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Augmenting the articular cartilage-implant interface: functionalizing with a collagen adhesion protein

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Abstract

The lack of integration between implants and articular cartilage is an unsolved problem that negatively impacts the development of treatments for focal cartilage defects. Many approaches attempt to increase the number of matrix-producing cells that can migrate to the interface, which may help to reinforce the boundary over time but does not address the problems associated with an initially unstable interface. The objective of this study was to develop a bio-adhesive implant to create an immediate bond with the extracellular matrix components of articular cartilage. We hypothesized that implant-bound Collagen Adhesion protein, CNA, would increase the interfacial strength between a poly(vinyl alcohol), PVA, implant and articular cartilage immediately after implantation, without preventing cell migration into the implant. By way of a series of *in vitro* immunohistochemical and mechanical experiments, we demonstrated that: free CNA can bind to articular cartilage, implant-bound CNA can bind to collagen type II and that implants functionalized with CNA result in a four-fold increase in interfacial strength with cartilage relative to un-treated implants at day zero. Of note, the interfacial strength significantly decreased after 21 days in culture which may be an indication that the protein itself has lost its effectiveness. Our data suggests that functionalizing scaffolds with CNA may be a viable approach towards creating an initially stable interface between scaffolds and articular cartilage. Further efforts are required to ensure long-term interface stability.

Introduction

Full-thickness articular cartilage ('chondral') defects secondary to a traumatic event are common in young active patients, with sports participation as the most commonly associated activity [1], and can often cause symptoms such as pain, swelling, a 'clicking' sensation and joint instability. These defects have limited intrinsic healing capacity [2], and once initiated, the damage can spread and lead to osteoarthritis (OA) [3], a condition which affects as many as 27 million Americans with societal costs greater than US \$15 billion annually [4-5].

While many surgical approaches and implantable materials have been developed in an effort to repair or replace the damaged articular surface and delay OA progression, all have been limited by an inability to integrate with the native tissue. For example, microfracture, the most commonly used clinical procedure for the treatment of full-thickness defects, results in poor integration between the fibrocartilage tissue that fills the defect site and the surrounding host tissue hyaline cartilage [6-14]. Osteochondral autograft transfers is another

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technique developed to fill osteoarticular defects in weight bearing regions of the knee [15-16]. However, chondrocyte death at the margins of the autograft [17-20] and persistent gaps between the graft and the host tissue [21-24] have been shown to lead to poor graft durability over time [25]. In cases where implants are used to fill the defect, margin integration is frequently characterized by gaps and fissuring in histologic sections [26-32].

Efforts to create a mechanically stable interface between an implant and the host articular cartilage have explored the use of partial enzymatic digestion of the host tissue [33-34] and the release of chemotactic agents [35-37] to increase the number of matrix generating cells at the interface. Such approaches may help to reinforce the boundary between the scaffold and the host tissue as a function of time, but they do not address the problems associated with an initially unstable interface. Newer approaches rely on the use of an adhesive agent as an intermediary that chemically binds the native tissue to the implant in an attempt to immediately “glue” the scaffold to the surrounding native cartilage. One such example involves using a functionalized chondroitin sulphate paste to create a covalent bond between a biomaterial and proteins in articular cartilage [38, 39]. While the technology has been demonstrated to increase the interfacial strength and percent tissue fill in scaffold implanted cartilage defects [38, 39], the addition of yet another interface (that between the glue and the implant and the implant and the cartilage) is far from ideal. A linker molecule that could form a direct bond between articular cartilage an implant/ scaffold would avoid these problems.

Collagen N Adhesion (CNA), is a bacterial surface protein, which is used by *Staphylococcus aureus* to attach to monomeric collagen and contribute to virulence [40-45]. CNA is a well characterized protein with demonstrated affinity for collagen type I, collagen type II (which is a major constituent of the extracellular matrix of articular cartilage), and collagen-like peptides [41-43, 45]. The full length CNA protein contains an N-terminal signal sequence, an A region containing N1, N2 and N3 domains, B repeats, and a cell wall anchoring region. Biochemical assays in combination with *x*-ray crystallography suggest a mechanism of binding where the N2 domain initiates contact with the collagen triple helix; subsequently the linker and N1 domain wrap around the helix [45]. As such, if it were possible to attach CNA to the periphery of an implant, it should allow for that implant to be attached to the collagen within articular cartilage, thereby creating an initial bond between the two materials.

