(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 May 2007 (03.05.2007)

PCT

(10) International Publication Number
WO 2007/050387 A2

(51) International Patent Classification:
A61K 9/14 (2006.01)

(21) International Application Number:
PCT/US2006/040673

(22) International Filing Date: 18 October 2006 (18.10.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/728,971 21 October 2005 (21.10.2005) US
11/549,748 16 October 2006 (16.10.2006) US

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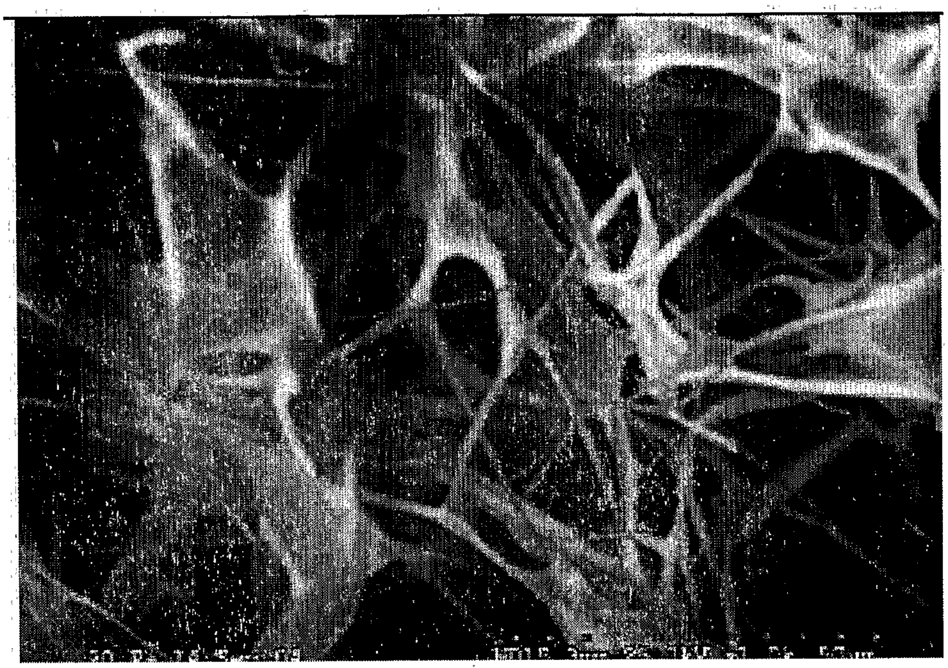
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: KERATIN BIOCERAMIC COMPOSITIONS



(57) Abstract: A malleable bone graft composition is described. The composition comprises: *(a)* keratose; *(b)* particulate filler;*(c)* antibiotic; and *(d)* water. The invention may be provided in sterile form in an container, and optionally lyophilized. Methods of treating a fracture with such compositions are also described.

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KERATIN BIOCERAMIC COMPOSITIONS

Mark E. Van Dyke

Related Applications

This application claims the benefit of United States Provisional Patent Application Serial No. 60/728,971, filed October 21, 2005, the disclosure of which is incorporated by reference herein in its entirety.

Field of the Invention

The present invention concerns bioceramic bone graft compositions and methods of using the same.

Background of the Invention

Bone loss following bone fracture is a significant problem. Indeed, bone loss in combination with infection can lead to chronic osteomyelitis and non-union. Since the first report of incorporating antibiotics into bone cement in 1970, many hospitals have adopted the practice for prophylactic treatment during arthroplasty, as well as for arrest of chronic infections (McQueen M et al., *Int Ortho* 1987; 11 :241-3; Fish DN et al., *Am J Hosp Pharm* 1992; 49: 2469-74; Hanssen AD and Osmon DR. *Clin Ortho Rel Res* 1999; 369(1): 124-38; Hanssen AD. J Arthroplas 2002; 17(4S1) :98-101).

The use of antibiotic-impregnated poly(methylmethacrylate) (PMMA) beads has also become widespread (Henry SL et al., *Ortho Rev* 1991; 20(3): 242-7; Popham GJ et al., *Ortho Rev* 1991; 20(4): 331-7; Klemm KW. *Clin Ortho Rel Res* 1993; 295: 63-76), although in the case of infected non-unions or bony defects, this technology is typically used in two stage operations. In these cases, the defect or non-union site is debrided as needed and the infection treated by placement of antibiotic-impregnated PMMA beads into the defect site. In the second stage, the beads are removed approximately six weeks later and a graft is used to repair the bone defect. The graft can be animal derived, allogenic, or autologous, such as COLLAGRAFT® bone graft matrix, demineralized bone matrix, or bone from the iliac crest, respectively. This two-stage methodology has also been widely adopted (Ueng SWN et al., *J Trauma* 1996; 40(3): 345-50; Ueng SWN et al., J Trauma 1997;43(2):268-74; Chen CY et al., *J Trauma* 1997; 43(5): 793-8; Swiontkowski MF et al., *J Bone Joint Surg Br* 1999; 81(B6):1 046-50).

In a logical progression of the technology, the two-stage method was soon followed by a one-stage procedure. In this technique, antibiotics are combined with the bone graft material in order to limit the intervention to a single surgery. Ideally, the resident antibiotic provides local delivery for prophylactic treatment of infection while the graft provides the environment to grow new bone. This methodology has also been widely adopted and used both with human autologous and bovine grafts as well as synthetic grafts (Chan YS et al., *J Trauma* 1998; 45(4): 758-64; Chan YS et al., *J Trauma: Inj Inf Crit Care* 2000; 48(2): 246-55; Winkler H et al., *J Antimicrobial Chemo* 2000; 46: 423-8; Sasaki S and Ishii Y., *J Ortho Sci* 1999; 4: 361-9; McKee MD et al., *J Ortho Trauma* 2002; 16(9): 622-7).

These approaches are not without their limitations. For example, the typical protocol for impregnation of antibiotic into PMMA is to heat the polymer to form a melt, then to mix powdered antibiotic into the liquid. The antibiotic must be heat stable to withstand the PMMA melt temperatures, which is often not the case so the number of potential antibiotics is limited. In addition, powdered antibiotic and liquid PMMA are not thermodynamically miscible; therefore the mixture is typically not homogeneous. This leads to uneven release of the antibiotic. Finally, the two-stage protocol subjects the patient to two surgeries and thereby, increased risk.

Similar limitations exist when impregnating graft materials with antibiotic. Typical graft materials are donor bone, demineralized bone matrix, or synthetic ceramic substitutes (e.g. hydroxyapatite), among others. These biomaterials are often not compatible with the antibiotic, and the resulting composite is non-homogeneous. Whether one employs impregnation into PMMA or a graft material, these methods, although clinically effective to some degree, are not controlled release systems and are by no means optimized for therapeutic dosing of antibiotics. They are osteoconductive, and in the case of autologous bone are certainly osteoinductive, but any approach that uses autologous bone subjects the patient to another wound. This increases risk to the patient and can lead to donor site morbidity, thereby compounding the original problem (Silber, JS et al., *Spine* 2003; 28(2): 134-9).

Obviously, healing bone defects is a challenging area of orthopaedic medicine. Current methods are not optimized for complete patient benefit. Ideally, bony defects should be healed with a graft material that provides both an osteoconductive and osteoinductive environment, and controlled, effective antibiotic treatment in a biomaterial that can be utilized in a single-stage operational protocol.

A recent review (Ludwig, SC et al., *Eur Spine J* 2000; 9(S1): S119-25) on the subject of bone graft substitutes listed the three most important elements of the ideal product as:

1. Osteoconductive in that it provides a scaffold conducive to vascular invasion, cell infiltration, and new bone formation;

2. Osteoinductive (i.e. capable of growth factor mediated differentiation of precursor cells into osteoblasts); and

3. Capable of delivering cells that will form new bone matrix.

Any effective regeneration scheme must seek to optimize all three of these parameters in order to recapitulate functional bone. Accordingly, there is a continuing need for new compositions useful as bone graft materials.

Summary of the Invention

A first aspect of the invention is a malleable bone graft composition, comprising, consisting of or consisting essentially of:

(a) from 1 to 90 percent by weight keratose;

(b) from 1 to 90 percent by weight particulate filler (*e.g.*, an osteoconductive filler);

(c) from 0.001 to 5 percent by weight antibiotic; and

(d) water to balance;

the composition having a viscosity of at least 3 centipoise at a temperature of 37°C.

in some embodiments the keratose is alpha keratose, gamma keratose, or mixtures thereof; in some embodiments the keratose is a mixture of alpha keratose and gamma keratose; in some embodiments the keratose comprises from 10 to 90 percent by weight alpha keratose and from 90 to 10 percent by weight gamma keratose; in some embodiments the said keratose is crosslinked keratose (*e.g.*, produced by the process of combining the keratose with transglutaminase in the presence of a calcium initiator).

In some embodiments the composition further comprises from 0.001 to 5 percent by weight bone morphogenic protein.

In some embodiments the composition is sterile, and in some embodiments the composition is packaged in a sterile container.

A further aspect of the invention lyophilized or freeze-dried composition which upon reconstitution with water or saline solution produces a composition as described herein.

The foregoing and other objects and aspects of the present invention are explained in greater detail in the drawings herein and the specification set forth below. The disclosures of all United States patent references cited herein are to be incorporated by reference herein in their entirety.

