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Development and characterization of a rapid polymerizing collagen for soft tissue augmentation

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Abstract: A liquid collagen has been developed that fibrilizes upon injection. Rapid polymerizing collagen (RPC) is a type I porcine collagen that undergoes fibrillization upon interaction with ionic solutions, such as physiological solutions. The ability to inject liquid collagen would be beneficial for many soft tissue augmentation applications. In this study, RPC was synthesized and characterized as a possible dermal filler. Transmission electron microscopy, ion induced RPC fibrillogenesis tests, collagenase resistance assay, and injection force studies were performed to assess RPC's physicochemical properties. An *in vivo* study was performed which consisted of a 1-, 3-, and 6-month study where RPC was injected into the ears of miniature swine. The results demonstrated that the liquid RPC requires low injection force (<7 N); fibrillogenesis and banding of collagen occurs when RPC is injected into ionic solutions, and RPC has enhanced resistance to collagenase breakdown. The *in vivo* study demonstrated long-term biocompatibility with low irritation scores. In conclusion RPC possesses many of the desirable properties of a soft tissue augmentation material. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 758–767, 2016.

Key Words: collagen, in vivo studies, polymerization, characterization

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INTRODUCTION

Skin changes and wrinkles are some of the obvious signs of aging. The process of skin aging is influenced by many intrinsic and extrinsic factors.[1] Intrinsic factors include inherent factors such as genetics, cellular metabolism, and hormone environment. Extrinsic factors include exposure of the skin to chemicals, toxins, pollutants, UV, and ionizing radiation. Even though these two types of skin aging process are different, collagen losses occur in both while both are responsible for skin aging.[2]

Cosmetic anti-aging therapies, including over-the-counter cosmetic products, microdermabrasion, laser surgery, chemical peels, muscle relaxers, dermal fillers, and surgical facelifts are available to improve the skin's appearance.[3] Most of these products do not contribute to long term outcomes, that is, they do not restore the reduced dermis structure; they only achieve transitory or short-term effects. However, compared with the other treatments, injectable soft tissue fillers can offer a promising rejuvenation to an individual's appearance providing that the correct filler and application are utilized.[4]

For clarification, soft tissue fillers are equivalent to dermal fillers. These materials are a type of medical device for multiple cosmetic and therapeutic indications. Most usages of soft tissue fillers are to correct various facial folds and wrinkles. As Murray et al.[5] concluded an ideal soft tissue filler should meet three basic requirements: safety, effectiveness, and practicality. Safety requires that the filler cause no potential of carcinogenesis, infection, disease-causing, with a minimal foreign body reaction. Effectiveness of the fillers is that it contains long-term benefits, no migration, natural feeling, and reproducible results. Practical means that the products should be cost-effective, easy to use, removable, if needed, and have a long shelf life.[5] Unfortunately, there is no current ideal soft tissue filler on the market which can meet all of the desired characteristics.[6,7]

For a soft tissue restoration, collagen possesses ideal properties. It is naturally in the dermis to cushion and support the epidermis; it gives skin its fullness, texture, and strength; therefore it is an ideal construct to recapitulate skin. While collagen soft tissue fillers were first introduced in 1981 with Zyderm®I, by 2010 most, if not all, collagen soft tissue fillers were discontinued. The collagen products such as Zyderm®I and II, Zyplast®, Evolence®, CosmoDerm®I and II, and CosmoPlast® have been withdrawn and no longer available in the United States.[8–12] There appeared to

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be no single adverse event that resulted in the discontinuation, but rather a combination of events. Approximately 3% of the population is allergic to bovine collagen which could cause an autoimmune response. Therefore for patients who received bovine collagen, sensitivity testing was required. This entailed injection of the bovine collagen under the skin followed by 4 weeks of observation of any redness, swelling, itching, or pain. If there was a mild reaction, then a second skin test was usually performed. Most patients did not want to wait 4 to 8 weeks to use the product, and this lag time led to a decrease in demand for the bovine products. Another drawback of the collagen was its poor durability.[13] While intramolecular and/or inter molecular crosslinking of the collagen fibrils did extend the durability, it was rare for collagen to last >9 months aesthetically even though histologically remnants of the injectable collagen can be found after 9 months.[14] Evolence® alleviated some of the collagen concerns when it was introduced in 2008; its novel crosslinking chemistry extended the clinical benefits up to 12 months while the use of porcine collagen did not require sensitivity testing and thus did not elicit an autoimmune response in patients as demonstrated in their clinical trials. However, Johnson & Johnson discontinued its manufacturing in 2010. This may be due to the introduction of hyaluronic acids (HA) and other dermal filler that claimed longer lasting clinical effects.

HA is basically a type of glycosaminoglycan; it is a large hydrophilic molecule which forms long hydrated chain arrangement and attracts or maintains water within extracellular space.[5] The concentration of hyaluronic acid or hyaluronan in the human body is very low compared with the concentration of collagen. For example, the average concentration of HA in all tissues is 0.02% or 0.2 mg/g but the average concentration in skin is greater, about 0.8 mg/g; therefore, the estimated concentration of HA is skin is between 0.03 and 0.09%, significantly less than the skin collagen concentration, which is 80% collagen by weight.[15]

Hyaluronic acid (HA) dermal fillers have replaced collagen in the U.S. and are the most popular in the market today because of its reversibility, good performance and low risk profile. A unique virtue of HA is that their chemical structures are uniform in all living species, therefore, there is a minimal chance of immune reaction to HA-based soft tissue fillers. Hyaluronic acid derived dermal fillers[16] were first available in Europe in 1996 followed by U.S. approval in 2003 of the product, Restylane®. Current data states that their durability is approximately 6 months.[17] They satisfy most criteria of ideal soft tissue filler characteristics. Hence, they are considered gold-standard filler instead of collagen-based fillers.[4–7] However, the big drawback of HA fillers is that they are only volume fillers and they work by inflating the dermis.