The objective of this study was to develop a bio-adhesive implant intended to create an immediate bond with the extra-cellular matrix components of articular cartilage through the action of CNA as a linker molecule. The implant of choice is a porous, non-degradable poly(vinyl alcohol) scaffold, PVA, previously demonstrated to allow for integration with articular cartilage through chondrocyte migration and matrix generation [46]. We hypothesized that CNA would increase the interfacial strength between a PVA implant and articular cartilage immediately after implantation, without preventing cell migration into the implant. To satisfy our objective and test our hypothesis, we explored the ability of (i) CNA to attach to articular cartilage, (ii) implant-bound CNA to bind to collagen type II, and (iii) implant-bound CNA to increase cartilage-implant interface strength at time zero as a function of time after implantation.

Methods

Expression, purification and labeling of recombinant CNA

The recombinant protein CNA35 was cloned into pQE30 expression plasmid and transformed into *E. coli* as previously described [45]. Expression and purification of recombinant CNA was performed as described in [47]. Briefly, bacterial lysates expressing

an N-terminal His-tag CNA were first purified by nickel chelate affinity chromatography using a 5-ml Hi-Trap chelating column in a fast protein liquid chromatography system (Amersham BioSciences, GE). Fractions, collected and analyzed by SDS-PAGE, containing protein of expected size were pooled and dialyzed in 25mM Tris-HCl, pH 8 and further purified in a 5ml Hi-Trap Q-Sepharose column (Amersham Biosciences, GE) using a linear gradient of 0 to 1M NaCl.

Ability of CNA to bind to articular cartilage

Recombinant CNA was labeled with fluorescent Texas-Red, as follows: CNA protein (2mg/ml) was first dialyzed in NaHCO₃ to 100mM final concentration (pH 8.2). The CNA protein (5 ml) was incubated with Texas Red dye (dissolved in DMF to 5μg/μl) for 1 hr at room temperature in shaker covered in aluminum foil. After the labeling reaction, the protein was dialyzed four times in 2L PBS buffer. Thin strips of articular cartilage approximately 1-2 mm thick (n=3) were cut from juvenile bovine knees, incubated with Texas-Red tagged CNA suspended in water at a concentration of 1mg/mL for one hour with agitation, and washed vigorously. Three samples were examined with fluorescence microscopy where CNA was detected using the fluorescent Texas Red tag. A further three articular cartilage samples were incubated with CNA (without a Texas-Red tag), suspended in water at a concentration of 1mg/mL for one hour with agitation, washed, fixed, paraffin embedded, and immunohistochemistry was performed using an antibody to the poly-histidine tag on the CNA (Alpha Diagnostic, TX).

Ability to activate the implants and functionalize them with CNA

Porous polyvinyl alcohol (PVA) scaffolds were manufactured as previously described [42]. In brief, rectangular collagen sponges were impregnated with 10% weight PVA in solution with deionized water and the construct was subjected to six freeze-thaw cycles over a period of five days. The impregnated sponges were cored into cylindrical geometries using a 5mm diameter biopsy punch, and sliced to the desired height to form cylinders. The cylindrical specimens were digested with a collagenase solution to remove the collagen sponge and result in an interconnected porous PVA scaffold [48].

