

In vitro assessment of the proinflammatory characteristics of two different hyaluronan based products

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Sammanfattning

Q-Med är ett snabbväxande företag som tillverkar medicinska implantat baserat på hyaluronsyra. Hyaluronsyra är en molekyl som finns i nästan alla kända arter med identisk kemi. Ren hyaluronsyra har en kort livslängd i kroppen. Q-Med har utvecklat en patenterad teknik som gör hyaluronsyra mer stabil i kroppen i form av en injicerbar gel.

Inflammation är kroppens svar på fysisk skada som kan bero på t.ex. en infektion eller mekanisk skada. Immunförsvaret är extremt känsligt för främmande ämnen innanför huden. Hyaluronsyraprodukter som inte ses som kroppsegna kan orsaka en inflammation.

Design av medicinska implantat baserat på hyaluronsyra medför alltså svårigheter. Implantatet måste vara stabilt för att inte brytas ned omedelbart men får heller inte avvika allt för mycket från ren hyaluronsyra.

Mitt examensarbete är en studie då inflammatoriska egenskaper hos två olika produkter baserad på hyaluronsyra från Q-Med utvärderas.

Examensarbete 20p
Civilingenjörsprogrammet Molekylär bioteknik
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Abstract

Q-Med is a rapidly growing biotechnology/medical company in Uppsala Sweden that develops, manufactures, markets and sells primarily medical implants. The majority of the products are based on the company's patented technology, NASHA™, which enables production of stabilized non-animal hyaluronan, a sugar molecule present in soft tissue. The degree project presented in this report was established between Q-Med and Dr Marjam Ott at the department of Physical and Analytical Chemistry, division Surface Biotechnology, Uppsala University Sweden in February 2008. The project includes *in vitro* assessment of the proinflammatory characteristics of two different products namely gelA and gelB which are both used on the market as wrinkle fillers.

Introduction

Inflammation appears as redness and swelling in tissue due to a reaction of vascularised living tissue to local injury. This serves to contain, neutralize, dilute or wall off an injurious agent or process. An inflammation occurs if the body is infected, mechanically damaged or subjected to antigens (substances that trigger the immune system). Increased blood flow in combination with higher capillary permeability in the vascular tissue allows for quick concentration of inflammatory cells and mediators to the site of inflammation. The inflammation will proceed until the stimuli is removed, and if not, it can lead to a chronic inflammation causing tissue damage¹.

Aim of the project

- The aim of the project was to develop models for *in vitro* inflammatory studies of hydrogels from Q-Med.
- To evaluate inflammatory responses. Examine for differences in between gel products.

Mononuclear phagocytes

Mononuclear phagocytes are the immune system's degraders of unwanted microbes and particles. The word phagocyte comes from Greek and means cell eater. Monocytes are important cells belonging to the mononuclear phagocyte system and are derived from pluripotent bone marrow stem cells under the influence of soluble hematopoietic growth

factors and physical interactions with stromal cells and extracellular matrix proteins. Monocytes remain very briefly in the bone marrow to then enter the blood stream where they circulate for 1 to 3 days being available throughout the body. Circulating monocytes can develop further into macrophages. Monocytes may become resident macrophages in the tissues and serous cavities² with specific functions acquired dependent on their localization. The circulating monocytes can be seen as the reservoir to recruit and/or replace macrophages to an inflamed area or wherever that cell number is too low. Macrophages can be found in organs such as lungs, spleen, liver and the skin where they play important roles because i) as residing cells they are always present for immediate response and ii) they will become specialized for that specific organ. Macrophages of the lung, *alveolar macrophages*, are highly phagocytic being able to rapidly degrade inhaled harmful substances whereas *Langerhans cells*, macrophages in the skin are specialized at antigen presentation to recruit other immune cells.

In the case of an inflammation monocytes will also migrate into tissue (see Figure 1) and become macrophages. A macrophage performing its functions is said to be an activated macrophage. The activating stimuli can come from cellular factors, intracellular activators, adhesion molecules or bacterial molecules such as lipopolysaccharide (LPS, a major component of the outer membrane of gram negative bacteria). The response of an activated macrophage is a change in gene expression, the cell becomes more adherent and metabolically active which leads to effector functions such as phagocytosis,

scavenger functions or cell killing. The efficient phagocytic properties of the

macrophages are dependent on the possession of hydrolytic enzymes, proteases and

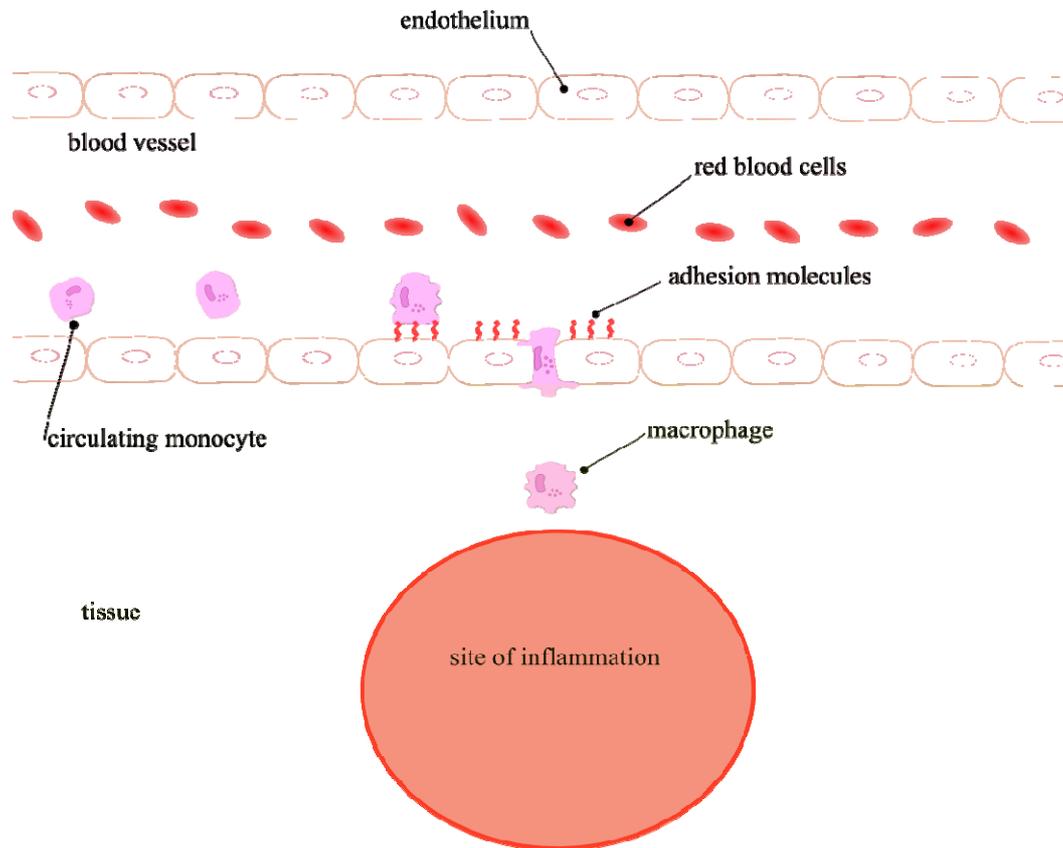


Figure 1: Circulating monocytes attracted to an inflamed site. The monocytes leave the blood stream migrating through epithelial by interactions of expressed adhesion molecules. Further stimuli leads to differentiation to macrophages, where they serve as one of the most important phagocytes.

myeloperoxidase (MPO), an enzyme which generates highly microbicidal halides. Particles that are to become phagocytosed are engulfed and simultaneously surrounded with a part of the cell membrane forming an intracellular vesicle called phagosome. The compartment of the phagosome will be degraded when exposed to the enzymes mentioned above. Additionally, macrophages are capable of generating reactive oxygen intermediates, reactive nitrogen intermediates, cytolytic proteases and cytokines such as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ that all have cell cytotoxic properties when being released out of the cell. Invading microorganisms, foreign cells, particulate matter and apoptotic cells are all targets for phagocytosis. These are either recognized as targets by receptors on the macrophage cell surface or opsonised with antibodies or complement factors.