While hyaluronic acid dermal fillers now dominate the dermal filler market, there are still adverse reactions and lack of clinical performance with these fillers.[7,18,19] Most current hyaluronic acid fillers are synthesized by fermentation of *Streptococcus equi*, therefore it is a non-animal origin which theoretically mitigates any possibility of hypersensitivity. Even though they are considered low immunogenic fillers, some significant uncommon adverse events have been recorded.[18] In addition, as the concentration of crosslinking increases to extend its durability, the gels become hard, resulting in higher extrusion forces, which can create more tissue trauma.[19] Also, these stiffer gels are not as biocompatible and can lead to a foreign body response that causes fibrous encapsulation thereby resulting in lumpiness at the injection site. Another concern is the Tyndall effect.[19] Superficial placing of hyaluronic acids can create bluish discoloration of the skin. Water absorption by the HA fillers can also create swelling, in particular under the eyes. Treatment of these adverse effects includes hyaluronidase, an enzyme which degrades HA.

Unlike collagen, there have been mixed results on whether hyaluronic acid induces cell proliferation or tissue regeneration. In one study, hyaluronic acid based dermal fillers were injected intradermally into a rat model.[20] While the study reported that HA stimulated the production of dermal collagen and elastin, it was unclear whether this was due to the biomechanical force stimulation from the bolus of viscous HA distorting the tissues.[21] Another study stated that HA does not provide a matrix or scaffold for cell migration and tissue host tissue integration.[16] For example, histologic examination of Restylane® implants have demonstrated little evidence of cellular infiltration into the HA.[16] In fact, histology images of HA gels demonstrate clumps of HA as individual islands while the proteolytic proteins degrade the HA. Hyaluronic acid remains as distinct boluses and not a structural constituent of skin. Therefore the mechanism of action of HA appears to be inflation of the dermal region by HA to bulk up or add volume; it does not appear to restore the natural extracellular matrix of the dermis. These boluses of HA are then degraded over time by the proteolytic enzymes and thus deflates the dermis, reintroducing wrinkles.

Collagen possesses many of the desired properties of a soft tissue filler. Its failure in the market was most likely due to the lack of duration, use of extensive and/or toxic crosslinkers, and/or difficulty in injecting the polymerized material. Our research has addressed these concerns by developing a liquid, noncrosslinked collagen called rapid polymerizing collagen (RPC) that fibrilizes upon injection. In this study, we investigated the physicochemical properties of RPC and its biocompatibility with a 1-, 3-, and 6-month implant study. It was predicated that RPC will meet many of the requirements of an injectable, soft tissue augmentation material.

MATERIALS AND METHODS

Materials

Type I porcine collagen powder (extracted from the hide) was purchased from Sewon Cellotech Inc. (Seoul, Korea). The following items were acquired from Fisher Scientific (Pittsburgh, PA): glacial acetic acid, ethylenediaminetetraacetic acid (EDTA), disodium salt dihydrate, deionized ultra-filtered water, glutaraldehyde, paraformaldehyde, and D-mannitol. Dialysis cassettes and Water for Injection were purchased from Thermo Fisher

TABLE I. Ionic Solutions Utilized to Determine Fibrillogenesis Times of RPC

Reagent	Concentration (*M*)	Average pH Before Fibrillogenesis	Average pH After Fibrillogenesis
KCl	0.03	7.09	7.20
NaCl	0.03	6.89	7.23
$CaCl_2$	0.02	7.04	3.92
$MgCl_2$	0.02	6.82	6.84
$ZnCl_2$	0.02	6.82	3.56
K_3PO_4	0.015	6.84	6.86
Na_2CO_3	0.02	7.19	8.26
Na_2SO_4	0.02	7.52	7.21
RO water		7.03	
HBSS		7.40	

Scientific (Waltham, MA). Sodium hydroxide solution (1*N* and 10*N*) was bought from Ricca Chemical Company (Arlington, TX).

Synthesis of RPC

Type I porcine collagen powder is solubilized in 0.2*M* acetic acid and subsequently diluted to prepare a 3 mg/mL collagen solution before filtration through a 0.22 μm filter. The resulting filtered collagen solution is precipitated while mixing at room temperature. Thereafter the precipitated material is collected by centrifugation and the pellets are transferred into dialysis cassettes with molecular weight cut-off (MWCO) 10,000 to target concentration of 30 mg/mL and solubilized in 0.5*M* acetic acid. Reproducibility and repeatability measurements were conducted to ensure that 30 mg/mL (±2 mg/mL) was consistently achieved. The RPC is resolubilized and systematically, in a stepwise manner, raised to neutral pH by dialysis in appropriately pH adjusted EDTA (∼35 m*M*) containing dialysis buffers. The purpose of the EDTA is to ensure stability of the resulting RPC and prevent spontaneous fibrillogenesis.

Ion induced RPC fibrillogenesis testing methods

An ion-induced RPC fibrillogenesis test was performed to assess the time-dependent ion-induced fibrillogenesis of RPC. The studies consisted of injecting ∼0.15 mL of RPC into various ionic solutions including Reverse Osmosis (RO) water, Hank's Balance Salt Solution (HBSS, composed of 1.25 m*M* $CaCl_2$, 5.37 m*M* KCl, 0.81 m*M* $MgSO_4$, 0.44 m*M* KH_2PO_4, 136.9 m*M* NaCl, Na_2HPO_4, 5.55 m*M* G-glucose, 4.15 m*M* $NaHCO_3$) and solutions containing physiologic cations including sodium and potassium. Table I displays the solutions, concentrations, and pH before and after fibrillogenesis of RPC. All solutions were heated to 37°C before RPC injection. The initial injection time was recorded as the start time; the onset time to fibrillogenesis was noted when the clear RPC started to become opaque; the final time was noted when RPC was completely opaque. The total time to fibrillogenesis was calculated as the difference from the injection time to the final time while the reaction time was calculated as the difference between the onset time and the final time.

A second set of studies was performed to determine the extent of fibrillogenesis upon exposure of RPC to physiological conditions. Domestic swine were euthanized following an exercise at the University of Missouri Medical School. Immediately after euthanization, 0.1 mL of RPC was injected into four different areas on the swine's ears. After 30 min and after 60 min, the RPC was harvested from the ear and placed in formalin. The extent of fibrillogenesis was determined using a transmission electron microscope (TEM; JEOL JEM-1400 TEM). The samples were prepared by fixation in 0.1*M* cacodylate buffer containing 2% glutaraldehyde and 2% paraformaldehyde. Following the fixative, the samples were sectioned for TEM. The RPC was analyzed at the center section of the injection as well as at the edge section of the injection to determine the extent of fibrillogenesis throughout the injected samples.