The scaffolds were functionalized using a sequential process to result in reactive carbonate groups on the scaffold's surface, which can covalently bond with any amino group-containing side chain residue [49]. The procedure is summarized in Figure 1 and the various processing steps are labeled from 1 to 6. PVA implants were lyophilized for 24 hours to remove all water without collapsing the porous structure. They were exposed to vapor of zirconium tetra(*tert*-butoxide) (**1**, Figure 1) as previously described [43]. The reaction chamber was then heated to 45 °C and was held at this temperature for 10 minutes. The chamber was back-filled with zero grade nitrogen, and the implant samples were then agitated to expose any surface that had not yet been coated. The newly exposed surfaces were then treated further with vapor of zirconium tetra(*tert*-butoxide) from **1**, figure 1 [43]. The chamber was again heated to 45 °C, and was held at this temperature for 10 minutes, to result in **2**, Figure 1. The chamber was again back-filled with zero grade N₂, and the implants were quickly transferred into a 0.1 mM solution of 11-phosphonoundecanol (**3**, Figure 1) in ethanol. After 12 hours the implants were removed from this solution, sonicated in ethanol, and dried in vacuo to give the surface-bound hydroxyalkylphosphonate. *p*-Nitrophenyl chloroformate (80 mg, 0.4 mmol) was dissolved under argon in 60 mL dry tetrahydrofuran in a 100 mL three-necked round bottom flask equipped with a dry stir bar. The implants activated with **4**, Figure 1 were then submerged in this solution; dry diisopropylethylamine (0.75 mL, 4 mmol) was added, and the suspension was stirred for 45 min. The implants were removed from this suspension and washed briefly with ethanol. These steps resulted in an implant functionalized with an organophosphonate terminated

with carbonate groups that are free to react with any nucleophile in solution (herein called “open carbonates,” OC). The implants were then soaked in a solution of CNA in water (pH 8.5) for 24 hrs (5, Figure 1), BSA in water (6, Figure 1), or not soaked at all. All samples were removed from the solution and washed sequentially with water and ethanol. These steps resulted in the following groups:

- i. an organophosphonate terminated with CNA (CNA);
- ii. the same phosphonate terminated with Bovine Serum Albumin (BSA), used as a control for functionalizing with a protein;
- iii. the organophosphonate terminated with carbonate groups that are free to react with any nucleophile in solution (called “open carbonates,” OC); and,
- iv. untreated control (UC)

Three samples functionalized with Texas-Red labeled CNA were frozen sectioned and imaged using fluorescent microscopy to visualize their Texas Red tag.

Ability of CNA-bound proteins to bind to free-collagen

This step was designed to ensure that functionalization of the scaffold did not interfere with the ability of CNA to bind to collagen. Five scaffolds from each group were incubated for three hours with a solution of collagen type II isolated from bovine articular cartilage and labeled with FITC (Sigma, MO) at 0.25mg/mL. Each scaffold was washed with three centrifugation steps at 14000 rpm for 3min each. The samples were frozen sectioned and imaged using fluorescence microscopy. Scaffolds that were not incubated with the collagen were also imaged to ensure that there was no auto-fluorescence. Images were captured using a microscope with exposure time consistent between groups by a blinded observer.

Interfacial strength between modified scaffolds and cartilage at day 0 (4 hours after implantation)

Middle zone cartilage discs were isolated from juvenile bovine knees (10mm diameter, 2mm thick) and perforated at the center with a 3.5mm diameter biopsy punch. Porous PVA scaffolds with a 5mm diameter functionalized with CNA, BSA, OC and UC as described above (n=20 per group) were press-fit into the central hole in the cartilage explant and incubated for 4hrs. Samples were subjected to a push-out test using a stainless steel indenter which was advanced at 10 $\mu\text{m/s}$ [50]. The maximum load was recorded and normalized to the surface area of the interface for each sample to compute the maximum stress.

Interfacial strength between modified scaffolds and cartilage and biocompatibility at day 21

An additional 13 scaffold-cartilage constructs per group were cultured in 30 mL ADMEM/F12 with 100 nM dexamethasone, 50 $\mu\text{g/mL}$ ascorbate-2-phosphate, and antibiotics (Sigma Aldrich) for 21 days, after which they were subjected to a push-out test where the maximum load was recorded. The scaffolds were digested using proteinase K (Sigma, MO), and assessed for glycosaminoglycan (GAG) content using a dimethylmethylene blue assay (DMMB) and DNA content using a quant-iT kit Pico green assay (Invitrogen, CA).

Statistical Analysis

Statistical analyses were performed using Graphpad Prism software. To test for significance, a 2-way ANOVA analysis was conducted with a post-hoc Bonferroni post-test. The two independent variables used were time (0 vs 21 days) and treatment type (BSA, CNA, UC, OCH).