U937, a monocytic cell line

Established monocytic cell lines are often used as models to investigate the differentiation and activation processes of monocytes. U937 is a monoblastic cell line grown as a single cell suspension that can be induced to monocytes/macrophages³. U937 is derived from human malignant cells. The cell line was established in 1974 by Prof. K. Nilsson at Rudbeckslaboratoriet in Uppsala⁴. U937 cells are frequently used in macrophage/monocyte studies and were therefore also chosen for our inflammatory studies.

U937 cells can be induced to differentiate to macrophages/monocytes by eg. phorbol ester (PMA) and Vitamin D_3 . After differentiation the U937 cells stop to proliferate and become arrested in the G_0/G_1 phase. Phenotypically, differentiated U937 changes morphology and increase expression

of certain cells-surface proteins i.e. Fc receptors and MHC molecules. U937 has been shown to increase IL-1 β secretion by LPS stimulation *in vitro*⁵. CD14 is the receptor that binds to LPS and triggers production of inflammatory cytokines, e.g. TNF- α , IL-1 β and IL-6. However, CD14 is only expressed by differentiated U937 cells.

Hyaluronan (also known as hyaluronic acid and HA)

A human body of 70 kg contains about 15 g hyaluronan of which one third is turned over every day⁶. The hyaluronan molecule illustrated in Figure 2 is an acidic glucosaminoglycan. One hyaluronan molecule consists of alternating *N*-acetyl glucosamine and glucuronic acid units linked by β -1-3 and β -1-4 glycosidic bonds⁷. Up to 30 000 monosaccharides can be linked in this way, thus giving very large molecules⁸ with molecular weights over 10⁷ Da. The average hyaluronan molecule consists of 10 000 units⁹. Hyaluronan was isolated for the first time in 1934 from the vitreous of the eye from cattle¹⁰.

Back then it was only identified as a novel, high molecular weight glycosaminoglycan. Its right structure was determined first in 1954¹¹. Hyaluronan has since then been studied extensively and is now used in many medical applications¹².

Hyaluronan is present in all soft tissues in the human body reaching its highest concentrations in the synovial fluid and in the vitreous humour of the eye. The molecule can be found in vertebrates and bacterias such as group C *Streptococcus*¹³. It can also be produced recombinantly in for example *Bacillus subtilis*¹⁴. Commercially hyaluronan is obtained from rooster combs or *Streptococcus*.

Hyaluronan possesses unusual physiochemical properties; it can form very large molecules that are able to form random coils. It also has a large capacity to interact with water molecules. Altogether, hyaluronan gives viscous and elastic solutions that makes the molecule suitable for filling, lubrication and filtering functions.

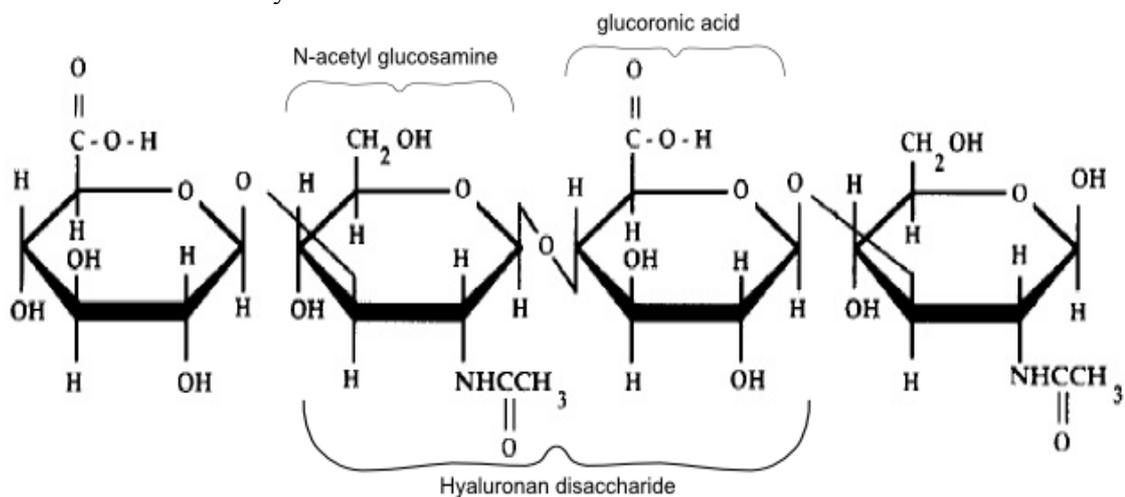


Figure 2: The structure of hyaluronan.

A commonly known feature of hyaluronan is that it is an extracellular filling molecule that hydrates the skin and lubrication in the synovial fluid. However, research on hyaluronan has revealed many more and complex roles of this molecule¹⁵, e.g. formation of extracellular matrices, regulation of cell motility, proliferation and cell-signalling and promotion of monocyte adhesion¹⁶. The speculations of its role in promoting cell motility suggest an indirect mode of action, making the extracellular or pericellular surroundings more conducive to migration.

Hyaluronan is involved in many interactions between proteins (both inside and outside the cell) and receptors. In the extra cellular matrix, hyaluronan is often bound to proteins from the link protein family. Mice lacking these proteins die shortly after birth due to defects in cartilage development which indicates the importance of these proteins¹⁷. An extensively studied receptor with ability to bind hyaluronan is CD44. The interaction between hyaluronan and CD44 activates several cellular responses, e.g. rapid reorganization of the actin cytoskeleton and induction of lamellipodia formation¹⁸. CD44 also plays a critical and complex role during inflammation, i.e. in recruitment of macrophages¹⁹. Studies have also shown that hyaluronan fragments of certain lengths can activate the nuclear transcription factor NF- κ B.

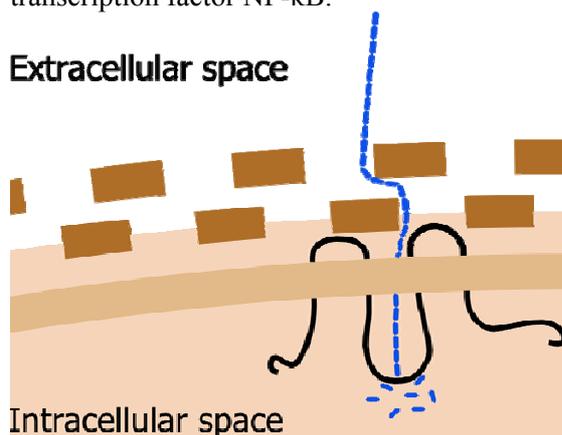


Figure 3: Illustration of bacterial HAS proteins located in the membrane extruding a hyaluronan molecule into the extracellular space.

(HAS). These are transmembranal proteins which synthesizes at the inner surface of the plasma membrane together with an extrudation of hyaluronan out to the extracellular matrix. An illustration of a bacterial HAS producing a molecule of hyaluronan is seen in Figure 3. This synthesis is unique since other glycosaminoglycans are synthesised by another mechanism in the Golgi apparatus. Production of hyaluronan is an energetically demanding process and is thereby tightly regulated by the majority of cells in vertebrates.

Recently, the role of hyaluronan inside the cell has gained increasing interest. It is clear that hyaluronan is extruded out of the cell during synthesis, but also that exogenous hyaluronan can be taken up by endocytosis. Hypotheses of the role of intracellular hyaluronan includes, maintaining cell shape, modifying and regulating the nuclear matrix, modifying the cytoskeleton and maybe even influencing elements of the chromosome. Hyaluronan are used in several treatments/products, i.e. arthritis treatment, in eye drops and wrinkle fillers.

Hyaluronan is synthesised by a group of enzymes called hyaluronic acid synthases

Materials and Methods

Cultivation of U937 cells

U937 cell line was cultivated in DMEM F12 (Invitrogen™), supplemented with 10% FCS, 100 IU penicillin/ml, 100 µg streptomycin/ml, 2nM L-glutamin and 1% non essential amino acids. The growth environment was an incubator with a humidified atmosphere with constant temperature at 37°C and 5% CO₂. Cells were grown in an aerated cell culture

flask and the cell concentration were kept between $0.2-0.8 \times 10^6$ cells/ml by replacement of about two thirds of the medium after agitation approximately every third day.

