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# Cellulose - A Biomaterial with Cell-Guiding Property

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Additional information is available at the end of the chapter

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## 1. Introduction

A biomaterial is defined as a material, either man-made or natural, intended to interact with biological systems. It does not have a chemical effect in the organism, nor thus it need to be metabolised to be active like for example drugs [1]. When inserted into the body, a local tissue inflammatory reaction called foreign body reaction is induced [2]. This reaction may either favour or adversely affect the tissue repair process.

Cellulose and its derivatives are well tolerated by most tissues and cells [3-5]. These non-toxic materials have good biocompatibility, therefore, they offer several possibilities in medical applications. Regenerated cellulose sponges have also been used in experimental surgery for decades as it does not affect the healing process, but acts as a chemoattractant inducing cells involved in the repair process to migrate towards it [6-8].

We have studied different biomaterials including cellulose in search for an optimal bone substitute. In bone defects, regenerated cellulose supported with cotton fibres was shown to allow new bone in-growth to some degree [9-11]. Oxidation with periodate and hydrogen peroxide, or carbamation further improved its biocompatibility but not enough to be used as bone substitutes. We also expected to increase the osteostimulating property of regenerated cellulose by coating it with a silica-rich hydroxyapatite (HA) as it resembles the mineral composition of bone. To our disappointment, the HA-coated cellulose did not promote bone formation but favoured instead inflammation and fibroplasia. Since the bone implant study revealed unexpectedly an enormous ability of the HA-implants to induce granulation tissue, the coated cellulose was tested subcutaneously as well. These studies showed that the HA-coated cellulose not only attracted inflammatory cells but also bone marrow-derived progenitor cells of both haematopoietic and mesenchymal origin (see box 1). In this chapter, we will discuss cellulose as implant material with emphasis on the cell guiding properties of regenerated cellulose coated with silica-rich HA.

## 2. Cellulose for medical applications and as a tissue engineering matrix

Cellulose, the most common organic compound on Earth, is degraded by microbial enzymes. Animal cells cannot cleave the  $\beta(1\rightarrow4)$ -bond between the two glucose moieties in cellulose. Thus, cellulose degradation in tissues takes place by a slow non-enzymatic hydrolysis of the  $\beta(1\rightarrow4)$ -bond and therefore cellulose can be regarded as an almost stable matrix. Despite this, cellulose and its derivatives are well tolerated by cells and tissues and induce a moderately strong foreign body reaction in the tissue [3-8].

### BOX 1. ADULT BONE MARROW-DERIVED STEM CELLS

Adult stem cells are immature cells, dispersed in tissues throughout the body after development. Like all stem cells, they are capable of either making identical copies of themselves or to differentiate depending on their local environment into mature cell types with characteristic morphology and function. Stem cells usually generate an intermediate, partly differentiated, cell type, called precursor or progenitor cell, before they achieve their fully differentiated state. Adult stem cells are rare, however. Their primary functions are to replenish dying cells, and with limitations, to regenerate damaged tissues.

The best characterised adult stem cells are those found in the bone marrow, which provides a unique niche for haematopoietic stem cells (HSCs) and the mesenchymal stem or stromal cells (MSCs). HSCs are responsible for the production and replacement of all blood cells during the entire lifetime [13]. The earliest haematopoietic precursor, the haemangioblast, is not only a precursor of haematopoietic cell lineages but also of cells that line all blood vessels and lymphatics, namely the endothelial cells [14].

Mesenchymal stromal cells are a heterogeneous population of stem/progenitor cells able to differentiate into several cell types such as chondrocytes, osteocytes, fibroblasts, myocytes, adipocytes, epithelial and neuron-like cells. When stimulated by specific signals, these cells can be released from their niche in the bone marrow into circulation and recruited to the target tissues where they undergo *in situ* differentiation and contribute to tissue homeostasis and repair [15]. MSCs also secrete factors that promote survival and differentiation of endogenous cells as well as angiogenic factors essential for blood vessel formation. MSCs possess remarkable immunosuppressive properties and can inhibit the proliferation and function of the major immune cell population [16, 17] as well as antimicrobial properties [18]. Furthermore, these multipotential stromal stem and progenitor cells at different stages of maturation contribute to the formation of HSC stem cell niche and play a critical role in haematopoiesis [19]. The characteristic of MSCs makes these cells exceptionally suitable for various therapeutic possibilities such as supporting tissue regeneration, correcting inherited disorders, dampening chronic inflammation, and delivering biological agents [15].

Cellulose is non-toxic and has good biocompatibility, therefore, it offers several possibilities in medical applications. Cellulose and its derivatives are used, among other things, as coating materials for drugs, additives of pharmaceutical products, blood coagulant, supports for immobilized enzymes, artificial kidney membranes, stationary phases for optical resolution, in wound care and as implant material and scaffolds in tissue engineering [3, 12].

### 2.1. Regenerated cellulose

Cellulose sponges can be manufactured by adding supportive strengthening fibres (8-10 mm long cotton fibres; about 20% of the weight of the cellulose) and sodium sulphate crystals as pore forming material to a cellulose viscose (sodium xantogenate) solution (4-6 g cellulose/100 g viscose). The cellulose is regenerated by heating the solution in a water bath after which the sponge is washed with hot water, treated with a dilute acid and sodium hypochlorite bleaching solution, and finally washed repetitively in distilled water before drying and sterilisation [20, 21]. When inserted subcutaneously, a vital and well vascularised repair tissue, called granulation tissue, grows rapidly into this cellulose sponge. Due to this good granulation tissue formation ability, cellulose sponges have been used in experimental surgery for decades [6, 7, 22] and the subcutaneous implantation of the cellulose sponge is widely accepted method for wound healing (see box 2) studies [8, 23]. Several cellulose products for wound healing purposes (e.g. Cellospon®, Cellstick®, Sponcal®, Visella®, and Absorpal®) are commercially available. These products are made from the sponge form viscose cellulose and have homogenous porous structure, characterized by thin pore walls with one or more inter-pore openings. They are elastic and can be compressed and expanded repeatedly with no damage to their internal structure, hence providing free entrance for the invading cells to the inner parts of the sponge [24].

Host reactions following implantation of biomaterials include injury, blood-material interactions, provisional matrix formation, inflammation, granulation tissue development, foreign body reaction, and fibrosis/fibrous capsule development [25]. When implanted subcutaneously, a blood-material interaction occurs with protein adsorption to the cellulose sponge and a blood-based transient provisional matrix, a blood clot; is formed on and around the sponge. The platelets, originated from the injured blood vessels, not only participate to haemostasis but also liberate bioactive agents like cytokines and growth factors that will attract inflammatory and phagocytosing cells. The first cells to arrive are polymorphonuclear leucocytes, i.e. neutrophils, which are characteristic for the acute inflammatory response. These cells secrete pro-inflammatory cytokines that, in turn, attract circulating monocytes, which are activated and converted in the tissue to macrophages that kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils. Biomaterial surface adherent macrophages can also fuse to form multinucleated foreign body giant cells. In their attempt to phagocytose the biomaterial, adherent macrophages become active [25]. By releasing a variety of chemotactic, neovasculogenic and growth factors that stimulate cell migration, proliferation and formation of new blood vessels and tissue matrix, macrophages mediate the transition from the inflammatory phase to the

proliferative phase. During the proliferative phase, the provisional extracellular matrix in the cellulose sponge is gradually replaced with granulation tissue, which is formed from infiltrated mature fibroblasts and rapidly proliferating mesenchymal stromal cells (MSCs) differentiating to fibroblasts *in situ*. The newly formed extracellular matrix is rich in blood vessels, which carry oxygen and nutrients to maintain the metabolic processes. The sponge is surrounded by a well-vascularised fibrous capsule, which becomes somewhat thinner during the final remodelling phase [38].

