Chapter 3

Calcification of Biomaterials and Diseased States

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Additional information is available at the end of the chapter

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Abstract

Calcification is one of the most common issues that arise concerning biocompatibility, known to affect many systems in the body. It is often associated with an increase in free phosphate and calcium particles in the serum that leads to mineral deposition. Calcification is problematic both in the naturally occurring state of the body, as well as when it exists as result of biomaterial implants. While calcification is prominent in many different forms, not all mechanisms and processes associated with the phenomenon are completely understood. In this chapter, materials affected by calcification, potential mechanisms of action, and potential treatments will be discussed. Both bioprosthetic and polymer heart valves and urinary implants will be evaluated for material composition, application, and failure. Current research on the assessment of these materials will be reported, with the associated chemical and biological mechanisms explained. The chapter will also detail diseased states of the arteries that induce calcification and what treatments can be used for both arterial and bioprosthetic calcification. Finally, the chapter will conclude by detailing future designs for biomaterials to prevent and treat calcification in both natural and synthetic applications.

Keywords: aortic valves, biomaterials, bioprosthetic, calcification, tissue engineering, urinary regeneration

1. Introduction

Calcification is one of the most common issues that arise concerning biocompatibility, known to affect many systems in the body. It is often associated with an increase in free phosphate and calcium particles in the serum that leads to mineral deposition [1]. Calcification is problematic both in the naturally occurring state of the body, as well as when it exists as result of biomaterial implants [2]. While recent research confirms that it is an active, cell-mediated

process rather than a passive association of age, the various mechanisms of calcification and related factors that are involved with this phenomenon are still not completely understood.

The cardiovascular system is one that is majorly affected by calcification, both naturally and via biosynthetic and bioprosthetic implants. Natural vascular calcification is associated with the stiffening of arterial walls and the deposition of free calcium and phosphate particles in the serum [3]. Vascular calcification is also seen to increase in patients on dialysis, due to serum being stripped of natural inhibitors of mineralization [4]. Valve implants, coronary stents, and balloon angioplasty are all affected by mineralization due to immune response of biomaterials used, calcium affinity, or even elastin/collagen injury post-implantation [5]. The consequence of this calcification is often associated with implant failure and stiffness of tissue. This also occurs in implants in the urinary system due to adhesion of various minerals and cells to the surface of the implant [6]. Implants such as urinary catheters and ureteral stents calcify as a result of interaction of the bacteria and the device.

While calcification is prominent in many different forms, not all mechanisms and processes associated with the phenomenon are completely understood. In this chapter, materials affected by calcification, potential mechanisms of action, and potential treatments will be discussed. Both bioprosthetic and polymer heart valves and urinary implants will be evaluated for material composition, application, and failure. Current research on the assessment of these materials will be reported, with the associated chemical and biological mechanisms explained. The chapter will also detail diseased states of the arteries that induce calcification and what treatments can be used for both arterial and bioprosthetic calcification. Finally, the chapter will conclude by detailing future designs for biomaterials to prevent and treat calcification in both natural and synthetic applications.

2. Heart valves

A major contributor to morbidity and mortality worldwide is valvular heart diseases (VHDs). Valvular dysfunction is related to an insufficient opening or closing of the valve caused by either stenosis, regurgitation or both [7]. Stenosis can be described as a stiffening of the leaflets, leading to improper opening and closing of the valves. Regurgitation occurs when blood flows back through the valve indicating inadequate valve closure [8]. Almost 2.5% of the U.S. population is affected by VHDs. With 300,000 surgeries completed annually, heart valve replacements come in second for the most common cardiovascular surgical procedure to treat this issue [7]. There are currently two strategies for this treatment: repair or valve replacement [7].

Valve replacements generally exist in two forms: mechanical heart valves (MHVs) or bioprosthetic (biological) heart valves (BHVs) [9]. There are five categories of biological heart valves: autograft, autologous, homografts, pericardial valves, and porcine xenografts [7]. Autograft heart valves are implanted using the Ross procedure, which replaces the problematic aortic valve with a healthy valve that is already within the patient [10]. To create an autologous heart valve, cells from a patient must be harvested and transplanted onto a scaffold using tissue engineering techniques. The resulting tissue that has formed within the scaffold is then placed back inside the same patient [11]. Homografts that used for valve replacements are typically taken from

organ donors. Grafts obtained from these donors, or sources other than the receiving individual are known as allografts. Pericardial valves are fabricated from bovine pericardium and are fixed onto a stented frame during implantation [12]. Xenografts are any valves transplanted from an animal source, including porcine and bovine pericardial valves [12].

Bioprosthetic valves can also be in one of three forms: stented, stentless, and percutaneous [13]. Mechanical valves are typically created from non-biological materials like polymers, metal, carbon, and various alternatives [9]. Of the two valve replacement types, roughly half of U.S. patients receive bioprosthetic valves. These are usually either porcine xenograft or bovine pericardial valves. Another 43% of patients undergoing heart valve surgery will receive mechanical prosthesis (**Table 1**) [7].

