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(54) **Title**: MULTIPOTENT STROMAL CELL LOADED MICROCARRIERS

(57) **Abstract**: The invention provides a recombinant collagen or gelatin micro-carrier crosslinked by hexamethylenediisocyanate (HMDIC) comprising stromal cells for use in the treatment of cardiovascular disease. The invention is also directed at applications in which such cell carriers loaded with adipose derived stromal cells are used, for example as injectable cell carrier, for treatment of cardiovascular disease or treatment of damage of other tissues and organs.

MULTIPOTENT STROMAL CELL LOADED MICROCARRIERS

This invention relates to cell carrier particles prepared from gelatin. More specifically the invention is directed to such micro-particles prepared from gelatins loaded with (human) stromal cells. The invention is also directed at applications in which such cell carriers loaded with adipose derived stromal cells are used, for example as injectable cell carrier, for treatment of cardiovascular disease or treatment of damage of other tissues and organs.

BACKGROUND

Cardiovascular disease is a major cause of morbidity and mortality in the Western world. Patients with myocardial infarction (MI) may develop heart failure despite improvements in reperfusion therapy. Cell therapy, e.g. with stem or stromal cells, has been described as a promising therapeutic option to improve cardiac function in patients with myocardial infarction. However, in the setting of myocardial infarction stem cell therapy is limited by the low stem cell retention and survival, and potential pro-arrhythmic effects of stem cells.

The efficacy of stem cell therapy is subject to cell survival and therefore indirectly dependent on their micro-environment. Only a small proportion of the administered cells survives and integrates into the host myocardium as differentiated myocardial tissue cells. Although most in vivo studies do show engraftment of the stem cells, only a few reported the actual percentage that survives.

Wei et al. showed in Cell Transplant 2012;21 (12) pages 2723-2733, that the percentage of cells in the engrafted region was only about 1.5 % three days after intra-myocardial injections of mesenchymal stem cells in an MI rat model. This percentage decreased to 0.39 % at day 7. The second limitation of stem cell therapy concerns its electrophysiological consequences and potential pro-arrhythmic effect. Numerous in vitro and in vivo studies show a potential pro-arrhythmic effect of stem cells.

WO 2005/079879 describes a process for producing a cell carrier or medical material consisting of cross-linked collagen fibres. Cross-linked gelatin layers are also used to coat implants. WO 00/6701 describes the use of gelatin scaffolds for cell adhesion in repairing myocardial scar tissue or for coating pacemakers.

EP 1801122 describes the use of gelatin particles as micro cell carrier for cell adhesion. The gelatin is produced recombinantly and comprises at least two outer lysine residues which are separated by at least 25 percent lysine residues.

US2013217118 provides adipose-derived stem cells, adipose-derived stem cells fractions, lattices and method for obtaining the cells, fractions and lattices. However this patent does not describe how to overcome the potential negative effects of the adipose derived stem cells on the electrophysiology of cardiomyocytes when brought into contact with cardiomyocytes and ways to address them and ways to neutralize these adverse effects.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a gelatin or collagen micro-carrier comprising stromal cells for use in the treatment of cardiovascular disease.

In one aspect the present invention provides a recombinant gelatin or collagen carrier loaded with adipose tissue derived stromal cells (ADSC). Additionally the present invention provides a recombinant gelatin tailored for cell attachment by regulating the presence of specific cell binding sequences such as the amino acid sequence –arginine-glycine-aspartic acid (herein referred to as RGD-motifs) comprising adipose tissue derived stromal cells. In a preferred embodiment the invention provides a particular beneficial way of crosslinking the carriers.

FIGURES

Figure 1 shows imaging of the different cultures on day 7 after plating.

A: Phasecontrast micrograph of neo rat ventricular myocytes (NRVM)- monolayer (10x).

B: Fluorescent micrograph (10x) of a co-culture with adipose tissue derived stromal cells (ADSC) only. NRVM are stained for sarcomeric-actinin (green), and ADSC for CD44 (blue).

C: Fluorescent image (10x) of a ADSC loaded microspheres (MS) of co-culture using recombinantly produced gelatin (SEQ ID NO 3 , 54 kDa).
ADSC are Invitrogen Vybrand® CFDA SE Cell Tracer Kit labelled (green).

Figure 2 shows the layout of the culture chamber and the organization of the 60 embedded electrode terminals in the core portion of the multi-electrode array (MEA). Each electrode has a diameter of 100 μm and an interelectrode distance of 700 μm. Numbers 1 through 4 represent the different stimulation positions, which are located outside the core portion of the MEA.

Figure 3 shows the conduction velocity of the different cultures illustrated in a bar-graph, for examples of an activation map measuring conduction velocity in a control monolayer (NRVM only), a monolayer of NVRM seeded with ADSC and a monolayer seeded with ADSC loaded MS of recombinantly produced gelatin (SEQ ID NO 3 , 54 kDa) respectively.

Figure 4 shows the conduction heterogeneity of the different cultures illustrated in a bar-graph for examples of an activation map measuring conduction heterogeneity in a control monolayer, a monolayer seeded with ADSC and a monolayer seeded with ADSC loaded MS recombinantly produced gelatin (SEQ ID NO 3 , 54 kDa) respectively. Conduction heterogeneity is measured by taking the range of the maximum difference in activation time between each quadrant.

Figure 5 shows the electrical mapping and fractionation.
A: Local electrogram from a control culture.
B: Local electrogram from a culture of NRVM and ADSC, arrows indicate three clear fractionation of the deflection.
C: Percentage of fractionation present in the different cultures illustrated in a bar-graph.

Figure 6 shows the resting membrane potential (RMP) and action potential duration at 20, 50 and 90% (APD20, APD50 and APD90).
A: example of a typical ventricular action potential demonstrating where RMP and APD20, APD50 and APD90 were measured.
B: RMP of the different cultures.

Figure 7 shows the binding time dependant crosslinking of the recombinant gelatin types to ADSC.

ADSC bind in a time dependent fashion to all types of cross-linked recombinant collagens.

A: ADSC showed higher adhesion capacity to (SEQ ID NO 3).

