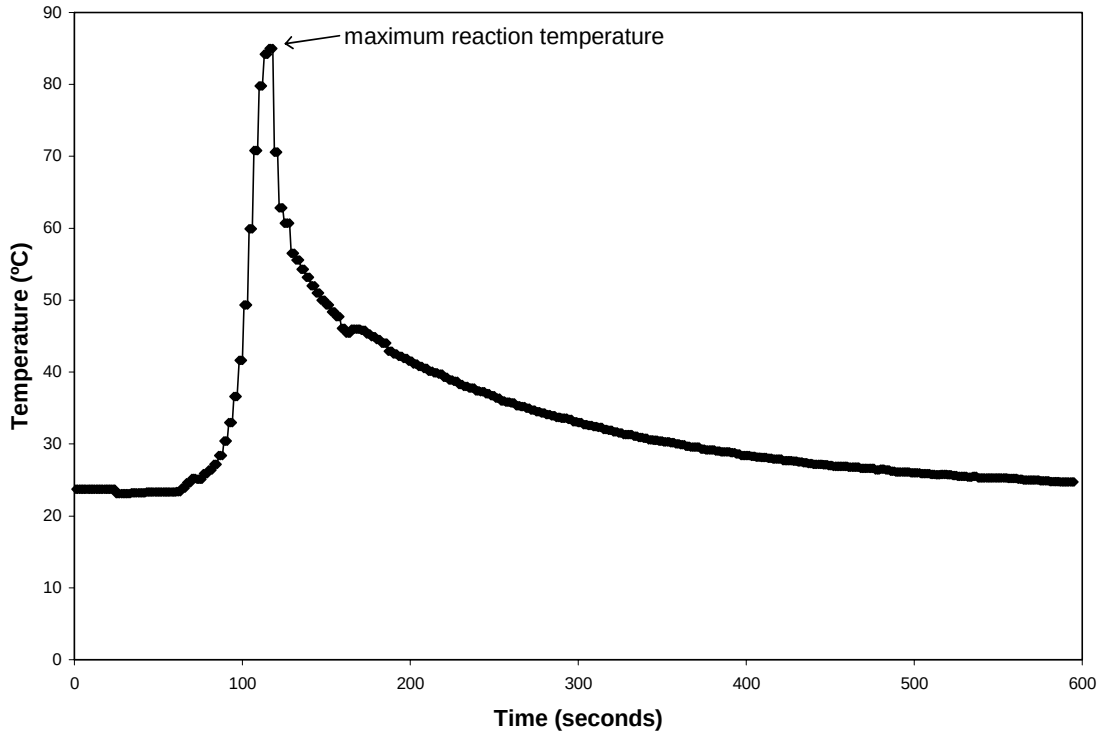


Figure 5. A representative temperature profile. The maximum reaction temperature for each temperature profile was determined.

3.2.7. Sol fraction and degree of swelling

A study of the EHD network sol fraction was performed using the EHD network disks, whose fabrication was described previously. Once the networks had gelled, they were patted dry with weigh paper and weighed (W_i). Each sample was placed in a vial containing 10 mL of acetone, as EHD monomers are soluble in acetone, but the EHD networks are not. The samples were then removed from the acetone after 24 h and the surface was patted dry with weigh paper and each sample was weighed (W_w). The networks were then placed in vials and were left in a fume hood for 24 h for the remaining acetone to evaporate. Next, the samples were

placed in an oven set at 60 ºC and were periodically weighed until their mass stabilized (W_d). The sol fraction was calculated using the formula:

$$\text{Sol fraction} = \frac{W_i - W_d}{W_d} \times 100\%$$

The degree of swelling was calculated using the formula:

$$\text{Swelling degree} = \frac{W_w - W_d}{W_w} \times 100\%$$

Each sample type was run five times; the reported values are the mean values and the associated errors are the standard deviations.

3.2.8. Cell Attachment

Bone marrow was harvested from the femurs and tibias of adult male Wistar Hannover GALAS rats (101-125 g). The cells were cultured in αMEM media + 10% FBS in a T-75 polystyrene flask. Osteoprogenitor cells adhere to the polystyrene. Media is changed every two days, and non-adherent cells are washed away, isolating the osteoprogenitor cells. Cells were grown until confluent and passaged cells were used in the cell attachment studies.