Freshly grown U937 cells were differentiated to monocytes by addition of 0.1 µM of Vitamin D₃ (1 α ,25-Dihydroxyvitamin D₃, SIGMA), followed by an incubation in 96 hours.

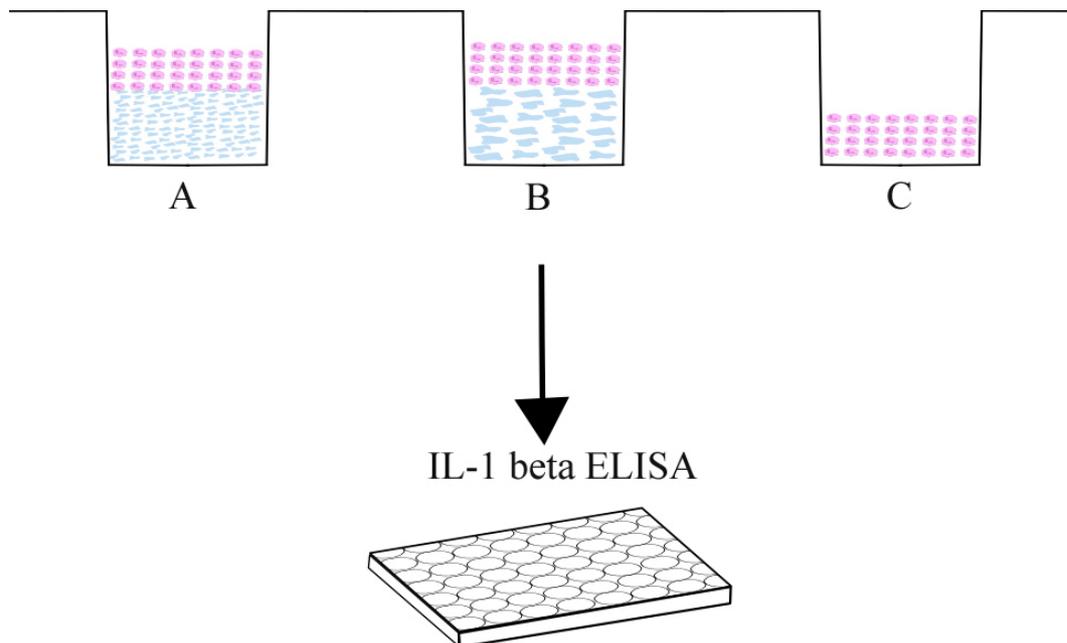


Figure 4: Overview of the experiment set up. Cells can become exposed to different hydrogels in separate wells **A** and **B**. Cells can also be added as controls in wells without any gel **C**. The inflammatory response can be measured with the ELISA method from samples collected from the wells.

Procedure to expose cells to hydrogels

For the cell experiments (illustrated in Figure 4) flat bottomed sterile 24 well plates (Figure 5) from Nunc with a culture area of 1.9 cm² was used. Exposure to hydrogel of the cells was achieved by covering the bottoms with gel before adding the cells. 0.33 ml of gel per well was used to ensure total coverage of the bottom area. Cells were added to the wells in cultivation medium. To cover the gel surface with liquid, at least 0.75 ml of culture (cell medium with cells) had to be added to hydrogel containing wells.

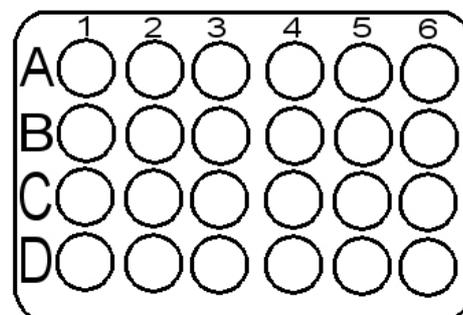


Figure 5: Illustration of a 24-well polystyrene plate (NUNC) used in this study

	1	2	3	4	5	6
A	GelB + Chondroitinase (0.51U in 50 μ l HBSS)	GelB + Chondroitinase (0.26U in 25 μ l HBSS)	GelB + Chondroitinase (0.13U in 13 μ l HBSS)	GelA + Chondroitinase (0.51U in 50 μ l HBSS)	GelA + Chondroitinase (0.26U in 25 μ l HBSS)	GelA + Chondroitinase (0.13U in 13 μ l HBSS)
B	HA solution + Chondroitinase (0.51U in 50 μ l HBSS)	HA solution + Chondroitinase (0.26U in 25 μ l HBSS)	HA solution + Chondroitinase (0.13U in 13 μ l HBSS)	GelB + Hyaluronidase (11kU in 140 μ l HBSS)	GelB + Hyaluronidase (5kU in 69 μ l HBSS)	GelB + Hyaluronidase (1kU in 14 μ l HBSS)
C	GelA + Hyaluronidase (11kU in 140 μ l HBSS)	GelA + Hyaluronidase (5kU in 69 μ l HBSS)	GelA + Hyaluronidase (1kU in 14 μ l HBSS)	Empty well	Empty well	Empty well

Table 1: Experimental set up of degradation study with the enzymes hyaluronidase and chondroitinase on a 24-well polystyrene plate (NUNC).

ELISA procedure

A general description of the ELISA method can be seen in the appendix **Fel! Hittar inte referensskälla.** In the experiments, ready-to-use ELISA-kit was used to measure IL-1 β secretion of cells exposed to hydrogel material. We used Quantikine® IL-1 β High sensitivity kit or corresponding kit from Bender MedSystems according to the manufacturer's protocol. Each sample was measured in duplicates on the ELISA plate.

Determination of particle size distributions of hydrogels

GelB and GelA were stained with Alcian Blue Staining Solution (ABSS). A 1 $\frac{w}{v}$ % of Alcian Blue (8GX Sigma Aldrich) stock solution in 3 $\frac{w}{v}$ % acetic acid was diluted to a final concentration of 0.5 $\frac{w}{v}$ % in 0.15 M NaCl. Incubation of ABSS with the respective gels was made at room temperature for 1 hour in a volume ratio of 1:1.

Monolayers of stained GelA and GelB particles were digitally photographed. The particles of GelA were photographed from a light microscope (4x magnification). Single particles (that had a gel/"jelly like" surface and were stained blue) on images were manually identified in ADOBE photoshop. Smaller fragments that did not have this attribute were considered as background present in both gel

types. The area for each particle was thereafter determined using MATLAB.

Degradation of hydrogels

GelB and GelA were added to wells on a 24-well polystyrene plate (NUNC) in the amount of 0.2-0.4 ml per well. 750 μ l of DMEM F12 (containing 10% FCS, 100 IU penicillin/ml, 100 μ g streptomycin/ml, 2nM L-glutamin and 1% non essential amino acids) was carefully added to the wells containing gel. The two hyaluronan degrading enzymes Chondroitinase ABC (Sigma) and Hyaluronidase (Sigma) were dissolved in Hank's balanced salt solution (HBSS) and prepared separately in different concentrations (Table 1). The plate was incubated at 37°C, 5% CO₂ in a humidified atmosphere. 50 μ l fractions of Alcian Blue stained gel was examined repeatedly every 15 minute for 2 hours using a light microscope. The particle sizes of the different gel types was compared optically thereby giving an approximate degradation rate.

Quantification of proteolytic activity of Hyaluronidase

Azocoll (CALBIOCHEM®) was used to analyze protease contamination in Hyaluronidase samples purchased from SIGMA. Azocoll was dissolved (10 mg/ml) in phenol red free Alpha Medium (GIBCO) supplemented with 10% FCS, 100 IU

penicillin/ml, 100 µg streptomycin/ml, 2nM L-glutamin and 1% non essential amino acids. 11.6 kU/ml of Hyaluronidase and 2.5 % Trypsine were added to the two azocoll solutions and blanks with only azocoll were used as references. Absorbance at 520 nm was measured after 15 and 50 minutes, 2.5 hours and after 24 hours.