Similar biocompatible regenerated cellulose developed for wound healing studies has also been tested as a scaffold for cartilage tissue engineering. Although the cellulose sponge provided a non-toxic environment for cartilage cells, the construct remained soft and lacked the extracellular matrix composition typical for normal articular cartilage [26]. When implanted into bone defects, regenerated cellulose strengthened by cotton fibres allowed new bone in-growth to some extent [9-11].

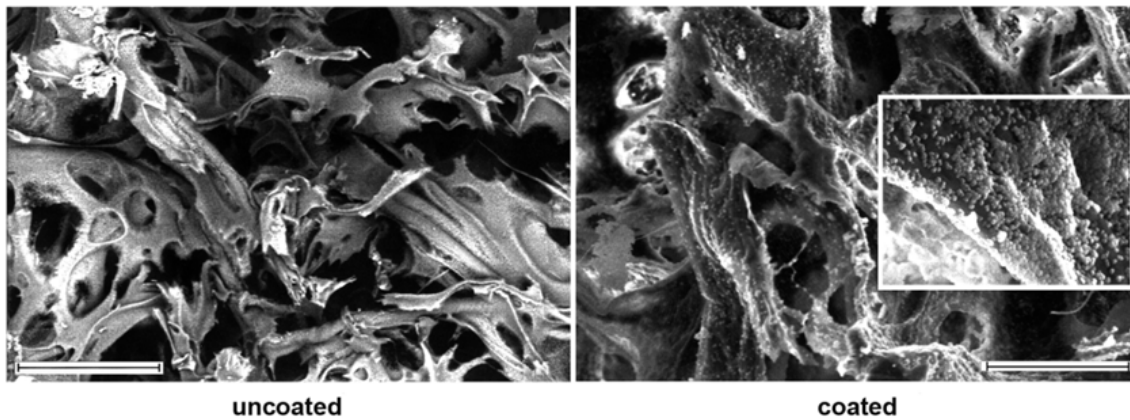
### 2.1.2. Hydroxyapatite-coating of regenerated cellulose

The number of cells and tissue in-growth are affected to a certain limit by the porosity, size of pores, and the thickness of the pore walls of the cellulose sponge [8]. We hypothesised that coating the regenerated cellulose with hydroxyapatite (HA) that resembles the mineral composition of bone, would improve its bone forming properties. The mineral originated from a specific bioactive glass, S53P4 (23% Na<sub>2</sub>O, 20% CaO, 4% P<sub>2</sub>O<sub>5</sub>, 53% SiO<sub>2</sub>) that has a good osteoconductivity and is in clinical use [27-32]. However, glass as such, is difficult to trim to the desired size and form. Furthermore, it is brittle and fragile, and therefore, not suited in sites subjected to load like in femoral and tibial bone defects.

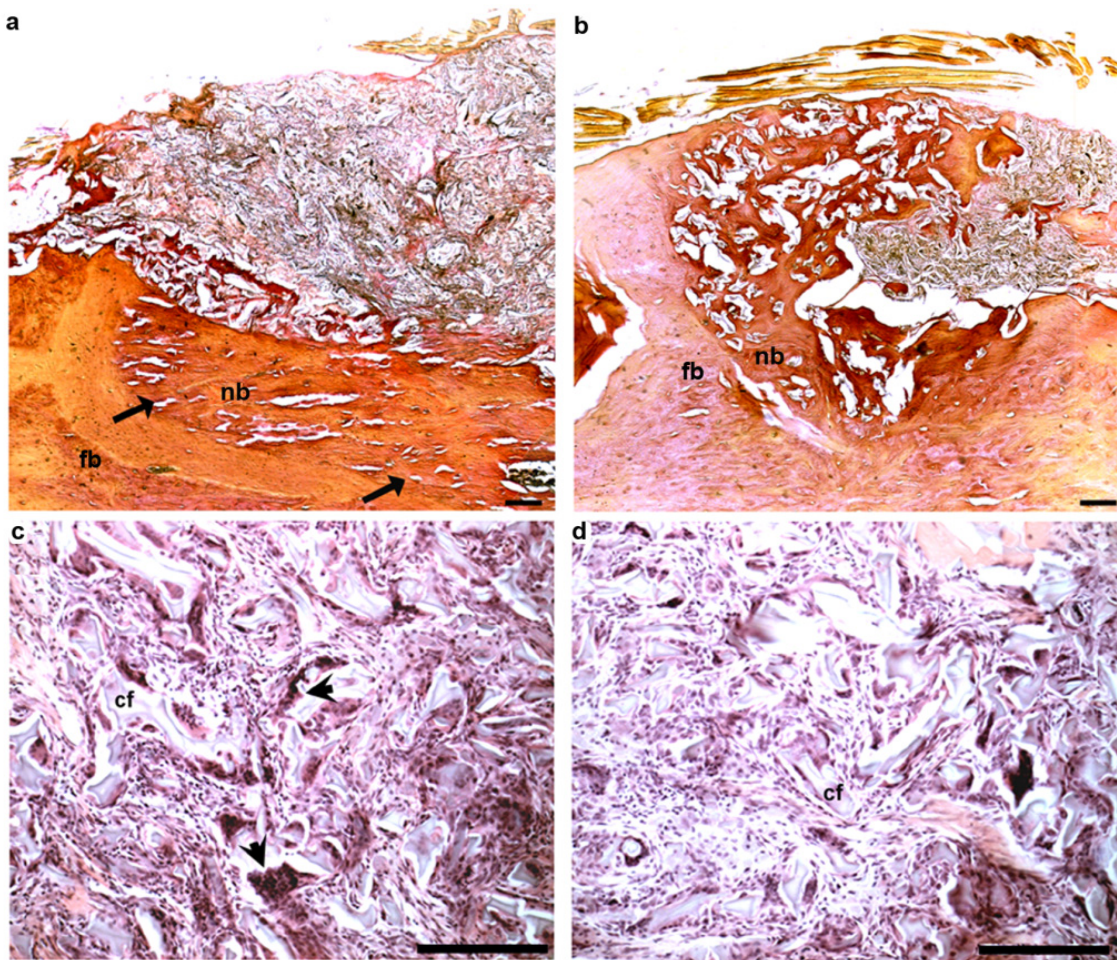
In our studies, the calcium phosphate layer was precipitated on cellulose sponges (10 x 100 x 100 mm) with average pore sizes between 50 and 350 µm by the biomimetic method of Kokubo et al [33]. Mineralisation was initiated in 500 ml of sterile simulated body fluid (SBF) supplemented with a 2.0 g of the bioactive glass at 37°C for 24h and was then grown in 500 ml sterile 1.5 x SBF for 14 days at the same temperature under continuous shaking. The SBF solution was changed every second day. The formed calcium phosphate layer rich in silica was verified by scanning electron microscope (figure 1) and characterised with Fourier transform infrared spectroscopy [11]. (1 x SBF = 136.8 mM NaCl, 4.2 mM NaHCO<sub>3</sub>, 3.0 mM KCl, 1.0 mM K<sub>2</sub>HPO<sub>4</sub> x 3H<sub>2</sub>O, 1.5 mM MgCl<sub>2</sub> x 6H<sub>2</sub>O, 2.5 mM CaCl<sub>2</sub> and 0.5 mM Na<sub>2</sub>SO<sub>4</sub>, pH 7.4; ion concentration close to that of human plasma)