B: Dehydrothermal treatment (DHT) cross-linked (SEQ ID NO 4) biomaterials in short term showed higher adhesion, however, after 4h both (SEQ ID NO 3) and (SEQ ID NO 4) showed same adhesion pattern.

C: Chemical cross-linked (SEQ ID NO 4) materials showed poor adhesion in comparison to (SEQ ID NO 3). Within chemically cross-linked materials, hexamethylenediisocyanate (HMDIC) showed advanced adhesion to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and even slightly higher number in comparison to TCPC.

Figure 8: The effect of chemical and thermal cross-linked (SEQ ID NO 3) on human (h)-ADSC proliferation.

(a) and apoptosis/necrosis.

(b) EDC-High cross-links induced cell apoptosis/necrosis in comparison to other conditions.

Expression of proteins associated with cell survival (Akt/pAkt) and adhesion (FAK/pFAK) showed no significant changes. Total proteins extracted from lysed h-ADSC in RIPA buffer on plastic and different materials.

Figure 9: 2D coatings of (SEQ ID NO 3) gene expression level by qRT-PCR. Expressions of mesenchymal, ECM, inflammatory cytokines and growth factors related genes by hADSC on chemical and thermal cross-linked coated plates in comparison to plastic. All results were normalized to GAPDH. Mesenchymal and ECM gene levels were not influenced by chemical cross-linked materials. Gene expressions of IL-1b and MCP1 were significantly lower in EDC-Low, HMDIC-Low and HMDIC-High coated plates. IL-6 gene expression level was lower in all chemical cross-linked materials in compare to plastic. Despite, TNFa showed down-regulation on EDC (Low and High) and HMDIC-High cross-linked materials. The chemical-cross-linked recombinant materials do not elicit negative influence on growth factors gene expressions, despite only IGF showed reduction on three out of four. On the other hand, IGF gene expression level of HMDIC-High was similar to plastic (A). Fibronectin, Col-III and

elastin were down-regulated, IL-8 and MCP-1. VEGF and FGF were decreased, however only Tb4 showed higher gene expression level (B).

Figure 10: Gene expression level of 3D cultured ADSC by qRT-PCR. SM22a, elastin and FGF were down regulated on ADSC cultured on Cultisphers (CS) and HMDIC-High crosslinked rough microspheres (A-C). However, findings of HMDIC-High microspheres were comparable to well established CS, which used widely in 3D cultured experiments. Col-I was down regulated on CS culture condition, which was not affected by HMDIC-High cross-linked material.

DETAILED DESCRIPTION

We have found that a gelatin or collagen micro-carrier comprising stromal cells may be advantageously used for the treatment of cardiovascular disease.

Although strictly speaking there is a difference between collagen and gelatin, these differences are not essential to the invention. In this respect "collagen" may also be read as "gelatin" and "collagen polypeptide" may also be read as "gelatin polypeptide" throughout this document.

Further in this document the "micro-carrier" can be read as "microbeads" or "microspheres (MS)" or "cell carriers" or "micro particles" and are used interchangeably in explanation of the invention.

It is further preferred that these micro-carriers have an uniform cell carrier particle size, since this results in a uniform cell density in in vitro cell culture applications and in medical applications. Too large particles will bind less cells relative to their volume, and are therefore less effective. Furthermore, too large micro-carriers hamper their administration by injection. Too small particles will bind no cells and are therefore unproductive. Furthermore, too small micro-carriers are phagocytosed by immune cells after their administration and thus cleared. Preferred are micro-carrier gelatin based cell carriers with a uniform particle size distribution, uniform physical and (bio-)chemical properties.

Uniform behaviour can be made by using recombinant gelatin, so it is preferred to provide a recombinant gelatin or collagen micro-carrier comprising stromal cells for the use of the treatment of cardiovascular disease. Uniform behaviour can be made by

using a recombinant gelatin that comprises at least two outer (also referred to as extreme) lysine residues separated by at least 25% of the total number of amino acids in the recombinant gelatin. In a preferred embodiment the recombinantly produced gelatin comprises at least one lysine residue between the outer (extreme) lysine residues.

In a further preferred embodiment of the present cell carrier particles the recombinantly produced gelatin polypeptides comprise at least two amino acid residues, said two amino acid residues being extreme amino acid residues, which independently are selected from an aspartic acid residue and a glutamic acid residue, wherein a first aspartic acid residue or glutamic acid residue is the aspartic acid residue or glutamic acid residue that is closest to the N-terminus of the polypeptide and the second extreme aspartic acid residue or glutamic acid residue is the aspartic acid residue or glutamic acid residue that is closest to the C-terminus of the polypeptide and said extreme aspartic acid residues and/or glutamic acid residues are separated by at least 25 percent of the total number of amino acids in the recombinant gelatin polypeptide. In yet a further embodiment the recombinantly produced gelatin polypeptides comprise at least one aspartic acid residue or glutamic acid residue between said extreme extreme aspartic acid residues and/or glutamic acid residues.

Gelatins and collagens can be cross-linked via the amine groups of lysine, via carboxyl groups of glutamic acid or aspartic acid, or a combination thereof. Suitable cross-linking agents are preferably those that do not elicit toxic or antigenic effects when released during biodegradation. Suitable cross-linking agents are, for example, one or more of glutaraldehyde, water-soluble carbodiimides, bisepoxy compounds, formalin, 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide, bis-hydroxy-succinimides, glycidyl ethers such as alkylene glycol diglycidyl ethers or polyglycerol polyglycidyl ether, diisocyanates such as hexamethylene diisocyanate, diphenylphosphorylazide, D-ribose, natural crosslinking agents such as genipin. Cross-linking techniques are also described by Weadock et. al. in Evaluation of collagen cross-linking techniques (Biomater. Med. Devices Artif. Organs, 1983-1984, 11 (4): 293-318). In a preferred embodiment water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is used. In another preferred embodiment hexamethylenediisocyanate is used. Other suitable cross-linking agents are triazines such as for example dichlorohydroxy-triazine. Other cross-linking compounds are divinyl sulfones, di-anhydrides, bifunctional imidates di- epoxides or dimaleiimidines. It is also possible to use bi-

functional cross-linking compounds that have different active groups such as a bifunctional cross-linking compound comprising an epoxyde and an anhydride in one molecule.