Collagenase resistance assay

A collagenase resistance assay was performed to determine if RPC had an enhance resistance to collagenase digestion compared with a control collagen sample. The RPC sample was prepared by adding 0.5 mL liquid RPC to 1 mL 0.9% NaCl solution (preheated to 37°C) and incubating for 2 h at 37°C. After 2 h, the NaCl solution was removed and the fibrilized samples were stored in RO water. The control sample was a fibrilized collagen sample prepared without dialysis and without the additional of EDTA. The control sample, which is referred to as the "pure collagen fibrils" was prepared by reacting 4 mL of Type I prefibrillized porcine collagen (purchased in 3 mg/mL quantities from Sunmax, Inc.) with 440 μL fibrillogenesis buffer as stated by the manufacturing protocol to ensure fibrillogenesis. After fibrillogenesis, the sample was washed and stored in RO water. TEM micrographs were obtained to confirm banding (data not shown). The digestion rate of the RPC to the control was determined by exposing the samples to collagenase. Equal masses of fibrilized RPC and porcine collagen fibrils were placed in 24-well plates and treated with 1 mL 200 U/mL bacterial collagenase (*Clostridium histolyticum*) in Hank's Balance Salt Solution (HBSS) and incubated at 37°C for 1 h, 3 h, and 5 h ($n = 3$). At the end of each time point, the liquid in the well was gathered and collagen concentration of the liquid was measured by hydroxyproline assay. The collagen loss resulting from collagenase digestion was calculated by the collagen concentration in the liquid solution of each test group.

Injection force study

The injectability of RPC was examined by an extrusion force study using an Instron 584 Universal Testing Machine (Norwood, MA). A 5 mL syringe was held by a custom-designed holder via its flange and the plunger was in contact with the compression platen. Vertical force was applied to the plunger by the platen to push the material through a 30-gauge needle. Force was measured over a constant rate of displacement. The syringes were run for 30 mm at a rate of 0.167 mm/sec with force measurements taken every 0.1 sec. Average force was acquired 20 sec poststart to 170 sec

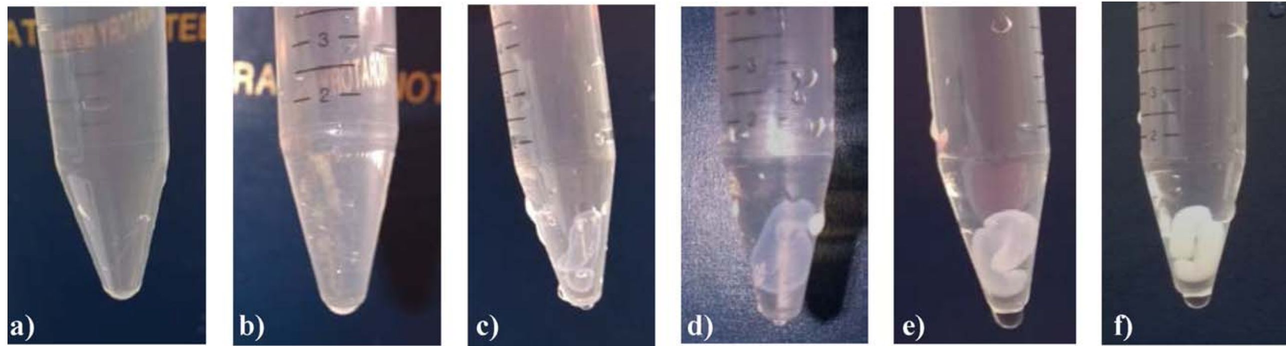

FIGURE 1. Visualization of RPC fibrillogenesis in HBSS; (a) within 30 sec of injection; (b) 5 min (onset time); (c) 10 min; (d) 15 min; (e) 20 min; (f) 25 min after injection (end time). (Important to note that the intact RPC bolus (gel) is evident at 10 min with surface opacity that gradually extends throughout the bolus by 25 min; the soluble collagen does not disperse following injection).

poststart in order to eliminate the spike in friction force necessary to initial the injection. The extrusion force of RPC was compared with several current dermal fillers. In addition, the extrusion force of RPC in commercial syringes were also tested. Since it is known that the injection force would increase with increase in collagen concentation, the RPC collagen concentration tested was consistently 30 mg/mL (±2 mg/mL).

In vivo swine study

The *in vivo* swine study evaluated the biocompatibility and local tissue reaction of RPC and control compositions that were injected into the subcutaneous space of Yucatan miniature pigs. The study was performed at Sinclair Research, (Auxvasse, MO) under an approved ACUC and under GLP conditions. Seven Yucatan miniature pigs were utilized. RPC, the test article, along with dermal filler controls (commercially available hyaluronic acid, Restylane™ (HA) and commercially available (in Europe) crosslinked porcine collagen, SunMax™ (Crosslinked Collagen)) were injected into the ear flaps at defined location (marked by dyes). Two minipigs were euthanized per time point ($n = 4$) with three euthanized at the 6-month time point ($n = 6$). After wiping with 70% isopropyl alcohol on the target dose site as labeled, the designated test or control articles were injected directly into subcutaneous space using a 25-gauge needle. Each site was injected with approximately 0.25 mL of designated material. At 1, 3, and 6 months, each time-point designated animal had the implant sites retrieved for histopathological examination.

The implants were collected and fixed in 10% neutral buffered formalin. The tissues were processed, embedded in paraffin, sectional and stained with H&E. A registered DVM, DACVP evaluated the slides according to 10993–6, 2007 Biological Evaluation of Medical Devices Part [6]: Tests for Local Effects After Implantation, which is commonly utilized for dermal fillers. The severity scale employed for the implant site evaluation was on a scale of 0–4 as follows: 0 = not present; 1 = minimal/slight—1% to 25% of the implant site is involved or 1–5 cells per high power field (400×); 2 = mild—26% to 50% of the implant site is involved or 5–10 cells per high power field (400×); 3 = moderate—51% to 75% of the implant site is involved or a heavy infiltrate of cells per high power field (400×); 4 = marked/severe—76% to 100% of the implant site is involved or the cells are packed per high power field (400×).