Results

Ability of CNA to bind to articular cartilage

After one hour of incubation in the presence of Texas-Red labeled CNA, the protein was detected on the edges of articular cartilage using fluorescent spectroscopy (Figure 2A). The non-tagged CNA was also detected on the surface of the articular cartilage using immunohistochemistry with anti-histidine antibodies (Figure 2B).

Ability to functionalize the implants

All PVA scaffolds that were functionalized with Texas-Red labeled CNA, were found to have fluorescent edges indicating that the process successfully bound CNA to the walls of the scaffold. (Figure 2C&D).

Ability of CNA-bound proteins to bind to free-collagen

The CNA-functionalized group could bind collagen type II as demonstrated by a qualitative detection of the FITC tag on the collagen via fluorescence microscopy, Figure 3. This result indicates that the processing steps to which the scaffold and CNA were subjected (Figure 1) did not interfere with the ability of CNA to bind collagen. Surprisingly, the OC group also demonstrated an ability to bind collagen type II ; while the UC and BSA groups did not bind collagen (Figure 3).

Interfacial strength between modified scaffolds and cartilage at day 0

The maximum interface strength was approximately three times higher for the CNA and OC groups than the BSA and UC groups, and these differences were statistically significant Figure 4. There was no significant difference between the push-out strength of the CNA and OC groups or between the BSA and UC groups.

Interfacial strength between modified scaffolds and cartilage and biocompatibility at day 21

The push out strength of the BSA and UC groups was significantly higher at day 21 vs. that at day 0. There was no significant change in the push-out strength of the OC group with incubation time; while there was a significant decrease in the push-out strength of the CNA group at day 21 (Figure 4). There were no significant differences in GAG (Figure 5A) content, DNA content (Figure 5B) after 21 days of incubation between the groups.

Discussion

In this study, we successfully developed a bio-adhesive implant capable of creating a bond with the extracellular matrix components of articular cartilage. We demonstrated that free CNA can bind to articular cartilage and that implant-bound CNA can bind collagen type II. Using these results as a platform, we tested and accepted the hypothesis that implant-bound CNA can increase cartilage-implant interface strength at time zero without adversely affecting the ability of cells to migrate into the scaffold. A surprising result was that the implants functionalized with the open carbonate groups (*OC*) – where the reactive sites remained free to interact with any protein/ molecule with which it comes in contact – also resulted in an ability to bind collagen type II and a corresponding increase in interfacial strength. The CNA-cartilage interfacial strength significantly decreased after 21 days in culture, relative to that of the time zero values. This may be an indication that the protein itself has lost its effectiveness. However, there was no significant difference in the number of cells that migrated into the scaffold, in the amount of new matrix deposited, or in the histological appearance of the samples as function of treatment, suggesting that the presence

of CNA does not have an adverse effect on chondrocyte migration. The finding that implant-bound CNA significantly increases the time-zero interfacial strength between an implant and articular cartilage, without adversely affecting cellular response, suggests that our concept is a viable approach towards creating an initially stable interface across which cells can migrate and lay down matrix. Further efforts are required to ensure long-term interface stability.

In a report of 35,516 arthroscopies, full-thickness articular cartilage defects occurred in a minimum of one of every 100 knee arthroscopies [51]. Laboratory investigations and preclinical animal studies have most recently focused on the use of biodegradable matrix scaffolds, alone and in combination with chondrogenic cells, in order to improve the quality of cartilage repair tissue after surgery [52]. Both natural and synthetic polymers have been fabricated for use as cell-seeded scaffolds, the chemistry and biology of which has taken a variety of forms, including fibrous structures, porous sponges, woven or non-woven meshes, and hydrogels [48, 52-55]. However, integration with the surrounding native cartilage remains a significant challenge that will negatively impact the translation of new technologies for cartilage repair into clinical use. Attempts to tackle the challenge of poor integration include implant surface modification techniques with the goal of inhibiting biofilm formation [56]. Other efforts have sought to improve scaffold biocompatibility by using cationized gelatin [57], RGDs [58], multiple peptides in combination (such as RGD, YIGSR, and IKVAV) [59], and extracellular matrix proteins with or without growth factors such as collagen II and Fibroblast Growth Factor [60]. While these modification strategies have shown interesting results, they are targeted at discouraging infection and encouraging cell migration into the implant to result in long term integration. However, the lack of integration in the immediate post-implantation period challenges not only initial implant stability, but the ability of cells to move across a mechanically unstable gap. Our concept attempts to address this shortfall.