Il-1 β production of U937 after 1 hour of incubation

Both differentiated and undifferentiated U937 cells were added to wells with no hydrogel present. Cells were added in the amount of 0.27×10^6 cells/well in 0.75 ml medium. Cells were either subjected to LPS (10 ng/ml) or left untreated at 37°C for 1 hour. Samples from the wells were stored in a freezer (-80°C). Interleukin-1 β levels of thawed samples were analysed with ELISA (Quantikine[®]) following the manufacturers protocol with the use of cultivation medium instead of the provided assay diluent as the only exception.

Il-1 β production of U937 after 24 hours of incubation with hydrogels

Differentiated U937 in the amount of 0.2×10^6 cells per well cells were added to wells with GelA or GelB hydrogels. In the presence of the different hydrogels, the cells were either subjected to LPS (10 ng/ml) in singlet wells or left untreated at 37°C for 24 hours in duplicates. Samples were collected from the supernatant covering the gel and from wells with gel degraded with Hyaluronidase (11.6 kU; 1 hour; 37°C). The samples were stored in a freezer (-80°C). Levels of interleukin-1 β in thawed samples were analysed with ELISA (Quantikine[®]) following the manufacturers protocol with the use of cultivation medium instead of the provided assay diluent as the only exception.

Il-1 β production of U937 after 24 hours of incubation with hydrogels and larger cell amounts

Differentiated U937 in the amount of 0.3×10^6 cells per well were added to wells with GelA or GelB hydrogels, hyaluronan (20 mg/ml) or just cultivation medium. Cells were either subjected to LPS (10 ng/ml) in singlet wells or left untreated in triplicates in 37°C for 24 hours. Samples were collected from the supernatant covering the gel and from wells with gel degraded with Hyaluronidase (11.6 kU; 1 hour; 37°C). The samples were stored in a freezer (-80°C). Interleukin-1 β levels of thawed samples were analysed with ELISA (Bender Med systems) following the manufacturers protocol with the use of cultivation medium instead of the provided assay diluent as the only exception.

Il-1 β production of U937 after 24 hours of incubation with pre-degraded hydrogels

Before addition of any cells wells were prepared with GelA or GelB hydrogels or hyaluronan (20 mg/ml). The gels were degraded (1 kU Hyaluronidase; 24 hours; 37°C) and differentiated U937 in the amount of 0.3×10^6 cells per well were thereafter added to all wells. Cells were either subjected to LPS (10 ng/ml) in singlet wells or left untreated in triplicates in 37°C for 24 hours. Samples from the wells were stored in a freezer (-80°C). Interleukin-1 β levels of thawed samples were analysed with ELISA (Bender Med systems) following the manufacturers protocol with the use of cultivation medium instead of the provided assay diluent as the only exception.

Results

Determination of particle size distribution of hydrogels

Several images of digitally photographed GelA (Figure 7) and GelB (Figure 6) were used to determine the sizes of 241 (GelB) and 232 (GelA) particles. Both GelB and GelA showed broad particle size distributions in the millimetre range. GelB particles are bigger (median: 2.77 mm²; average: 3.27 mm²) than GelA (median: 0.0929 mm²; average: 0.126 mm²). The GelB particles are 26 times bigger in average than GelA particles. The particle distributions of GelB and GelA are shown in Figure 8 and Figure 9 respectively.



Figure 6: Image of Alcian Blue stained GelB particles. The image is photographed without any magnification.

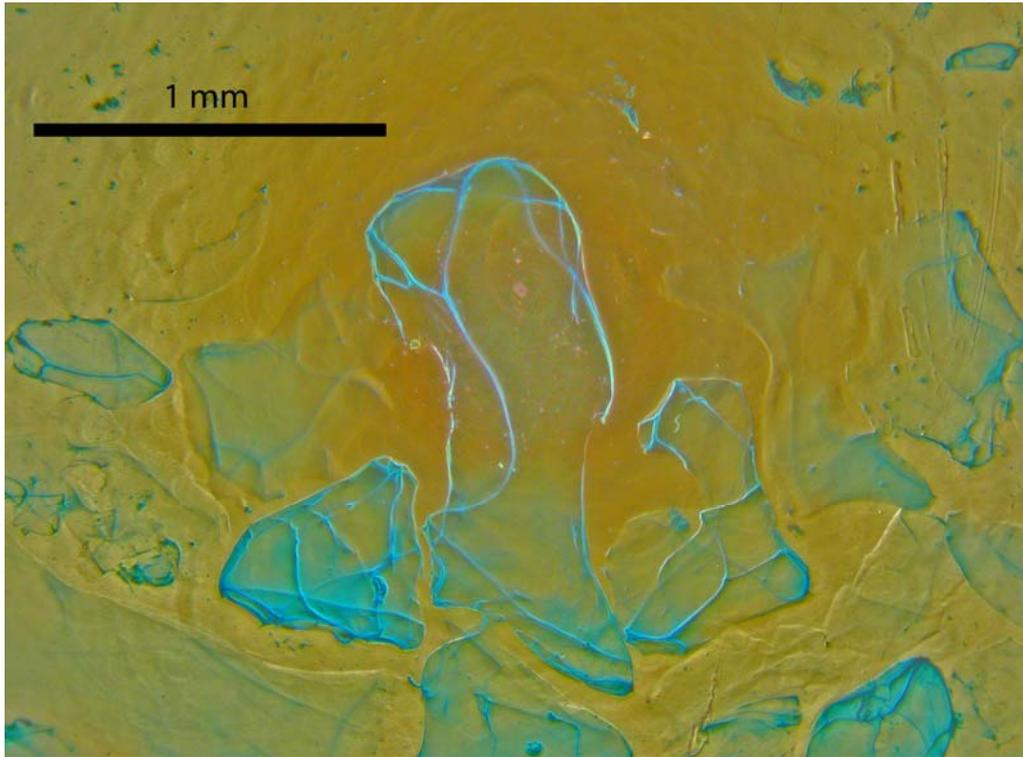


Figure 7: Microscope Image (4x) of Alcian Blue stained GelA particles.

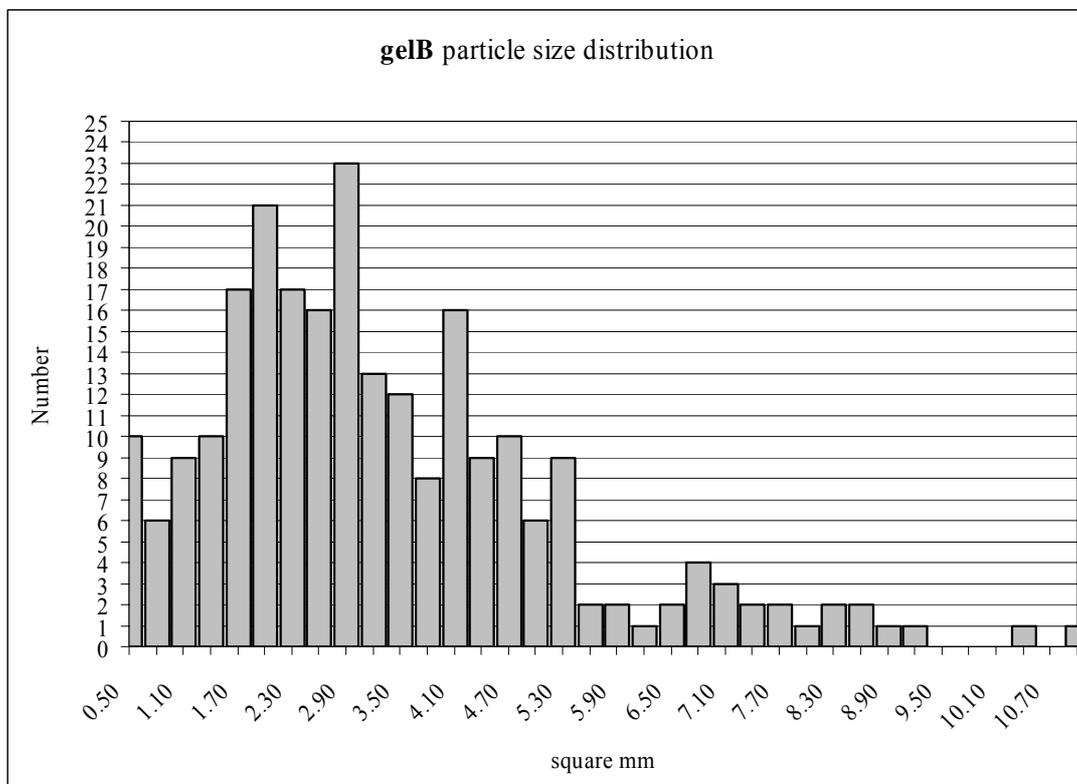


Figure 8: The area distribution based on the two-dimensional projection of 241 Alcian Blue stained GelB particles.