Total irritancy scores were calculated for each implant site for each animal via the following formula: [(sum of inflammation scores) × 2]. The components that were summed for the inflammation scores included mast cells, macrophages, eosinophils, lymphocytes, multinucleated giant cells, heterophils/neutrophils. The average irritancy scores were calculated for each implant material (control or test article) by averaging the total irritancy scores for each individual implant material. The ranked irritancy score for the test article group was applied an irritancy conclusion as determined to be a non-irritant, slight, moderate, or severe irritant by the following scale present in the ISO 10993 part 6 guidelines: nonirritant = 0.0 up to 2.9; slight irritant = 3.0 up to 8.9; moderate irritant = 9.0 up to 15.0; severe irritant = >15.

In addition, total tissue response scores were calculated for each implant site for each animal by summing the tissue response components. The components that were summed for the tissue response scores included neovascularization and granulation tissue. The average tissue response scores were calculated for each implant material (control or test article) by averaging the total tissue scores for each implant material.

RESULTS

Ion induced fibrillogenesis results

Figure 1 displays a visual representation of the start time, onset time, and final times for RPC in HBSS while Figure 2(a,b) provide graphs of the onset time/final time and the reaction times. Different solutions displays different onset and final fibrillogenesis times, but the reaction times were not significantly different (one-way ANOVA with Tukey multiple comparison; 95% confidence level). $CaCl_2$ and $ZnCl_2$ solution resulted in non-fibrillogenesis. The reaction appeared to be initiated, but then dissolved in the $CaCl_2$ and $ZnCl_2$ solutions.

The second part of the study involved injections of RPC into euthanized swine ears and determine their *in situ* fibrillogenesis.

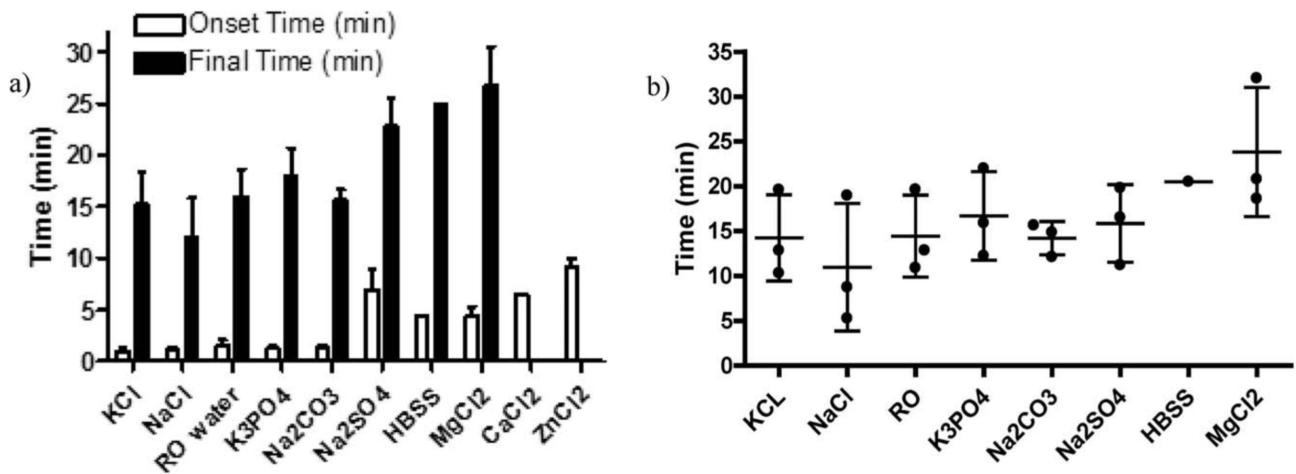

FIGURE 2. (a) Onset time and end time of the fibrillogenesis of RPC in different solutions; (b) reaction times of the solutions to achieve fibrillogenesis.

Figure 3(a,b) displays TEM images of a 30 min fibrillogenesis time at a center cut and edge cut respectively. Figure 3(c,d) are TEM images 60 min fibrillogenesis at a center and edge cut respectively. The 60 min edge cut demonstrated banding structures. The 60 min center cut also demonstrated banding; however, while the 30 min edge cut resulted in banding structures, the 30 min center cut demonstrated little if any banding structures of collagen.