Sterile HA-cellulose and untreated cellulose sponges, sized 2.3 x 3 x 8 mm, were implanted into femoral bone defects of male rats aged 10-13 weeks (for further details see [11]) and were followed up for 52 weeks. The implants were analysed histologically and with biochemical and molecular biologic methods. The HA layer did not improve the bone in-growth into the cellulose sponge. In fact, the new bone was instead mainly formed beneath the implant at the bottom of the defect leaving the implant filled with a well vascularised fibrous tissue rich in inflammatory cells (figure 2). The inflammatory reaction was much stronger than in the uncoated cellulose indicated by the larger number of inflammatory cells





**Figure 1.** SEM micrograph of regenerated uncoated and HA-coated cellulose sponges (bar = 50  $\mu\text{m}$ ). The hydroxyapatite layer was initiated in sterile 1  $\times$  SBF with bioactive glass at 37  $^{\circ}\text{C}$  for 24 h and was then grown in sterile 1.5  $\times$  SBF at the same temperature for 14 days under continuous shaking.



**Figure 2.** HA-coating of cellulose prevents bone in-growth. One year after implantation into rat femoral bone defect, new bone (nb) growth is mainly observed beneath (arrows) the HA-implant (a), which has been pushed out from the defect area. The HA-implant itself (b) is mostly filled with soft connective tissue containing abundant giant cells (arrow heads). Uncoated cellulose implant (c) allows new bone in-growth and the non-ossified parts contain less inflammatory cells. (a and c; van Gieson stain; b and d haematoxylin-eosin stain; cf = cellulose fragment; scale bar = 100  $\mu\text{m}$ , modified from [11]).

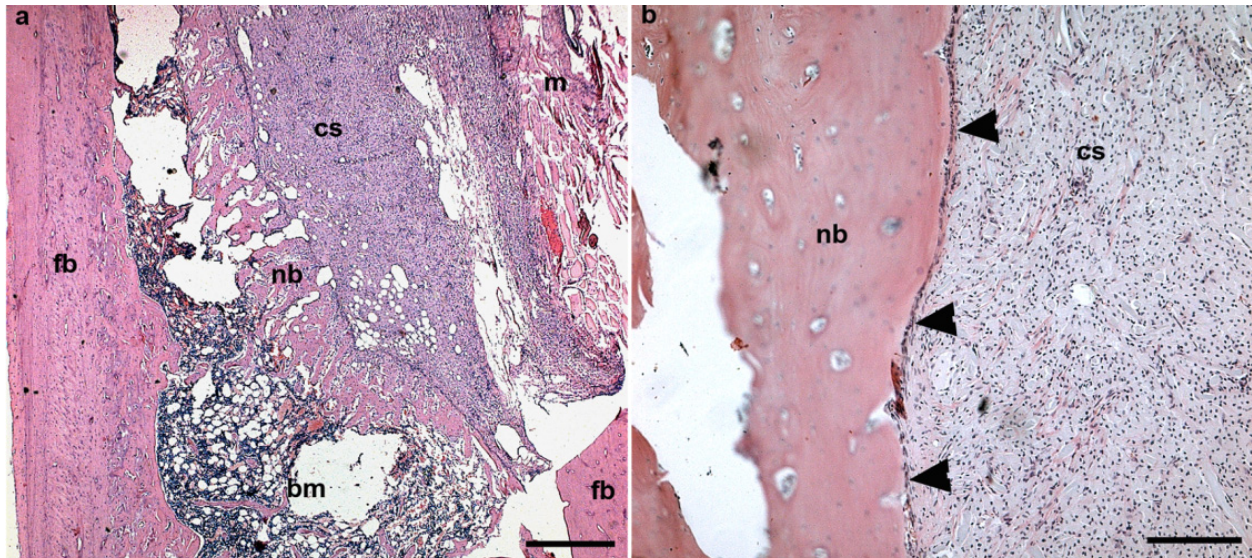
including macrophages and foreign body cells, which also is a sign of chronic inflammation. Activated inflammatory cells produce many pro-inflammatory bioactive agents, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), which is known to interfere with the bone specific transcription factor Cbfa1 and to depress the function of differentiated osteoblasts [34,35]. Continuous exposure to these agents may, thus, inhibit differentiation of the progenitor cells into bone forming osteoblasts explaining, as least partly, the less osteoid tissue in HA-coated cellulose implants. Furthermore, the HA layer did intensify the attachment of transforming growth factor beta 1 (TGF $\beta$ 1) [11], a growth factor involved in fibroplasia. Hence, the HA surface did not offer any advantages in comparison with untreated cellulose in cortical bone defect healing.

## 2.2. The effect of increased biodegradability of cellulose

Another approach to improve the biocompatibility of cellulose was to alter its chemical structure in order to increase its biodegradability. The mild bleaching and oxidation of regenerated cellulose with sodium hypochlorite carried out during the preparation of cellulose sponge does not cleave the glucose ring and the resultant cellulose is not biodegradable, which probably prevented complete ossification of the implanted sponge. Therefore, in the search for suitable bone defect fillers, we extended the material development with a two sequential oxidation steps. Firstly, the cellulose was oxidated by periodate for 1-3 hours. This treatment opens some glucose molecules and should theoretically make them more susceptible to glucosidases and other enzymes capable for carbohydrate degradation. Excess periodate was washed by ascorbate or thiosulphate and water before the second oxidation by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 3 or 4 hours. As the oxidation reactions were not complete, the resultant materials are combinations of 2,3-dialdehyde and 2,3-dicarboxyl celluloses. The biogradability of the celluloses was tested in SBF for 7, 15 and 30 days. Oxidations for 3 h in periodate followed by 4 h in H<sub>2</sub>O<sub>2</sub> turned out to be the best combination as 70% of the material was dissolved. Therefore, this material was used for further testing. No cytotoxicity was observed in fibroblast cultures. The material has to be sterilised by 70-95 % ethanol or ethylene oxide because autoclaving destroys the porous structure of the scaffolds.

The results of the bone implantation experiments (figure 3 a, b) showed that oxidised scaffolds were flattened, their pores had disappeared and the material was completely replaced by cells so that no visible cellulose fibrils were observed in the implantation sites. The degradation was not complete as the phagocytosing cells were full of homogenous material. It is conspicuous, however, that no giant cells were observed in the oxidised samples, whereas normal cellulose always induces a number of foreign body giant cells. If the sponges were oxidised more extensively their structures collapsed and the material could not be used for implantation. The implanted scaffolds did not show, on the other hand, any significant bone in-growth. Instead they consisted of cell masses that histologically were strikingly homogeneous. New bone had been grown on the opposite site of the implant strengthening the defect site. Despite improved biodegradability, oxidised cellulose was considered to have no value as a bone substitute.