Brief Description of the Drawings

Figure 1. Keratin biomaterial scaffold (a) was formed spontaneously by a self-assembly mechanism. Notice the fibrous architecture, high porosity, and homogeneity similar to that of native ECM (b; bladder submucosa ECM shown). This is in dramatic contrast to synthetic scaffolds that claim to have "interconnected" pores (c). These types of synthetic scaffolds are difficult to seed at best and must rely on degradation of the matrix for tissue infiltration. This results in slower, less complete healing.

Figure 2. Viscosity curves for α-keratose (bottom curve) and α-SCMK (top curve). Samples were formulated at 5 weight percent and below in RL solution to provide high porosity. Low solids content hydrogels are desired to provide biocompatibility and to accommodate the in growth of osteoblasts.

Figure 3. SEM micrographs of keratose formulations (as defined in Table 1). Samples were created from lyophilized solutions in order to investigate the underlying microstructure of the hydrogels. Those gels showing the most fibrous microstructure also demonstrated the greatest increase in apparent viscosity.

Figure 4. Kill curves for an antibiotic containing keratin biomaterial. These data demonstrate the efficacy of a keratin biomaterial DDS on S. aureus. Effective arrest of the bacteria was noted at each concentration of Cefazolin.

Figure 5. Release kinetics for KBAP formulations containing Cefazolin were measured in a modified Franz diffusion cell (a). The antibiotic was simply added to the keratin hydrogel and was not encapsulated or chemically conjugated. Consequently, the release kinetics show rapid delivery in the first 24 hours, followed

by much lower release during the subsequent 3 days. Encapsulation and conjugation methods are currently being developed to provide an MIC for up to 2 weeks.

Figure 6. Growth of bovine osteoblasts in the presence of six different KBAP formulations compared to control conditions (media alone). Optical density values (Y axis) are proportional to the total number of viable cells. These data suggest that KBAP formulation nos. 3, 4, 5, 8, and 9 are compatible with osteoblasts ($p > 0.05$, $n=5$). Formulation nos. 6 and 7 reached near significance with p values of 0.030 and 0.041, respectively ($n=5$).

Figure 7. Micrographs showing the effect of the KBAP extract on osteoblast migration.

<u>Detailed Description of the Preferred Embodiments</u>

The compositions described herein are intended for use in the treatment of human subjects (including males and females, and including infant, juvenile, adolescent, adult and geriatric subjects) as well as animal subjects, particularly other mammalian subjects such as dogs, cats, horses, etc., for veterinary purposes.

"Bone" as used herein includes any bone, such as: the pelvis; long bones such as the tibia, fibia, femur, humerus, radius, and ulna, ribs, sternum, clavicle, etc.

"Fracture" or "break" as used herein with respect to bones includes any type thereof, including open or closed, simple or compound, comminuted fractures, and fractures of any location including diaphyseal and metaphyseal. "Fracture" as used herein is also intended to include defects such as holes, gaps, spaces or openings, whether naturally occurring or surgically induced (*e.g.*, by surgical removal of undesired tissue from bone).

"Antibiotic" as used herein includes any suitable antibiotic, including but not limited to cefazolin, vancomycin, gentamycin, erythromycin, bacitracin, neomycin, penicillin, polymycin B, tetracycline, biomycin, chloromycetin, streptomycin, ampicillin, azactam, tobramycin, clindamycin, gentamicin and combinations thereof. *See, e.g.*, US Patent No. 6,696,073. In some embodiments the antibiotic is preferably a water soluble antibiotic.

"Particulate fillers" used to carry out the present invention can be formed from any suitable biocompatible material, such as a ceramic. In some embodiments, the particulate filler is preferably osteoconductive. Examples of suitable materials from which the filler may be formed include but are not limited to tetracalcium phosphate, tricalcium phosphate, calcium alkali phosphate ceramic, calcium phosphorus apatite, bioglass, calcium carbonate, calcium hydroxide, calcium oxide, calcium fluoride, calcium sulfate, magnesium hydroxide, hydroxyapatite, calcium phosphorus apatite, magnesium oxide, magnesium carbonate, magnesium fluoride, collagen, allograft bone, other resorbable biocompatible materials and mixtures thereof. *See, e.g.,* US Patent No. 6,869,445; 5,281,265. In some embodiments the particulate filler comprises hydroxyapatite, tricalcium phosphate, or a mixture thereof.

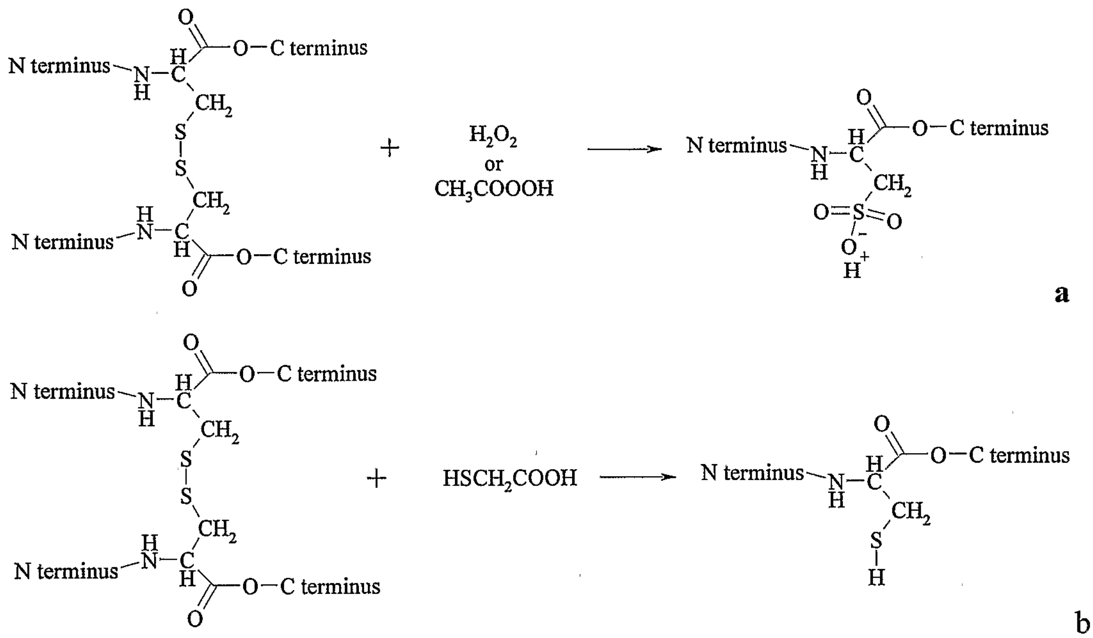
The particulate filler content of the composition of the present invention may be in a range from about 0.1 percent to about 200 percent of the keratin content of the composition. In some embodiments, the particulate filler content of the composition may be in a range from about 10 percent to about 100 percent of the keratin content. In other embodiments of the invention, the particulate filler content of the composition may be in a range from about 20 percent to about 90 percent of the keratin content. In further embodiments, the particulate filler content may be in a range from about 40 percent to 80 percent of the keratin content. In additional embodiments, the particulate filler content of the composition may be in a range from about 25 percent to about 50 percent of the keratin content. As an example, in one embodiment, when the keratin concentration in 100 g of gel is 20 percent (i.e., 20 g keratin per 80 g water) then the particulate filler content may be in a range from about 2 g to about 20 g.

In particular embodiments, the composition of the present invention has a consistency similar to toothpaste or modeling clay. Further, in representative embodiments, the viscosity of the composition is fluid and malleable and able to hold a form or shape without a supporting structure.

The composition of the present invention may be provided to the user in a dry form, which can be rehydrated for later use.

Keratin materials. Keratin materials are derived from any suitable source including but not limited to wool and human hair. In one embodiment keratin is derived from end-cut human hair, obtained from barbershops and salons. The material is washed in hot water and mild detergent, dried, and extracted with a nonpolar organic solvent (typically hexane or ether) to remove residual oil prior to use.

Scheme 1 below provides general representations of (a) oxidation and (b) reduction of disulfide crosslinks in keratin. These reactions cleave the sulfur-sulfur bond in cystine residues, thereby destroying the superstructure and rendering the keratins soluble in the reaction media.



Scheme 1.

Keratose Fractions. Keratose fractions are obtained by any suitable technique. In one embodiment they are obtained using the method of Alexander and coworkers (P. Alexander et al., *Biochem. J.* **46**, 27-32 (1950)). Basically, the hair is reacted with an aqueous solution of peracetic acid at concentrations of less than ten percent at room temperature for 24 hours. The solution is filtered and the alpha-keratose fraction precipitated by addition of mineral acid to a pH of ca. 4. The alpha-keratose is separated by filtration, washed with additional acid, followed by dehydration with alcohol, and then dried under vacuum. Increased purity can be achieved by redissolving the keratose in a denaturing solution such as 7M urea, aqueous ammonium hydroxide solution, or 20mM tris base buffer solution, re-precipitating, re-dissolving, dialyzing against deionized water, and re-precipitating at pH 4.

The gamma-keratose fraction remains in solution at pH 4 and is isolated by addition to a water-miscible organic solvent such as alcohol, followed by filtration, dehydrated with additional alcohol, and dried under vacuum. Increased purity can be achieved by redissolving the keratose in a denaturing solution such as 7M urea, aqueous ammonium hydroxide solution, or 20mM tris buffer solution, reducing the pH to 4 by addition of a mineral acid, removing any solids that form, neutralizing the supernatant, re-precipitating the protein with alcohol, re-dissolving, dialyzing against deionized water, and reprecipitating by addition to alcohol. The amount of alcohol consumed in these steps can be minimized by first concentrating the keratose solution by distillation.