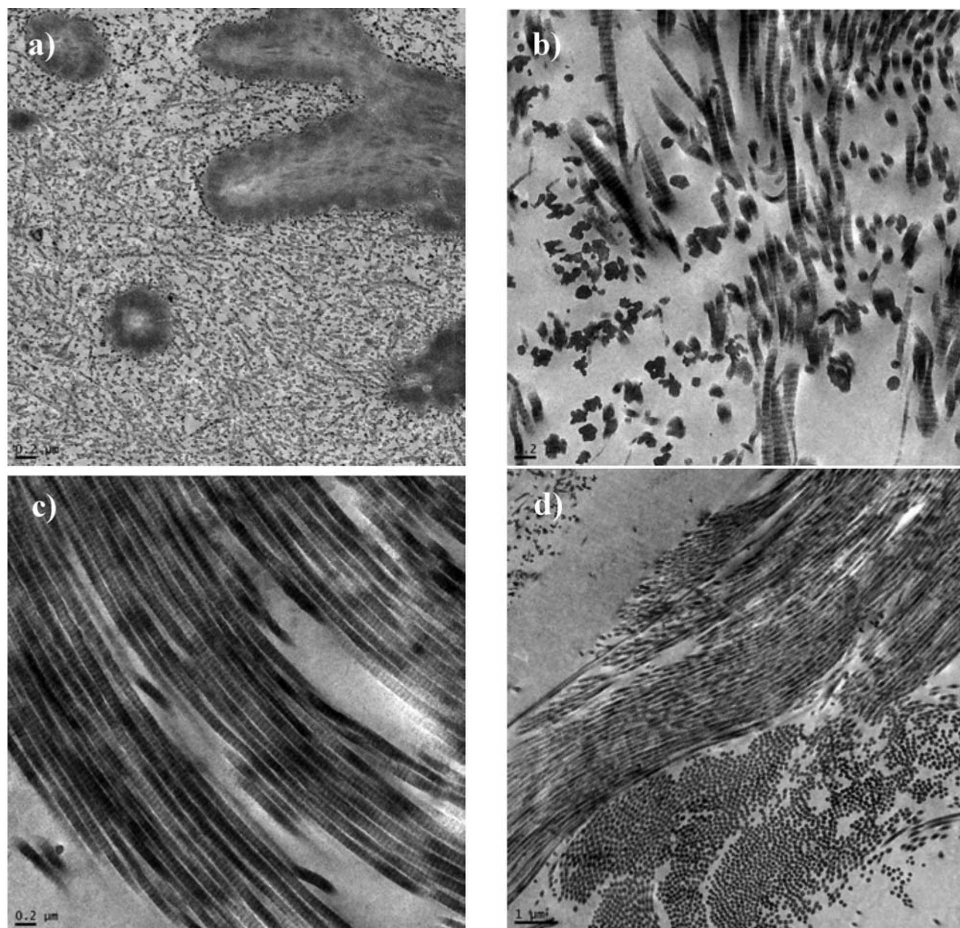

FIGURE 3. TEM images of (a) 30 min center cut; (b) 30 min edge cut; (c) 60 min center cut; (d) 60 min edge cut. Scale 0.2 μm for (a–c) and 1 μm for (d).

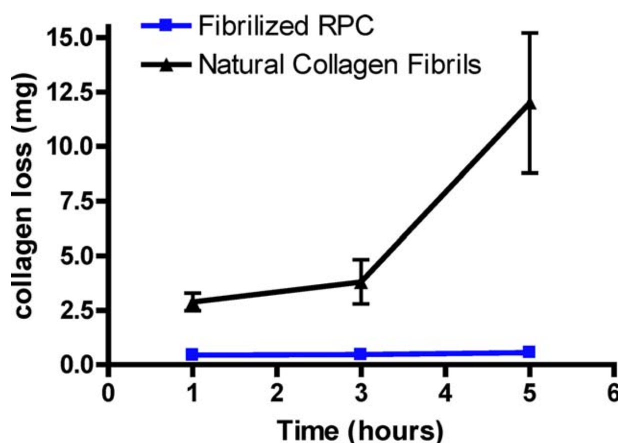

FIGURE 4. Collagen loss at time 1, 3, and 5 h.

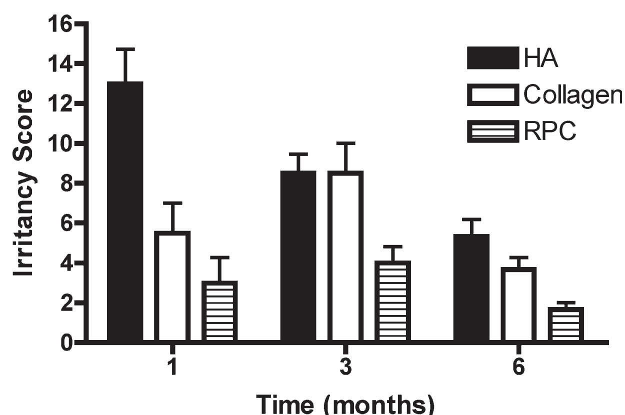

FIGURE 5. Total irritancy scores of the RPC and control articles at 1, 3, and 6 months.

Collagenase resistance assay

The collagen degradation rate of RPC fibrils was much lower than that for standard or natural collagen fibrils at three time points: 1 h, 3 h, and 5 h. As shown in Figure 4, RPC exhibited resistance to collagenase digestion compared with "natural" collagen fibrils. The natural collagen fibers had a 12.05% loss, 15.86% loss, and 50.12% loss after 1, 3, and 5 h, respectively. RPC displayed only a 1.87%, 1.94%, and 2.23% loss over 1, 3, and 5 h, respectively. One-way ANOVA with Tukey multiple comparison (95% confidence level) demonstrated significant difference ($p < 0.0001$) between the RPC and collagen fibrils at the 5-h time point ($n = 3$). The collagenase resistance of RPC is attributed to its EDTA shielding.

Injection force study

Results as shown in Table II indicate that the average injection force of RPC extruded through a 30-guage needle is approximately 7 N. This extrusion force is much lower than that of commercially available hyaluronic acids such as Perlane™, Restylane™, Juvederm Ultra,™ and Juvederm Ultra Plus™. SunMax™, a porcine Crosslinked Collagen product, displayed an extrusion force of 10 N. To account for any variability in syringe/needle designs, RPC was also extruded through some of the commercial product syringes. Average force results for RPC ranged from 5 N in a Juvederm Ultra syringe to 10 N in the Restylane syringe.

TABLE II. Average Extrusion Force for a Number of Injectable Collagen and Hyaluronic Acid Products Through a 30-Gauge Needle

Sample	Extrusion Force (N)	Extrusion Force (N) of RPC Through Commercial Needles
RPC	7	
SunMax™ porcine collagen	10	
Restylane™	14	6
Perlane™ (Restylane)	34	10
Juvederm Ultra™	18	5
Juvederm Ultra Plus™	21	

In vivo swine study

Figure 5 displays the ranked irritancy scores for RPC, cross-linked collagen, and hyaluronic acid (HA) at the 1-, 3-, and 6-month time points. At the one month time point, there is a significant difference between HA and Crosslinked Collagen ($p < 0.01$) as well as HA and RPC ($p < 0.001$). HA is considered to be a moderate irritant while Crosslinked Collagen and RPC, a slight irritant. At 3 months, HA and Crosslinked Collagen are considered to be a moderate irritant while RPC, a slight irritant. There were no significant differences in irritancy scores at the 3-month time point. At the 6-month time point, only RPC is considered to be a non-irritant while Crosslinked Collagen and HA, a slight irritant. However, there were no significant differences in irritancy score at the 6-month time point between the controls and test articles.