Also useful are enzymatic cross-linking compounds such as trans-glutaminase.

Also cross-linking compounds that can attach to more than 2 cross-linkable amino acid residues such as for example lysine residues can be applied such as for example cyanuric chloride. In that respect compounds that combine three or more reactive groups are envisaged such as a compound comprising two epoxide- and an anhydride group.

In one embodiment cross-linking of the collagenous polypeptide is achieved by addition of one or more cross-linking agents. These comprise agents that start cross-linking spontaneously upon addition to collagenous polypeptide solution, or after adjusting for example, pH, or by photo initiation or other activation mechanisms.

A well-known cross-linker is for example glutaraldehyde, which cross-links two lysine residues. Another well known biocompatible cross-linker is EDC, which couples an amine and a carboxyl group. Also hexamethylenediisocyanate is frequently used as cross-linker.

Another well-known way to crosslink peptide is by heat. This so-called dehydrothermal treatment (DHT) forms crosslinks between amine and carboxy groups by removal of water thereby forming peptide bonds, and proceeds at temperatures above 120°C.

To contribute to particle formation, the recombinant gelatin comprises at least two lysine residues. Preferably the recombinant collagen polypeptide comprises at least 3, or at least 4, 5, 6, 7, 8, 9, 10, 11 or at least 12 lysine residues. In a further embodiment the recombinant gelatin polypeptide comprises in addition to the lysines also at least two amino acid residues selected from aspartic acid and glutamic acid, more preferably the recombinant gelatin polypeptide comprises at least 3, or at least 4, 5, 6, 7, 8, 9, 10, 11 or at least 12 aspartic acid and glutamic acid residues.

For contributing to the three dimensional network structure the lysines, aspartic acid and/or glutamic acid residues should have a spatial distribution over the polypeptide. Thus in one embodiment each stretch of 50 amino acids comprises at least 1, preferably at least 2, lysine residues or at least 1, preferably at least 2, aspartic acid or glutamic acid residue or at least 1 lysine residue and at least 1 aspartic acid or

glutamic acid residue. Preferably each stretch of 40 amino acids comprises at least 1 lysine residues and/or at least one aspartic- or glutamic acid residues, even more preferably each stretch of 25 amino acids. Preferably the cross-linkable amino acid residues are not adjacent to each other. More preferably they are separated by at least 5 amino acids, more preferably by at least 10 amino acids.

Especially in case of recombinantly produced gelatin, the number of lysine residues can be increased as desired. Many cross-linking agents bind to lysine residues and/or N-terminal amines. Natural gelatins contain 25 to 27 lysine residues and 112-133 glutamic- and aspartic acid residues per 1000 amino acids. In recombinant gelatins the number of lysines can be reduced to for example equal to or less than about 20, 15, 10 or 5 lysines per 1000 amino acids or increased to for example equal to or more than about 30, 40 or 50 lysines per 1000 amino acids. The number of glutamic- or aspartic acid residues can be decreased to for example equal to or less than about 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 or 5 residues per 1000 amino acids or can be increased to for example equal to or more than 150 residues per 1000 amino acids.

When a part of a human collagen sequence is expressed, both asparagine and aspartic acid and both glutamine and glutamic acid may be present in the recombinant polypeptide. When desired, the glutamine and asparagine residues can be de-aminated, converting them to aspartic acid and glutamic acid residues.

In one embodiment the recombinant gelatin of the cell carrier particles is cross-linked by adding between 0.02 and 1.0 millimol cross-linking compound(s) per gram recombinant gelatin. Thus, the cross-linking compound(s) may be present in an amount of about 0.02, 0.05, 0.1, 0.25, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 millimol / gram gelatin. In another embodiment the cell carrier particles are cross-linked by adding between 0.5 and 5.0 millimol cross-linking compound(s) per gram recombinant gelatin (or radiation induced cross-linking which is equivalent hereto), preferably about 1.0 to 2.5 millimol/g. Thus, the cross-linking compound(s) may be present in an amount of about 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0 and 5.0 millimol / gram recombinant gelatin. In yet another embodiment the recombinant gelatin is cross-linked by adding between 0.25 and 2.5 millimol cross-linking compound(s) per gram gelatinous polypeptide.

Thus, the number of cross-linkable amino acid residues together with the amount of cross-linking compound that is applied can be used to determine or customize the physical properties of the recombinant collagen particles. A high number

of cross-linkable residues and/or a high concentration of cross-linking compound can yield particles that are suitable for cell cultures, in which the particles are subjected to mechanical stress. Lower numbers of cross-linkable amino acid residues and/or low concentrations of cross-linking compounds can yield particles that are easily deformable and can be applied for injectable collagen particles for medical or pharmaceutical applications. In one embodiment the cross-linking agent is added to the gelatin during preparation of the cell carrier particles. In another embodiment particles are formed, and cross-linking compound is added during the final stage of particle formation or after particles have been formed, to produce particles that are surface-cross-linked, but have essentially no internal cross-linking.

Recombinant production of gelatin makes it possible to obtain a monodisperse molecular weight distribution and also a uniform amino acid composition of the gelatin polypeptides. When hydrolyzing natural gelatin for preparing cross-linked cell carrier particles, low molecular weight fractions can be present that contain none or only one lysine residue, or a cluster of lysine residues that are too close together to effectively contribute to cross-linking. Such structures do not contribute to the formation of particles. It is likely that many low molecular weight fractions from natural gelatins do not have the amino acid sequence arginine-glycine- aspartic acid (-RGD-) which may be advantageous required for cell attachment. So, even if a small cell carrier particle is formed it may not contribute to cell attachment. In cases where the absence of an RGD-motif is preferred, a skilled person may be hesitant to use natural gelatins. Recombinantly produced collagens that do not comprise the RGD sequence can be applied in that case.