2.1. Bioprosthetic vs. polymer valves

When choosing between a bioprosthetic and a mechanical valve, there are some important factors that should be taken into consideration. These include the patient's age, preference, life expectancy, comorbidities, and indication/contraindication for warfarin therapy [13]. MHVs and BHVs not only have different compositions, but also differ in features like thrombogenicity, durability, and hemodynamic properties [15]. MHVs have superb durability but require lifelong anticoagulation therapy because of their increased risk of thromboembolism, thrombotic obstruction, and hemorrhage. In contrast, bioprosthetic valves do not require anticoagulation therapy because they are less thromboembolic; however, due to calcific

Bioprosthetic heart valves	**Material**	**Purpose**	**Implantation methods**	***In vivo* response**
Stented	Porcine valve leaflets and bovine pericardium fabricated into pericardial valves are both mounted onto a polymer or metallic supporting stent	Unlike mechanical valves, stented valves are not susceptible to thrombo-embolic effects	Requires open heart surgery	These biological valves do not present the patient with thrombo-embolic problems but they do lead to calcification and tissue hardening due to immune response
Stentless	Made from bovine pericardium or porcine aortic valves	Used to improve hemodynamics and durability of the valves	Requires open heart surgery	These present the same problems *in vivo* as stented valves but have been shown to have a 10% larger effective valve area compared to stented valves
Percutaneous	Biologic porcine or bovine pericardium is affixed to a supporting stent or cage	A less invasive surgery for valve replacement in patients with high operative risks	Implanted into the body by a percutaneous transfemoral method	Presents same problems as stented and stentless but is a very novel technique and needs further investigating

Table 1. Table summarizing the differences between stented, stentless and percutaneous bioprosthetic heart valves [12–14].

tissue degradation, their durability is finite [13]. MHVs and BHVs last around 20–30 years and 10–15 years, respectively. Biological valves are used more often than mechanical valves because of their ease of implantation, safety, functionality, and the fact that they do not require anticoagulant therapy [7, 15].

Most BHVs used are fabricated from porcine heart valves or from bovine pericardium. While bioprosthetic valves are competent, they are still lacking in that they have significant structural deterioration due to calcification [16]. Younger age, renal insufficiency, mitral valve position, and hyperparathyroidism are all predictive factors thought to be associated with structural valve deterioration (SVD). Patient age is a major factor of SVD in bioprostheses. Implant failure ten years after application occurs in less than 10% of elderly patients, while reaching 20–30% in patients less that forty years old [13].

Other factors contributing to calcification are the pre-implantation techniques used on bioprosthetic valves. For example, prior to implantation, most bovine or porcine valves are decellularized which make them less antigenic; however, this process removes all the endothelial cells present. Therefore, adjoining tissue and/or circulating cells cannot then be reseeded after decellularization occurs [7, 17]. Along with decellularization, stabilization of the extracellular matrix (ECM) components, and masking of xenogeneic epitopes are important. For this reason, all animal pericardium must be treated with specific crosslinking agents such as glutaraldehyde prior to implantation. However, glutaraldehyde stimulates many destructive effects such as structural damage, cytotoxicity, and calcific deterioration [18]. Because of problems associated with prosthetic valves, approximately 60% of all patients receiving heart valve replacements will need to have a revision surgery [7]. Also, all studies thus far have neither confirmed nor rejected the use of pericardial valves over porcine valves or *vice versa* (**Figure 1**) [13].

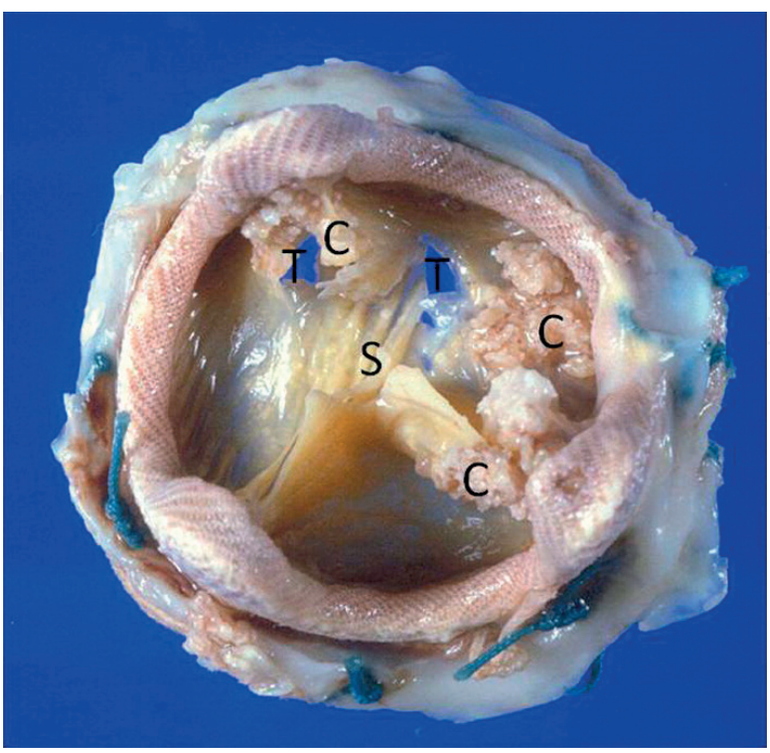

Figure 1. Image of a porcine bioprosthetic heart valve. (C) is showing calcification, (T) is showing cusp tears, and (S) is showing stenosis of the valve [16].

2.1.1. Mechanism

2.1.1.1. Modes and mechanisms of valve failure

The specific mechanisms leading to VHD are not fully known, meaning it is unclear how important genetics, cellular characteristics, and microenvironmental characteristics are in this disease. However, in light of recent evidence, it is believed that alterations in developmental morphogenesis signaling pathways could play a role in VHD [8]. One affected pathway is that of Notch1. The Notch1 pathway is engaged in numerous cell-to-cell communication processes. With this pathway being an intercellular signaling mechanism, it is believed that the loss of Notch1 results in deformation of leaflet morphology throughout embryo development and the inability to suppress calcification during adulthood [19].