In use the compositions may be rehydrated if necessary, and used to treat fractures in a subject (*e.g.* filling bone defects) in accordance with known techniques by contacting the composition to the fracture in a treatment-effective amount. Fractures may be of any bone, including but not limited to: ethmoid, frontal, nasal, occipital, parietal, temporal, mandible, maxilla, zygomatic, cervical vertebra, thoracic vertebra, lumbar vertebra, sacrum, rib, sternum, clavicle, scapula, humerus, radius, ulna, carpal bones, metacarpal bones, phalanges, ilium, ischium, pubis, femur, tibia, fibula, patella, calcaneus, tarsal bones or metatarsal bones, etc. Indeed the compositions may be used for any suitable purpose for which bone graft or osteogenic implants are used, as described in US Patent No. 6,863,694 to Boyce et al.

The present invention is explained in greater detail in the following non-limiting Examples.

EXPERIMENTAL

In this study, a keratin bioceramic antibiotic putty (KBAP) that provides osteoconductivity, osteoinductivity, and controlled antibiotic release is tested. The putty is comprised of a keratin hydrogel with ceramic filler and antibiotic. The KBAP is malleable and can be formed into shapes and pressed into a bony defect site with no additional preparation. It provides immediate and prophylactic antibiotic release as well as an osteoconductive and osteoinductive environment for bone regeneration in a single-stage operational protocol.

Keratin hydrogel is a proteinaceous network that is highly hydrated; therefore any water soluble antibiotics (e.g. Cefazolin, Gentamicin, and Vancomycin) can be used. The hydrophilicity of the hydrated keratin promotes cell attachment and in growth. The ceramic component may have the osteoconductive properties of products currently on the market (e.g., COLLAGRAFT®), but may not require aspirated bone marrow. The keratin matrix provides a highly biocompatible environment, as keratins are a class of proteins that elicit one of the lowest foreign body reactions among all biomaterials (Ito H et al., Kobunshi Ronbunshu 1982;39(4):249-56; Blanchard CR et al., US Pat No 6461628. October 8, 2002; Tachibana A et al., J Biotech 2002;93:165-70).

When processed correctly, keratin proteins have a unique capability of molecular self-assembly, a process by which they reconstruct some semblance of their original tertiary structure (Sauk JJ et al., J Cell Bio 1984;99:1590-7; Thomas H et al., Int J Biol Macromol 1986;8:258-64; van de Löcht M. Melliand Textilberichte 1987;10:780-6). This is a particularly useful characteristic for a bone graft substitute for two reasons. First, self-assembly results in a highly regular structure with reproducible architectures, dimensionality, and porosity. Second, the fact that these architectures form of their own accord under benign conditions allows for the incorporation of cells as the matrix is formed. These two features are critically important to any system that attempts to mimic the native ECM. The keratin scaffold shown in **Figure 1** was prepared by spontaneous self-assembly of a hydrogel and demonstrates the type of architecture conducive to cell infiltration and tissue regeneration.

Native ECM is a regular structure created around the cells, by the cells. In a tissue damage scenario, the ECM is an interactive medium for cell recruitment, growth, and differentiation, leading to the formation and maturation of new functional tissue. The ECM helps to orchestrate these processes by providing architectural support, growth factor delivery, and sites of molecular recognition whereby cells can bind and receive information.

Cellular recognition is facilitated by the binding of cell surface integrins to specific amino acid motifs of the ECM (Buck CA and Horwitz AF. Annu Rev Cell Biol 1987;3:179-205; Akiyama SK. Hum Cell 1996;9(3):181-6). The predominant ECM proteins are collagen and fibronectin, both of which have been extensively studied with regard to cell binding (McDonald JA and Mecham RP (editors). Receptors for extracellular matrix (1991). Academic Press, San Diego). Fibronectin contains several regions that support attachment by a wide variety of cell types. Mould *et al.* showed that in addition to the widely know Arginine-Glycine-Aspartic Acid (RGD) motif, the "X"-Aspartic Acid-"Y" motif on fibronectin is also recognized by the integrin α4β1 where X equals Glycine, Leucine, or Glutamic Acid and Y equals Serine or Valine (Mould AP et al., J Biol Chem 1991;266(6):3579-85). Inexpensive, biocompatible scaffolds from keratin biomaterials contain these same binding motifs.

A recent search of the NCBI protein database revealed sequences for 71 discrete, unique human hair keratin proteins (Data from the National Center for Biotechnology Information (NCBI) database. http://www.ncbi.nlm.nih.gov). Of these, 55 are from the high molecular weight, low sulfur, alpha-helical family. This group of proteins is often referred to as the alpha-keratins and is responsible for imparting toughness to human hair fibers. These alpha-keratins have molecular weights greater than 40 kDa and an average cysteine (the main amino acid responsible for inter- and intramolecular protein bonding) content of 4.8 mole percent. Importantly, analysis of the amino acid sequences of these alphakeratin proteins showed that 78% contain at least one fibronectin-like integrin receptor binding motif, and 25% contain at lease two or more. A recent paper has highlighted the fact that these binding sites are present on keratin biomaterials by demonstrating excellent cell adhesion to a keratin foam (Tachibana A et al., J Biotech 2002;93:165-70). Although this paper uses fibroblasts to demonstrate the principle, the osteoconductivity of a keratin bioceramic was later demonstrated by these same authors (Tachibana A et al., Biomaterials 2005;26(3):297-302).

Some studies are beginning to show mounting evidence that a number of growth factors are present in end-cut human hair, and that the keratins may be acting as a highly effective delivery matrix. It has been known for more than a decade that growth factors such as bone morphogenetic protein-4 (BMP-4) and other members of the transforming growth factor-β (TGF-β) superfamily are present in developing hair follicles (Jones CM et al., *Development* 1991; 111: 531-42; Lyons KM et al., *Development* 1990; 109: 833-44; Blessings M et al., *Genes and Develop* 1993; 7: 204-15).39-41 In fact, more than 30 growth factors and cytokines are involved in the growth of a cycling hair follicle (Stenn KS et al., *J Dermato Sci* 1994; 7S: S109-24). Many of these molecules have a pivotal role in the regeneration of a variety of tissues (Clark RAF (editor). *The molecular and cellular biology of wound repair* (1996) Plenum Press, New York). It is highly probable that a number of growth factors become entrained within human hair when cytokines bind to stem cells residing in the bulge region of the hair follicle (Panteleyev AA et al., *J Cell Sci* 2001; 114: 3419-31). We have recently analyzed extracts of human hair and shown the presence of growth factors such as vascular endothelial growth factor (VEGF) in these samples. We are currently assaying keratin biomaterials for the presence of several other growth factors including BMP, TGF-β, and nerve growth factor.

The preceding discussion demonstrates several key advantages that the KBAP has over conventional bone graft substitutes, and the ways in which we are leveraging our expertise to achieve the objective of developing a superior product. A keratin biomaterial with antibiotic filler provides sustained antibiotic release and has the potential to achieve this goal for the following reasons:

•Keratin biomaterials are easily obtained and processed

•Keratins are highly biocompatible

•Keratins self-assemble into architectures that are conducive to cell attachment and growth

•Keratins contain sites of cellular recognition and are effective ECM surrogates

•Keratins are able to act as encapsulants or conjugates of drug compounds such as antibiotics and control their release kinetics

•Keratin biomaterials contain growth factors such as BMP that modulate cell growth and differentiation and therefore have the potential to impart osteoinductivity

Results

1. Prepare a bone graft putty using a keratin matrix with a mineral component that can incorporate antibiotic microcapsules.

There are many published methods that describe the extraction of keratins from hair fibers. In general, there are three procedures used to chemically break down the resilient structure of the hair fiber and impart aqueous solubility to the cortical keratin proteins of interest: 1) oxidation, 2) reduction, and 3) sulfitolysis (*see, e.g.*, Crewther WG et al., Advances in protein chemistry (1965). Anfinsen CB Jr., Anson ML, Edsall JT, and Richards FM (editors). Academic Press. New York:191-346; Goddard DR and Michaelis L., *J Bio Chem* 1934; 106: 605-14; Kelley RJ et al., PCT Patent Application No. WO 03/011894; Zackroff RV and Goldman RD. *Proc Natl*

Acad Sci 1979; 76(12): 6226-30). Efficient extraction depends first on breaking the disulfide bonds using one of these three methods, and second on gently denaturing the free proteins and affecting their dissolution. The importance of this second step cannot be over emphasized due to the existence of a competing reaction, hydrolysis of the peptide bonds in the keratin backbone. Hydrolysis of the protein backbone destroys many of the useful properties of keratins and must be avoided. We have evaluated all three of these methods as described more fully below, with the goal of creating a malleable putty that can be used to repair bony defects.