Because of the variation in particle size, amino acid composition and hence degree of cross-linking when cell carrier particles are made from natural gelatins, the degree of biodegradability is also variable. In applications such as cell delivery in medical applications this may impose a further imbalance in the healing process, so that in one place healing may proceed as intended, reaching full effect of treatment, while in other places beneficial effects may be impaired and may not go to completion. In case of tissue augmentation or plastic surgery an imbalanced biodegradability may cause the injected area to become irregular in shape, which is cosmetically unacceptable.

Now that according to the present invention particles with a uniform size and uniform properties can be made using recombinant gelatin polypeptides it is also

possible to tune the biodegradation speed, and more important, to obtain a uniform biodegradation speed and behaviour.

Thus, according to the invention with recombinant gelatins cell carrier particles can be produced that have more uniform properties than particles prepared from natural gelatin. The size distribution will be narrower. No extreme small or extreme large particles will be formed. Thus it can be expected that less than 10% of the particles in a population prepared from recombinantly produced gelatins will have a size that deviates more than 20% from the average size, and it can further be expected to have essentially no particles that deviate more than 50% from the average size. Preferably less than 5% of the particles have a size that deviates more than 20% from the average particle size. More preferably less than 5% of the particles have a size that deviates more than 10% from the average particle size. Most preferred is that less than 2.5% of the particles have a size that deviates more than 5% from the average particle size.

The cell carrier particles may be porous or non porous or may comprise cavities to increase both the quality and quantity of adherence cells, as described in for example WO 2003/104313. Porous particles thus increase cell adhesion because of 1) increment in surface area, and 2) providing opportunities for invagination of cell protrusions.

Suitable gelatin polypeptides to make the recombinant cell carrier particles according to the invention are gelatins (or collagens) from recombinant sources. Although strictly speaking there is a difference between collagen and gelatin, these differences are in principle not essential to the invention, although specific requirements may make the selection of collagen or gelatin for a certain application obvious.

A gelatin or collagen or collagenous or gelatinous polypeptide is thus defined as being a polypeptide in which at least one GXY domain is present of at least a length of 5 consecutive GXY triplets and at least 20% of the amino acids of the gelatinous polypeptide are present in the form of consecutive GXY triplets, wherein a GXY triplet consists of G representing glycine and X and Y representing any amino acid. Suitably at least 5% of X and/or Y can represent proline and in particular at least 5%, more in particular between 10 and 33% of the amino acids of the GXY part of the collagenous polypeptide is proline.

The gelatinous polypeptide preferably has an average molecular weight of less than 150 kDa, preferably of less than 100 kDa. Ranges of between 50 and 100 kDa are

suitable or gelatinous polypeptides of less than 75 kDa but more than 20 kDa or between 5 and 40 kDa may be used. Preferably the collagenous polypeptides have an average molecular weight of at least 5 kDa, preferably at least 10 kDa and more preferably of at least 30 kDa. Lower molecular weights may be preferred when higher concentrations of gelatinous polypeptides are required because of the lower viscosity.

In the present invention the gelatin as a micro-carrier is comprising stromal cells.

Stromal cells are connective tissue cells that form the supportive structure in which the functional cells of the tissue reside. Multipotent stromal cells (MSC) can be isolated from virtually all tissues including blood, fat or bone marrow.

In vitro, MSC can differentiate into osteoblasts, chondrocytes, adipocytes, but also myogenic cells, and pericytes. The historically used term mesenchymal stem cells is, and has been used, to denote the multipotent stromal cells too. In the context of this document and invention the terms “stem cell”, “stromal cell” and “multipotent stromal cell” are used interchangeably.

More preferred in the present invention the gelatin as micro-carrier is comprising therapeutically potent cells, such as multipotent stromal cells and even more preferred these cells are adipose tissue derived.

The most preferred embodiment is the use of recombinant gelatin comprising adipose derived human (h) stromal cells (abbreviated further as ADSC).

The present invention involves the use of these stromal cell comprising microcarriers for the treatment of cardiovascular diseases. The limitation of the use of stem/stromal cells, including ADSC, in stem cell therapy concerns its electrophysiological consequences and potential pro-arrhytmic effect. This results in conduction slowing, conduction heterogeneity and unidirectional block facilitating e-entrant arrhythmia. We have now found surprisingly that this conduction slowing is not present when ADCS are administered loaded onto recombinant gelatin (or collagen) micro-carriers while the slowing is visible when administered as ADSC alone.

This effect is shown in the electrophysiology of an underlying confluent layer of neonatal rat cardiomyocytes and is mediated via the interaction between ADSC and the cardiomyocyte monolayer through electrotonic and/or paracrine mechanisms.

The method of making recombinant gelatinous polypeptides has been described in detail in patent applications EP 0 926 543 and EP 1 014 176 by the same applicant,

the content of which is herein incorporated by reference. The methodology is described in the publication 'High yield secretion of recombinant gelatins by Pichia pastoris', M. WT. Werten et al., Yeast 15, 1087-1096 (1999). Suitable recombinant gelatins are also described in WO 2004/85473.

In one embodiment the recombinant gelatinous polypeptide does not form stable triple helices, specifically not at temperatures of higher than 5 degrees Celsius, or at temperatures higher than 25 degrees Celsius. Such gelatinous polypeptides have preferably an amount of prolines present in GXY triplets that is comparable to collagen or gelatin originating from mammals or collagens originating from cold-blooded animals such as fish. To prevent stable triple helix formation less than 2 number percent, preferably less than 1 number percent, of the amino acids present in the gelatinous polypeptide are hydroxylated. Occurrence of hydroxyprolines can be reduced to be practically zero by expression in micro-organisms that do not co-express a prolylhydroxylase or fulfill that function in another way. Practically zero means that the presence of hydroxyprolines in the growth medium of for example yeasts may result in incorporation of some of these amino acids into the gelatinous polypeptide. Recombinant gelatinous polypeptides that are not hydroxylated and have the advantage of avoiding the occurrence of anaphylactic shock are described in EP 1 238 675.