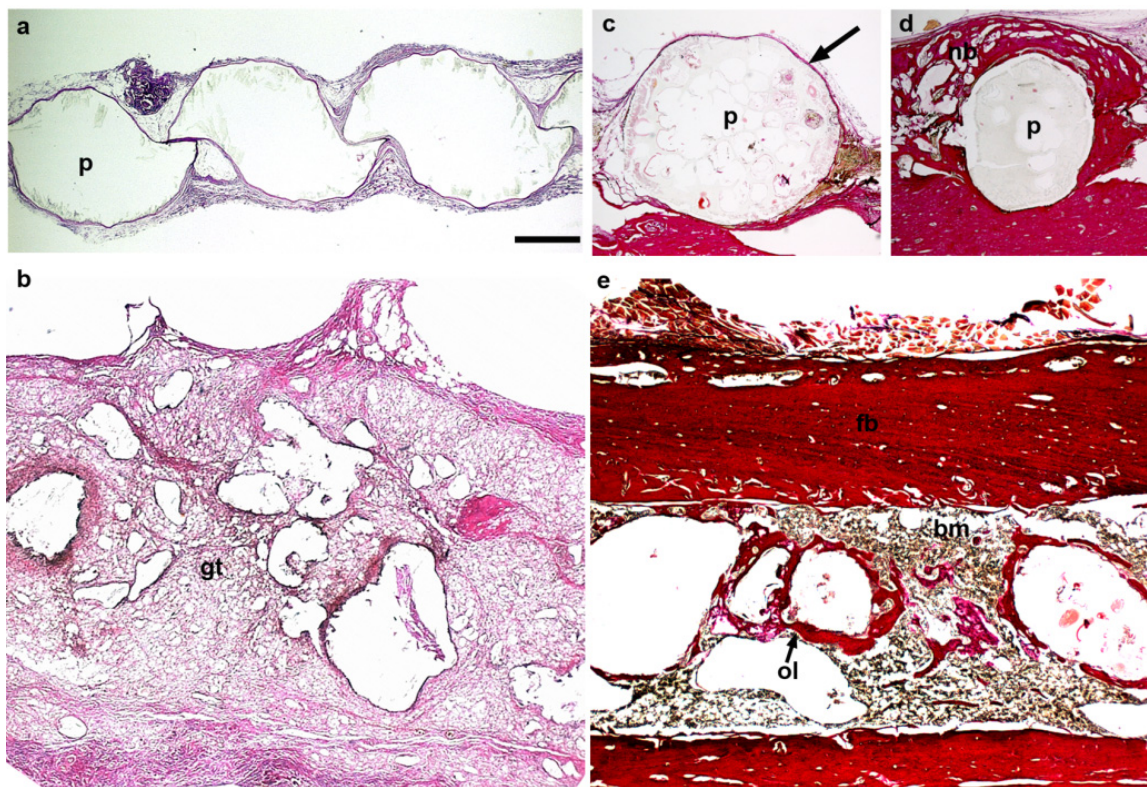


**Figure 3.** Oxidations with periodate and  $\text{H}_2\text{O}_2$  increase the biocompatibility and degradation of cellulose. Oxidised cellulose (a, b) allows new bone (nb) formation when implanted into femoral bone (fb) defects of rat. (cs = cellulose scaffold, bm = bone marrow, m=muscle overlaying the implant site, arrow heads point at osteoblasts lining the new bone; haematoxylin-eosin stain; scale bars = 100  $\mu\text{m}$  (a), and 25  $\mu\text{m}$  (b)).

Biodegradation of cellulose can also be improved by treating it with urea. The resultant carbamino cellulose showed increased solubility that could be regulated by the duration of treatment. The fundamental aim was to develop material that could be used as a vehicle for drugs in tablets, or perhaps for subcutaneous long-lasting administration of drugs. Small, round or oval cellulose pearls with 50-500  $\mu\text{m}$  diameters can be manufactured from regular or carbamino cellulose by dropping viscose into a solution containing 100 g  $\text{H}_2\text{SO}_4$  and 200 g  $\text{Na}_2\text{SO}_4/\text{l}$  at  $20^\circ\text{C}$  followed by centrifugation [36]. Four and six per cent viscose solutions were used to make the 0.5 mm diameter cellulose pearls. The material was collected, washed with distilled water and 5g  $\text{H}_2\text{SO}_4/\text{l}$  and dried for 24 hours at  $40^\circ\text{C}$ . Sterilisation was carried out by autoclaving or with 70% ethanol.

For implantation studies, several pearls were glued together with alginate [37] in moulds. The results from the subcutaneous implantation experiment (Figure 4 a, b) were encouraging as implanted 4% cellulose pearls were infiltrated with new granulation tissue and most of the pearls showed signs of nearly complete degradation where as 6%-pearls were more resistant during the observation period of two weeks. Intramedullary implantation into rat femoral bone (figure 3 c-e) showed similar behaviour: many of the 4 %-pearls were infiltrated by new granulation tissue and some were surrounded by new osteoid tissue. There was some variation in the degree of degradation; while some pearls had been digested completely, some remained almost intact. No foreign body giant cells were observed, however. We do not know whether alginate surroundings affected the degradation of pearls in the bony environment, but to make the carbamino cellulose more useful in medical applications, the structure should be further altered to become even more vulnerable to hydrolytic enzyme attacks, especially if used for subcutaneous administration of drugs.





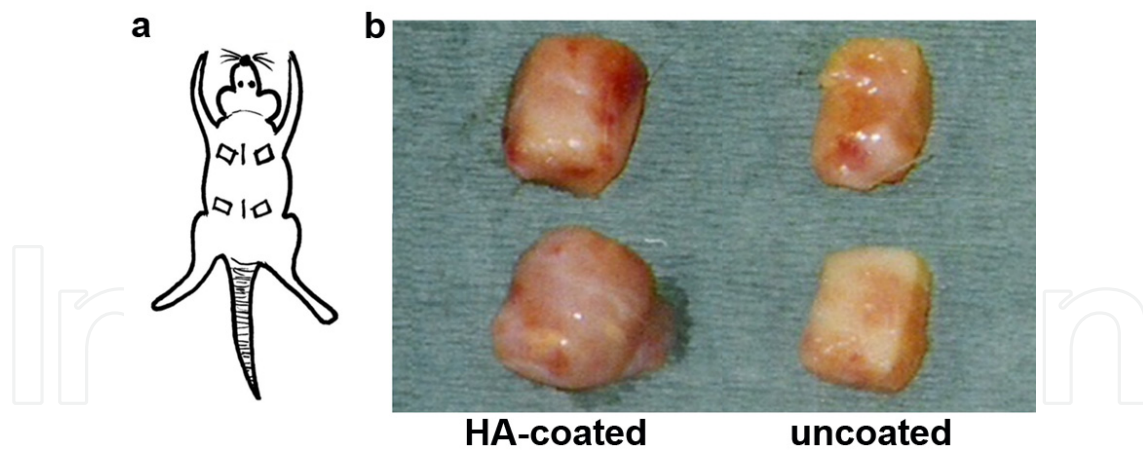
**Figure 4.** Tissue reactions of carbamino cellulose two weeks after implantation. Subcutaneously implanted 6%-cellulose pearls (p) stayed intact and showed only modest degradation (a), whereas b) 4%-cellulose pearls were degraded and infiltrated with new granulation tissue (gf). Similar behaviour was observed in bone implants: c) 6%-cellulose pearls were surrounded by a thin connective tissue capsule (arrow) whereas about half of the b) 4%-cellulose pearls were partially degraded and surrounded by bone (nb) or a thin osteoid layer (ol) even in the bone marrow (bm) area. (van Gieson stain; equal magnifications; scale bar 200  $\mu\text{m}$ ).