Oxidation: Human hair was obtained from a local salon, washed with mild detergent (Fisher Scientific, Pittsburgh, PA), degreased with ethyl ether (Sigma-Aldrich, St. Louis, MO), and dried in air. In a typical reaction, 20 grams of clean, dry hair was treated with 400 mL of a 2 weight/volume (w/v) % solution of peracetic acid (PAA, Sigma-Aldrich, St. Louis, MO) in deionized (DI) water. The oxidation was conducted in a closed polypropylene container maintained at 37°C for 12 hours with gentle agitation. The oxidized hair was recovered and rinsed with copious amounts of DI water. The wet, oxidized hair was extracted with successive volumes of 0.2M tris base (Sigma-Aldrich, St. Louis, MO), 0.1M tris base, and DI water (500, 500, and 1000 mL, respectively). The extracts were combined and the α-keratose precipitated by drop wise addition of 12M hydrochloric acid (HCL; Fisher Scientific, Pittsburgh, PA) to a final pH of 4.2. The α-keratose was re-dissolved in 20 mM tris base with 20 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO), re-precipitated by drop wise addition of HCL to a final pH of 4.2, and again re-dissolved in tris base +EDTA. The resulting protein solution was dialyzed against DI water for three days with twice daily water changes (LMWCO 12.4K; Sigma-Aldrich, St. Louis, MO). After dialysis, the α-keratose powder was isolated by reducing the liquid volume via vacuum distillation at 50°C and freeze-drying the concentrate. This sample was formulated at 5, 4, 3, 2, 1, and 0.5 weight percent in Ringer's lactate (RL) and analyzed for viscosity on a Brookfield cone and plate viscometer (Brookfield Engineering, Middleboro, MA) at 37°C. The viscosity data are shown in **Figure 1.**

Reduction: A second sample of keratin was obtained using a different extraction protocol. Human hair was obtained from a local salon, washed with mild detergent, degreased with ethyl ether, and dried in air. In a typical reaction, 20 grams of clean, dry hair was treated with 400 mL of a 1.0M thioglycolic acid (Sigma-

Aldrich) in DI water that had been titrated to pH 10.2 using saturated sodium hydroxide solution (Fisher Scientific). The reduction was conducted in a closed polypropylene container maintained at 37°C for 12 hours with gentle agitation. The reduced hair was recovered and rinsed with copious amounts of DI water. The wet, reduced hair was extracted with three successive 500 mL volumes of 0.1M tris base with 0.1M thioglycolic acid. The extracts were combined and the α-kerateine precipitated by drop wise addition of 12M HCL to a final pH of 4.2. The α-kerateine was re-dissolved in 20 mM tris base with 20 mM EDTA, re-precipitated by drop wise addition of HCL to a final pH of 4.2, and again re-dissolved in tris base + EDTA. The protein solution was dialyzed against DI water for three days with twice daily water changes (LMWCO 12.4K). After dialysis, the α-kerateine was derivatized by reacting the cysteine residues with iodoacetic acid (Sigma-Aldrich) by adding 0.25 mg per mL of dialyzate. The reaction was performed in a closed polypropylene container maintained at 37°C, pH 9.0, with occasional stirring over 24 hours. Excess iodoacetic acid and other contaminants were removed by dialysis against DI water (LMWCO 12.4K). The dialyzate was concentrated by reduced pressure evaporation and the α-s-carboxymethylkerateine (α-SCMK) obtained by freeze-drying. Solutions of α-SCMK at 5, 4, 3, 2, 1, and 0.5 weight percent in RL were prepared and analyzed for viscosity as described previously. These data are also shown in **Figure 2.**

Although these data trend toward acceptable viscosity values, formulating the hydrogels at 10 weight percent or less is desired to maintain biocompatibility and provide a porous matrix that can be populated by osteoblasts. Biomaterials with low porosity or pores that are too small (i.e. <85% and <100 μm, respectively) are difficult for cells to populate without first degrading them; this slows healing and can lead to fibrosis. The viscosities shown in **Figure 1** for formulations below 5% keratin were deemed to be unacceptably low. It was determined that the extraction conditions employed resulted in excessive hydrolysis, consequently, we modified our oxidation protocol to minimize this side reaction.

Oxidation (low hydrolysis method): In a typical procedure using this protocol, 50 grams of clean, dry hair was treated with 1,000 mL of a 2 weight/volume (w/v) % solution of PAA in DI water. The oxidation was conducted in a closed polypropylene container maintained at 37°C for 12 hours with gentle agitation. The oxidized hair was recovered and rinsed with copious amounts of DI water. The wet, oxidized hair was extracted with 1,000 mL of 0.1M tris base and subsequently extracted with successive 1,000 mL volumes of DI water. The extracts were combined and concentrated 10-fold by reduced pressure evaporation at 50°C. The α-keratose was precipitated by drop wise addition of the concentrated solution to cold ethanol. The precipitate was re-dissolved in a minimum amount of DI water and re-precipitated by drop wise addition of 12M HCl to a final pH of 4.2. The α-keratose was isolated by centrifugation, re-dissolved in DI water, adjusted to a pH of 7.0, dialyzed against DI water for three days with twice daily water changes (LMWCO 12.4K), concentrated, and freeze dried. Solutions of the low hydrolysis (LH) α-keratose were prepared at 10 and 5 weight percent in phosphate buffered saline (PBS) and analyzed for viscosity as described previously. At 10 weight percent, the viscosity was too high to be measured by the viscometer. At 5 weight percent, the viscosity (analyzed at lower torque than previous measurements) was 460 centipoise at 37°C. From these data, we concluded that the LH method substantially reduced hydrolysis during keratin extraction and downstream processing.

Recognizing that additional improvements could potentially be made in our keratin production process, a small change was made to the LH extraction protocol described above. Rather than separate α- and γ-keratose by isoelectric precipitation, the crude extract was dialyzed to remove trace contaminants and residual processing chemicals, and the dialyzate concentrated and freeze dried. This results in a mixture of both α- and γ-keratose that has demonstrated improved viscoelastic properties. In addition, we attempted to improve the viscosity of the hydrogel formulation by crosslinking the keratose mixture. The details of this method are as follows: In a typical procedure, 50 grams of clean, dry hair was treated with 1,000 mL of a 2 weight/volume (w/v) % solution of PAA in DI water. The oxidation was conducted in a closed polypropylene container maintained at 37°C for 12 hours with gentle agitation. The oxidized hair was recovered and rinsed with copious amounts of DI water. The wet, oxidized hair was extracted with 1,000 mL of 0.1M tris base and subsequently extracted with successive 1,000 mL volumes of DI water. The extracts were combined and concentrated 10-fold by reduced pressure evaporation at 50°C. The concentrated solution was dialyzed against DI water (LMWCO 12.4K), concentrated, and freeze dried. 5 w/v % solutions of α+γ-keratose and α-keratose in DI water were prepared and their viscosities measured. These solutions were also reacted with a solution of transglutaminase (1 mg/mL; Sigma-Aldrich, St. Louis, MO) in an attempt to further increase their viscosity through glutamine-lysine crosslinking (both amino acids are prevalent in keratins). Interestingly, while the initial viscosity of the α+γ-keratose samples was lower than the α-keratose, the former achieved higher viscosity than the latter after only one hour of incubation at 37°C. Based on this observation, several formulations were prepared and crosslinked with transglutaminase, and the viscosities of the resulting gels measured. These data are shown in **Table 1.**

These data must be interpreted carefully as the formulations contained less keratose than would normally be used for a KBAP formulation simply to ensure their viscosities were within the range of our equipment. Also, addition of the transglutaminase and a small amount of calcium initiator decreased the apparent weight percent keratose. These data suggest that in forming a high viscosity hydrogel a mixture of α+γ-keratose and the use of transglutaminase crosslinking may be useful. Qualitatively, the α-keratose solution decreased in viscosity after 1 hour of incubation at 37°C while a similar α+γ-keratose solution increased. This phenomenon may be due to complex formation between the alpha and gamma forms of keratin.

Table 1. Viscosity of keratose formulations measured at 30 rpm and 37°C

No.	Type	Vol. of keratose solution used	Transglutaminase (vol. of 1mg/mL solution)	Viscosity (cP)	
				Initial	After 1 hr @ 37°C
1	1% α-keratose	400 μL	100 μL	2.80	1.36
2	1% α-keratose	300 μL	300 μL	2.80	1.23
3	1% α+γ-keratose	400 μL	200 μL	1.71	2.24
4	1% α+γ-keratose	300 μL	200 μL	1.71	2.69
5	2% α+γ-keratose	300 μL	0 μL	2.43	1.86
6	2% α+γ-keratose	300 μL	200 μL	2.43	1.93
7	5% α+γ-keratose	800 μL	100 μL	5.72	3.72
8	5% α+γ-keratose	800 μL	200 μL	5.72	3.08
9	5% α+γ-keratose	700 μL	200 μL	5.72	2.65
10	5% α+γ-keratose	700 μL	400 μL	5.72	3.03

In addition to viscosity analysis, the underlying microstructure of the first six of these gel formulations was investigated. Each formulation was recovered from the viscometer and freeze dried. The structures of the resulting samples were characterized by scanning electron microscopy (SEM; Model S-2600N; Hitachi High Technologies America, Inc., Pleasanton, CA) and are shown in **Figure 3**. These images show the fibrous nature of the hydrogels and demonstrate the effect of this microstructure on viscosity. Formulation no. 4, for example, demonstrated one of the largest increases in viscosity after enzyme crosslinking; it also shows the most developed fibrous architecture. We believe the fibrous architecture is mediated by a process of molecular self-assembly, a unique characteristic of keratin-based biomaterials.

2. Optimize and validate the antibiotic formulation

In order to demonstrate antibiotic delivery, we conducted an initial investigation using the keratin as a drug carrier. In these experiments, Cefazolin sodium was dissolved in aqueous α-kerateine solution at target concentrations of 0, 10, 20, 40, and 80 μg. The solutions were lyophilized and the solid samples ground into fine powders. The powders were formed into 500 mg disks using a manual press. The disks were used to generate kill curves for suspensions of *Staphylococcus aureus* ATCC 29213. Mueller Hinton broth cultures were prepared from an overnight sheep blood agar (SBA) culture of *S. aureus* so as to contain approximately 105 cfu per mL final concentration. Keratin disks were placed into culture tubes containing 2 mL final volume of *S. aureus* culture and incubated at 37°C. A 10 μL aliquot of each culture was removed at 0, 4, and 8 hours, diluted into 10 mL of saline, of which 100 μL were plated onto SBA using a glass spreader and inoculating turntable. Each concentration of antibiotic was tested in triplicate. The average number of colonies was calculated from the triplicate samples and compared to a positive control culture without antibiotic and a negative control culture without organisms. These data, shown in **Figure 4,** demonstrate the efficacy of the keratin biomaterial DDS.