CNA was chosen for this experiment because it is a well characterized protein with a known binding affinity to monomeric collagen. The full length CNA protein contains an N-terminal signal sequence, an A region containing N1, N2 and N3 domains, B repeats, and a cell wall anchoring region. The CNA (CNA35) minimal binding region demonstrates the highest affinity for collagen [43, 45] and was therefore used for this study. CNA35 constitutes the N1 and N2 domains separated by a linker sequence with each domain exhibiting an IgG-like fold. Biochemical assays in combination with x-ray crystallography suggest a mechanism of binding where the N2 domain initiates contact with the collagen triple helix and subsequently the linker and N1 domain wrap around the helix [45] using a so-called hug mechanism. While glycosaminoglycans as present within articular cartilage, might pose a steric hindrance to CNA binding of collagen, in theory, fibrillar collagen in freshly cut or damaged areas of cartilage should provide a site for the attachment of CNA. By using FITC-labeled collagen type II that was incubated with cut strips of articular cartilage, we determined that CNA could indeed bind to the frayed edges of the cartilage strips. This finding suggests that the cartilage defect edges should be freshly cut prior to scaffold implantation to enable CNA-collagen binding.

To explore the ability of CNA to create an immediate bond between articular cartilage and an implant, we chose to work with a previously developed poly(vinyl alcohol) scaffold, which was press-fit into a cartilage defect [46]. The degree of press-fit between the scaffold and the cartilage was chosen based on a previous study [48], in which macroporous PVA scaffolds of three different diameters (4.0, 5.0, 6.0 mm) were inserted into 3.5mm diameter cylindrical articular cartilage defects and cultured in vitro. Immediately after implantation (time zero), the 4.0 mm scaffold had the lowest initial interfacial strength and exhibited no changes in interface strength or cellularity over time; the largest scaffold diameter tested

(6mm) led to the highest initial interface strength, but at 46 days led to cell death and matrix loss in the surrounding cartilage and a lack of chondrocyte migration into the scaffold. The Ø5.0 mm scaffold had interfacial strength that was in-between that of the 4 and 6mm diameter scaffolds at time zero; but was reinforced with time due to the inward migration of chondrocytes leading to a 10-fold increase in interface strength at day 42. On the basis of these results, we chose to implant a 5mm diameter scaffold into a 3.5mm diameter defect.

The technique used to attach the CNA to the PVA scaffold relied on anchoring the protein to the implant using its N-terminus. Based on prior work, this type of anchoring was predicted not to interfere with CNAs ability to bind collagen [45], and this was supported by the fact that the tethered form of CNA successfully bound FITC-labeled collagen type II. When the CNA-tagged implants were placed in contact with articular cartilage, the resulting construct exhibited increased interfacial strength at day 0 in comparison to both BSA-coated and unmodified implants. A surprising result was that the “open” surface-bound carbonate groups (OC) - where the reactive sites remain free to interact with any amino group side chain-containing protein/ molecule with which it comes in contact- also resulted in an ability to bind collagen type II and a corresponding increase in interfacial strength. This result suggests that the OC groups may provide an alternative mechanism for tissue adherence.

The functional significance of the improvements in interfacial strength shown in the OC and CNA groups at time zero is difficult to interpret. On the one hand, the interfacial strengths measured are lower than that reported by Moretti et al. (2005) -71-161 kPa - and an order of magnitude below that reported by van de Breevaart Bravenboer et al., 2004, 1.32 ± 0.15 MPa. However in a relative sense, the CNA and OC processed scaffolds resulted in maximum interfacial strengths at time zero that were three-fold higher than that of the untreated controls; and significantly higher than that created by manipulating the degree of press fit of the PVA implant (1.4 ± 0.2 kPa for 6mm diameter un-functionalized scaffold [48] vs. 3.5 ± 1.5 kPa for 5mm diameter CNA functionalized scaffold). While such relative increases in interfacial strength are impressive, it is as yet unclear if they will result in any improvement in the functional mechanics of the interface. Indeed, deciding on the interfacial strength required to ensure a mechanically robust interface is complex. It is envisaged that immediately post-operatively the joint will be protected from loading (i.e. that the patient will be non- or minimally-weight bearing), and one might argue that maximizing the interfacial bond at time zero is not critical to the long-term behavior of the interface. However, it is our belief that even in the situation of minimal loading, without a connected and mechanically stable interface between the implant/scaffold and the adjacent articular cartilage at time zero, cells will never have the ability to cross the implant-tissue junction to result in a mechanically robust interface.