One known major cause of failure in bioprosthetic heart valves is calcification [17]. The exact mechanism of tissue degeneration leading to calcification is not fully known. However, IgM/IgG antibodies entering the valve matrix initiate the process. This then leads to deposition of macrophages on the valve surface which is followed by collagen breakdown and calcification [15]. These macrophages are critical factors in the innate immune response. Macrophages are in charge of inducing phagocytosis and killing bacteria. When these macrophages become overwhelmed, they induce an inflammatory response [20]. This inflammatory response causes an increase in inflammatory cytokines that cause calcification [13]. For this reason, the immune system is thought to be a key factor in the initiation of calcification (**Figure 2**) [15].

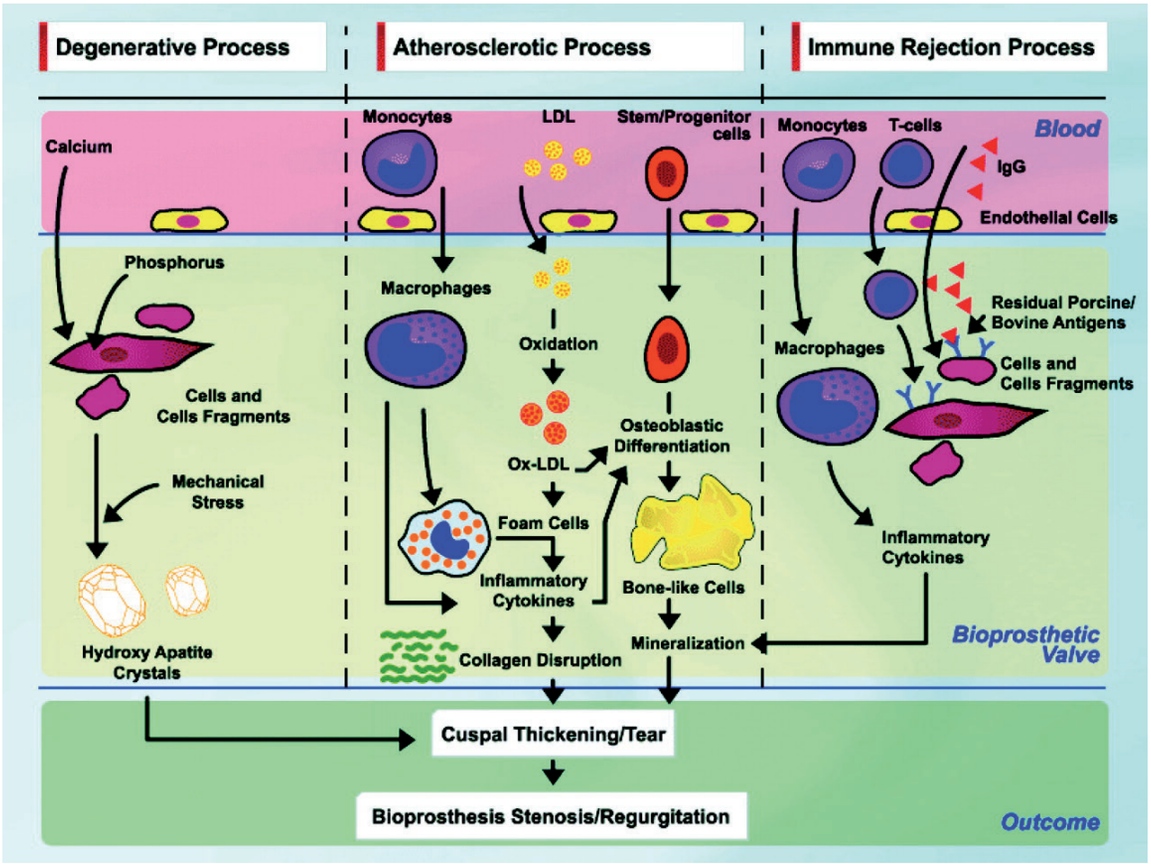

Figure 2. This is a theoretical model showing the degenerative, atherosclerotic, and immune rejection processes involved in the structural degradation of bioprosthetic heart valves [13].

To further determine the reason for calcification involved with BHVs, their composition must be examined. BHVs are fixed in glutaraldehyde to reduce immunogenicity and ameliorate the mechanical strength of the heart valve; however, this fixation reduces antigen presentation and chemical stabilization by concealing antigens and eventually leading to an influx in calcium [9]. Consequently, glycoproteins and other substances are lost during glutaraldehyde fixation which allows for the formation of a calcium phosphate precipitate that would not occur under normal cardiac conditions [17]. This glutaraldehyde fixation is also thought to cause chemical interactions between aldehyde groups, phospholipids and circulating calcium ions which can also cause calcification in bioprosthetic valves [15].

Surface heparin has been used as a preventative method for dealing with tissue calcification in heart valve replacements. This heparin treatment is meant to replace glutaraldehyde fixation. In one study, it was discovered that porcine aortic valves that were pretreated with surface heparin showed a decrease in the accumulation of calcium in valve tissue [17]. While the exact mechanisms of heparin are unclear, it is thought that the heparin molecules block calcium phospholipid-binding sites that glutaraldehyde fixation targets. Ingrowth and antiproliferative effects are also characteristics of heparin which may potentially influence small muscle cell growth during implantation which would indirectly inhibit calcification [17].

In addition to glutaraldehyde fixation causing calcium influx and tissue degradation deterioration, recent studies have suggested that SVD is also due to active mechanisms such as atherosclerosis and immune rejection. This immune rejection could be due to bioprosthetic valves not being "immunologically inert" [13]. This results in humoral and cellular immune responses that lead to tissue disruption and/or mineralization. This would explain why younger patients with a more vigorous immune system might experience faster SVD.