Confluent flasks were rinsed with 2 mL of phosphate buffered saline (PBS). Cells were incubated at 37 ºC for 8 minutes with 2 mL of trypsin to release the cells from the flask. The trypsin was then neutralized with 4 mL of αMEM media + 10% FBS. The cell solution was then placed in a 50 mL Falcon tube and 100 μL of the

solution was removed to count the cells, using a hemacytometer. The cells were centrifuged for 5 minutes at 1500 rpm. The cell pellet was resuspended in αMEM media + 10% FBS.

Cells were seeded at a density of 150,000 cells/well in a 12 well plate. The experimental group was the EHD networks of formulations 1, 4, 7, and 10. The EHD networks were prevented from floating by a 16 mm inner diameter stainless steel ring. There were two controls: an empty well and a well with the stainless steel ring inserts.

Cell attachment was determined at 4 hours and 8 hours after initial seeding. Each well was rinsed with 0.2 mL of PBS. Cells were incubated at 37 ºC for 8 minutes with 0.2 mL of trypsin to release the cells. The trypsin was the neutralized with 0.4 mL of αMEM media + 10% FBS. Cells were counted using a hemacytometer. Both controls at each time point were run ten times and the experimental groups at both time points were run five times; the reported values are mean values and the associated errors are standard deviations.

3.2.9. Statistics

The results of the 2 x 2 x 3 factorial design were studied by an analysis of variance (ANOVA). Three factors were investigated, and therefore a total of seven effects could be identified. These include three main factor effects (the effect of BP, the effect of diluent, and the effect of DMT), three two-factor interactions (the effect of BP and diluent, the effect of BP and DMT, and the effect of diluent and DMT), and one three-factor interaction (the effect of BP, diluent, and DMT). An *F*

value, *F* critical value, and *p* value were calculated for each of the effects. The resulting *p* values are reported. A significance level of 95% ($\alpha = 0.05$) was chosen, therefore an effect with a *p* value of <0.05 is considered to be significant. While all seven effects were studied in this way, only the main effects will be discussed.

3.3. Results

3.3.1. Cell viability

As a preliminary study, the cell viability on the EHD networks was determined as a function of the amount of DMT used in formulating the networks. Because DMT is toxic, it was important to determine that the cells would not die when allowed to grow on EHD networks that contain DMT. The cell viability on the EHD networks containing DMT varied between 87.9% and 89.1% (Figure 10). There is no significant difference in the viability between the experimental groups and the control group, which had a viability of 92.0%