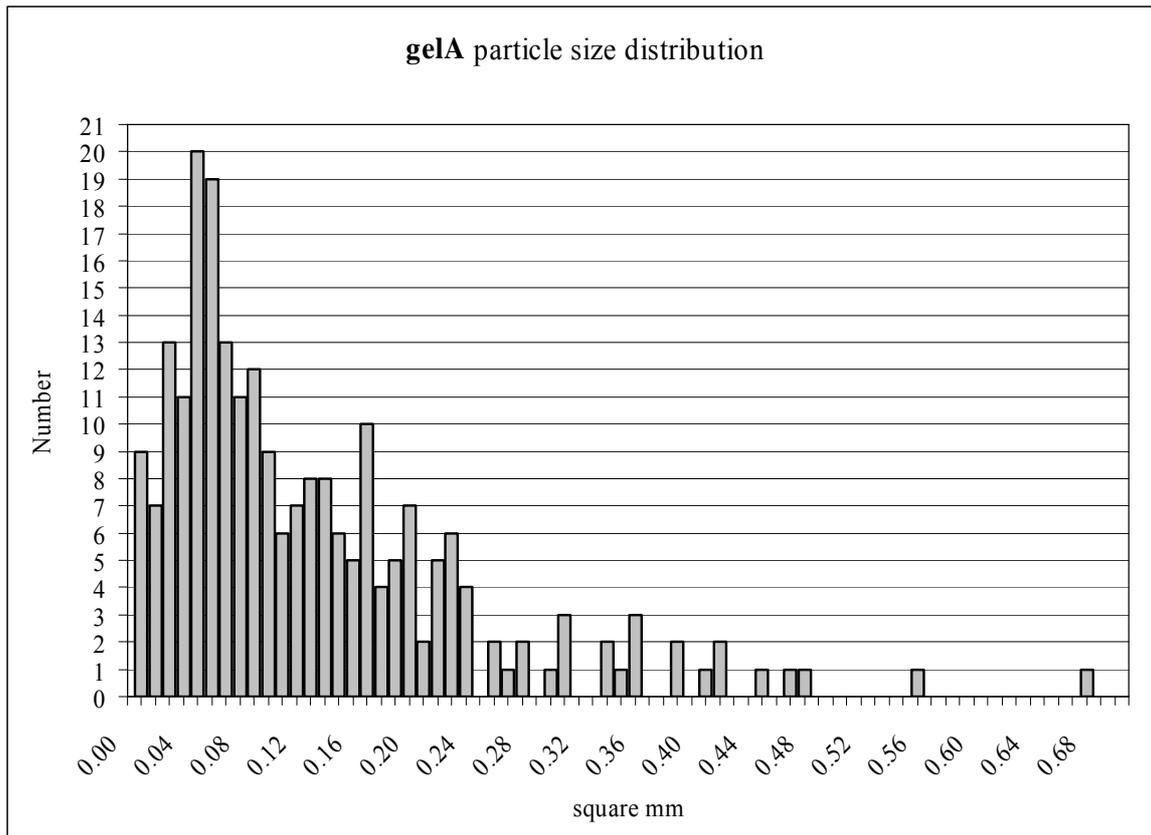


Figure 9: The area distribution based on the two-dimensional projection of 232 Alcian Blue stained Gela particles.

Degradation of hydrogels

Low concentrations of the enzymes (0.26 and 0.13 U Chondroitinase ABC; 5 and 1 kU Hyaluronidase) failed to degrade the hydrogels within one hour. 30 min was however enough to turn the gels into a fluid like form when subjected to high enzyme concentrations (0.51 U Chondroitinase ABC; 11 kU Hyaluronidase). Complete degradation, defined as degraded into a fluid like form with no visual differences between the gel types, was achieved after 1 hour of degradation. As

shown in Figure 10, there was no visible difference between GelB and Gela after 1 hour of degradation (11 kU Hyaluronidase).

There is a slight tendency of proteolytic activity in the hyaluronidase purchased from Sigma (see **Fel! Hittar inte referenskölla.**). The effect is however not significant during the first 60 min, which is the time used for degradation of the hydrogels, and can therefore be disregarded.

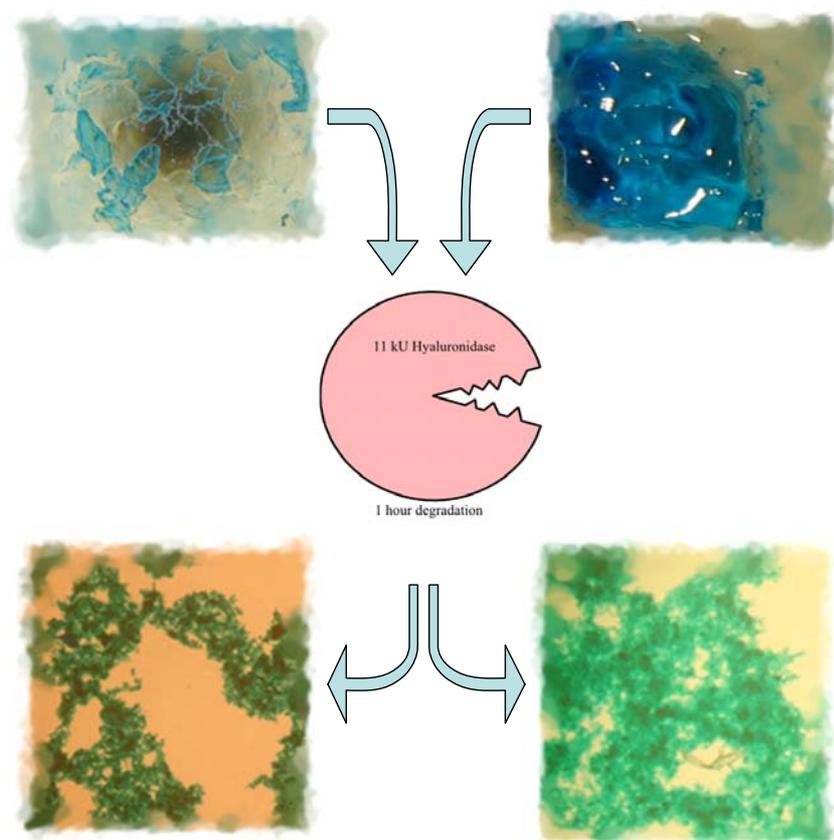


Figure 10: GelA (upper left) and GelB (upper right) were degraded into a fluid like form with 11 kU Hyaluronidase for 1. After degradation, there is no visual difference between GelA (lower left) and GelB (lower right).

IL-1 β production of U937 after 1 hour of incubation

All samples showed Interleukin-1 β levels below detectable range (< 0.125 $\mu\text{g/ml}$).

IL-1 β production of U937 after 24 hours of incubation with hydrogels

For all cells activated with LPS, IL-1 β levels were so high, that they were out of range (> 8 $\mu\text{g/ml}$ independently of the gel type. For the unstimulated cells more IL-1 β was secreted when incubated with gelA (1.260 (sup.) $\mu\text{g/ml}$; 0.308 (deg.) $\mu\text{g/well}$) compared to GelB (0.174 (sup.) $\mu\text{g/ml}$; 0.266 (deg) $\mu\text{g/well}$). This difference was more prominent for supernatants (see Figure 11) from both hydrogels compared with samples from degraded gels (see Figure 12).