In a preferred embodiment the cell carrier particles comprise gelatinous polypeptides with excellent cell attachment properties, and which do not display any health related risks. Advantageously this is achieved by production of RGD-enriched gelatinous polypeptides in which the percentage of RGD motifs related to the total number of amino acids is at least 0.4. If the RGD-enriched gelatinous polypeptide comprises 350 amino acids or more, each stretch of 350 amino acids contains at least one RGD motif. Preferably the percentage of RGD motifs is at least 0.6, more preferably at least 0.8, more preferably at least 1.0, more preferably at least 1.2 and most preferably at least 1.5.

A percentage RGD motifs of 0.4 corresponds with at least 1 RGD sequence per 250 amino acids. The number of RGD motifs is an integer, thus to meet the feature of 0.4%, a gelatinous polypeptide consisting of 251 amino acids should comprise at least 2 RGD sequences. Preferably the RGD-enriched recombinant gelatinous polypeptide comprises at least 2 RGD sequence per 250 amino acids, more preferably at least 3 RGD sequences per 250 amino acids, most preferably at least 4 RGD sequences per

250 amino acids. In a further embodiment an RGD-enriched gelatinous polypeptide comprises at least 4 RGD motifs, preferably at least 6, more preferably at least 8, even more preferably at least 12 up to and including 16 RGD motifs.

The term 'RGD-enriched gelatinous polypeptide' in the context of this invention means that the gelatinous polypeptides have a certain level of RGD motifs, calculated as a percentage of the total number of amino acids per molecule and a more even distribution of RGD

Natural gelatins are known to comprise RGD sequences. Only a fraction of the polypeptides in a natural hydrolyzed gelatin will have an RGD sequence. It is important however that a recombinant particle does not contain too large parts without RGD motifs. Too large parts without RGD sequence reduce the possibility of cell attachment. Apparently not all RGD sequences in a gelatinous polypeptide are under all circumstances available for cell attachment. It was found that cell attachment was remarkably improved in gelatinous polypeptides according to the invention comprising at least one RGD motif in each stretch of 350 amino acids when compared to gelatins having a stretch of amino acids of more than 350 without an RGD sequence. For gelatinous polypeptides of less than 350 amino acids it is sufficient to have a percentage of RGD sequences of at least 0.4.

Recombinant gelatinous polypeptides used in this invention are preferably derived from collagenous sequences. Nucleic acid sequences encoding collagens have been generally described in the art. (See, e. g., Fuller and Boedtker (1981) Biochemistry 20: 996-1006; Sandell et al. (1984) J Biol Chem 259: 7826-34; Kohno et al. (1984) J Biol Chem 259: 13668-13673; French et al. (1985) Gene 39: 311-312; Metsaranta et al. (1991) J Biol Chem 266: 16862-16869; Metsaranta et al. (1991) Biochim Biophys Acta 1089: 241-243; Wood et al. (1987) Gene 61 : 225-230; Glumoff et al. (1994) Biochim Biophys Acta 1217: 41-48 ; Shirai et al. (1998) Matrix Biology 17: 85-88; Tromp et al. (1988) Biochem J 253: 919-912; Kuivaniemi et al. (1988) Biochem J 252: 633640; and Ala-Kokko et al. (1989) Biochem J 260: 509-516).

For pharmaceutical and medical uses, recombinant gelatinous polypeptides with amino acid sequences closely related to or identical to amino acid sequences of natural human collagens are preferred. More preferably the amino acid sequence of the gelatinous polypeptide is designed by a repetitive use of a selected amino acid sequence of a human collagen. A part of a natural collagen sequence comprising an RGD motif is

selected. The percentage of RGD motifs in such a selected sequence depends on the chosen length of the selected sequence, selection of a shorter sequence results in a higher RGD percentage. Repetitive use of a selected amino acid sequence results in a gelatin with a higher molecular weight, which is non-antigenic and with an increased number of RGD motifs (compared to natural gelatins or collagens).

Thus in a preferred embodiment the recombinant gelatinous polypeptide comprises a part of a native human collagen sequence. Preferably the RGD-enriched gelatinous polypeptide consists for at least 80 % of one or more parts of one or more native human collagen sequences. Preferably each of such parts of human collagen sequences has a length of at least 30 amino acids, more preferably at least 45 amino acids, most preferably at least 60 amino acids, up to e.g. 240, preferably up to 150, most preferably up to 120 amino acids, each part preferably containing one or more RGD sequences. Preferably the RGD-enriched gelatinous polypeptide consists of one or more parts of one or more native human collagen sequences.

An example of a suitable source of a gelatinous polypeptide for preparing the recombinant particles according to this invention is human COL1A1-1. A part of 250 amino acids comprising an RGD sequence is given in WO 04/85473. RGD sequences in collagenous polypeptides can adhere to specific receptors on the cell surface called integrins. These integrins differ in their specificity in recognizing cell binding amino acid sequences in molecules in the natural extracellular matrix. Although both natural gelatin and, for example, fibronectin may contain RGD sequences, gelatin can bind cells that will not bind to fibronectin and vice versa. Therefore fibronectin comprising RGD sequences cannot always replace gelatin for cell adhesion purposes.

As already mentioned RGD-enriched gelatinous polypeptides can be produced by recombinant methods as disclosed in EP-A-0926543, EP-A-1014176 or WO 01/34646. For the production and purification of gelatinous polypeptides that are suited for preparing cell carrier particles of this invention reference is made to the examples in EP 0 926 543 and EP 1014 176. The preferred method for producing an RGD-enriched gelatinous polypeptides is by starting with a natural nucleic acid sequence encoding a part of the collagen protein that includes an RGD amino acid sequence. By repeating this sequence an RGD-enriched gelatinous polypeptide is obtained.

If X-RGD-Y is a part of the natural collagen amino acid sequence, a (part of a) gelatinous polypeptide with three RGD amino acid sequences would have the structure

-X-RGD-Y- (GXYG)m-X-RGD-Y-(GXYG)n-X-RGD-Y-, with m and n being integers starting from 0. By varying n the number of RGD sequences on the total amino acids the percentage of RGD motifs can be controlled. A clear advantage of this method is that the amino acid sequence remains most natural and thus has the lowest risk of inducing immunological response in clinical applications.