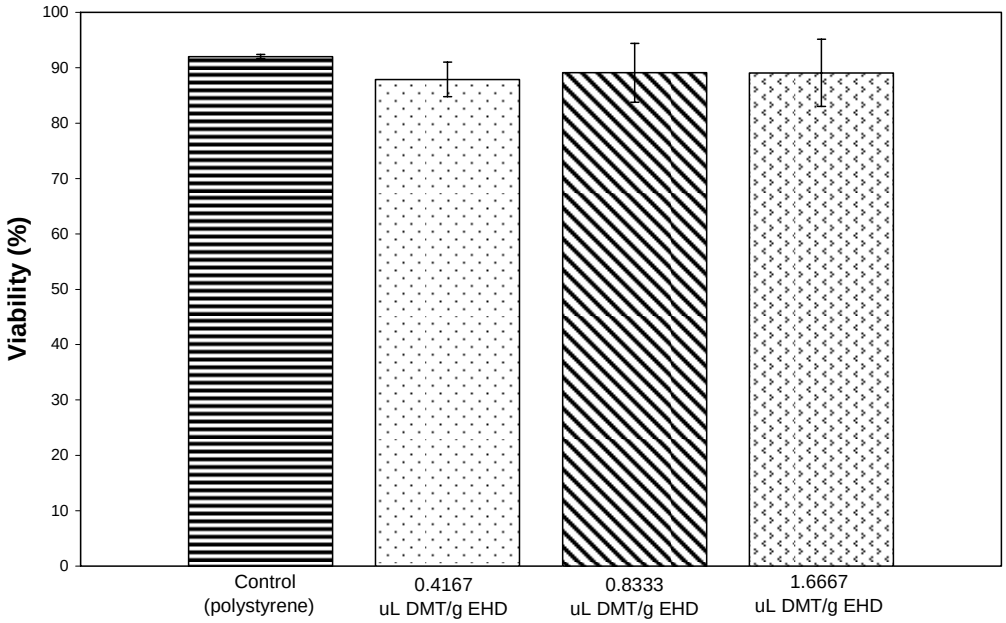


Figure 6. Osteoprogenitor cell viability on EHD networks containing 0.4167, 0.8333, and 1.6667 μL DMT / g EHD. Results are compared with viability results on a polystyrene control. No significant difference was found between the experimental groups and the control.

3.3.2. Gelation time

In order to measure the rate of the EHD polymerization reaction, the gelation time for each of the EHD network formulations was found. The gelation time was determined by monitoring a change in the EHD solution viscosity with time. The time that the networks take to gel decreases from 193.9 s to 33.3 s as the volume of initiator, DMT, increases (Figure 6). Analysis of the results from the factorial design study showed the main effect of BP, the main effect of diluent, and the main effect of DMT to be statistically significant in determining the gelation time. DMT was found to be the parameter that most affects the gelation time.

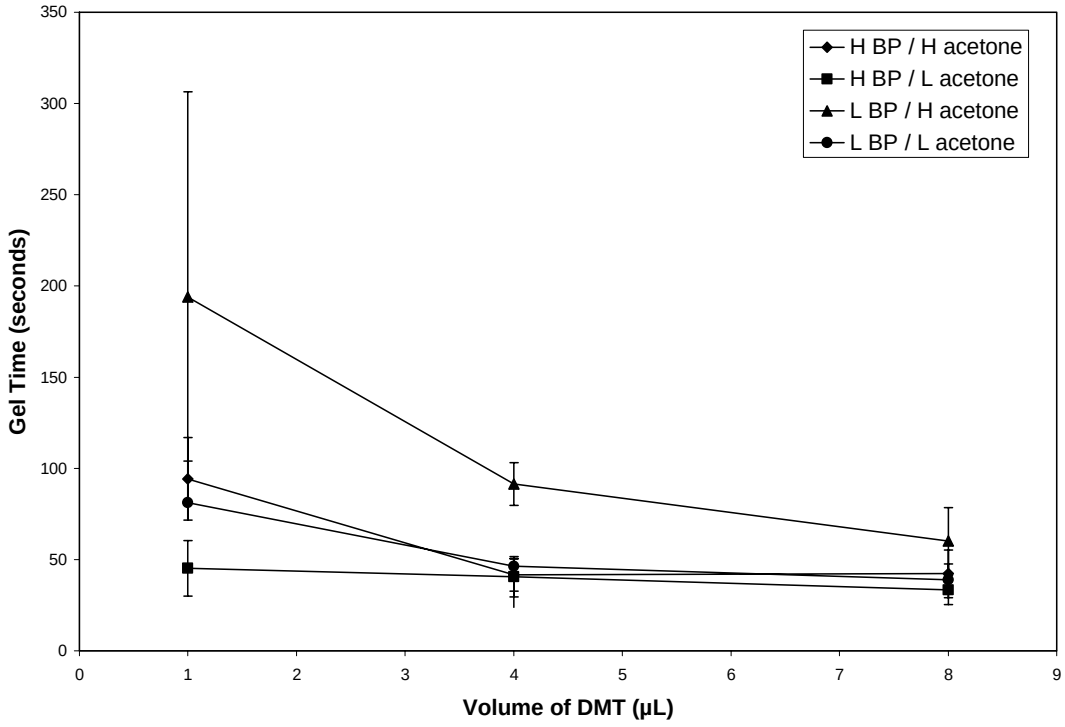


Figure 7. The effect of BP content, volume of diluent, and volume of DMT on the EHD network gelation time. All factors, BP content ($p = 2.7 \times 10^{-4}$), volume of diluent ($p = 6.9 \times 10^{-5}$), and volume of DMT ($p = 3.9 \times 10^{-6}$) were found to be significant in determining the gelation time.