Bioprosthetic SVD might also be due to atherosclerotic processes from associated risk factors [13]. The oxidation and infiltration of low-density lipoproteins within bioprosthetic tissue might trigger an inflammatory process. This would result in osteoblastic differentiation of stem/progenitor cells caused by the oxidized low-density lipoproteins and inflammatory cytokines [13]. Another reason for bioprosthetic valve failure is calcific deposits found in tears in the commissural and basal areas of the cusp. Within 15 years of implantation, over 50% of porcine valves show some form of functional degradation, usually due to regurgitation caused by these cusp tears [15].

2.1.2. Prevention

2.1.2.1. Biomaterial alterations and coatings

One of the major reasons that implants calcify is due to the biocompatibility of the material. In several studies, either altering the chemical makeup and properties of the biomaterial or coating the material with anticalcification agents have been used to reduce these effects.

Crosslinking surface material of various implants has become a topic of interest in current research, specifically because of the mechanical properties it supplies to implants. Crosslinking chemistry provides protection to various extracellular matrix components in bioprosthetic heart valves in order to retain structural strength [21]. Several crosslinking methods, specifically crosslinking with glutaraldehyde, provide strength by preventing degradation, but the

elastin in the ECM is not protected. This leads to stiffness, tears, and deformations in the surface of the material, a condition known as "permanent set" [5]. A modified form of cross-linking involving treating the surface of biomaterial implants with pentagalloyl glucose does not cause damage to collagen or surface deformations. In a study using bovine pericardium tissue, the combined cross-linking was prepared by first soaking the tissue in neomycin trisulfate and a buffer, then incubating it in a cross-linking carbodiimide solution with pentagalloyl glucose. The treated leaflets of tissue were then tested *in vivo* in rats to determine calcification after a thirty day period. Little to no calcification was found on any of the leaflets. The study showed the potential benefits of using this cross-linking method to prevent or delay calcification in implants, though further data should be collected (**Figure 3**) [5].

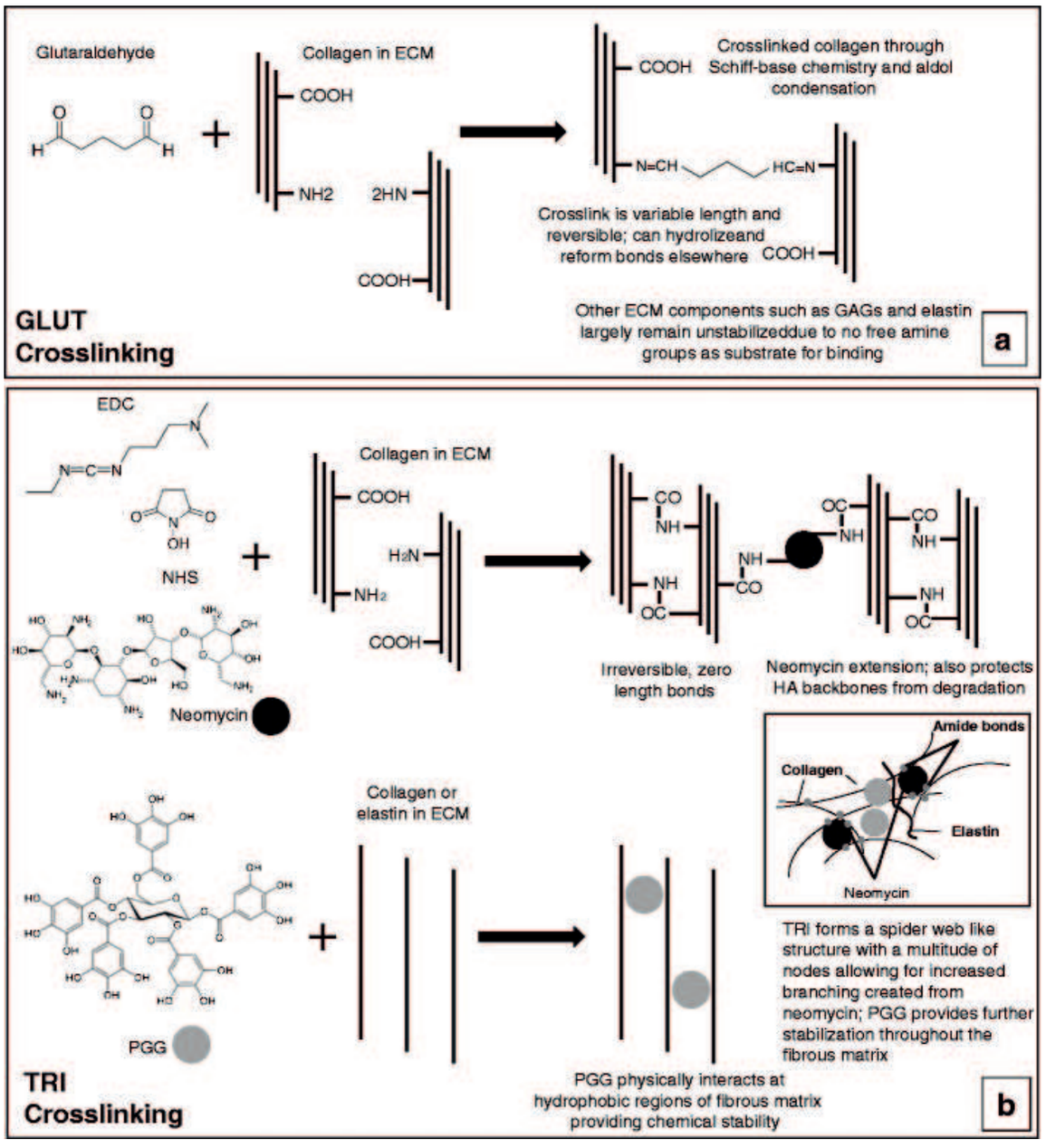

Figure 3. Treating the biomaterial with POSS to form POSS-PCU changes existing receptors so that free calcium ions are no longer able to bind, preventing deposition and mineralization [5].