In a more recent experiment, a high viscosity KBAP formulation (LH method) was used to incorporate Cefazolin at 1000, 500, and 250 μg antibiotic by vortexing. The gel was cross-linked using 100μl of 0.1% transglutaminase at 37 °C for 1 hour and the formulation was lyophilized to form a free standing disc. The Cefazolin release from the KBAP disk was measured in PBS using a modified Franz diffusion cell). The pellet (15 mg) was placed in the donor compartment, while the acceptor compartment was filled with PBS. The donor compartment was separated from the acceptor compartment by a cellulose membrane (100 μm pore size). This diffusion cell was then placed in an incubator at 37 °C. The solution in the acceptor compartment was periodically removed and replaced with and equal amount of fresh buffer solution. The Cefazolin released through the cellulose membrane was analyzed by UV/Vis spectrometry (Thermo Spectronic, USA, UV/VIS) at 285 nm.

As shown in **Figure 5,** the release curves revealed a controlled release of Cefazolin from the KBAP formulation. Approximately 60 ~70 % of the incorporated antibiotic was released from KBAP in one day and sustained the release for 4 days.

3. Biocompatibility, osteoconductivity, and osteoinductivity *in vitro.*

A sample of bovine osteoblasts was reconstituted from frozen stock by rapid thawing and plating onto a 10 cm tissue culture dish. The cells were cultured in 10 mL of low glucose DMEM containing 10% FBS (Invitrogen, Carlsbad, CA) with 0.05 mg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO) and antibiotics added. Media was changed three times per week and the cells were expanded by subculturing every 3-5 days. After at least three subculture cycles ("passages"), the cells were trypsinized and seeded in 96-well tissue culture plates at a density of approximately 3,000 cells per well. To each of five replicate wells was added approximately 1 mg of one of the KBAP formulations shown in **Table 2**. The KBAP had been lyophilized and gamma sterilized prior to placing in the media. After approximately 72 hours of incubation at 37°C, 5% CO_2, and 95% humidity, 200 μL of 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 1 mg/mL; Sigma-Aldrich, St. Louis, MO) was added to the culture wells, the cells were incubated for 4 hours, and the metabolic reduction product, MTT formazan, dissolved in a known amount of dimethylsulfoxide. The number of cells was measured indirectly by measuring the color intensity of metabolically reduced MTT using a microplate reader at 540 nm (model Elx800; Bio-Tek Instruments, Inc.; Winooski, VT). The optical density (OD) data from these cultures are shown in **Figure 6.**

Table 2. KBAP formulations used in osteoconductivity testing

No.	Cefazolin (mg)	Vol. of 10% α+γ-keratose gel used (μl)	Vol. of 5% chitosan lactate used (μL)	Vol. of 2% sodium alginate used (μL)	HA + TCP (mg)	Vol. of 0.5 mg/mL transglutaminase added (μL)	Vol. of 0.15% $CaCl_2$ used (μL)
4	2	500	50	50	40 + 10	100	50
5	1	500	50	50	40 + 10	100	50
6	0	500	50	50	40 + 10	100	50
7	0	500	50	50	40 + 10	100	50
8	0	500	50	50	40 + 10	100	50
9	0	500	50	50	40 + 10	100	50

These data indicate that the KBAP formulations are capable of acting as osteoconductors as they will support the growth of osteoblasts in culture.

The ability of KBAP to recruit osteogenic cells through a chemotactic mechanism (i.e. osteoconduction) was assessed using a porous cell membrane insert. This technique determines the extent of cell migration through the porous membrane in response to a concentration gradient of chemotactic agent(s). In these experiments, KBAP was placed in serum free media at 37°C and the supernatant removed at different time points such as 1, 3, 6, and 24 hours after immersion. This KBAP "extract" was placed in culture wells and the inserts place on top (not shown). Bovine osteoblasts were seeded at approximately 2 x 10^4 cells/well into the upper chamber and incubated at 37°C, 5% CO_2, and 95% relative humidity.

Cells cultured in the presence of serum free media alone was used as a control. After 6 and 20 hours of incubation, the membrane was fixed with glutaraldehyde and dehydrated with an alcohol gradient. The morphologic characteristics of the cells was examined by an environmental scanning electron microscopy (SEM; model N-2600 Hitachi, Japan).

The effect of the KBAP extract on osteoblast migration is apparent in the micrographs shown in **Figure 7.** These data suggest that the KBAP possesses soluble molecules that are capable of affecting osteoblast migration. Media with no KBAP extract showed few cells on the top ("face") surface of the membrane and none on the bottom ("back"). Media that had been used to extract KBAP for 6 and 24 hours showed more cells on the top surface, with 24 hours of extracting appearing to be slightly more effective. More importantly, cells migrated through the pores to the bottom surface of the membrane in the presence of KBAP extract after 20 hours of culture. These data indicate the chemotactic potential of KBAP's soluble fraction and the latent osteoinductivity of keratin biomaterials.

4. Conclusions. We evaluated three forms of keratin biomaterials for suitability in a bone graft substitute formulation termed keratin bioceramic antibiotic putty or KBAP. A low hydrolysis method for the extraction of keratins from human hair fibers was developed such that the protein possessed the desired viscoelastic characteristics. We discovered that when high molecular weight keratins were obtained, they were capable of self-assembling into fibrous micro-architectures that are conducive to cell infiltration and growth.

We further improved the physical characteristics of this self-assembled hydrogel by crosslinking strategies. From this keratin-based hydrogel, we developed a malleable KBAP prototype formulation that contained a drug delivery system capable of releasing antibiotics.

The KBAP prototype formulation was tested for antibiotic release in an *in vitro* model using *S. aureus* and was shown to effectively kill this species of bacteria. We further characterized the antibiotic release by determining the *in vitro* release kinetics. The biocompatibility of several different KBAP formulations was demonstrated in vitro using bovine osteoblasts, and the osteoinductivity of human hair keratins was shown using a cell migration assay. These data show the feasibility of formulating a malleable bone graft substitute with antibiotic release from a keratin biomaterial.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A malleable bone graft composition, comprising:

(a) from 1 to 90 percent by weight keratose;

(b) from 1 to 90 percent by weight particulate filler;

(c) from 0.001 to 5 percent by weight antibiotic; and

(d) water to balance;

said composition having a viscosity of at least 3 centipoise at a temperature of 37°C.

2. The composition of claim 1, wherein said keratose is alpha keratose, gamma keratose, or mixtures thereof.

3. The composition of claim 1, wherein said keratose is a mixture of alpha keratose and gamma keratose.

4. The composition of claim 1, wherein said keratose comprises from 10 to 90 percent by weight alpha keratose and from 90 to 10 percent by weight gamma keratose.

5. The composition of claim 1, wherein said keratose is crosslinked keratose.

6. The composition of claim 1, wherein said crosslinked keratose is produced by the process of combining said keratose with transglutaminase in the presence of a calcium initiator.

7. The composition of claim 1, further comprising from 0.001 to 5 percent by weight bone morphogenic protein.

8. The composition of claim 1, wherein said particulate filler is osteoconductive.

9. The composition of claim 1, wherein said particulate filler is selected from the group consisting of tetracalcium phosphate, tricalcium phosphate, calcium alkali phosphate ceramic, bioglass, calcium carbonate, calcium hydroxide, calcium oxide, calcium fluoride, calcium sulfate, magnesium hydroxide, hydroxyapatite, calcium phosphorus apatite, magnesium oxide, magnesium carbonate, magnesium fluoride, collagen, other resorbable biocompatible materials and mixtures thereof.

10. The composition of claim 1, wherein said particulate biocompatible filler comprises hydroxyapatite, tricalcium phosphate, or a mixture thereof.

11. The composition of claim 1, wherein said antibiotic is selected from the group consisting of cefazolin, vancomycin, gentamycin, erythromycin, bacitracin, neomycin, penicillin, polymycin B, tetracycline, biomycin, chloromycetin, streptomycin, ampicillin, azactam, tobramycin, clindamycin, gentamicin and combinations thereof.

12. The composition of claim 1, wherein said composition is sterile.

13. The composition of claim 12 packaged in a sterile container.

14. A lyophilized or freeze-dried composition which upon reconstitution with water or saline solution produces a composition of claim 1.

15. A method of treating a fracture in a subject in need thereof, comprising contacting a composition of claim 1 to said fracture in a treatment-effective amount.