3.3.3. Temperature profiles

As the EHD networks could potentially be formed *in vivo*, it is imperative to examine the temperature profiles of the networks as they form. An important piece of information obtained from these profiles is the maximum temperature reached by the networks as they formed. The maximum reaction temperature increases from 31.9 ºC to 109.0 ºC as the BP content increased from 2 wt% to 5 wt% and the volume of DMT increased from 1 µL/g EHD to 8 µL/g EHD (Figure 7). Analysis of the results from the factorial design study showed the main effect of BP, the main effect of diluent, and the main effect of DMT to be statistically significant in

determining the gelation time. The volume of diluent was found to be the parameter that most affects the maximum temperature reached by the networks as they formed.

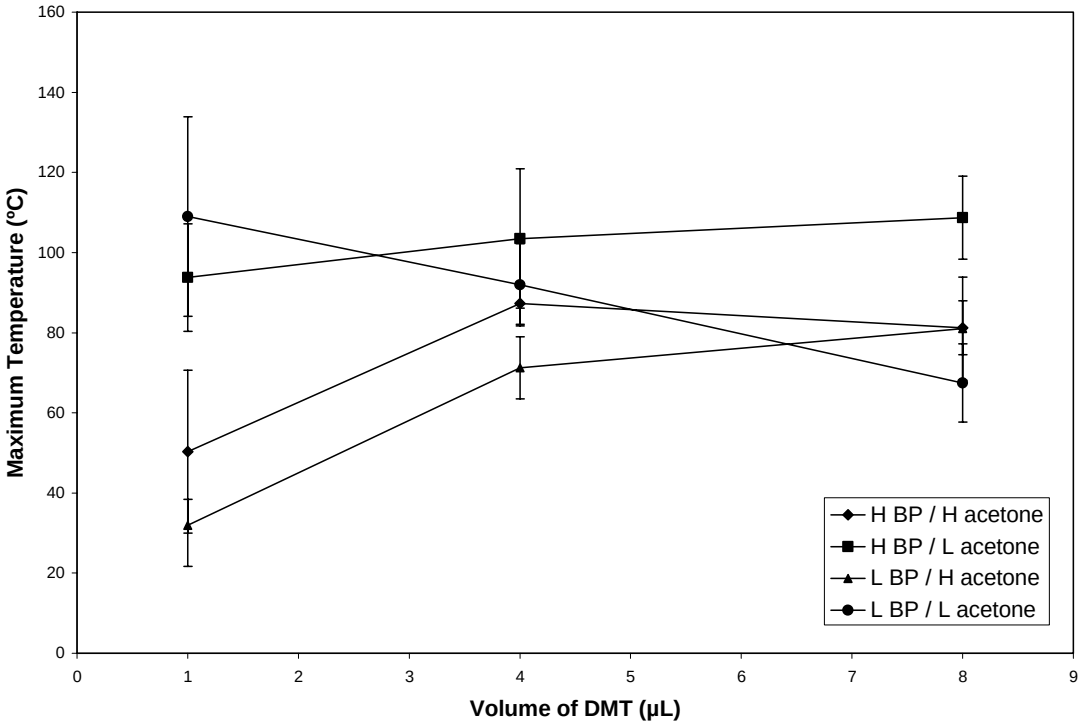


Figure 8. The effect of BP content, volume of diluent, and volume of DMT on the maximum temperature reached during EHD network formation. All factors, BP content ($p = 1.1 \times 10^{-3}$), volume of diluent ($p = 9.6 \times 10^{-11}$), and volume of DMT ($p = 2.4 \times 10^{-10}$) were found to be significant in determining the maximum temperature.

3.3.4. Sol fraction

The sol fraction of a material is a measure of the amount of unreacted components. A decrease in sol fraction corresponds to a decrease in the amount of unreacted material. The sol fraction of the EHD networks decreases from 45.0% to 22.0% as the BP content increased from 2 wt% to 5 wt% and the volume of diluent

decreased from 1 mL/g EHD to 0.5 mL/g EHD (Figure 8). Analysis of the results from the factorial design study showed the main effect of BP, the main effect of diluent, and the main effect of DMT to be statistically significant in determining the sol fraction. The volume of diluent was found to be the parameter that most affects the sol fraction.