Another method of preventing calcification is by targeting the free aldehydes present in bioprosthetic tissue. As previously mentioned, glutaraldehyde is used to set tissue to be used for implants because of the strength it provides, and the aldehydes remaining on the tissue are thought to promote calcification [22]. When bovine pericardium is treated with alternatives to cap the free aldehydes, the tissue shows significantly reduced levels of calcium content and mineralization [23]. Reducing agents such as glycine, glutamate, and sodium bisulfite can form a Schiff base and effectively neutralize the aldehydes present [23]. This process allows the tissue to maintain its mechanical strength but inhibits the formation of calcification [23].

Nanocomposites are growing in popularity for use in biomaterials due to their biocompatibility and anticalcification properties [24]. Using polyhedral oligomeric silsesquioxane (POSS) nanoparticles with poly(carbonate-urea)urethane (PCU) has proven to increase mechanical strength, and potentially work as a calcification-resistant material. **Figure 3** illustrates the treatment of POSS-PCU [24]. These composites have been shown to significantly decrease deposition compared to glutaraldehyde-fixed bovine pericardium tissue. The treated tissue shows decreased platelet adhesion to its surface compared to typical bovine pericardium, a mechanism thought to be associated with calcification resistance [25]. These nanocomposites show increased promise for use in biomaterials.

2.2. Tissue engineered and ion-loaded scaffolds

Because of their biocompatibility and regenerative capabilities, tissue engineered scaffolds are becoming a popular source for heart valves replacements. There are three different types of scaffolds: porous, fibrous, and hydrogels. These can be either acellular or seeded with autologous cells to promote regeneration and avoid a negative immune response [7]. A key component in the scaffold is its ability to degrade in a controlled time period [7]. The tissue can then be regenerated while the synthetic scaffold degrades or is remodeled, leaving behind growth and proliferation resembling natural tissue [26].

Tissue-engineered scaffolds seeded with vascular interstitial cells (VICs) have been shown to regenerate valvular tissue, while still retaining the alpha-smooth muscle actin (α-SMA) marker expressed in smooth muscle cells [7]. The tissue engineered scaffolds have a lower risk of ECM damage than the decellularized tissue used in many heart valve implants; therefore, the collagen and fibers behave in a more normal manner, retaining their smooth muscle phenotype. Porosity can be controlled, compared to the unintentional porosity created in decellularized tissue, making the scaffolds more resistant to calcification [7].

These scaffolds can be manipulated before entering the body, from seeding them with autologous cells to promote new growth to loading them with ions as a form of drug delivery to mitigate negative responses post-implantation. Metal ions, including iron, aluminum, and magnesium, are gaining popularity in current research because of their ability to bind to forming hydroxyapatite crystals in the serum and prevent further deposition [27]. Though the mechanism is not fully understood, it is hypothesized that ions such as magnesium bind where calcium typically would, preventing calcium deposits [28]. They also are thought to interrupt alkaline phosphatase activity [27]. These ions often exist naturally in the body for

this process, but by loading tissue-engineered scaffolds with different metals (aluminum, magnesium, iron) they can specifically target calcium deposition at the site of the implant to prevent failure. The problem that rises with this design is that, after implantation, there is no way to reload the matrices with more ions, so it cannot act as a long term inhibitor.

3. Urinary

3.1. Urinary tract materials

In the entire urinary system, the organs most commonly and significantly affected by calcifications are the kidneys, followed by ureters, and then the urinary bladder. Urinary tract calculi are formed when the urine is supersaturated with salt and minerals such as calcium oxalate, struvite (ammonium magnesium phosphate), uric acid, and cysteine [29]. This supersaturation can be caused by a variety of genetic and dietary factors, urinary calcium excretion, and environmental triggers [30]. Urinary calculi are solid particles in the urinary system that cause pain, nausea, vomiting, hematuria, and chills/fever due to secondary infection.

Urinary tract calcification is organized in three different categories: renal calcification, ureteral calcification, and bladder calcification. These are further classified by their orientation, position, shape, size, mobility, opacity, chemical composition, and location in the kidneys, ureters, or bladder, and their relation to pathologic conditions [31]. Calcification in the urinary tract can also occur from infection of the implants. Healthcare associated infections are the fourth leading cause of disease [33]. Studies indicate that biofilm infections cause up to 80% death [33]. One of the leading causes of infection is the insertion of catheters and ureteral stents [33].

3.1.1. Mechanism

3.1.1.1. Urinary infection mechanisms

Catheters and stents used in patients are subject to biofilm formation when different particles in the urine, blood, or surrounding tissue attach to the surface of the implant. Biofilms begin to form when colonized bacteria attaches to the surface of the device, altering the surface properties [32]. Once attached, the bacteria binds with target molecules and, after an extended period of time, the attachment becomes permanent and the process is irreversible. Overtime, as the biofilm becomes more developed, it will repeat these processes to form a new biofilm formation on an unpopulated area of the implantation device [34].

One of the main reasons for encrustation of a device is infection due to bacteria that produce urease. This enzyme uses urea to create an alkaline environment from ammonia, raising the pH [35]. *E. faecalis, Proteus mirabilis, Staphylococcus aureus*, and *Candida tropicalis* are considered the strongest strains to form biofilms; however, *P. mirabilis* is hydrolyzes urea ten times faster than the rates of other strains [34]. Under these conditions, hydroxyapatite and struvite crystals form on the surface of the device, resulting in encrustation [34]. This process will continue and repeat until the flow of urine is blocked due to encrustation resulting in the complete device failure [35].