Starting from a natural nucleic acid sequence encoding (part of) a gelatinous polypeptide, also point mutations can be applied so as to yield a sequence encoding an RGD sequence. Based on the known codons a point mutation can be performed so that an RGX sequence after mutation will yield an RGD sequence, alternatively also an YGD sequence can be mutated to yield an RGD sequence. Also it is possible to carry out two mutations so that an YGX sequence will give an RGD sequence. Also it may be possible to insert one or more nucleotides or delete one or more nucleotides giving rise to a desired RGD sequence.

Thus the gelatinous polypeptides can be produced by expression of nucleic acid sequence encoding such polypeptide by a suitable micro-organism. The process can suitably be carried out with a fungal cell or a yeast cell. Suitably the host cell is a high expression host cells like Hansenula, Trichoderma, Aspergillus, Penicillium, Saccharomyces, Kluyveromyces, Neurospora or Pichia. Fungal and yeast cells are preferred to bacteria as they are less susceptible to improper expression of repetitive sequences. Most preferably the host will not have a high level of proteases that attack the collagen structure expressed. In this respect Pichia or Hansenula offers an example of a very suitable expression system. Use of Pichia pastoris as an expression system is disclosed in EP 0 926 543 and EP 1 014 176. The microorganism may be free of active post-translational processing mechanism such as in particular hydroxylation of proline and also hydroxylation of lysine. Alternatively the host system may have an endogenic proline hydroxylation activity by which the gelatinous polypeptide is hydroxylated in a highly effective way. The selection of a suitable host cell from known industrial enzyme producing fungal host cells specifically yeast cells on the basis of the required parameters described herein rendering the host cell suitable for expression of gelatinous polypeptides which are suitable for use as artificial skin in combination with knowledge regarding the host cells and the sequence to be expressed will be possible by a person skilled in the art.

In another embodiment the recombinant gelatinous polypeptides for making cell carrier particles have a higher glass transition temperature than natural occurring gelatins. Such sequences are described in WO 05/11740.

In a further embodiment, the gelatins used to make the cell carrier particles are low in glycosylation. There are various methods for ensuring that glycosylation is low or absent. Glycosylation is a posttranslational modification, whereby carbohydrates are covalently attached to certain amino acids of the protein or polypeptide. Thus both the amino acid sequence and the host cell (and enzymes, especially glycosyltransferases, therein) in which the amino acid sequence is produced determine the glycosylation pattern. There are two types of glycosylation: N-glycosylation begins with linking of GIcNAc (N-actylglucosamine) to the amide group of asparagines (N or Asn) and O-glycosylation commonly links GaINAc (N- acetylgalactosamine) to the hydroxyl group of the amino acid serine (S or Ser) or threonine (T or Thr).

Glycosylation can, therefore, be controlled and especially reduced or prevented, by choosing an appropriate expression host, and/or by modifying or choosing sequences which lack consensus sites recognized by the hosts glycosyltransferases. Obviously, chemical synthesis of proteins or polypeptides results in unglycosylated proteins. Also, glycosylated proteins may be treated after production to remove all or most of the carbohydrates or unglycosylated proteins may be separated from glycosylated proteins using known methods.

In yeasts N-linked glycosylation of asparagine occurs on the consensus sites Asn-X-Thr or Asn-X-Ser, wherein X is an amino acid. Commonly glycosylation in yeast results in N-linked and O-linked oligosaccharides of mannose. Thus, for expression in yeast the nucleic acid sequence may be modified or selected so that consensus sites are reduced or preferably absent. The Asn codon and/or the Thr codon may be modified, e.g. by mutagenesis or de novo synthesis. Preferably Asn and/or Thr is replaced by another amino acid. Also Asp may be replaced by another amino acid. In one embodiment the polypeptide sequence contains no Ser and/or no Asn. To analyze the degree of post-translational modification or to determine the content of glycosylation mass spectrometry, such as MALDI-TOF-MS (Matrix Assisted Laser Desorption Ionization mass spectrometry) can be carried out as known in the art.

Alternatively the amount of glycosylation can be determined using the titration method described by Michel Dubois et al, "Colorimetric Method for Determination of

Sugars and Related Substances", Analytical Chemistry, vol 28, No.3, March 1956, 350 356. This method can be used to determine simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl ethers with free or potentially free reducing groups.

The content of glycosylation of the gelatinous polypeptide used is preferably equal to, or less than about 2 (m/m) %, more preferably less than about 1 (m/m) %, most preferably less than about 0.5 (m/m) %, 0.2 (m/m) % or 0.1 (m/m) %. In a preferred embodiment the degree of glycosylation is zero. The degree of glycosylation refers to the total carbohydrate weight per unit weight of the collagenous polypeptides as determined by for example MALDI-TOF-MS (Matrix Assisted Laser Desorption Ionization mass spectrometry) or the titration method by Dubois referred to above. The term 'glycosylation' refers not only to monosaccharides, but also to polysaccharides such as di-. tri- or tetra saccharides.

Cells that can be adhered and / or grown on the micro particles of the invention can be any living, genetically modified or malignant living cell. Preferred are human (or mammalian) cells, in particular of the mesenchymal lineage such as (myo)fibroblasts, smooth muscle cells, pericytes, stromal cells (including multipotent/mesenchymal stem/stromal cells), and epithelial lineages such as epithelial (stem) cells from lung, gut, stomach, exocrine glands, liver, skin, kidney to mention a few, but also cardiac and striated muscle cells, endothelial (precursor) cells as well as neuronal lineages such as oligodendrocytes, neurons and neural stem cells and astrocytes. In a preferred embodiment the cells are obtained from the subject to be treated.

In one embodiment, the particles contain no RGD-motifs. These can be used in cases where a-selective attachment of cells occurs, or where attachment is not dependent of RGD- sequences. Competition from RGD-anchorage depending cells can be avoided on such particles, which is not possible with particles made from a natural gelatin. Preferably such particles are macroporous particles.