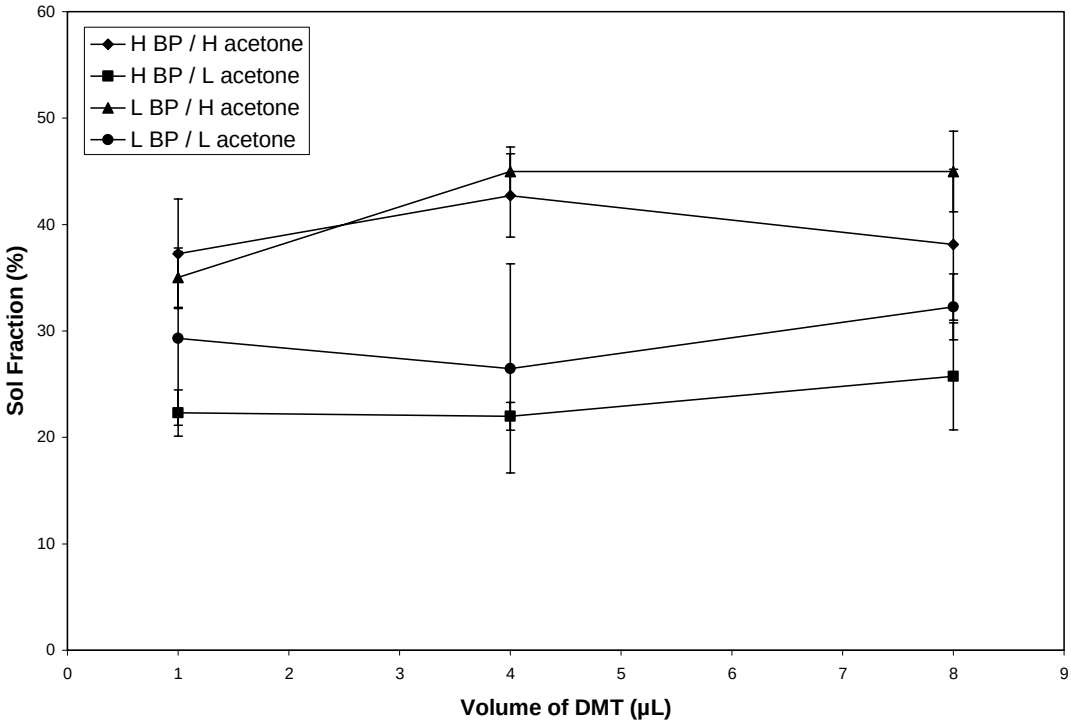


Figure 9. The effect of BP content, volume of diluent, and volume of DMT on the sol fraction of the EHD networks. All factors, BP content ($p = 3.3 \times 10^{-3}$), volume of diluent ($p = 4.2 \times 10^{-14}$), and volume of DMT ($p = 3.4 \times 10^{-2}$) were found to be significant in determining the sol fraction.

3.3.5. Degree of swelling

The swelling degree of a material is a measure of its ability to uptake liquid molecules and hold them between the polymer chains. The swelling degree of the EHD networks increases from 29.9% to 48.3% as the BP content decreases from 5 wt% to 2 wt%, the volume of diluent increases from 0.5 mL/g EHD to 1 mL/g

EHD, and the volume of DMT increases from 1 μL/g EHD to 8 μL/g EHD (Figure 9). Analysis of the results from the factorial design study showed the main effect of diluent and the main effect of DMT to be statistically significant in determining the degree of swelling. The volume of diluent was found to be the parameter that most affects the swelling degree.