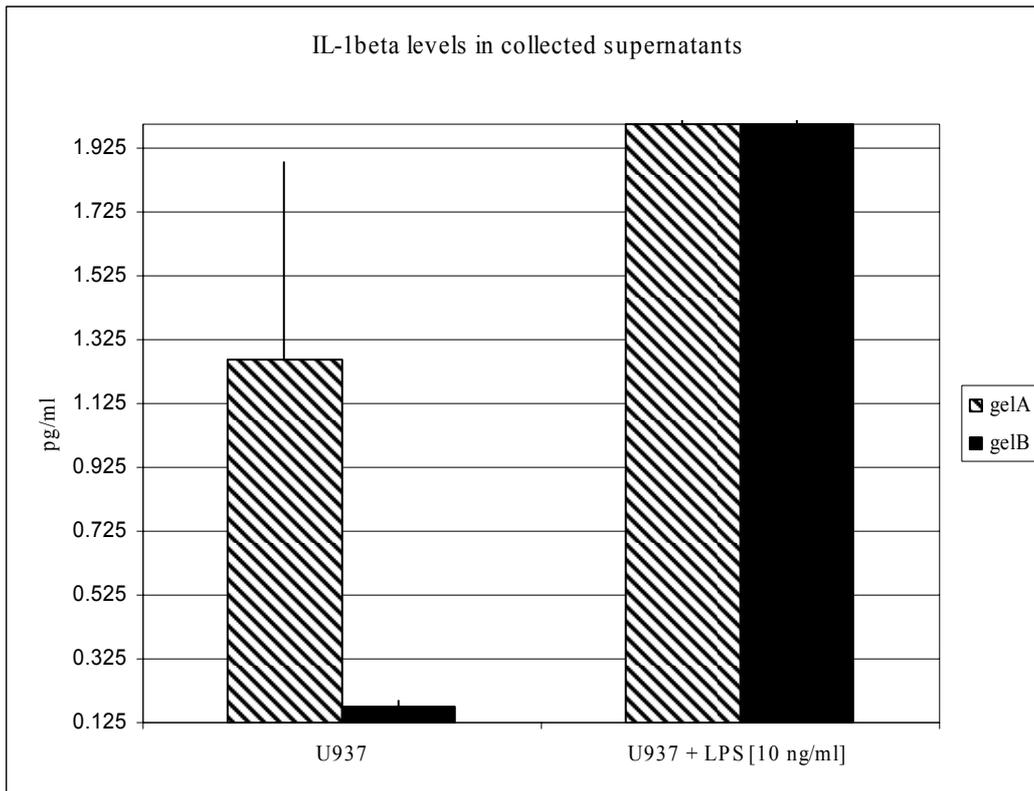


Figure 11: IL-1 β secretion collected from supernatants of 0.2×10^5 differentiated U937 cells per well incubated with gelA or GelB. LPS stimulated cells gave IL-1 β levels over 8 μ g/ml with both gel types.

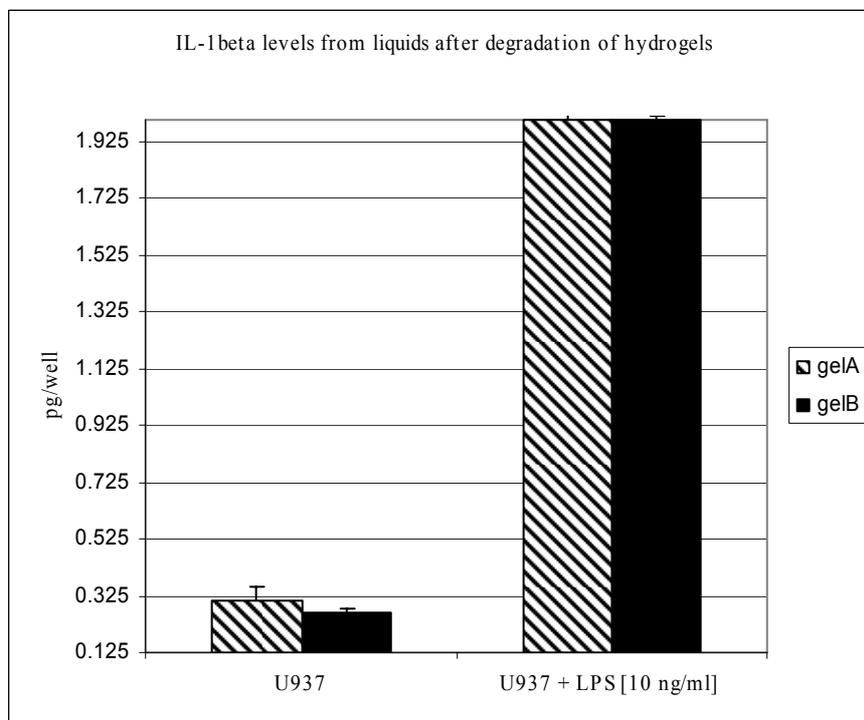


Figure 12: Total amount of secreted IL-1 β per well of differentiated U937 cells incubated with gelA and GelB. The hydrogels were degraded (11.6 kU Hyaluronidase; 1 hour; 37°C) before sample collection and ELISA measurement. LPS stimulated cells gave IL-1 β levels over 8 μ g/ml with both gel types.

IL-1 β production of U937 after 24 hours of incubation with hydrogels and increased cell amounts

By increasing the cell number to 0.3×10^6 cells per well a decrease in the amount of IL-1 β production was seen. All wells that contained unstimulated cells showed levels below detectable range (< 0.156 $\mu\text{g/ml}$). For the LPS

stimulated cells, IL-1 β levels from supernatants can be seen in Figure 13 and from degraded hydrogels in Figure 14. The detected levels of IL-1 β were higher for GelA (1.52 (sup.) $\mu\text{g/ml}$; 3.939 $\mu\text{g/well}$) and GelB (2.947 (sup.) $\mu\text{g/ml}$; 2.1593 $\mu\text{g/well}$) as compared to hyaluronan (0.286 (sup.) $\mu\text{g/ml}$; 0.50625 $\mu\text{g/well}$). The highest amounts were found in activated cells without any gel 6.877 $\mu\text{g/well}$.

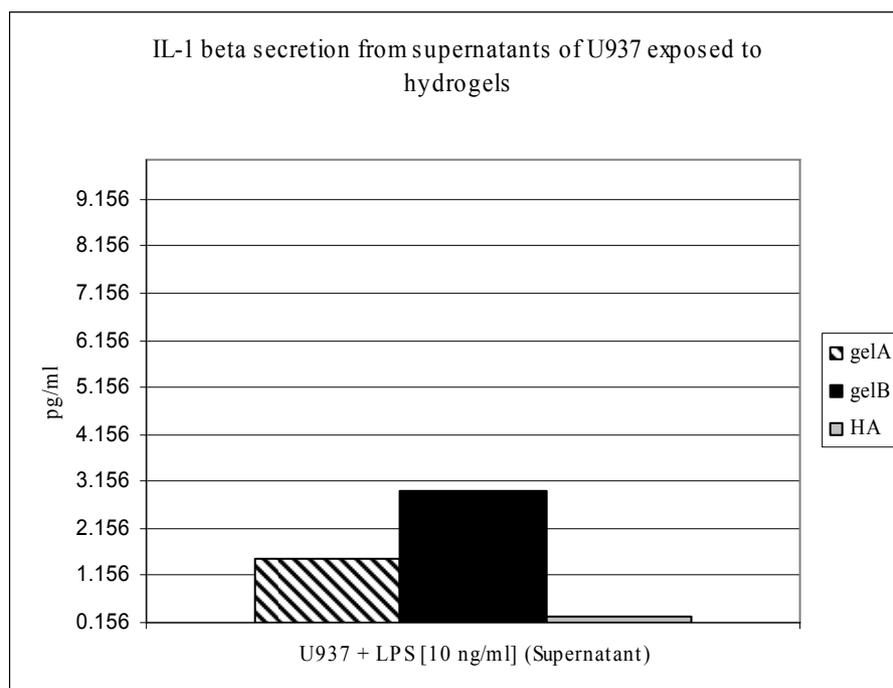


Figure 13: IL-1 β secretion from supernatants of 0.3×10^5 differentiated U937 cells per well stimulated with LPS and incubated together with GelA, GelB or hyaluronan. Unstimulated cells gave values below 0.156 $\mu\text{g/ml}$ for each gel type.