The recombinant gelatin polypeptide cell carriers may further comprise one or more bioactive compounds such as hormones, growth promoters, antibiotics, immune-suppressors, and the like. Further the cell carrier particles may comprise one or more compounds that can aid in wound healing processes. A "bioactive compound" is any compound (either a natural compound or a synthetic compound) which exerts a

biological effect on other cells that contributes to processes involved in healing and regeneration such as suppression of apoptosis, improvement of perfusion, remodelling of the tissue, restoration of the tissue parenchymal cells as well as suppression of adverse processes such as fibrosis. Such compounds are widely available in the art. The compound may be incorporated in the particles during its manufacture or, alternatively, it may be added subsequently to the particles. In one embodiment two cell carrier batches comprising recombinant particles are provided in which each cell carrier batch carries a different cell or bioactive compound which can be injected after mixing said two batches or which can be injected subsequently. The cell carrier particles can have an average size of from 1 to 500 micron. Alternatively, therapeutic cells that are loaded onto the micro-carriers themselves may be instructed by said carriers to produce factors that augment or suppress, wherever appropriate, above mentioned processes.

In one embodiment the particles are suitable as injectable cell carriers and have an average particle size of less than or equal to 200 μm, e.g. between 10 and 200 μm, or between 10 and 175 μm, or between 10 and 150 μm or between 10 and 125 μm. Alternatively such particles have an average particle size of less than or equal to 100 μm, e.g. between 10 and 75 μm. Injectable cell carriers can for example be applied for delivery of tissue repairing cells without the need of invasive surgery such as the delivery of adipose tissue derived stromal cells in myocardial scar tissue.

In one embodiment the cell carrier batch comprises a monodisperse recombinant particle population. In another embodiment the cell carrier is a mix of two or more monodisperse recombinant particle populations, each population having a different average size.

The micro particles according to the invention can be prepared from recombinant gelatin with methods known in the art and in which the particles are formed out of a starting solution of the recombinant gelatin. Such methods comprise particle formation using oil/water emulsion techniques which may comprise phase inversion such as described in for example EP 222718 or WO 2003/104313 or precipitation techniques such as described in for example SU1161548.

EXAMPLES

Micro-carrier preparations

Recombinant gelatin types

An RGD-enriched gelatine was produced based on a nucleic acid sequence that encodes for a part of the gelatine amino acid sequence of human COL1Al-I and modifying this nucleic acid sequence. The methods as disclosed in EP-A-0926543, EP-A-1014176 and WO01/34646 were used. This RGD-enriched gelatine is recombinant gelatin having the following acid sequence as in SEQ ID NO: 3 and SEQ ID NO: 4. ID SEQ 4 shown below is identical to SEQ ID NO 3 but then RGD's are replaced by DGR.

Amino acid sequence of SEQ ID 3:

GAPGAPGLQGAPGLQGMPGERGAAGLPGPKGERGDAGPKGADGAPGAPGLQ
GMPGERGAAGLPGPKGERGDAGPKGADGAPGKDGVRGLAGPIGPPGERGAA
GLPGPKGERGDAGPKGADGAPGKDGVRGLAGPIGPPGPAGAPGAPGLQGMPG
ERGAAGLPGPKGERGDAGPKGADGAPGKDGVRGLAGPPGAPGLQGAPGLQG
MPGERGAAGLPGPKGERGDAGPKGADGAPGAPGLQGMPGERGAAGLPGPKG
ERGDAGPKGADGAPGKDGVRGLAGPIGPPGERGAAGLPGPKGERGDAGPKGA
DGAPGKDGVRGLAGPIGPPGPAGAPGAPGLQGMPGERGAAGLPGPKGERGDA
GPKGADGAPGKDGVRGLAGPPGAPGLQGAPGLQGMPGERGAAGLPGPKGER
GDAGPKGADGAPGAPGLQGMPGERGAAGLPGPKGERGDAGPKGADGAPGKD
GVRGLAGPIGPPGERGAAGLPGPKGERGDAGPKGADGAPGKDGVRGLAGPIG
PPGPAGAPGAPGLQGMPGERGAAGLPGPKGERGDAGPKGADGAPGKDGVRG
LAGPPG;

SEQ ID 3 has 571 amino acids in length comprising 12 RGD motifs.

Amino acid sequence of SEQ ID 4:

GAPGAPGLQGAPGLQGMPGERGAAGLPGPKGEDGRAGPKGADGAPGAPGLQ
GMPGERGAAGLPGPKGEDGRAGPKGADGAPGKDGVRGLAGPIGPPGERGAA
GLPGPKGEDGRAGPKGADGAPGKDGVRGLAGPIGPPGPAGAPGAPGLQGMPG
ERGAAGLPGPKGEDGRAGPKGADGAPGKDGVRGLAGPPGAPGLQGAPGLQG

MPGERGAAGLPGPKGEDGRAGPKGADGAPGAPGLQGMPGERGAAGLPGPKG
EDGRAGPKGADGAPGKDGVRGLAGPIGPPGERGAAGLPGPKGEDGRAGPKGA
DGAPGKDGVRGLAGPIGPPGPAGAPGAPGLQGMPGERGAAGLPGPKGEDGRA
GPKGADGAPGKDGVRGLAGPPGAPGLQGAPGLQGMPGERGAAGLPGPKGED
GRAGPKGADGAPGAPGLQGMPGERGAAGLPGPKGEDGRAGPKGADGAPGKD
GVRGLAGPIGPPGERGAAGLPGPKGEDGRAGPKGADGAPGKDGVRGLAGPIG
PPGPAGAPGAPGLQGMPGERGAAGLPGPKGEDGRAGPKGADGAPGKDGVRG
LAGPPG;

SEQ ID 4 has 571 amino acids in length.

For both types of recombinant gelatin smooth and rough micro-carriers were prepared as described below.

Smooth Microspheres

Microspheres with smooth surface were prepared via water in oil emulsification. Briefly, an aqueous solution of 20 w/w% recombinant collagenous peptide solution was emulsified in corn oil by thoroughly mixing for 20 minutes. Beads were harvested by cooling to 4 ºC and were rinsed with acetone for several times to remove the oil. Finally, the microspheres were dried overnight at 60 ºC

Beads were subsequently cross-linked by either 1 v/v % HMDIC in ethanol for 24 hours (=HMDIC high, 6,25 mmol HMDIC/g beads) or via dehydrothermal treatment at 160 ºC for 96 hours under vacuum conditions. Beads were sieved to yield microspheres sized between 50 and 100 µm.