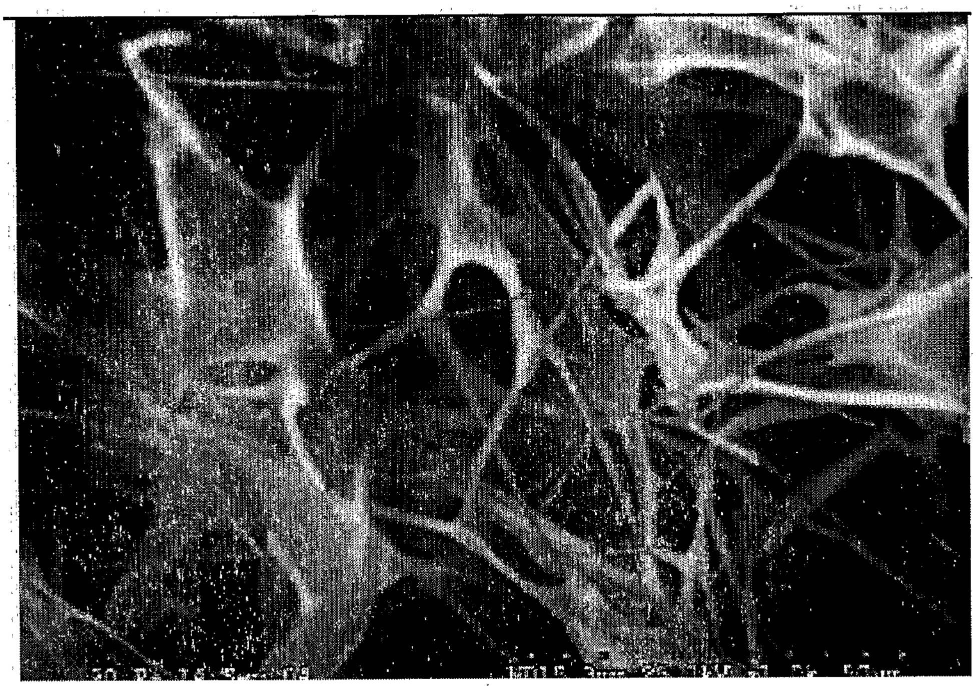


Figure 1A

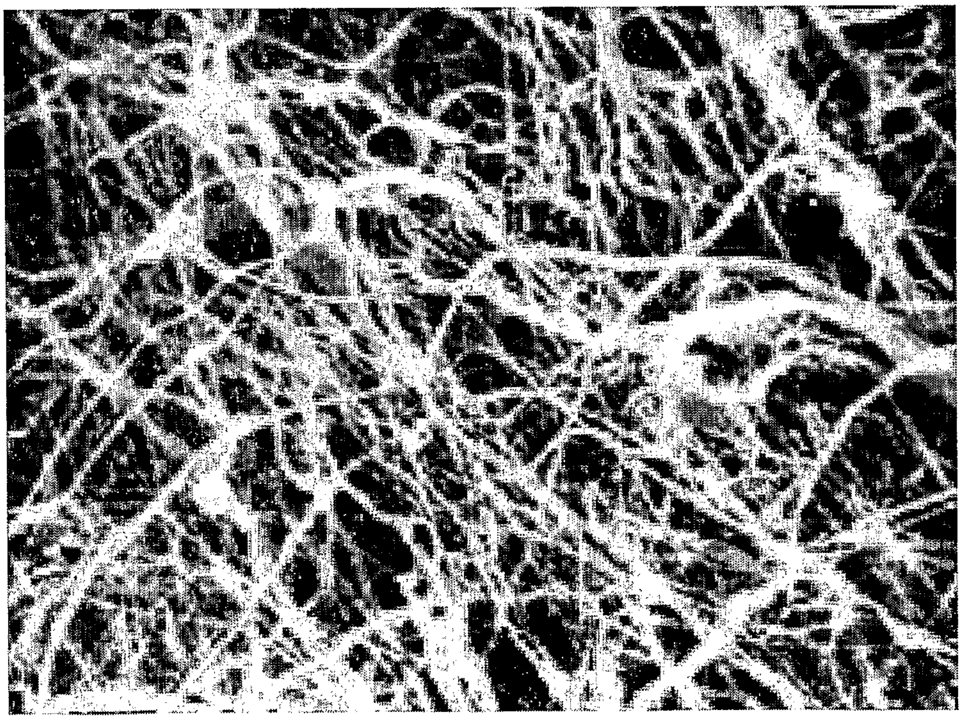


Figure 1B

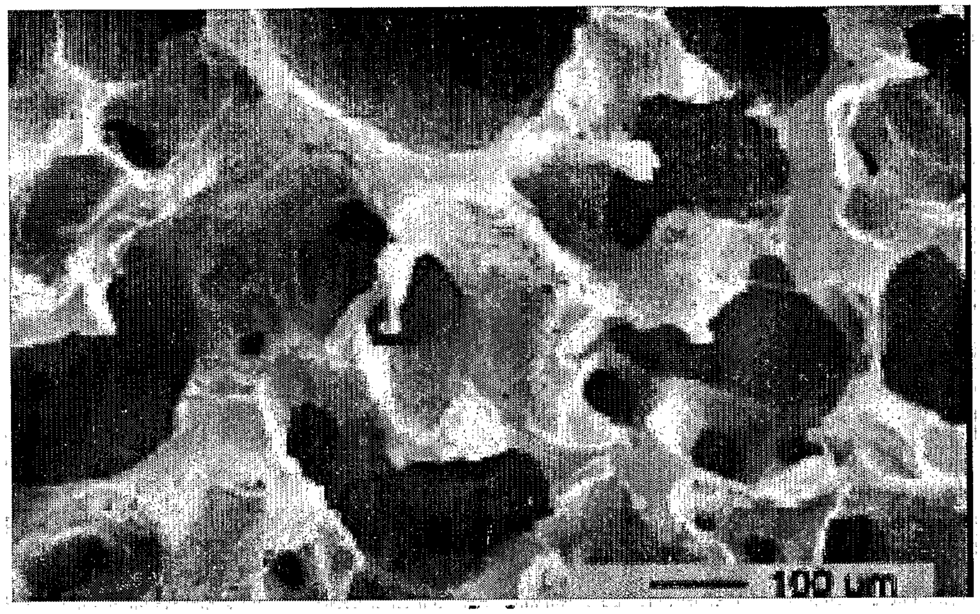


Figure 1C

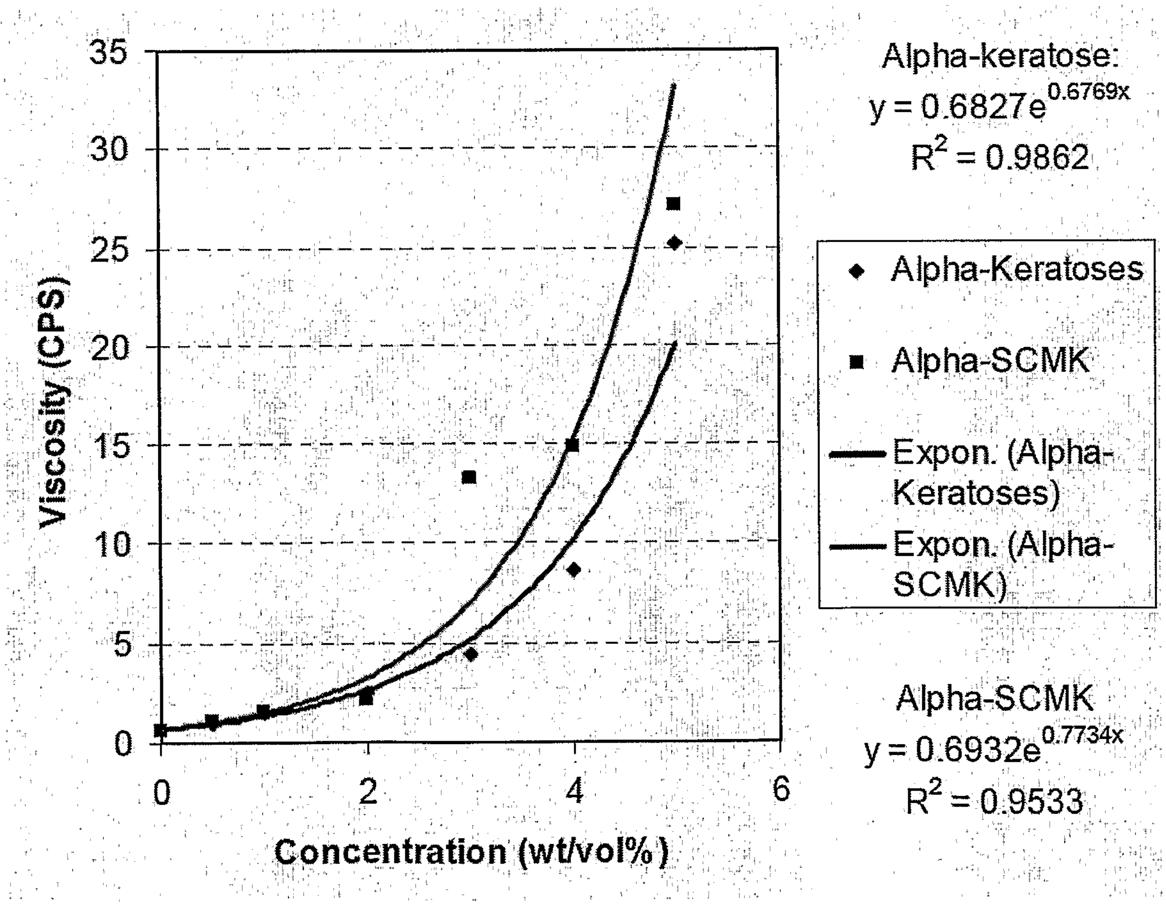


Figure 2.

Viscosity curves for α-keratose (bottom curve) and α-SCMK (top curve).