Encrustation of ureteral stents occurs for a variety of reasons. One reason is the failure of patients to return for stent removal after surgery or inadequate counsel by professional healthcare [36]. The material of the stent may also contribute to encrustation. Silicone containing stents seems to be more resistant to encrustation, followed by polyurethane, silitek, percuflex, and hydrogel coated polyurethane [36]. Stents fracturing after being *in situ* for a long period due to hardening and loss of tensile strength can also be another reason for encrustation. Other factors that contribute may include urinary composition (hypercalciuria, hyperoxaluria, hypocitraturia, homocystinuria, and hyperuricosuria), history of urolithiasis, and congenital urinary tract anomalies [37].

3.1.2. Prevention

3.1.2.1. Urinary implant coatings

Coatings of the urinary implantation devices are one of approaches that prevents bacterial adherence. Surface coatings of the devices inhibit bacterial biofilm formation to prevent infection and encrustation [38]. The coating needs to have certain properties to inhibit bacterial adherence which includes biocompatible, resists biofilm formation, and antimicrobial [39].

Hydrogels are hydrophilic, cross-linked polymers capable of absorbing large amounts of liquid. They form a thin layer of water on the surface of the device, preventing biofilm formation and bacterial adherence [34]. Studies have shown hydrogel-coated catheters have less bacterial adherence compared to non-hydrogel coated catheters [40]. In addition, hydrogel-coated catheters also cause less irritation and inflammation [34].

Similar to hydrogels, antimicrobial peptides (AMPs) are hydrophilic polymers that have antibiotic resistance which inhibits bacterial adhesion [39]. In one study, AMPs were coated on titanium implants and inhibited bacterial adhesions both *in vitro,* and *in vivo* using rat models. In addition to inhibiting bacterial growth, it also has wound healing benefits. However, there are some conditions with AMPs that include potential local toxicity, pH sensitivity, susceptibility to proteolysis, and high cost of synthesis [34].

Polyvinylpyrrolidone (PVP) is also hydrophilic and has excellent lubricant properties. Therefore, the implantation device has less bacterial adhesion and encrustation *in vivo* compared to uncoated catheters [34]. Heparin is a glycosaminoglycan which is a natural inhibitor of crystallization. Naturally, heparin is considered to prevent bacterial attachment and encrustation; however, studies concluded that there is not a significant decrease in bacterial adherence despite its overall good quality [38].

On the contrary, hyaluronic acid shows promising results *in vitro*. Hyaluronic acid is a type of glycosaminoglycan that inhibits nucleation, growth, and aggregation of salts. Covalently bound hyaluronic acid catheters increase hydration, while decreasing adsorption of proteins and bacterial adhesion. Even though hyaluronic acid coating shows promising results, it has yet to be fully analyzed. Gendine is another antimicrobial coating that contains gentian violet and chlorhexidine [34]. Compared to uncoated controls, devices that are coated in gendine are resistant to the adherence of multi-drug-resistant bacteria [34].

Researchers are continuously searching for an ultimate biocompatible material that can substitute segments of the urinary tract [41]. This involves urinary system cells or other cell sources that can be seeded onto biodegradable scaffolds [42]. Experts have reported that cells isolated from urine can express smooth muscle, endothelial and interstitial cells, and markers of urothelial [42].The ideal biomaterial has to be biocompatible and biodegradable, promote vascular regeneration, nerve regeneration, and cellular differentiation; also, it should be watertight and stretchable, resist encrustation and biofilm formation, and regain its shape [41]. However, most biomaterial includes natural collagens, and natural collagens scaffolds cannot maintain their physical properties in an *in vivo* environment resulting in graft failure or formation of fibrosis [43]. There is no ideal biomaterial available yet, but they can be modified to enhance biological properties for cellular integration. Smart polymers are also optimal for use in urinary construction [41].

4. Diseased state

In addition to affecting different biomaterials and biosynthetic implants, calcification also occurs naturally throughout the cardiovascular and the urinary system due to various states and diseases.

Cardiovascular disease is the leading cause of death in the United States, with a high mortality rate among end stage renal disease (ESRD) patients [44]. ESRD and other forms of kidney disease are marked by elevated levels of calcium phosphate in the serum, leading to mineral deposition and calcification of the arterial wall. This occurs as vascular smooth muscle cells differentiate from their typical phenotype to osteoblast-like cells that cause bone formation in atypical regions [45]. This risk increases with patients on dialysis, due to the fact that important calcification-inhibitory molecules, such as Fetuin-A, are stripped from the body [45]. This high level of phosphate also leads to the activation of the Wnt signaling pathway. When high levels of phosphate accumulate in smooth muscle cells, β-catenin is upregulated [46]. This leads to an increased expression of bone-morphogenetic protein 2 (BMP-2) and runt-related transcription factor-2 (Runx2) in smooth muscle cells, though the two factors are typically only seen in bone cells [47].

Primary hyperparathyroidism (PHPT), often associated with cardiovascular disease, is another condition potentially linked to calcification. It has been observed that as levels of parathyroid hormone increase in PHPT patients, there is an associated increase in abdominal aortic calcification [48].

Another form of calcification associated with imbalance of minerals in the body is nephrocalcinosis. When calcium intake increases and it begins to build up in the kidneys, it leads to the deposition of minerals in the renal parenchyma and tubules [49]. These calcified regions are also formed through an osteopontin deficiency. This calcification in the urinary system can contribute to renal dysfunction and potentially lead to ESRD [49].