### 2.3. The biological effect of subcutaneously implanted hydroxyapatite-coated cellulose

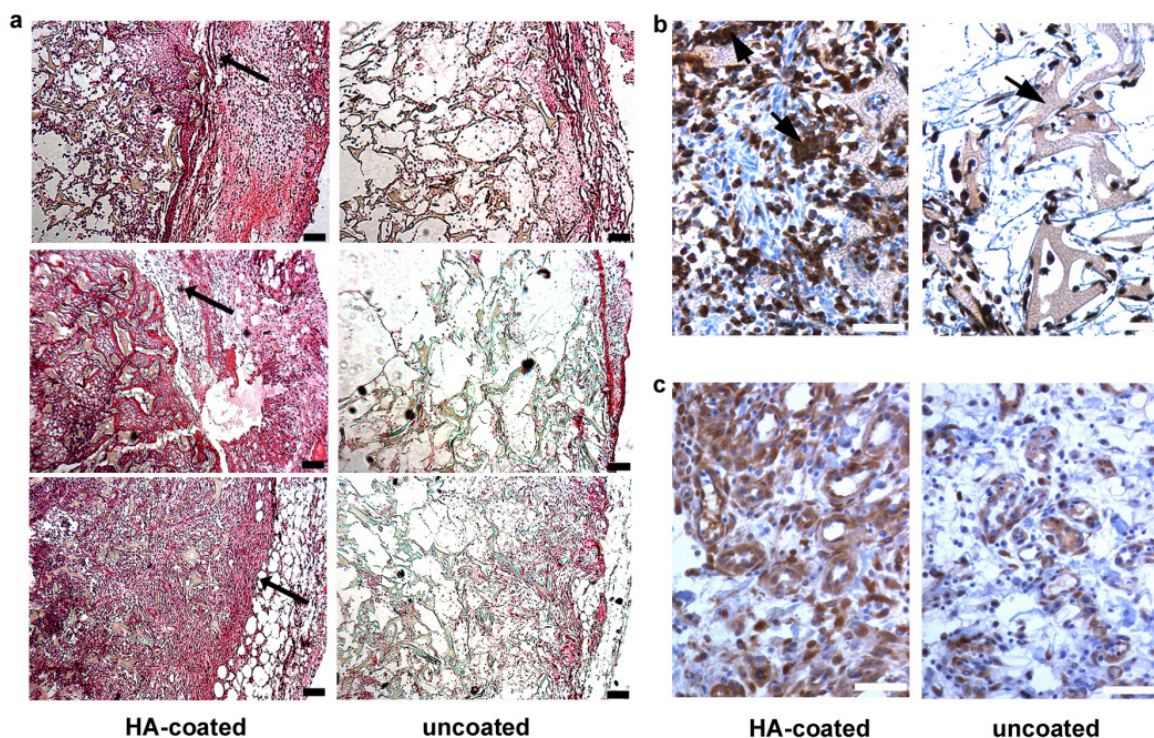
The bone defect study showed that HA-coated cellulose favoured rapid fibrous tissue proliferation instead of bone formation [11]. Therefore, it was considered to have no value as a bone replacement material but might be useful in other applications in which accelerated granulation tissue formation is needed. Subcutaneously (figure 5 a, b) implanted silica rich HA-implants showed a massive inflammatory reaction with an intense foreign body reaction and increased invasion of fibrovascular tissue already 1-3 days after implantation. Such strong tissue reaction was not seen with any other subcutaneously implanted cellulose sponge. Tissue growth into uncoated regenerated cellulose was much slower and took place mainly on their surface (figure 6). [38]

Subcutaneously implanted HA-sponges activate the inflammatory response and the secretion of cytokines and growth factors important to wound healing, such as TGF- $\beta$ 1, TNF- $\alpha$ , vascular endothelial growth factor (VEGF) and platelet derived growth factor A (PDGF-A). The long-term study revealed, however, that the excessive connective tissue



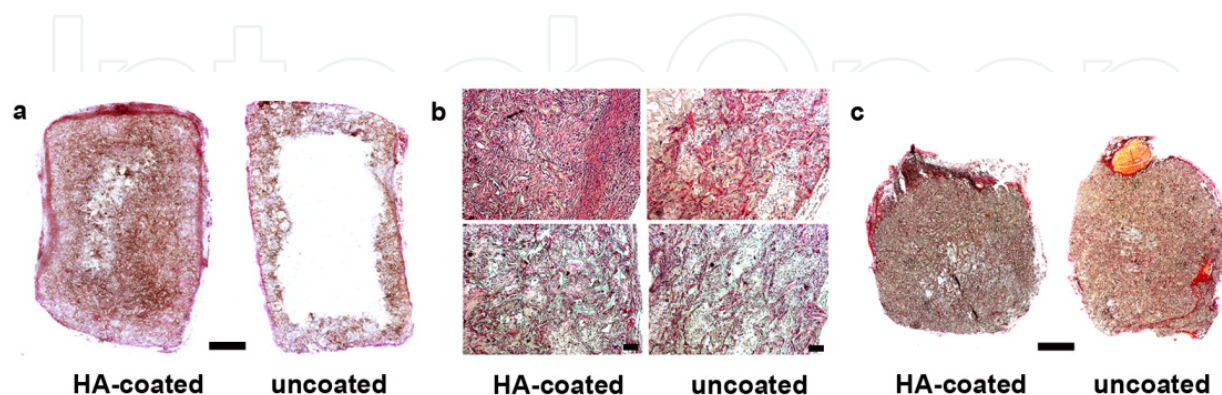


**Figure 5.** a). A schematic presentation of the subcutaneous implantation model used in our studies. Two midline incisions were made on the back of the rats, and sterilised, moistened sponge implants (10 × 5 mm) were inserted bilaterally into subcutaneous pockets under general anaesthesia. b). Subcutaneously implanted cellulose sponges 7 days after implantation. HA-coated implants are darker in colour as a sign of high cellularity and rich neovascularisation, whereas the uncoated implants are pale.