Figure 6 displays the ranked tissue response scores for RPC, crosslinked collagen, and HA at the 1-, 3-, and 6-month time points. The first month time point resulted in no significant differences in tissue response. Neither test articles nor controls demonstrated any adverse tissue effects such as

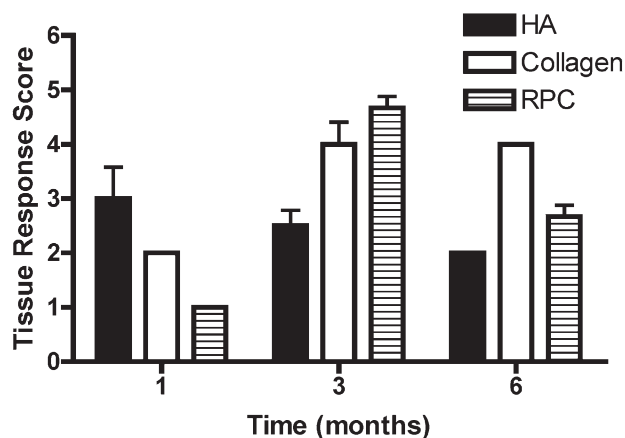

FIGURE 6. Total tissue response scores of the RPC and control articles at 1, 3, and 6 months.

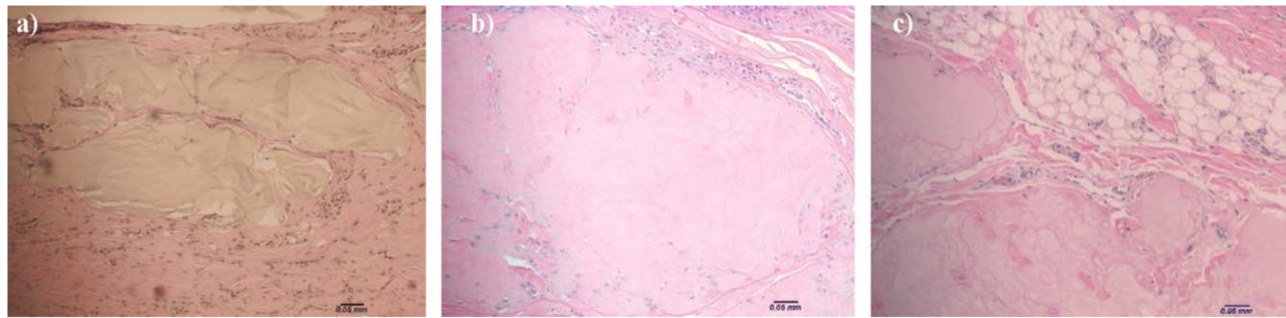

FIGURE 7. H&E slides of (a) HA; (b) Crosslinked Collagen; (c) RPC at 1 month implantation. ×10 (scale bar 50 μm).

mineralization or necrosis. At three months, again the samples demonstrated no adverse tissue effects. However, there is a significant tissue in-growth response between HA and Crosslinked Collagen ($p < 0.01$) and between HA and RPC ($p < 0.01$) with no significant differences between RPC and Crosslinked Collagen. At 6-month, the samples displayed no adverse effects. There is a significant difference between HA and Crosslinked Collagen ($p < 0.001$) and between HA and RPC ($p < 0.05$) with no significant differences between RPC and crosslinked collagen.

More detailed information on the inflammation and tissue response is demonstrated in the H&E slides as shown in Figure 7 of the 1 month HA, crosslinked collagen, and RPC samples. The histopathological examination of RPC at 1 month demonstrated few if any eosinophils, mast cells, lymphocytes, plasma cells, macrophages, and multinucleated giant cells (scores 0–1) while the HA demonstrated moderate inflammation scores from 0 to 3. Crosslinked Collagen demonstrated mild inflammation scores from 0 to 2. Tissue in-growth was observed in the RPC, HA, and Crosslinked Collagen constructs along with neovascularization and granulation tissue. Importantly, there was no fibrosis evident with RPC while HA and collagen scored in the 1–2 and 0–1 range, respectively.

Figure 8 details the H&E slides of HA, Crosslinked Collagen, and RPC at 3 months. At this time point, again RPC demonstrated few if any eosinophils, mast cells, lymphocytes, and plasma cells; there was a mild response in the number of macrophages (scored 0–2). HA demonstrated inflammation scores of 0 to 2, slightly lower average than at 1 month while Crosslinked Collagen demonstrated moderate inflammation scores from 0 to 3 with an increase in lymphocytes noted. There was a mild response in the number of blood vessels and granulation tissue for RPC while Crosslinked Collagen and HA were moderate and minimal, respectively. Tissue ingrowth into the constructs was noted. There was also very mild fibrosis response for RPC and HA with Crosslinked Collagen demonstrating a minimal fibrosis response.

Figure 9 details the H&E slides of HA, Crosslinked Collagen, and RPC at 6 months. At this time point, RPC demonstrated no presence of mast cells, lymphocytes, plasma cells, macrophages, and multinucleated giant cells. There was only a mild response noted with the presence of eosinophils. HA demonstrated a mild response in the number of eosinophils, lymphocytes, multinucleated giant cells, and a moderate response with macrophages. Crosslinked Collagen demonstrated to mast cells, plasma cells, and multinucleated giant cells, and a mild response to eosinophils, and lymphocytes. A moderate response was noted with macrophages. There was a mild response in the number of blood vessels and granulation tissue for RPC; a minimal response for HA, and a moderate response for crosslinked collagen. Tissue ingrowth into all the constructs was noted. There was also very mild fibrosis response for all the constructs.