Calcification of both the vascular and urinary system in the body can be driven by various diseases and conditions, typically due to some sort of mineral or chemical imbalance in the

serum. Taking this into account, as well as the increased rate of calcification seen in bio-implants, it is critical for current research to move toward both prevention and treatment of this phenomenon.

4.1. Mechanism

4.1.1. General mechanisms

Calcification is a pathological process that occurs with an imbalance of several genetic, chemical, and physical properties. The process can depend on the levels of proteins and ions present in the serum, like metal ions that bind with hydroxyapatite or proteins that chaperone free calcium and phosphate particles. It can also be induced by physical damage to cells and tissue, whether by chemical means or foreign implants in the body. Though many individual factors are associated with the formation of mineral deposits and calcification in the body, the mechanisms inducing calcification are still being researched and understood.

Vascular calcification (VC) is a prominent issue affecting both the intimal and medial layers of the arterial wall. Intimal calcification is usually associated with plaque rupture and thickening of the endothelium layer in the vessels while medial calcification occurs as smooth muscle cells differentiate into osteoblast-like cells, which are associated with bone growth. This phenotypic switch in the medial layer is often associated with various osteogenic predecessors [50].

Osteoprotegerin (OPG) is a glycoprotein that works by inhibiting bone resorption, and an increase in OPG is often associated with an increase in calcification [51]. It participates in the OPG/RANK/RANKL pathway to act as a decoy receptor binding to RANKL, where RANK is supposed to bind. This in turn prevents RANK's intended mechanism of osteoblast differentiation into osteoclasts [51].

BMP-2 is also thought to play an important role in VC, since it is expressed in higher levels in chronic kidney disease patients, and is a part of the Wnt/β-catenin pathway [52]. Typically, it is associated with bone and tooth formation, but the high phosphate levels in uremic patients activate this pathway and causes BMP-2 expression.

Apart from the osteogenic markers and factors associated with calcification is another major protein, matrix Gla protein (MGP). In its activated form, it antagonizes BMP-2 signaling as a negative feedback regulator due to its carboxylated glutamate residues [53]. It also acts by binding to forming hydroxyapatite crystals to prevent deposition and calcification. However, it must be carboxylated by vitamin-K in order to be active, which may be why vitamin-K deficient kidney disease patients show calcified vessels [54].

In biomaterials, the surface structure of the material can determine the post-implantation calcification. Materials with a higher porosity have the increased potential for calcification, because the larger pores allow for more calcium deposition [55].

4.2. Prevention

4.2.1. Dietary changes

Because most causes of calcification are rooted in a mineral imbalance, dietary modifications or supplementation are currently being studied for potential use in attenuating the effects of calcification in different regions in the body. Magnesium ions are known to inhibit calcium deposition, though the mechanism is not clearly understood [56]. When supplied to vascular smooth muscle cells, the rate of cell damage by apoptosis is significantly decreased as the magnesium levels increase in the media [57]. Decreasing the rate of apoptosis decreases arterial stiffness and calcification since apoptosis of smooth muscle cells often leads to the disruption and remodeling of plaque in the arteries [58]. Increased magnesium levels also decrease the expression of Runx2, inhibiting the differentiation of smooth muscle cells into osteoblast-like cells [57].

In a Framington Heart Study of 2695 participants, a dietary assessment was used to measure magnesium intake levels and determine whether adding the supplement could prevent or inhibit calcification. It was found that both coronary artery and abdominal aortic calcification decreased as magnesium intake increased, with a 22% decrease in coronary artery calcification for every 50 mg increase in daily magnesium intake, and significant decrease in abdominal aortic calcification with magnesium increase [59]. Even though the mechanism is not fully understood, the metal does correlate with an inhibition of calcification.

MGP is another inhibitor of calcification that prevents the differentiation of vascular smooth muscle cells into osteoblasts [60]. However, MGP can only inhibit calcification if it is activated via carboxylation, making it a vitamin K-dependent protein. In patients with chronic kidney disease, there is a vitamin K deficiency and MGP remains inactivated [54]. For this reason, vitamin K was investigated as a dietary supplement to activate circulating MGP and inhibit calcification.

In a randomized, controlled trial, male and female patients were given either a control or vitamin K supplement, and CT scans were used for analysis of calcification levels [61]. Blood samples were also taken and analyzed with a radioimmunoassay to determine MGP levels in the serum. While results showed that the supplement reduced the levels of calcification currently existing, it did not prevent the new formation of calcium deposits. MGP levels also showed no significant difference between the control and vitamin K group [61]. This shows that vitamin K could be used as a supplement to slow the progression of existing calcium deposits, though it has not been proven to prevent the formation of new calcification.

4.2.2. Protein therapy

Several naturally occurring proteins in the body act as inhibitors of calcification. MGP, as previously mentioned, is a naturally occurring inhibitor of calcification, requiring carboxylation to prevent osteoblastic-differentiation. Fetuin-A, also known as alpha-2-Heremans-Schmid glycoprotein, is another protein that acts by binding to free calcium and phosphate particles in the serum and preventing deposition [62]. In dialysis patients, fetuin levels in the body are significantly lower than in healthy patients, correlating with an increase in vascular

calcification [63]. Because of this correlation, fetuin has been considered as a potential therapeutic protein, as treatment for vascular calcification [4].

Osteopontin (OPN) is a protein associated with bone remodeling and resorption. When phosphorylated, it can easily bind with calcium ions to prevent calcification [49]. It resists calcification in a dose-dependent manner when supplemented to smooth muscle cells to protect their phenotype, and is currently being researched to determine its therapeutic abilities [49].