**Figure 6.** The HA-coating accelerated tissue growth into subcutaneously implanted cellulose sponges as well as the inflammatory response and blood vessel formation. a) Haematoxylin-eosin-stained sections 1 (upper), 3 (middle), and 7 (lower) days after implantation. The arrows in HA-coated sponges point at the border between the implant and the surrounding capsule (scale bar = 100  $\mu$ m). b) HA-coated sponges contain large clusters (arrows) of accumulated macrophages (brownish coloured cells). Macrophages favour gathering near to cellulose fibres (arrow head) (day 5; scale bar = 50  $\mu$ m). c) More blood vessels, as indicated by CD31-staining, can be seen in 5-day-old HA-coated sponge compared to uncoated one (scale bar = 50  $\mu$ m).

formation, which is histologically normal, does not disturb the animals in any way. After 14 days postoperatively, the foreign body reaction in HA-coated sponges starts to diminish. At one month, the difference between the HA-coated and uncoated cellulose had levelled off and at the end of the study, at one year no obvious histological difference between the coated and uncoated were detected (figure 7). [38]



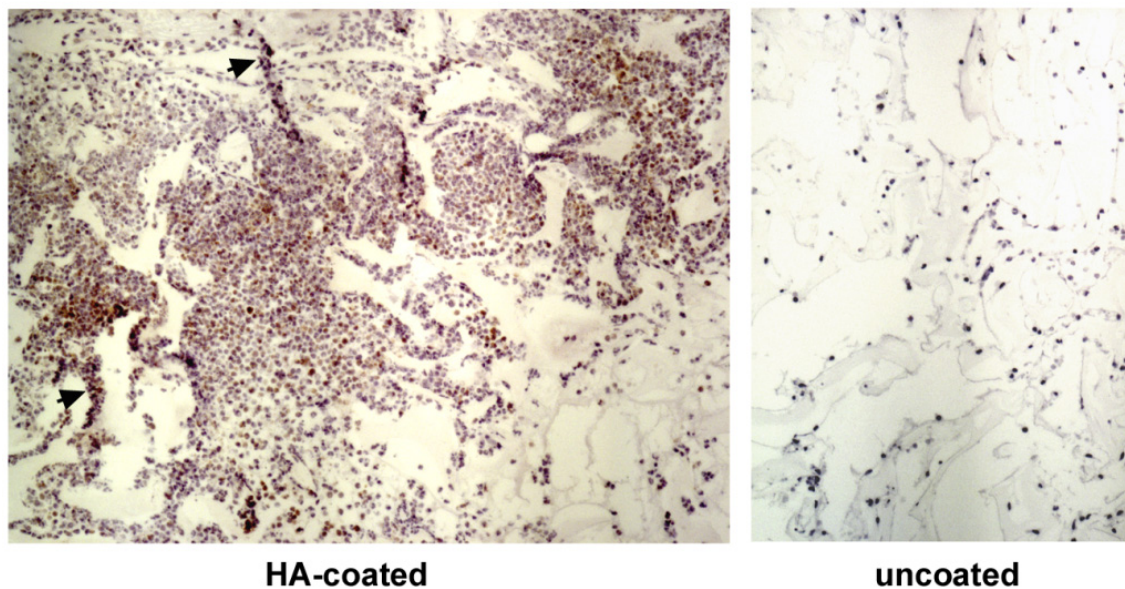
**Figure 7.** Histology of subcutaneous cellulose implants. a) At 14 days HA-coated sponge is filled with granulation tissue (van Gieson-stained whole implants, scale bar = 1000 µm). b) Haematoxylin-eosin-stained sections one and three months after implantation, scale bar 100 µm. c) At one year no significant difference can be observed between HA-coated and uncoated sponges (van Gieson-stained whole implants, scale bar = 1000 µm. Modified from [38]).

### 2.3.1. Cell trafficking and homing to regenerated cellulose

Cellular movement and re-localisation are essential for many fundamental physiologic properties, not only during embryonic development, but also during wound healing and organ repair. At the wound site, local and infiltrated cells release chemokines that recruit blood-circulating stem and progenitor cells. These bioactive agents also increase bone marrow cell mobility, thus facilitating cell mobilisation into the peripheral blood and consequently into the sites of wound healing [39]. Stromal-derived factor-1 (SDF-1) is one powerful chemokine in stem cell trafficking that regulates both haematopoietic, endothelial and mesenchymal progenitor cells. The biological effects of SDF-1 are mediated by the chemokine receptor CXCR4 [40-43]. During the early stages of wound healing, SDF-1 seems to be up-regulated by the influence of pro-inflammatory factors like TNF- $\alpha$ , which creates a SDF-1 concentration gradient that triggers the recruitment of CXCR4-expressing cells from the blood stream to the site of injury, where these cells further differentiate into other functional repair cells [44].

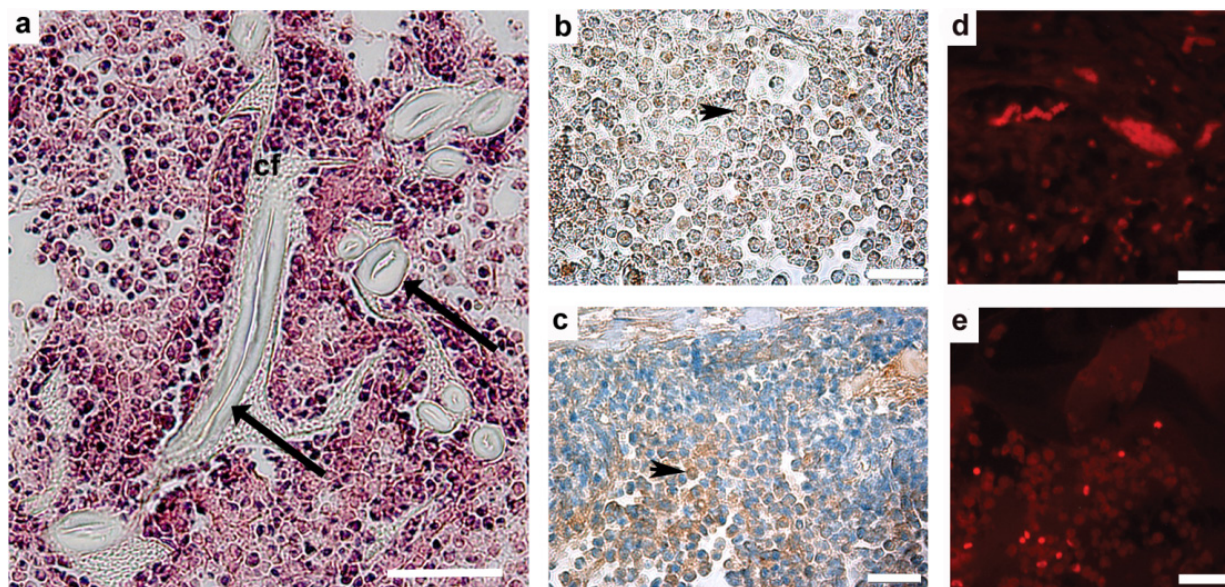
Mineralised cellulose implant not only attracts more inflammatory cells than uncoated cellulose but also circulating bone marrow-derived stem cells of both haematopoietic and mesenchymal origin [45]. SDF-1 expression (GEO series accession no. GSE19748 and GSE19749; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxx>) is upregulated in HA-sponges together with its receptor CXCR4 (figure 8). This strongly indicates that the HA-coated implant has a better homing capacity of circulating bone marrow-derived stem cells than the uncoated one.





**Figure 8.** HA-coated cellulose contain large amount of CXCR4-positive cells. Numerous clusters (arrow heads) of and individual CXCR4-positive (brownish coloured) cells are detected throughout the HA-coated sponge at day 7.