Of note, there were no significant differences in the number of cells that migrated into the PVA scaffold, in the amount of new matrix deposited, or in the histological appearance of the samples as function of treatment. This is an indication that although the groups have been functionalized in different ways, migrating chondrocytes do not appear to be negatively affected by these changes. The lack of substantial changes in the presence of CNA will allow us to introduce new factors that may support cell migration or matrix deposition and evaluate them with regard to their specific contribution.

Our study is not without limitations. While the proposed mechanism by which CNA acted to increase the interfacial strength is suggested as its ability to bind to collagen type II, to definitively prove that this is the case would require that we repeat our experiment with a negative control in which CNA is manipulated so that it lacks a collagen binding ability. Furthermore, while we have demonstrated that CNA can result in increased implant-

cartilage interface strength, we have not necessarily optimized the strength: for example, we do not know if all possible binding sites on the implant are quenched with CNA. Furthermore, while the increase in interfacial strength seen in the CNA and OC groups is statistically significant relative to the UC and BSA groups, it is unclear if such an increase will be sufficient to create an interface stable enough to allow for the inward migration of chondrocytes in the dynamically loaded environment of the knee joint (for example). Of note, the interfacial strength in the CNA group significantly decreased after 21 days in culture. This result may be an indication that CNA has lost its ability to bind monomeric collagen. Protein conformation can be drastically affected by pH or salinity change, and since the microenvironment between the implant and cartilage is rather unknown and may be ever changing, it is difficult to predict whether CNA may be affected. The decrease in strength may be due to simple degradation of CNA by proteases within the tissue or culture media. It is also possible that as the implant and cartilage become integrated, there may be conversion of monomeric collagens to fibrils which would lead to a lack of monomeric collagen available for binding and a subsequent decrease in interfacial strength.

In conclusion, we have demonstrated that the surface of a scaffold can be successfully modified to increase its initial interfacial strength with articular cartilage. We envisage that our approach can be used equally well with a range of materials that are intended to be used with and to bind to tissue in which collagen is present. Future studies will focus on understanding if an immunogenic response to implant-bound CNA occurs, on optimizing the strength of the interface, and on evaluate the technology over longer time points.

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Figure 1.

A representation of the series of dipping steps required to functionalize the scaffolds used in this study.

Figure 2.

A) Texas Red labeled CNA attaches to full thickness bovine articular cartilage (red fluorescence). B) Anti-histidine antibodies detect CNA with immunohistochemistry on bovine articular cartilage. C & D) Texas Red labeled CNA covalently bonded to the scaffold.

Figure 3. Fluorescence microscopy images of FITC-labeled collagen II incubated with scaffolds CNA, OC, BSA, and UC. The presence of collagen II is only detected on the CNA and OC scaffolds, indicating that those two scaffolds have the ability to bind collagen.

Figure 4.

A) Bovine articular cartilage discs were biopsy punched forming a central cylindrical 3.5mm defect. A 5mm diameter scaffold was press-fitted into the central defect. B) The interface strength was measured using a 2mm indenter advancing at a rate of 10um/s. C) The interface strength was significantly higher for the CNA and OC treated groups at day 0; but significantly reduced for the CAN functionalized samples at day 21.

Figure 5.

A) The GAG content was measured by DMMB assay and normalized to the wet weight of the scaffold. There were no significant differences between the groups. B) The DNA content was measured by a pico-green assay and normalized to the wet weight of the scaffold. There were no significant differences between the groups.