DISCUSSION

The research described characterized a novel liquid collagen that fibrilizes *in situ*. *In situ* polymerized materials have been

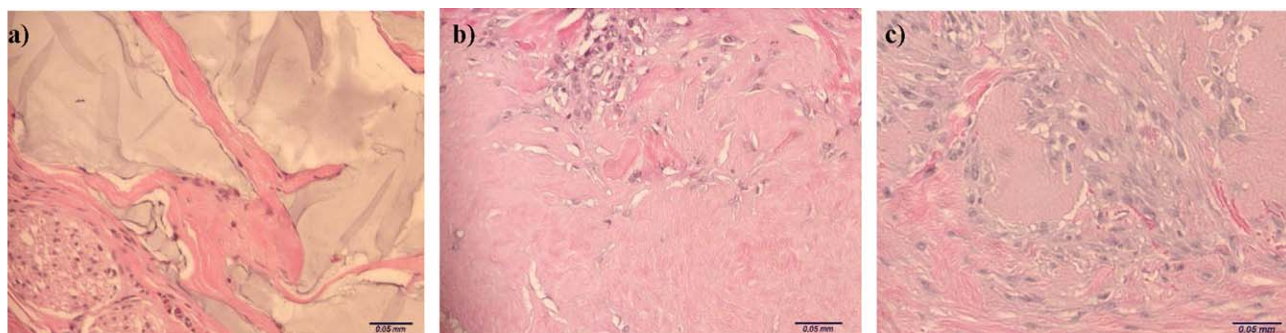

FIGURE 8. H&E slides of (a) HA; (b) Crosslinked Collagen; (c) RPC at 3 months implantation. ×20 (scale bar 50 μm).

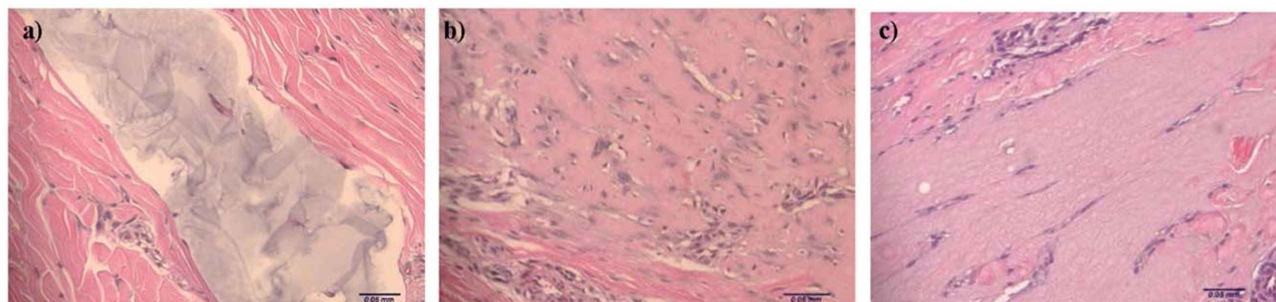

FIGURE 9. H&E slides of (a) HA; (b) Crosslinked Collagen; (c) RPC at 6 months implantation. ×20 (scale bar 50 μm).

gaining popularity due to the innate benefits. Implantation can be minimally invasive and easier to deploy in difficult areas; the *in situ* polymerization can conform and adhere to the tissue defect with good alignment; an added benefit is the capability of encapsulating cells are/or bioactive molecules with high survivability.[22] The applications for such in-situ forming biomaterials are broad from the treatment of damaged articular cartilage, tendon/ligament repair to dermal augmentation.[23,24]

While there are many benefits of injectable constructs, the development of *in situ* forming biomaterials is challenging. There have been many strategies utilized to design *in situ* forming biomaterials. The most popular *in situ* forming biomaterial utilizes synthetic hydrophilic polymer networks. These are typically polymerized *in situ* via chemical crosslinking or physical crosslinking. Chemical crosslinking involves radical polymerization, which is one of the most commonly used techniques, as well as enzymatic crosslinking, and peptide ligation.[22] However, concerns about the toxicity of the crosslinking reagent, initiators, and/or byproducts from the reactions has also led to the use of physical crosslinkers. Physical crosslinkers involved the use of hydrophobic interactions, ionic interactions, and stereocomplexation. Unfortunately, physical crosslinking *in situ* can result in mechanical weak and unstable structures. Temperature, ionic strength and pH of the surrounding tissue can lead to dissolution of the gel network.[22]

While synthetic hydrophilic polymer networks are popular, hybrid *in situ* forming hydrogels composed of both natural and synthetic materials are also being developed.[25–28] The advantage of a hybrid design is the use of biologically relevant material (such as collagen or hyaluronic acid) combined with a synthetic polymer to allow tunable properties. In one study, a collagen-PEG hydrogel was developed for *in situ* polymerization. Porcine collagen Type I and multi-armed PEG with reactive succinimidyl esters for the crosslinking chemistry were developed and characterized. It was determined that a range of physiomechanical and biochemical properties could be achieved. A drawback to the hybrid designs is the use of crosslinkers or reactive chemistry to initiate the polymerization.

In vitro fibrillogenesis of collagen from solution was discovered >50 years ago and its mechanism of fibrillogenesis has been examined as a model of formation of native collagenous tissues during development.[29–31] However, we are one of the first groups to develop and characterize an injectable liquid collagen where fibrillogenesis can occur *in vivo* with no cytotoxic effects. A variety of different ionic solutions were investigated to assess the time-dependent ion-induced fibrillogenesis of RPC. The time for fibrillogenesis is a critical parameter that requires controllability and predictability in order to develop injectable constructs. Our Rapidly Polymerizing Collagen (RPC) is a concentrated solution of triple helical collagen molecules at neutral pH. The presence of EDTA may be surrounding the triple helical structures or there may be ionic interactions at several different regions of collagen molecules and subsequent collagen fibrils, which help inhibit the *in vitro* fibrillogenesis. In this prefibrilized state, RPC appears clear and transparent in solution, making it ideal for optimal extrudability resulting in patient comfort and physician application control. Fibrillogenesis occurs due to the interaction of ions in physiological tissue fluids, which may be displacing the EDTA and/or ions; that is, mitigating the shielding effects, allowing fibrillogenesis to occur. As shown in Figure 1, a clear gel then transforms into an opaque, off white color, and a rigid consistency gel comprised of fully formed collagen fibrils is formed. In our study, as shown in Figure 2(a), there were differences in the onset time and final time of fibrillogenesis for the different solutions. However, determination of the reaction times for each of the solutions results in no significant difference; that is, once the reaction was initiated, the time to complete fibrillogenesis did not significantly varied between solutions. RPC fibrilized in all of the ionic solutions examined except for the $CaCl_2$ and $ZnCl_2$ solutions which resulted in non-fibrillogenesis. It appeared that the reactions were initiated but then the RPC was re-dissolved. This phenomena most likely was due to the EDTA chelating the Ca^{2+} or Zn^{2+} ions, which dropped the pH of the solution (due to the free H^+ ions). As shown in Table I, the pH in these solutions drop to under 4, which would prevent fibrillogenesis.