Haematopoietic stem cells seem to be the first stem cells to invade the empty centres of the HA-coated cellulose implants (figure 9 a-c). The more abundant occurrence of HSCs is most probably responsible for the augmented blood vessel formation in HA-coated cellulose. The earliest haematopoietic precursor, the haemangioblast, is namely the precursor for both haematopoietic and endothelial cell lineages, not only during embryogenesis but also in adults [14, 46]. The haematopoietic progenitors, especially in the HA-coated implants, were located in close contact with the cellulose fragments (figure 10 a). Hence, the coating of cellulose with HA creates an environment that facilitates stem cell homing more efficiently than uncoated cellulose. In the bone marrow, undifferentiated HSCs are detected near the inner surface of the medullary cavity, i.e. the endosteum, in the so-called endosteal stem cell niche. At this site, the bone is in constant turnover: bone is formed by the osteoblasts and removed by specific macrophages, the osteoclasts. Due to bone degradation, soluble calcium ions ( $\text{Ca}^{2+}$ ) are released into the bone marrow fluid. Various cells, including primitive HSCs, respond to extracellular ionic calcium concentrations through a calcium sensing receptor, CaSR. This receptor seems to have a function of holding HSCs in close physical nearness to the endosteal surface [47]. The mineral layer on the cellulose resembles that of bone. When the numerous foreign body giant cells/macrophages gathered around the mineralised cellulose try to get rid of the foreign material,  $\text{Ca}^{2+}$  is released generating a beneficial milieu for the primitive HSCs as it resembles the endosteal stem cell niche in the bone marrow. This theory is supported by the numerous CaSR-positive cells near the mineralised cellulose fibres, in the same areas as cells positive for CD34, a common marker for endothelial cells, are observed. These cells are not only found in the granulation tissue but also in the central parts of the implant. Similar cells are seen in uncoated cells, but in remarkably less quantity (figure 9 d-e).



**Figure 9.** Stem cells are located near the cellulose fragments. a) General histology showing cells gathering around the cellulose fragments (cf) at day 7. The arrows point at pores (scale bar = 50  $\mu\text{m}$ ). b). HA-coated implants contains numerous cells (arrowhead) positive for c-kit, a marker for premature cells (day 7; scale bar = 25  $\mu\text{m}$ ). c) Small rounded cells (arrowhead) positive for CD34, a commonly used marker for HSCs (day 7; scale bar = 25  $\mu\text{m}$ ). More CaSR-positive cells (red fluorescence) are observed in HA-coated implants at day 7 (d) than in uncoated (e) sample (scale bar = 25  $\mu\text{m}$ ).

In the cellulose implants, mesenchymal stem cells are mainly found in the forming granulation tissue [45] in line with the fact that these primitive cells home to the wound site and differentiate into connective tissue cells that produce the extracellular matrix of the granulation tissue [48]. In addition, MSCs secrete signals that limit systemic and local inflammation, decrease apoptosis in the threatened tissue, stimulate neovascularisation, activate local stem cells, modulate the immune cells, and exhibit direct antimicrobial activity [18, 49, 50]. Therefore, the more abundant occurrence of MSCs in the HA-coated cellulose sponge most probably contribute to the enhanced blood vessel formation compared to uncoated cellulose and to the declining of the foreign body reaction during the second week of implantation. MSCs also secrete many cytokines that stimulate haematopoiesis, mainly the myeloid cell lineage, but MSCs seems to have a supportive effect on erythropoiesis, the process of red blood cell formation, as well [51].

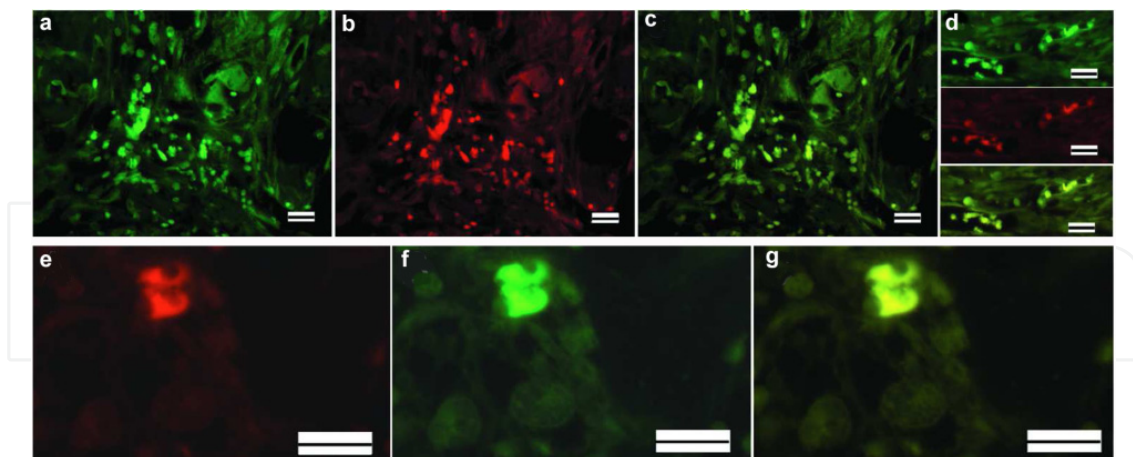
### 2.3.2. Experimental granulation tissue expresses haemoglobin

An unexpected finding was that the granulation tissue induced by cellulose sponge contains haemoglobin producing glycophorin A-positive cells (figure 10 a-d) indicating that the haematopoietic precursor cells are also able to differentiate into the erythropoietic lineage [52]. This, in turn, suggests that this repair tissue is capable of making blood. In healthy adults, globin has been considered to be expressed only in the bone marrow area by immature erythropoietic precursors. When the mature red blood cell or erythrocyte emerges from the bone marrow, it has lost its nucleus, ribosomes and mitochondria, which means that the cell is no longer capable of gene expression. As in bone marrow, where erythroid



progenitors mature in association with macrophages [53, 54], the plentiful macrophages, especially in the HA-coated implants, might further back up the erythropoietic differentiation of HSCs in the granulation tissue. Microarray data (GEO series accession no. GSE19748 and GSE19749; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxx>) revealed many genes related to erythropoiesis like erythropoietin and its receptor EpoR, the transcription factors Hif-1 $\alpha$ , gata-1 and -2, and particularly Alas2, which is exclusively expressed in developing red blood cells called erythroblasts and is required for the expression of  $\beta$ -globin [55].

Haemoglobin has traditionally been thought to serve as the main oxygen transporter in erythrocytes. Many studies, including ours [56-65], show, however, that haemoglobin expression is much more versatile than previously has been assumed. During granulation tissue formation in the cellulose sponges, the haemoglobin expression pattern showed a biphasic pattern [52]. The first peak appeared during the most intense inflammatory response in the initiation of the healing process before invasion of HSCs, indicating that also another cell type is participating in the haemoglobin expression. Since active macrophages are known to express globin [64], these cells (figure 10 e-g) are most likely responsible for the early globin expression in the granulation tissue. In macrophages, the globins are most probably involved in processes different from oxygen transport and delivery to tissues. There is accumulating evidence that haemoglobin also binds, stores and transports nitric oxide. Nitric oxide is an important gaseous signalling molecule in wound healing [66] involved, among other things, in the formation of granulation tissue and new blood vessels [67-69]. While nitric oxide is a prerequisite for successful wound healing, an excess of this signalling molecule may be as harmful as its underproduction [67]. The fact that an intense expression of inducible nitric oxidase synthase (iNOS), an enzyme that catalyses the