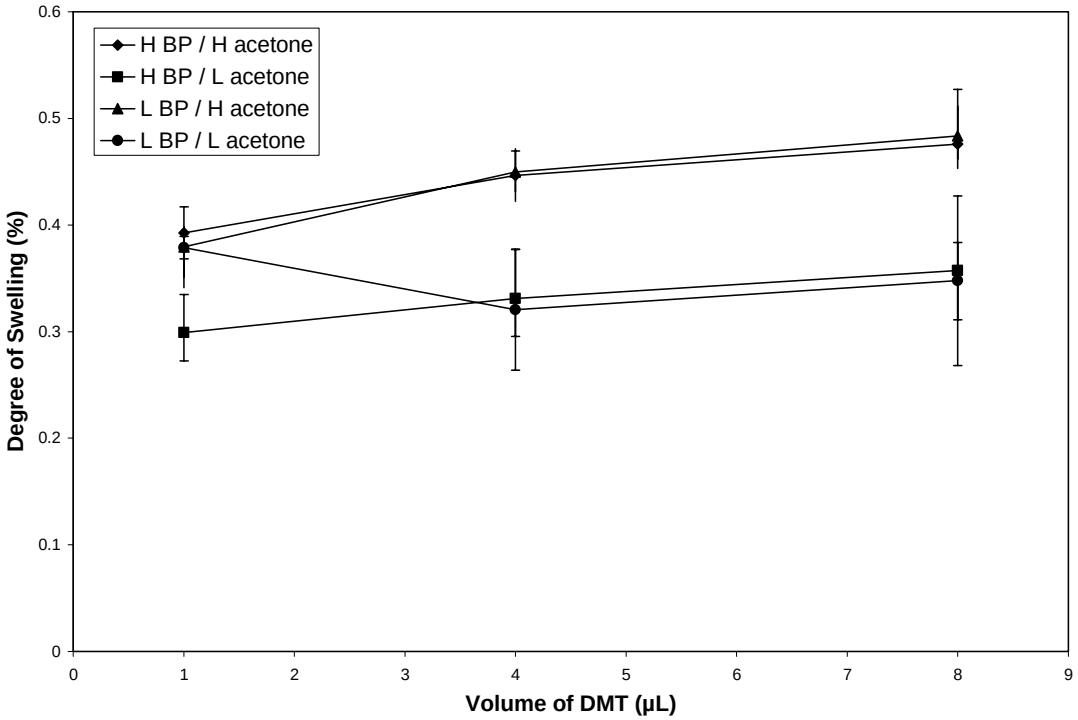


Figure 10. The effect of BP content, volume of diluent, and volume of DMT on the degree of swelling of the EHD networks. The volume of diluent ($p = 9.3 \times 10^{-13}$) and the volume of DMT ($p = 4.8 \times 10^{-5}$) were found to be significant in determining the swelling degree. BP content ($p = 0.36$) was not found to be significant in determining the swelling degree.

3.3.6. Cell Attachment

As the EHD networks could be used as prefabricated materials with osteoprogenitor cells seeded on the material before implantation, it is important to determine the cell attachment properties of the material. Cell attachment varied

between 7.3% and 78.3% at 4 hours (Figure 11). At this time point, it was determined that there was a significant difference in the cell attachment between the empty control and all other groups, between the insert control and all other groups, between Formulations 1 and 7, between Formulations 1 and 10, between Formulations 4 and 7, between Formulations 4 and 10, and between Formulations 7 and 10.

At 8 hours, the cell attachment varied between 14% and 77.3% (Figure 11). At this time point, it was determined that there was a significant difference in the cell attachment between the empty control and the insert control and Formulations 1, 4, and 7, between the insert control and Formulations 1, 4, and 7, between Formulations 1 and 10, between Formulations 4 and 10, and between Formulations 7 and 10.

Finally, it was also determined that there was no significant difference in the cell attachment between 4 and 8 hours in the empty control group, the insert control group, Formulation 1, and Formulation 4. There was a significant difference in the cell attachment between the 4 and 8 hour time points for Formulation 7 and Formulation 10.