TEM was utilized to determine if fibrillogenesis, that is, formation of banded fibrils, occurred after injecting into a swine's ear. As shown in Figure 3(c,d), a 60 min incubation time in the euthanized swine model was sufficient to form banded fibrils with a repeat period range from 53 to 66 nm. At 30 min incubation [Fig. 3(b)], the edges of the injected bolus indicate on-going fibrillogenesis with blurred

banding structure, but it is still growing while the central portion of RPC [Fig. 3(a)] is mostly in its non-fibril state. While the results appear to demonstrate that RPC may need at least 30 min to fully fibrilize *in vivo*, other factors such as temperature need to be taken into account. For example, the injections occurred after euthanasia of the swine, hence the temperature of the ear would not have been at 37°C by the time the 60 min and even the 30 min samples were removed. While fibrillogenesis time is an important parameter in determining the stability of the construct, many of the research articles on injectable *in situ* polymerized materials do not address this important parameter.[22–28,32]

Another important parameter of injectable tissue engineered constructs is the stability. Collagenase assays were performed to determine the stability or predicted longevity of RPC. While EDTA was included to prevent spontaneous fibrillogenesis of the RPC, it was also predicted that the presence of EDTA, associated with the triple helical structures, would result in enzymatic shielding. EDTA has long been shown to inhibit collagenase activity in an early study by Bar-Shavit et al.[33] In another study, it was stated that the metal-binding agents, EDTA and Ca-EDTA, are efficient inhibitors of collagenase and have been implemented for use in preventing corneal ulceration.[34] Our collagenase study correlated with these previous studies; however, collagenolytic activity of the enzyme used in this study has a very different mechanism than the mammalian collagenases that are present in humans. As shown in Figure 4, the loss of collagen caused by collagenase degradation in RPC was lower than natural porcine collagen fibrils at the 1 h and 3 h time points. At the 5 h time point, RPC demonstrated a significant difference ($p < 0.0001$) between the natural collagen fibrils. The *in vitro* results indicated that fibrilized RPC was resistant to collagenase digestion and EDTA may help with shielding from enzymatic degradation, but *in vivo* performance studies will be needed to ascertain the abilities of EDTA to shield mammalian enzymes. This important property would allow a longer-lasting construct thereby allowing recapitulation of tissue.

By nature, injectable materials should have less tissue trauma due to the small bore needles used to inject the material.[35,36] However, if the extrusion force is high, then the patient is at risk for additional trauma due to the added force exerted by the physician. Examining of extrusion force of RPC compared with other commercially available products demonstrated a lower extrusion force for RPC. Additionally, when RPC was injected through the commercial needle/syringe systems, RPC demonstrated lower extrusion forces than the commercial products. RPC in its prefibrilized state is very easy to inject which would translate into low risk of tissue trauma upon injection. In addition, the ease of injection would allow the physician to be more selective and specific with the location and volume of injection thus leading to preferred results.

In vivo animal studies were performed to assess the biocompatibility of the injected collagen. Since there are no toxic by-products from fibrillogenesis or any crosslinking reactions, it was hypothesized that RPC would be a nonirritant. As shown in Figure 5 and in the histology images [Figs. 7(c), 8(c), and 9(c)] RPC demonstrated little irritation and by 6 months was considered a nonirritant. Conversely, both commercial products, HA and Crosslinked Collagen, were noted as a slight irritant at the 6-month time point. Additionally, tissue in-growth was observed in the RPC constructs along with neovascularization and granulation tissue. At the 6-month time point, the RPC implant material blended somewhat imperceptibly with native, pre-existent collagen and accurate reaction zone measurements of the implant and associated host tissue response could not be made as shown in Figure 9(c). The Crosslinked Collagen also demonstrated tissue in-growth and vascularization with no adverse tissue effects. However, there was a significant difference between HA and the commercial Crosslinked Collagen product as well as between HA and RPC in that the Crosslinked Collagen and RPC demonstrated significantly higher vascularization than the HA. While HA appeared to have some tissue in-growth and vascularization, this growth did not appear within the HA gel; it appeared surrounding the HA as shown in Figures 7(a), 8(a), and 9(a).

The lack of true tissue integration for HA may be due to the fact that *in vivo* stimulation of neocollagenesis is due to mechanical stimulation cause by active stretching of the dermis upon injection of the bolus of HA and not due to the scaffold properties of HA. In a study by Wang et al.,[21] it was concluded that HA stimulated the activation of dermal fibroblasts in human subject resulting in collagen synthesis. In our study, we injected $\sim$0.25 mL, which is much less than a typical 1 to 2 mL injection for humans. It is very possible that we did not stimulate stretching of the fibroblast cells which would induce neocollagenesis in the HA. However, our collagen injections did provide a construct with an open micro structure for cellular attachment and proliferation, and thus led to enhanced tissue response.

CONCLUSIONS

An easy-to-inject liquid collagen that fibrilizes upon interaction with ions has been developed and characterized. Ion induced RPC fibrillogenesis tests along with TEM demonstrated the ability of liquid RPC to undergo fibrillogenesis in different ionic solutions and *in vivo,* resulting in strong organization and banding of the collagen. It was concluded that the presence of EDTA used in RPC may be surrounding the triple helical structures and/or there may be ionic interactions at several different regions of collagen molecules and subsequent collagen fibrils, which would help inhibit the *in vitro* fibrillogenesis contributing to the long-term, liquid state. Fibrillogenesis then occurs due to the interaction of ions in physiological tissue fluids, which may displace some of the EDTA and/or ions; allowing fibrillogenesis to occur, but also continuing to provide shielding effects for long-term stability. The swine *in vivo* study demonstrated long-term biocompatibility with low irritation scores. In conclusion, RPC, a natural collagen scaffold with open microstructure, possesses many of the desirable properties of a soft tissue augmentation material.

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