4.2.3. Drug-coated stents and balloon angioplasty

There are two main classifications of stents: bare metal stents and drug-eluting stents. The latter has been used to treat calcification and prevent restenosis by incorporating anti-proliferation and anti-inflammatory agents into the material of the stent [64]. However, in regions of high calcification, it is common for the stent to improperly deploy within the vessel, leading to further plaque build-up and implant failure [64].

In order to prevent improper placement of the stent and reduce adverse effects, drug-coated angioplasty balloons are often favorable to stents. Balloon angioplasty is a common treatment for calcification in the arteries, working as an immediate clearing of vessels to allow blood flow [65]. By modifying this design and using drug-coated balloons, obstructions in the vessels can be immediately broken up while also delivering various agents to prevent the return blockage without leaving a permanent implant behind [66]. Paclitaxel-coated balloons have been used because of the drug's ability to stop cell division so that when it is delivered to regions with increased plaque buildup, further growth is inhibited. The drug is delivered uniformly to the arterial wall with immediate release and incorporation into the tissue [67].

5. Assessment

5.1. Assessment of biomaterials calcification

Many different techniques are used to investigate and examine the calcification of biomaterials. This can be done with either morphologic or chemical techniques. Morphological testing yields important qualitative information like the detection, characterization, and distribution sites of calcific deposits as detailed below, but still lacks quantitative information. While chemical techniques reveal more qualitative data such as identification of elemental composition and determination of crystalline mineral phases, they require a complete ruination of the tissue specimen [68]. Furthermore, techniques such as microcomputer tomography (micro CT) are recent technologies available for both *in vitro* and *in vivo* samples that are non-invasive and non-destructive [68].

Morphological assessment of calcification uses many different techniques, including scanning electron microscopy (SEM), radiographs (X-rays), light microscopy, transmission electron

microscopy (TEM), and microcomputer tomography (micro CT) [68, 69]. Calcific deposit dispersal can be seen from X-rays, and most calcification is studied using morphological techniques done outside of the body once the implantation is removed. As mentioned previously, calcific deposit morphology, quantification, and localization can be seen from micro CT. Both X-ray and CT techniques require gross specimen sample preparation. Light microscopy is used in conjunction with various staining techniques to identify mineral deposits with either a calcium or phosphorus-specific stains. Alizarin red is a calcium-specific stain and von Kossa is a phosphate-specific stain [68]. Hematoxylin/eosin, Mallory's trichrome and alcian blue stains are known as histological stains associated with light microscopy, both readily available and easily applied to tissue [69].

Two types of microscope techniques mentioned previously, SEM and TEM, are electron microscopes that use a highly focused electron beam contained in a vacuum to passes the specimen [68]. In one study, SEM was used to analyze bovine pericardium samples in vitro for calcification using SEM. To prepare the samples for analysis, they were first soaked in a simulated body fluid containing ionic concentrations similar to natural body plasma fluid, then placed in a controlled environment. After seven days, samples were rinsed, deionized, and frozen in liquid nitrogen. Finally, samples had to be lyophilized before SEM analysis could be performed [70]. Other methods of calcification testing include Fourier transform infrared spectroscopy, which is used to determine structure coatings and x-ray diffraction of lyophilized samples using a diffractometer with Cu-Kα radiation [70].

6. Future works

There are many important factors to review when looking at heart valve replacements. Cost should be considered since valvular heart disease is prominent worldwide, especially in underdeveloped countries. Post-implantation failure is another factor, largely due to age of the patient given that children and young adults have a more competent immune system and experience a higher rate of BHV failure. In some countries lacking adequate ways of monitoring patients, mortality is an increased risk [9]. Calcification is also a major cause of deterioration in BHV replacements. The complications associated with calcification of artificial heart valves can lead to the need for revision surgery in patients. Mechanical valve replacements potentially require additional surgery due to thrombosis, thromboembolism, or spontaneous bleeding can occur; additionally, these replacements require lifelong anticoagulation therapies [9].

For these reasons, experts are trying to further understand the mechanism of biomaterial calcification and exploring more biocompatible materials. As mentioned before, there is not a specific mechanism that leads to VHD, so further understanding of the various processes involved will improve treatment strategies that include tissue engineering and drug-coated biomaterials [8]. Some studies have reported that tissue engineering scaffolds have similar uniaxial mechanical properties but need more investigation with biaxial mechanical properties that are more related to soft tissue. There are also clinical studies that combined both

synthetic and natural polymers to construct a scaffold that could be similar to the native mechanical properties of a heart valve which may improve their biocompatibility [7]. Another approach to prevent calcification is to modify the surface of the device; for example, heparin can be used to inhibit tissue calcification [69].

In addition, urinary stents and catheters need more attention to overcome the two main causes that lead to calcification: infection and encrustation. Currently, studies are focusing on innovating stent designs, biomaterials, and surface coatings [41]. Many studies have attempted to combine multiple antimicrobial agents into one coating, for example using several antibiotics. Another approach that most researchers have recently used is constructing urinary tissue from organ-specific stromal cells resulting in better biomechanical properties similar to human than non-specific stromal cells [71]. However, most biomaterials include natural collagens that are unable to maintain the same physical properties, resulting in graft failure [43]. Further investigation and clinical studies are needed to introduce the ideal biomaterials and coating [34].

In conclusion, further development will include better understanding of VHD to improve our treatment strategies. More trials and clinical studies are needed to create an "ideal" biomaterial for tissue engineering and drug-coated biomaterials. Additional experiments will be needed to test innovating stent designs, heart valves, and surface coatings to treat implantation calcification.

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