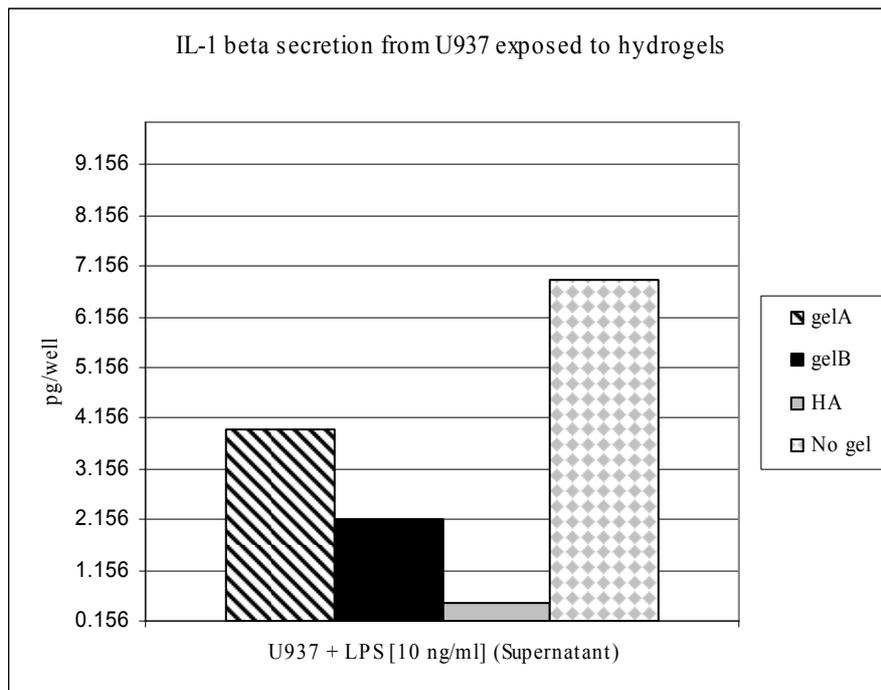


Figure 14: IL-1 β secretion of 0.3×10^5 differentiated U937 cells per well stimulated with LPS and incubated together with GelA, GelB, hyaluronan or just cell medium as a control. The hydrogels were degraded (11.6 kU Hyaluronidase; 1 hour; 37°C) before samples were collected.

IL-1 β production of U937 after 24 hours of incubation with pre-degraded hydrogels

When gelA, gelB and HA were degraded by Hyaluronidase before addition of U937 cells, there was no significant differences in IL-1 β

secretion between the different hydrogel types (Figure 15). LPS stimulation gave none or very little activation in the presence of degraded gelA and gelB, while a small increase in secretion was detected from activated cells in the presence of degraded hyaluronan.

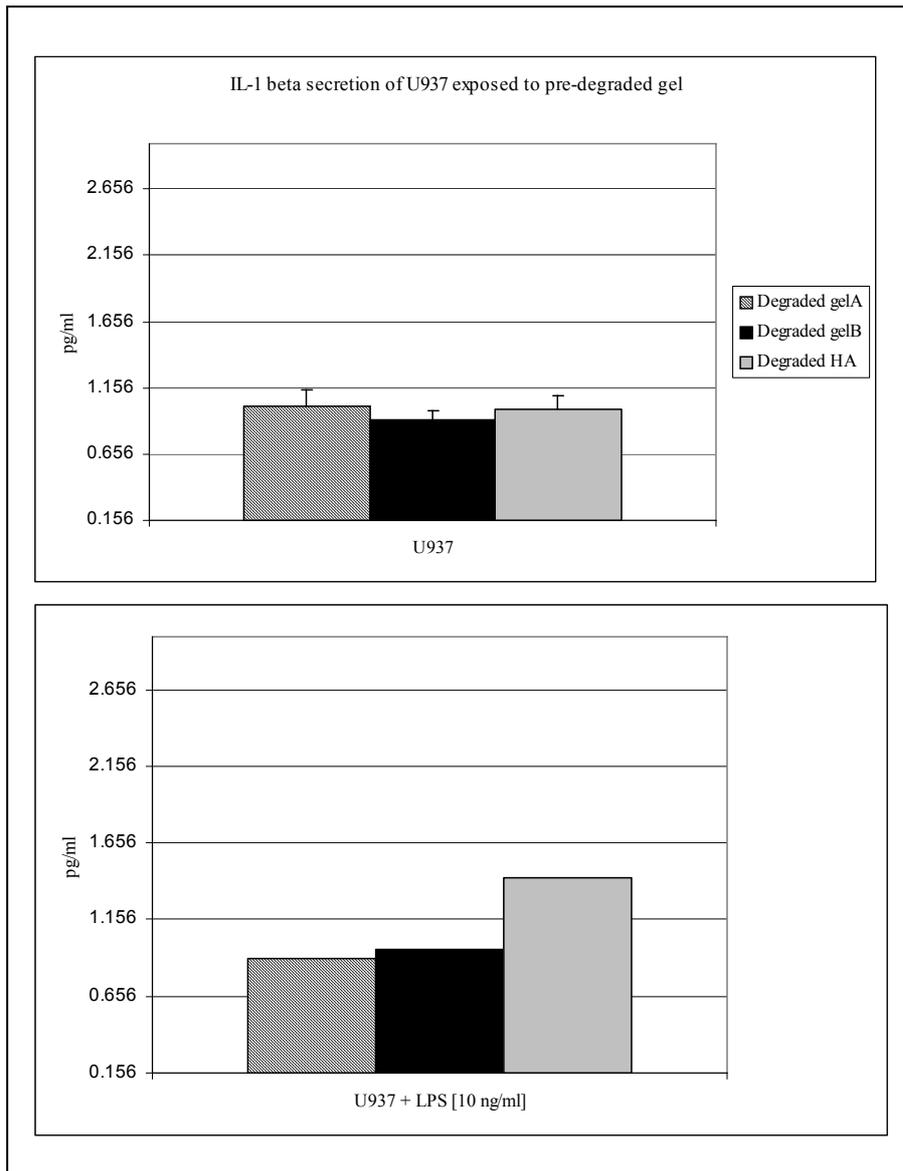


Figure 15: IL-1 β secretion of 0.3×10^5 differentiated U937 cells per well incubated with pre-degraded GelA, GelB or hyaluronan. The above figure represents the average values of triplicate samples of unstimulated cells incubated with hydrogels while the figure below shows IL-1 β values of LPS stimulated cells.