**Figure 10.** Double staining confirmed different haemoglobin positive cell types in cellulose implants. The granulation tissue in cellulose sponges contains haemoglobin (a) -producing glycophorin A-positive cells (b) implying that haematopoietic precursor cells are able to differentiate into red blood cells. c) Merged image of haemoglobin- and glycophorin A-positive cells. d). Red blood cells in a blood vessel in the capsule area of HA-implant; haemoglobin (upper) positive, glycophorin A (middle) and merge image (lower). e) CD-68 positive cells indicating macrophages. The same cells are also positive for haemoglobin (f). (g) Merged image of CD-68- and glycophorin A-positive cells (scale bar 20  $\mu$ m, modified from [52]).



formation of nitric oxide, which reflects the production of nitric oxide observed in 3-day-old HA-implant, but not at day 10 [52], coincides with the strong inflammatory reaction that starts to decline during the second week of implantation [38]. The production of haemoglobin during this phase might eliminate the excess nitric oxide and prevent its negative effect on matrix deposition, neovascularisation and apoptosis. In uncoated cellulose implants, iNOS is detected at day 10, which supports the observation of slower sequence of events in the granulation tissue formation in these uncoated implants

### 3. Conclusions and future perspectives

Regenerative medicine involves tissue formation and healing in order to restore the functionality of damaged organs or tissues. As tissue repair and regeneration after injury involve the selective recruitment of circulating or resident stem cell populations, stem cell therapy is often employed as one mean to promote tissue regeneration. Its success might, nevertheless, be complicated by strong immune-rejection of transplanted cells or shortage of autologous cell supply. Furthermore, if a scaffold, with or without bioactive agents, is used to administrate the stem cells, poor integration between the scaffold/implant and the host tissue might affect the outcome.

An interesting tissue engineering concept is cell guidance aimed at total *in vivo* tissue engineering without the need of adding bioactive agents or cells. Numerous studies have shown that cellulose itself functions as a chemoattractant and is able to stimulate granulation tissue formation. Uncoated cellulose sponge has been tested in treatment of chronic leg ulcers (Pajarre, unpublished data) and in severe burn injuries (Lagus, unpublished data) in the 1990's with good results. The cellulose sponge adsorbs debris and bacteria from the wound site and attracts inflammatory cells. In these cases, a short-term, powerful inflammatory response is actually necessary. After cleaning the wound bed, the cellulose induces vital granulation tissue formation, and smoothes and prepares the wound bed for successful skin transplantation.

The fascinating property of HA-coated cellulose sponge is its ability to even further amplify the healing mechanisms of the body. The HA-coated cellulose acts as a cell-guiding material, attracting stem cell reserves. The novel finding of haemoglobin expression during wound healing brought into daylight new data concerning blood formation and development of neovascularisation. The clinical relevance of this is the production of more vascularised granulation tissue in the critical early phases of wound healing.

We hypothesise that the cell guiding property of the HA-coated cellulose is due to the combination of silica and calcium phosphate. Preliminary results (Stark et al, unpublished) from our on-going study show that a mineral layer induced by dipping the cellulose sponge in a calcium phosphate solution has not the same beneficial feature on granulation tissue formation (not shown) than the mineral layer induced by the bioactive glass in simulated body fluid. Although there was a somewhat stronger inflammatory reaction when compared to uncoated cellulose it was not as intense as in HA-coated cellulose. Our deduction is that the silica elevates the inflammatory reaction with enhanced level of bioactive agent production

that attracts more circulating bone marrow-derived progenitor cells whereas the calcium phosphate layer contributes to hastened stem cell homing to the cellulose sponge.

Due to the cell-guiding property of silica rich HA-coated regenerated oxidised cellulose in combination with the capacity to promote proliferation of richly vascularised connective tissue, this material might have potential in clinical situations when rapid granulation tissue growth is needed as in treatment of poorly healing wounds. The contact with the HA-cellulose sponge would be local and temporary, therefore minimizing any possible disadvantages. In addition to safety issues, the manufacturing process of coating cellulose with HA is relatively simple and cheap, and the HA-coated cellulose sponge is easy to handle, form and sterilise.

## BOX 2. BIOLOGY OF WOUND HEALING

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in the body. The physiological and coordinated response to injury is practically similar in all tissues and involves three distinct but overlapping phases that can be divided into inflammation, new tissue formation and remodelling [70]. In turn, these three phases comprehend coordinated series of events that includes chemotaxis, phagocytosis, neocollagenesis, collagen degradation, and collagen remodelling. Furthermore, neovascularisation, epithelisation, and the production of new glycosaminoglycans (GAGs) and proteoglycans are vital during wound healing process.

The key initiators of the healing process are the platelets, which within minutes after injury aggregate and form fibrin clot in aim to control bleeding. In addition to their important role in hemostasis, platelets also liberate growth factors that will attract inflammatory and phagocytosing cells. The first cells to arrive are polymorphonuclear leucocytes, i.e. neutrophils that secrete proinflammatory cytokines. Shortly thereafter circulating monocytes will appear, are activated and converted in the tissue to macrophages that kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils. Macrophages also mediate the transition from the inflammatory phase to the proliferative phase by releasing a variety of chemotactic agents and growth factors that stimulate cell migration, proliferation and formation of tissue matrix.

The second phase of wound healing is often called the proliferative phase or the granulation tissue formation phase. This stage starts normally two to three days after injury and lasts approximately two to three weeks. During this phase the provisional extracellular matrix is gradually filled with granulation tissue. The phenomenal feature is to diminish the area of tissue loss by contraction and fibroplasia. The infiltrated cells produce a new extracellular matrix, rich in blood vessels, which carry oxygen and nutrients to maintain the metabolic processes. Although new collagen and other extracellular matrix proteins are continuously actively synthesised, the earlier formed

fibrin clot is enzymatically degraded. This process allows the proceeding of re-epithelisation that is needed to control the growth of the repair tissue and wound closure. The proteolytic activity is also a prerequisite of the neovascularisation.

Usually by three weeks after injury, new tissue formation starts to decrease, and the emphasis of wound healing process turns to the remodelling and maturation. The main objective of this phase is to achieve maximum tensile strength by reorganisation, degradation and re-synthesis of the extracellular matrix. This final process may last even several years, before the new granulation tissue rich in cells and vascular capillaries has matured into a relatively acellular and avascular scar that lacks appendages, including hair follicles, sebaceous glands, and sweat glands [70].

apoptosis	programmed cell death
biocompatibility	the ability of a material to perform with an appropriate host response in a specific application
chemoattractant	a chemical (chemotactic) agent that induces an organism or a cell to migrate toward it
chemokine	small chemotactic pro-inflammatory cytokine
chemotaxis	directional movement in response to the influence of chemical stimulation
cytokine	a small cell-signalling protein molecule secreted by numerous cells; involved in intercellular communication
endosteum	a thin layer of connective tissue lining the medullary cavity of bone
fibroplasia	the process of forming fibrous tissue
growth factor	a naturally occurring substance capable of stimulating cell growth, proliferation and cell differentiation
haemostasis	the process that causes bleeding to stop
haematopoiesis	production of all types of blood cells including formation, development, and differentiation of blood cells
phagocytosis	an important defence mechanism against infection by microorganisms (e.g. bacteria) and the process of removing cell debris (e.g. dead tissue cells) and other foreign bodies
receptor	a structure on the surface of or inside a cell that selectively receives and binds a specific substance
stem cell niche	a local tissue microenvironment that maintains and regulates stem cells
transcription factors	molecules, usually proteins, which are involved in regulating gene expression

Table 1.



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