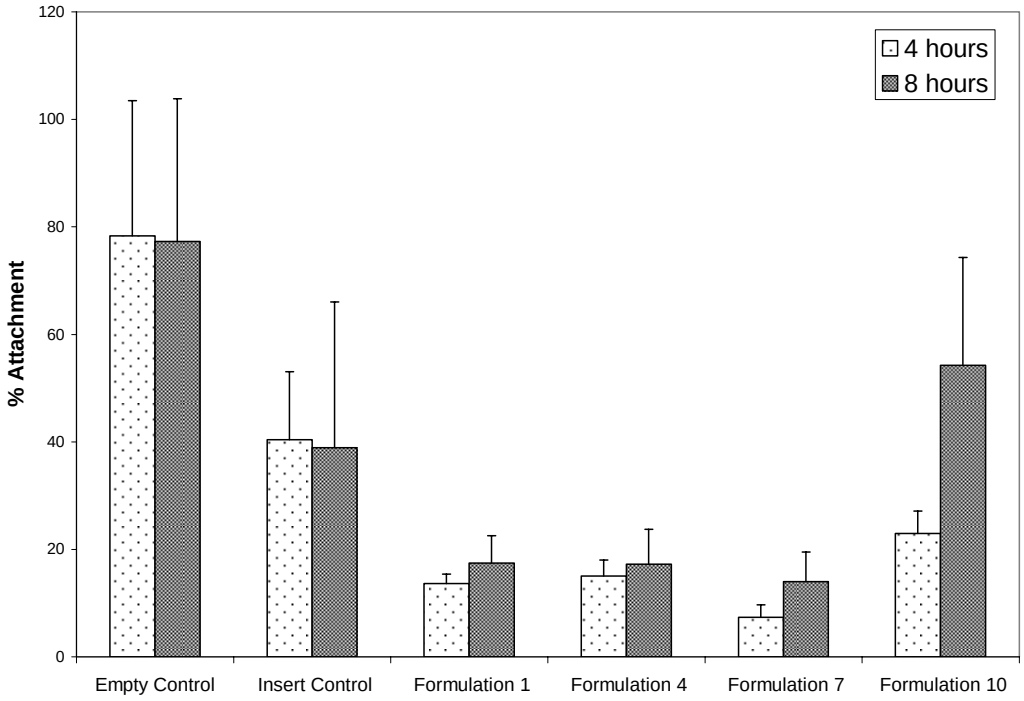


Figure 11. Cell attachment properties of osteoprogenitor cells on EHD networks fabricated with Formulations 1, 4, 7, and 10. This property was determined at 4 and 8 hour time points. The results were compared with the cell attachment properties on an empty control (tissue culture polystyrene) and an insert control (tissue culture polystyrene with the stainless steel ring).

3.4. Discussion

We are proposing here the use of the novel cyclic acetal biomaterial EHD as a bone tissue scaffold for the repair of cleft palates. The fabrication parameters, including the amount of initiator, BP, the amount of diluent, acetone, and the amount of accelerator, DMT, used each have an influence on the properties of the material. In this study, these components were varied in a factorial design to measure their main effects on the gelation time, maximum temperature reached, sol fraction, swelling degree, and cell attachment. By examining these properties for each fabrication scheme, the most desirable EHD formulations could be identified.

As a preliminary study, the viability of osteoprogenitor cells on the EHD networks was determined. As we propose using EHD as a biomaterial for bone tissue engineering, it is imperative that cells are viable on the material. We found that there was no decreased viability of the cells on the EHD networks as compared to a control of tissue culture polystyrene.

The first goal of this work was to determine the effect that the initiator content has on the properties of the novel EHD networks. The amount of initiator, here BP, which is present in the reaction system is linked to the rate of the reaction. Increasing the amount of initiator should increase the rate of the reaction. The gelation time of the EHD networks varied between 33.3 and 193.9 s. According to the results of the gelation time experiments, the gelation time could be reduced by increasing the BP content. Increasing the amount of BP causes more free radicals to form, which in turn makes it easier for the free radical on one EHD molecule to find the free radical on another EHD molecule, thus reducing the gelation time. The maximum reaction temperature of the EHD polymerization reaction increases from 31.9 ºC to 109.0 ºC as the BP content increased from 2 wt% to 5 wt%. As the amount of BP increases, the EHD networks form faster, and the reaction temperature is increased. The sol fraction of the EHD networks showed a minimum value for all formulations of 22.0%, with a maximum value of 45.0%.The sol fraction generally increases as the BP content decreases. As the BP content decreases, fewer free radicals are formed and smaller EHD chains are made, These small chains are easily dissolved and washed away, leading to an increase in sol fraction. The degree of swelling of the EHD networks varied between 29.9% and

48.3%. However, BP was not found to be a determining factor of the EHD network swelling degree.