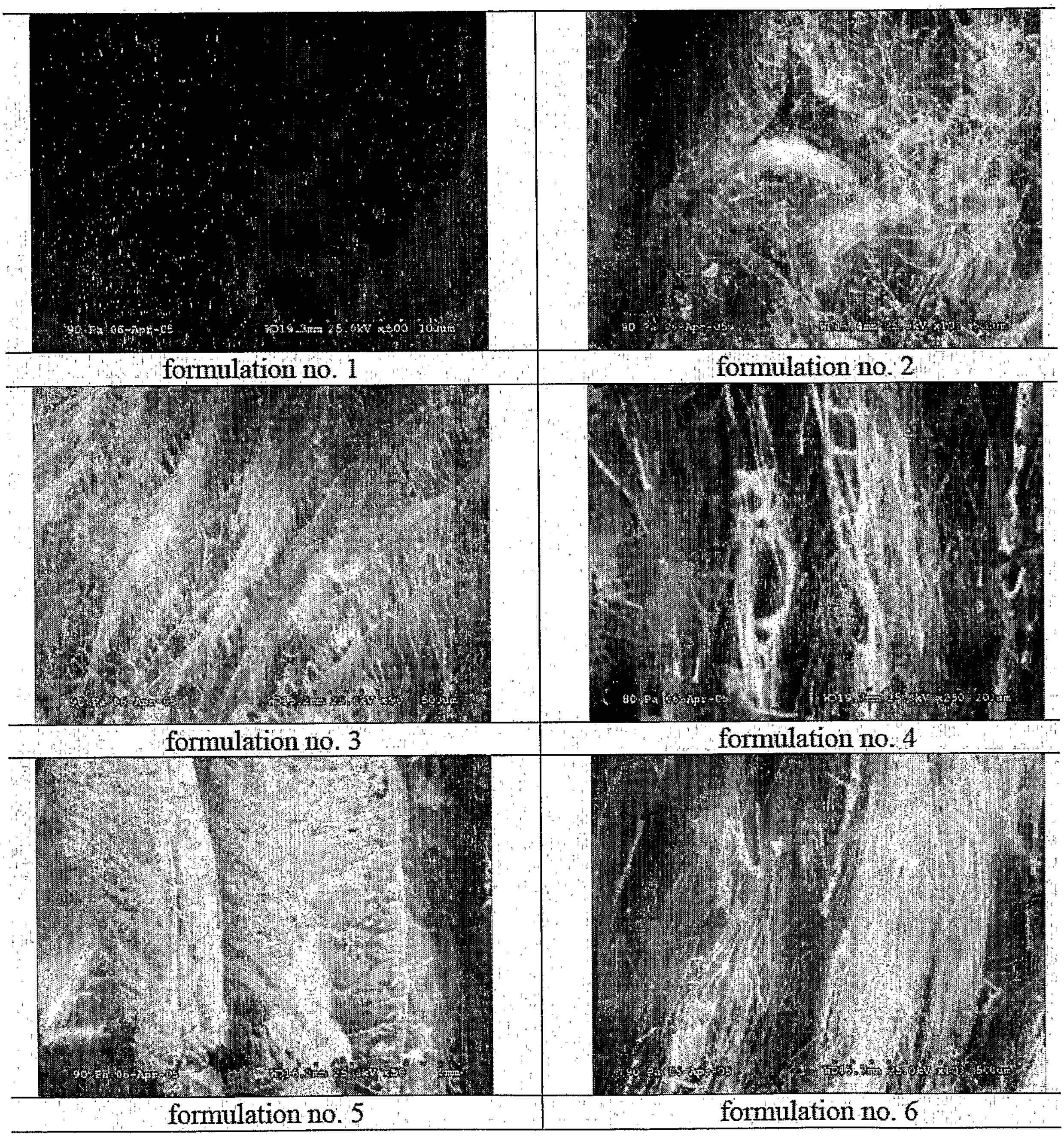


Figure 3. SEM micrographs of keratose formulations (as defined in Table 1).

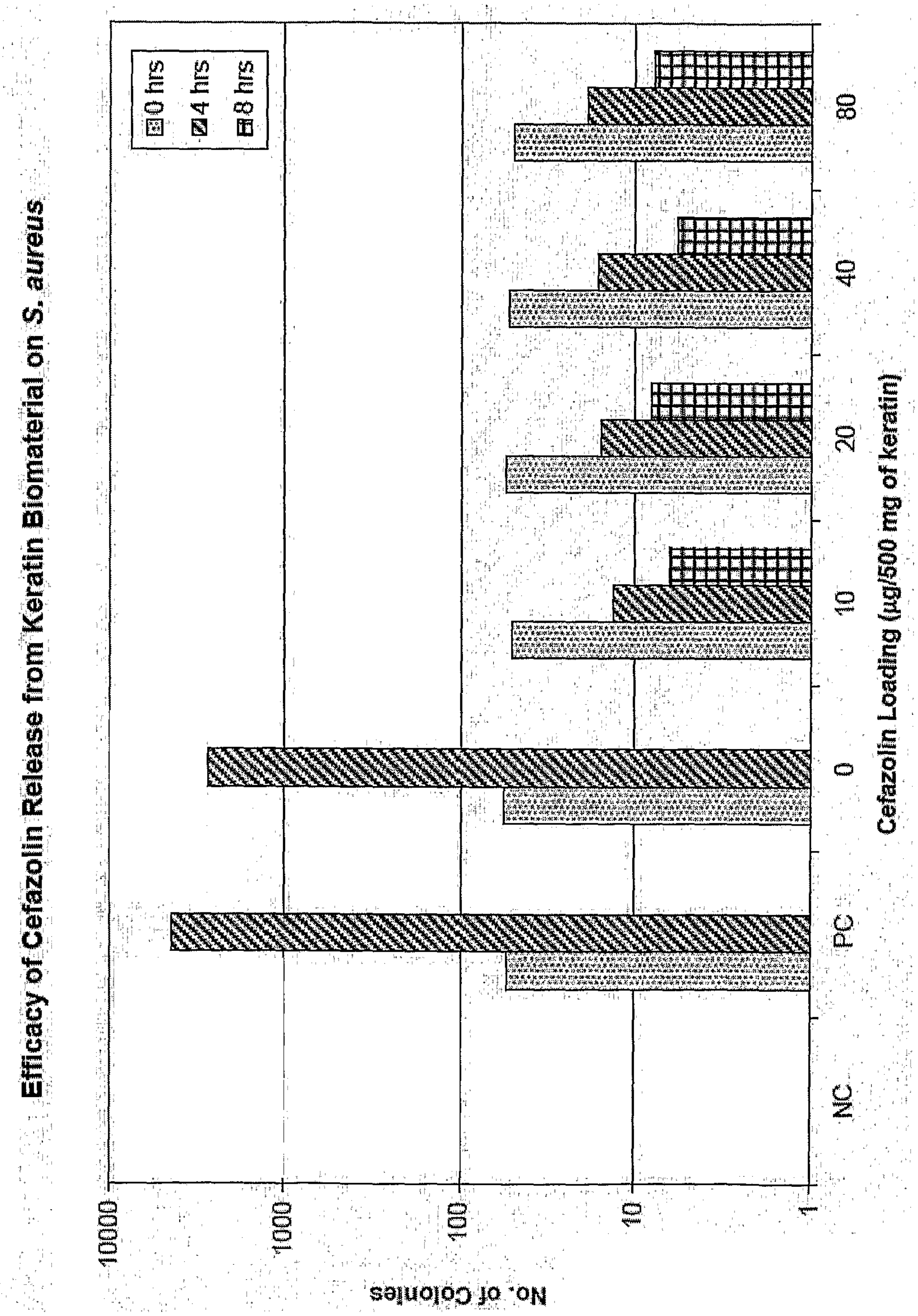


Figure 4. Kill curves for an antibiotic containing keratin biomaterial.