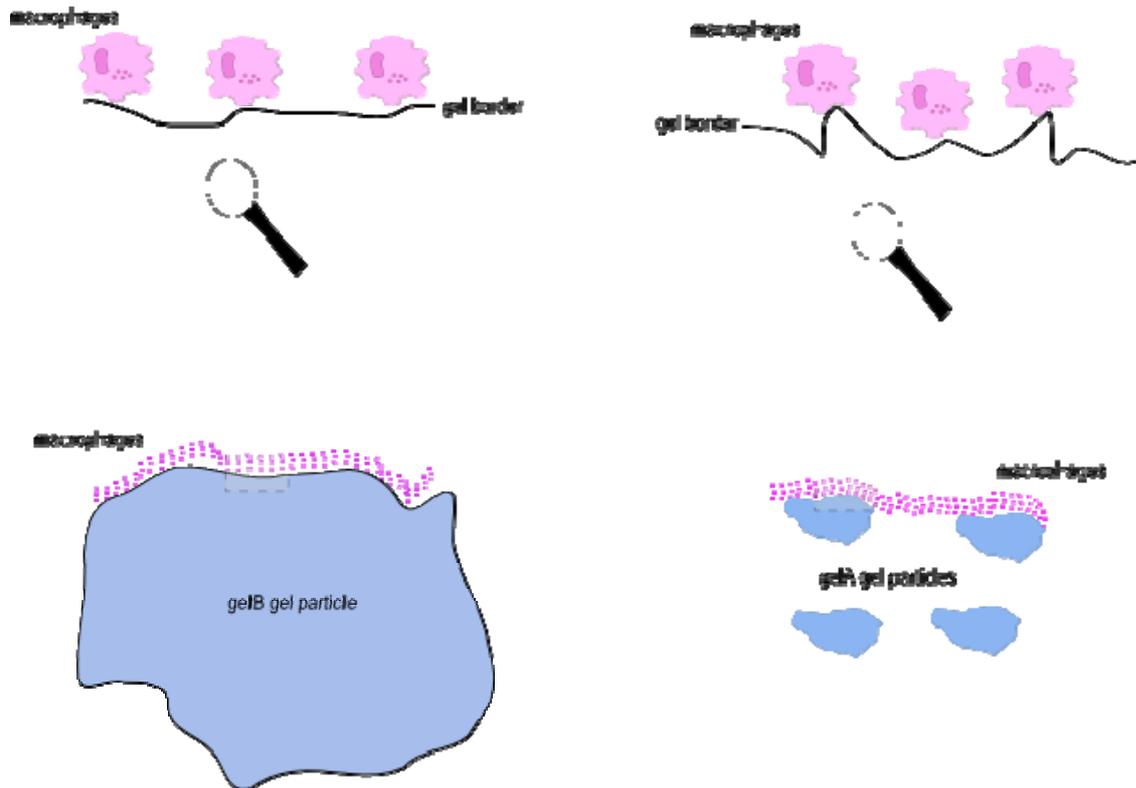


Figure 16: Macrophages in contact with GelB (to the **left**) and Gela (to the **right**). Gel with smaller size fraction could constitute more nano/micro topography, that can affect the macrophages.

Discussion

Determination of particle size distributions of hydrogels

GelB particles are approximately 26 times larger than Gela particles. Biologically both Gela and GelB can be considered as very large particles (i.e. compared with a macrophage $\sim 10 \mu\text{m}$). A lot of studies are made regarding inflammation versus particle size and composition but it mostly concerns particles smaller than the millimetre size range. Particles have been shown to trigger inflammation differently depending on particle size²⁰. Since macrophages are much smaller than the gel particles (~ 20 to 200 times smaller) the difference in size of these particles might be hard for inflammatory cells to recognize. However, a smaller particle fraction results in a larger exposure of the particle's surface nano/micro topography to the cells see Figure 16.

Quantification of proteolytic activity of Hyaluronidase

As gel degrading component we use Hyaluronidase (SIGMA) isolated from bovine testes*. Since contaminants with proteolytic activity in the product might be present and in turn might affect the IL-1 β determination, azocoll was used to determine protease activity. The proteolytic activity of Hyaluronidase was compared with trypsin, a serine protease²¹.

As the results showed, the proteolytic activity in hyaluronidase was not significant during the 60 min assay period and could therefore be disregarded.

IL-1 β production of U937 after 1 hour of incubation

* As gel degrading component we use Hyaluronidase (SIGMA) isolated from bovine testes. This is a source rich in Hyaluronidase. For successful fertilization the sperm has to degrade the matrix surrounding the oocyte therefore it is equipped with degrading enzymes such as Hyaluronidase*.

We were convinced that the U937 cells would secrete large amount of IL-1 β within an hour due to LPS activation. This was however not the case and a 24 hour experiment was therefore performed.

IL-1 β production of U937 after 24 hours of incubation with hydrogels

In contrast to the 1 hour experiment without gels, the 24 hour incubation showed detectable IL-1 β levels.

LPS activated cells gave significantly higher values thus indicating that activation of U937 cells can be achieved in the presence of hydrogels. Compared to non activated cells, the LPS stimulated cells showed significantly higher IL-1 β levels. Notable is the relatively larger differences of IL-1 β secretion between GelA and GelB exposed cells when collecting supernatants instead of degrading the gels and thus collecting “the entire” sample. This could mean that IL-1 β might partly be “trapped” in the gel particles of GelB. It is however also likely that particles might still be present from collected supernatants and might therefore interfere with the ELISA.

IL-1 β production of U937 after 24 hours of incubation with hydrogels and larger cell amounts

The increase in cell amount gave lower values of secreted IL-1 β . This may be effects from i) to high cell densities, ii) difference in cell batch or iii) the use of an other ELISA kit (with lower sensitivity) for the IL-1 β measurement. Again, the secreted amounts of IL-1 β cytokine is much higher when the cells are activated with LPS. Since the unstimulated cells did not produce detectable levels of IL-1 β , no individual differences between gel types could be noted. However, for the LPS stimulated cells there seemed to be a trend in activation due to gel particle size with hyaluronan showing the least activation followed by GelA and thereafter GelB. This might mean that hyaluronan gels have the possibility of inhibiting macrophage activation. This ability seems to decrease with increasing particle size. Cells that were LPS stimulated without any gel showed the largest amounts of IL-1 β and thus support this theory. This result might be a consequence of physical barrier or

cell affects of molecule interactions i.e. receptor stimulation.

IL-1 β production of U937 after 24 hours of incubation with pre-degraded hydrogels

There is no difference in IL-1 β secretion of cells exposed to different hydrogels that are degraded. The optical examination of degraded hydrogels showed no difference between gel types, see Figure 10. These results thus indicate that there is no chemical difference between the degraded gel types, at least not enough to cause a difference in the IL-1 β secretion. Another interesting observation is the difficulties of LPS activation in the presence of degraded hydrogels. We have seen a trend that a decrease of gel particle size more efficiently inhibits LPS activation to an increasing degree (see Figure 13 and Figure 14). Degraded gel particles are of course even smaller, hence the observed low degree of activation. This might be a consequence of gel interacting either with cells or LPS molecules. Activation of cells with another activator that acts inside the cells, i.e. Phorbol 12-myristate 13-acetate (PMA)²² would therefore be of great interest.

Conclusions

Whether it is a wrinkle filler or a new heart that is introduced to the human body an inflammation will take place. It is therefore important to find out how severe this inflammation may be, i.e. if it is likely that it will lead to a chronic inflammation with tissue damage or not. Hydrogels injected under the skin might be experienced as toxic for cells and biomolecules, hinder processes with its physical presence or do damage by other unexpected effects. In this report, the inflammatory response was quantified by measuring the secretion of the pro-inflammatory cytokine IL-1 β by U937 macrophages.

The hydrogels do not seem to cause an increased inflammatory response *in vitro*. The IL-1 β measurements were either very low or even below the detectable range with high

sensitivity ELISA kits. Hydrogels in combination with LPS simulated cells gave however deviations between different gel types.

The hydrogels seemed to have an inhibitory affect on the activation dependent on particle size. Pure hyaluronan seemed to be the best “inhibitor”. These are results have two sides in one way the fact that one can use hyaluronan to inhibit inflammation seems good but on the other hand to fully inhibit cell activation can cause severe effects in case of an infection needed to be “stopped” in the hydrogel environment.

Hyaluronan, as well as gelA and gelB, will be degraded and replaced in the human body meaning that cells will be exposed to the degradation product. LPS activation was strongly suppressed in the *in vitro* environment with degraded gel. This might in turn disturb important cell functions.

Future work

There are additional ways to quantify *in vitro* inflammatory responses.

Phagocytic cells of the immune systems (i.e. monocytes) have the ability to create reactive

oxygen species (ROS) which have efficient microbial functions. Generation of ROS starts with reduction of oxygen (O_2) to superoxide anion ($\cdot O_2^-$) that further reacts to create hydroxyl radicals, singlet oxygen and hydrogen peroxide. This reaction is catalysed by the NADPH oxidase system²³ located in the phagosome membrane.

ROS production can be triggered by common microbial components and can be regarded as an early response during inflammation. Monocytes and macrophages are also important producers of cytokines and other signal molecules of the immune system.

The production of ROS by THP-1 has been quantitatively determined by the use of chemiluminescence²⁴. Neutrophils, also known as PMN (polymorphonuclear neutrophils), are also phagocytic cells of the immune system with the ability of ROS production. These cells have been used in biocompatibility studies by measuring ROS production with chemiluminescence²⁵

Other cytokines can be used in similar studies as performed. $TNF-\alpha$ is a pro-inflammatory cytokine that can be used analogous to $IL-1\beta$ as a complement.

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