Like the initiator content, the amount of accelerator, here DMT, which is present in the reaction system is associated to the rate of reaction. Increasing the amount of initiator should increase the rate of the reaction. Therefore, another goal of this work was to determine the effect that the accelerator content has on the physical properties of the fabricated EHD networks. Increasing the accelerator content was found to reduce the gelation time of the EHD networks. Increasing the amount of DMT causes the polymerization reaction of EHD to be accelerated, thus reducing the gelation time. The main effect of DMT was found to be the most significant parameter in determining the gel time. The temperature profile experiments showed that as the volume of DMT increased, the maximum temperature of the reaction also increased. This is because increasing the DMT content causes the EHD networks to form faster. In turn, this violent polymerization causes the temperature of the reaction to increase. The DMT content was found to be a minor determining factor of the EHD network sol fraction. As the volume of DMT increases, the sol fraction of the networks was also found to increase. The DMT content of the formulation was found to be a significant determinant of the swelling degree of the EHD networks. The swelling degree was generally found to increase as the volume of DMT increased.

A third goal of this work was to determine the effect that the diluent content has on the properties of EHD networks. The amount of diluent, here acetone, which is present in the reaction system is related to the extent of completion of the

reaction. Reducing the diluent content causes a decrease in the gelation time of the networks. Increasing the amount of diluent increases the distance between EHD molecules. This makes it more difficult for the free radical on one EHD molecule to find another free radical to react with, thereby increasing the gelation time. The maximum reaction temperature was most affected by the diluent content. As the volume of diluent decreases, the maximum reaction temperature generally increases. From the factorial design, it is apparent that the volume of diluent in the formulation is the also the most significant factor in determining the sol fraction. Increasing the volume of diluent in the formulation causes the sol fraction to decrease. Again, an increase in the amount of diluent causes the EHD monomers to be further spread apart, leading to smaller polymerized EHD chains. These smaller chains are more easily dissolved in the diluent than larger EHD chains, leading to a decrease in the sol fraction. The swelling degree was found to be most significantly determined by the volume of diluent in the formulation. As the diluent content is increased, the swelling degree of the network is also increased. As previously described, increasing the diluent volume causes a separation of EHD monomers, leading to an increase in the amount of void space in the network once the residual diluent has evaporated. When the networks are then replaced in acetone, the solvent easily fills the void spaces, leading to a higher degree of swelling.

The final goal of this work was to determine the cell attachment properties of the networks. This property was only studied in the formulations containing the lowest volume of DMT because we would not want a patient to be subjected to any level of DMT that is greater than that needed for the EHD networks to form. There

was a significant difference between the cell attachment properties of all experimental groups and the empty control, and between all experimental groups and the insert control at 4 hours. However, at 8 hours, the attachment on Formulation 10 was not significantly different from that of the empty or insert controls. As the cells would be seeded on the biomaterial more than 8 hours before implantation, these results are promising. Formulation 10 is the most promising formulation in regards to the cellular attachment properties.

In selecting the most attractive fabrication scheme for the EHD networks, it is important to identify the formulation that has the fastest rate of reaction while also having the most complete reaction. Therefore, we believe that the most desirable EHD network to be used as a biomaterial is one that is formulated with either a high or low BP content, a low DMT content, and a high acetone content. These combinations lead to networks with a reasonable gelation time, a low maximum reaction temperature, a low sol fraction, a low swelling degree, and a high degree of cellular attachment. The formulations that fit these requirements are formulations 4 and 10.

3.5. Conclusions

This work investigated the properties of a novel cyclic acetal biomaterial that could be used in bone tissue engineering applications, and specifically for the regeneration of alveolar bone that is often missing in patients with a cleft palate. Monomers of EHD are reacted to form EHD networks. This study was designed to determine the effects of the EHD network formulation scheme on several properties

of the networks. Specifically, the effects of initiator content and volumes of diluent and accelerator on several properties of the EHD networks were examined. The results show that the EHD networks can be optimized for a fast gelation time, low maximum reaction temperature, low sol fraction, low swelling degree, and maximum cell attachment by varying the amounts of BP, diluent, and DMT used in their formulation.

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