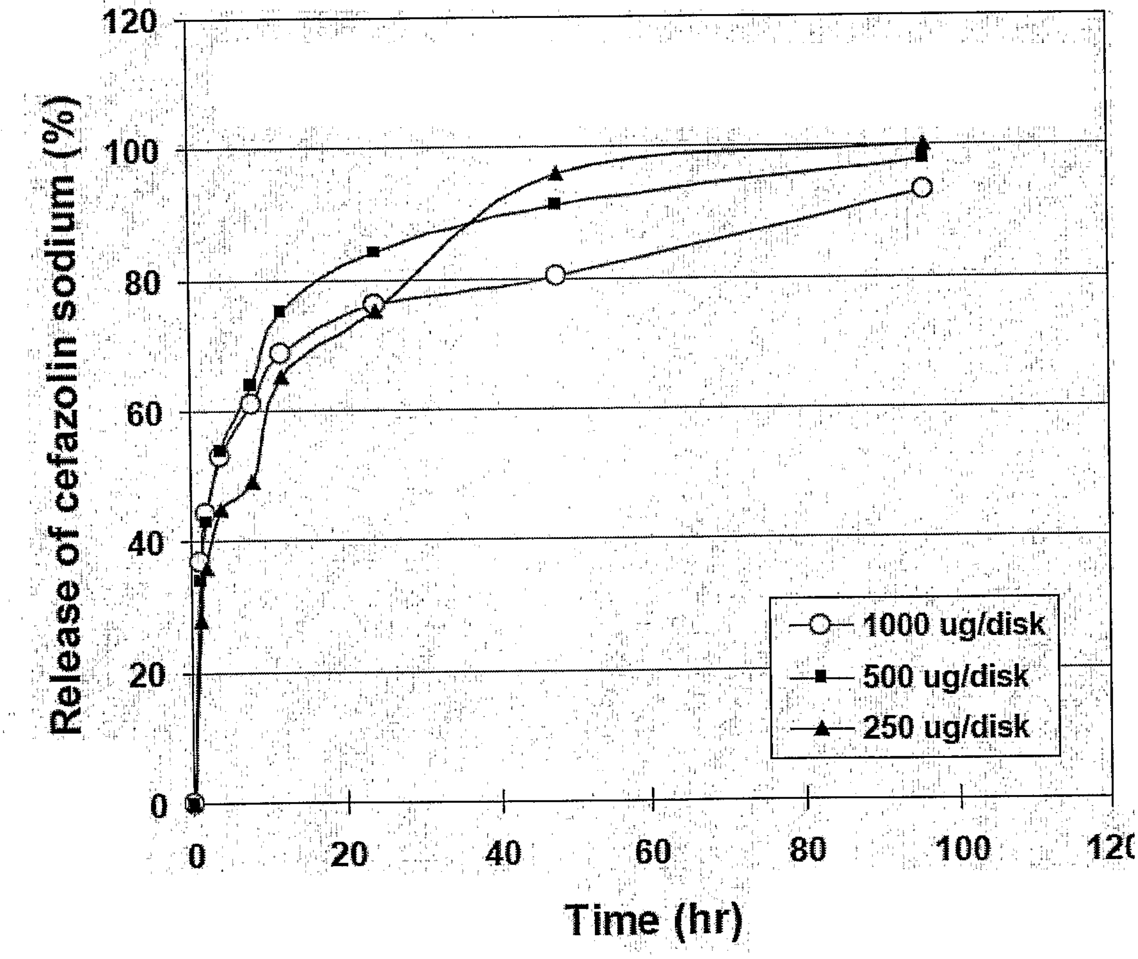


Figure 5. Release kinetics for KBAP formulations containing Cefazolin.

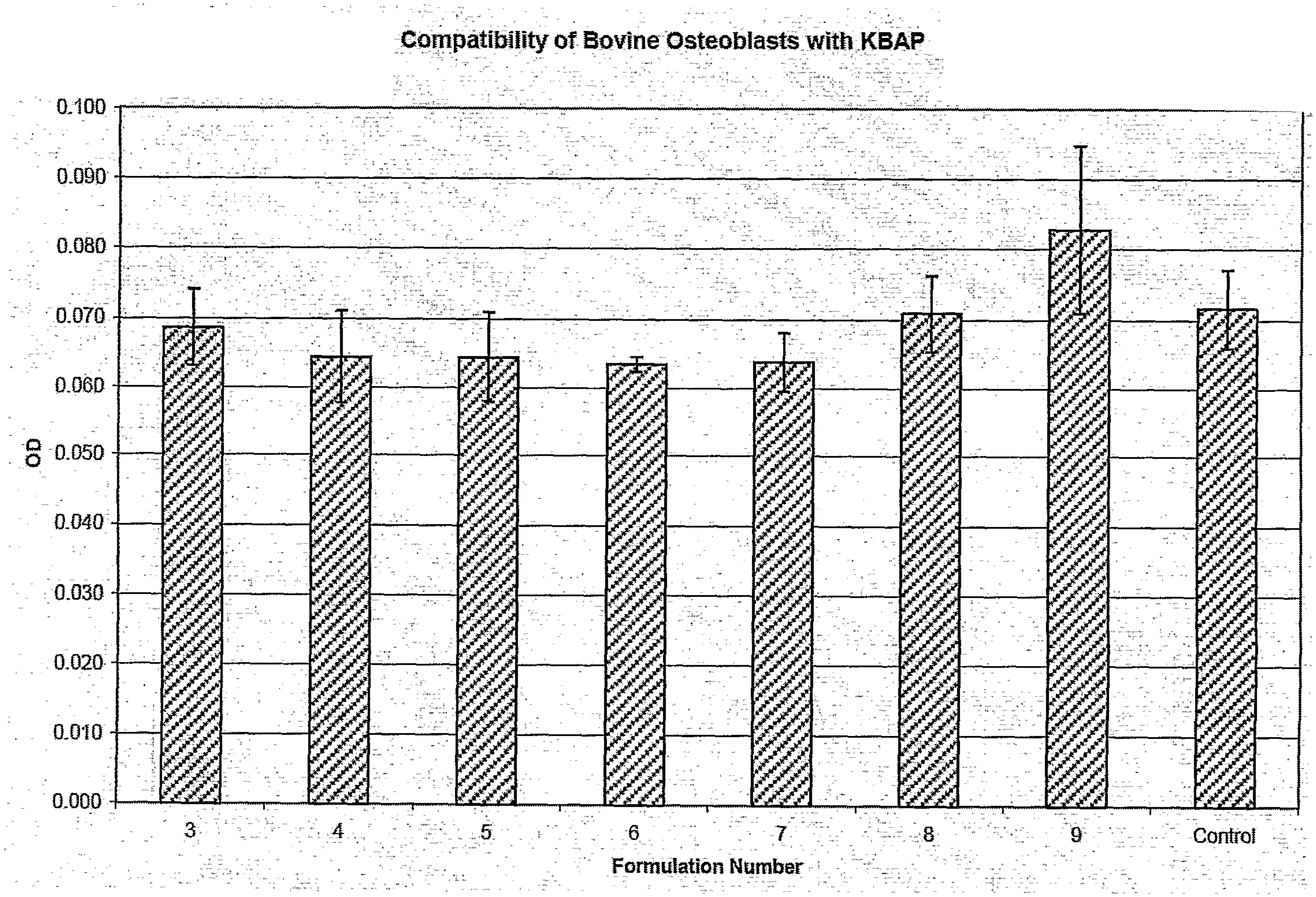


Figure 6. Growth of bovine osteoblasts in the presence of six different KBAP formulations compared to control conditions (media alone).

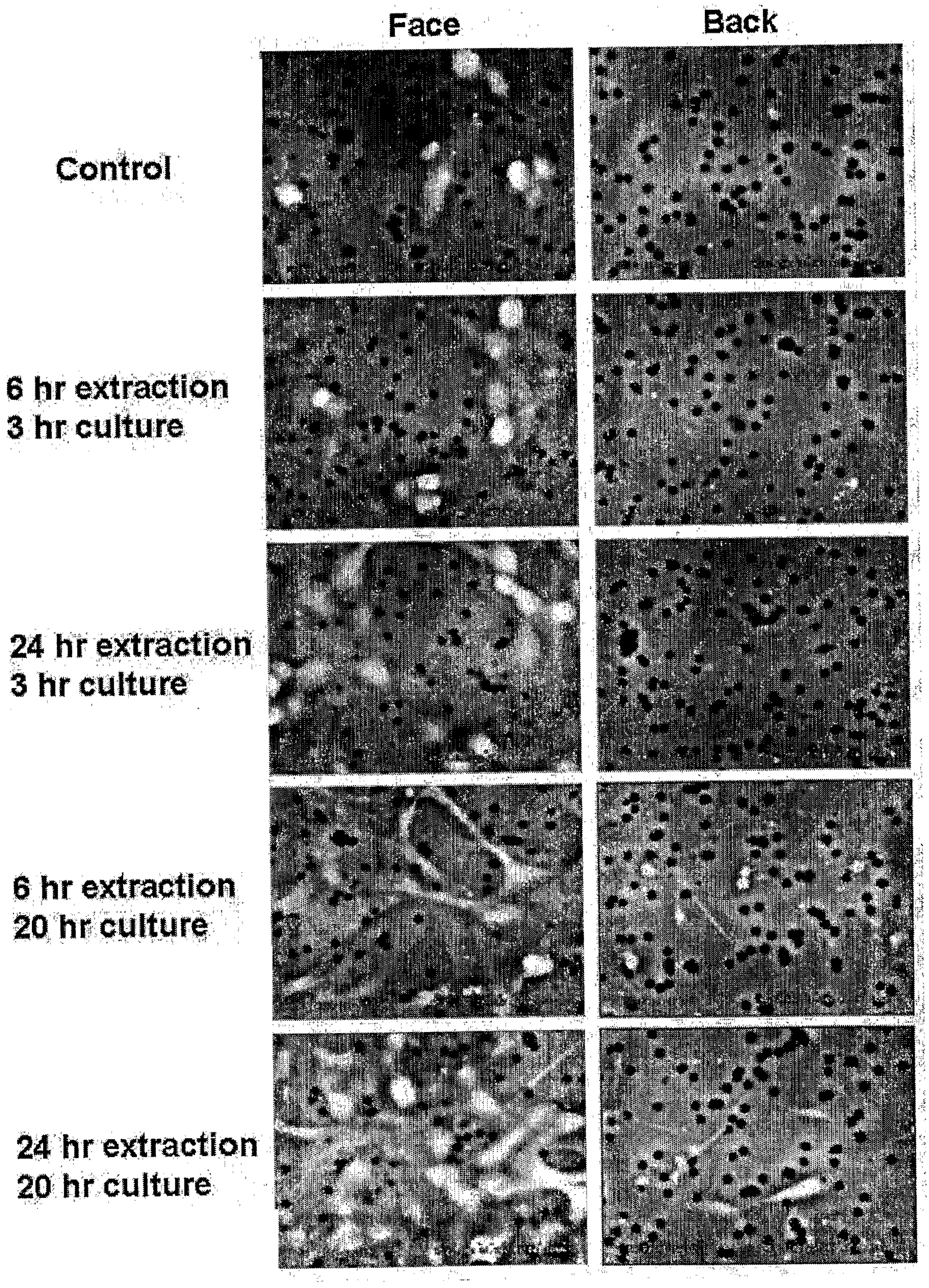

Face
Back
Control
6 hr extraction
3 hr culture
24 hr extraction
3 hr culture
6 hr extraction
20 hr culture
24 hr extraction
20 hr culture

Figure 7