Rough Microspheres

Rough microspheres were prepared via water in oil emulsification using calcium carbonate as a porogen. Briefly, calcium carbonate with a crystal size of 1 µm (Acros) was mixed in a 1:1 ratio with 20 w/w% recombinant collagenous peptide solution. This mixture was subsequently emulsified in corn oil by thoroughly mixing for 20 minutes. Beads were harvested by cooling to 4 ºC and were rinsed with acetone for several times to remove the oil. Finally, the microspheres were dried overnight at 60 ºC. Beads were subsequently cross-linked by either 1 v/v % HMDIC in ethanol for 24 hours (=HMDIC high, 6,25 mmol HMDIC/g beads), 0.2 v/v % HMDIC in ethanol for 24 hours (=HMDIC low, 1,25 mmol HMDIC/g beads) or via dehydrothermal treatment at 160 ºC for 96 hours under vacuum conditions. Calcium carbonate was removed by acid

washing steps with 1M HCl. Beads were again washed with buffer and water to remove residual crosslinking agents and dried overnight at 60 °C. Beads were sieved to yield microspheres sized between 50 and 100 µm.

Example 1: **Adhesion of adipose tissue derived stromal cells to 2D-coatings of recombinant gelatins with various crosslinkers**

Methods

Crosslinking of recombinant collagen peptide coatings was performed by; 1) amine-amine crosslinking using hexamethylene diisocyanate (HMDIC), 2) carboxy-amine crosslinking using 1-ethyl-3-(3-dimethylaminopropyl carbodiimide (EDC), and 3) dehydrothermal treatment (DHT).

Briefly, 32.5 mM EDC (EDC low) or 130 mM (EDC high) was added to 10 w/w % recombinant collagen peptide solution, initiating direct crosslinking. This mixture was immediately coated on the wells, followed by overnight crosslinking. Wells were washed three times with PBS to remove residuals. For HMDIC crosslinking, 24 wells were coated with 10 w/w% recombinant collagen peptide/ 15 w/w% glycerol solution and dried overnight to evaporate water. Coatings were cross-linked for 6 hours using 1 ml 15 w/w% glycerol in isopropanol with either 0.2 v/v% HMDIC in ethanol (HMDIC low) or 1 v/v% HMDIC in ethanol (HMDIC high) per 24 well. Residual HMDIC was removed by three additional washing steps with 15 w/w% glycerol in isopropanol. For DHT, Silane prep slides (Sigma) were coated with 10 w/w% recombinant collagen peptide followed by overnight drying. Coated glass slides were cross-linked at 160 °C under vacuum conditions. All coatings were washed 3 times with cell culture medium before actual cell culturing experiments were started.

Adipose tissue derived stromal cells isolation, characterizations and culturing

Adipose tissue derived stromal stem cells (ADSC) were isolated from human subcutaneous liposuction materials (Bergman Clinics, The Netherlands). For isolation of ADSC, collected adipose tissue was extensively washed with PBS to remove red blood cells, then tissue was enzymatically digested with 0.1% Collagenase A, (Roche Diagnostic, Mannheim, Germany) 1:1 in PBS, containing 1% bovine serum albumin (BSA; Sigma-Aldrich, Boston, MA) shaking at 37°C for 1 h. Digested tissue was

filtered and washed with 1% PBS/BSA. Collected cells were suspended and cultured at 37°C at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (Thermo Scientific, Hemel Hempstead, UK), 1% L-glutamine e (Lonza Biowhittaker, Verviers, Belgium), and 1% penicillin/ streptomycin (Gibco, Invitrogen, Carlsbad, CA). Medium was refreshed three times per week until cells get enough confluence for experiments. ADSC between passages 3-6 were used for experiments. Freshly isolated cells and cultured cells were examined by Flow cytometry for mesenchymal markers positive for CD90, CD105, CD44, CD29 and negative for CD45 and CD31.

Cell Seeding

Pooled human ADSC from three different donors were used for both 2D and 3D experiments. Briefly, for 2D experiments 20,000 cells/cm^2 were cultured overnight (16-18h) on different cross-linked (chemical and thermal) recombinant gelatin materials and tissue cultured plastic plates (TCP) were used as a control. For adhesion experiment ADSC were culture on biomaterials with either RGD-rich recombinant gelatins SEQ 3, or SEQ 4 with scrambled RGD for 2h and 4h. Non adherent cells were washed away, whereas attached cells were fixed with 2% PF and stained with DAPI (4', 6-diamidino-2-phenylindole). Number of attached cells was analyzed by TissueFAXS microscopy.

ADSC conditioned medium was collected after 18h of culture. Concisely, ADSC were cultured on plastic or different biomaterials with endothelial cell medium (ECM) supplemented with 2% fetal bovine serum, 1% L-glutamine, and 1% penicillin/ streptomycin) at 37°C with 5% CO_2 and 21% O_2. All the conditioned media was filtered through a 0.4 µm filter to exclude any cell residue and stored at -80°C.

Quantitative reverse transcriptase PCR

Cultured ADSC on different conditions were lysed by RNA isolation solvent (TEL-TEST, INC). Total RNA was isolated, according to manufacturer instructions, and total RNA concentration was measured by Nano drop. One µg of total RNA was used in cDNA synthesis with RevertAid First Strand cDNA synthesis kit (Fermentas UAB, Vilnius, Lithuania). Resulting cDNA was used for quantitative RT-PCR with established primer to investigate mesenchymal marker (SM22a), extra cellular matrix

(ECM) (Collagen I, III – Elastin – Laminin- Fibronectin), inflammatory factors (MCP-1/ IL-6/IL-1b / IL-8/ TNFa) and Growth factors (Tb4, FGF-2, HGF, IGF, and VEGF).

Flow cytometer Analysis

ADSC cultured on plastic and different cross-linked materials were stained for Ki67 (Abcam) and Annexin V/Ethidium Iodide using the Apoptosis necrosis detection kit (Promogen). Briefly, cells were harvested by enzymatic method (accutase), collected in 15 ml tubes and washed three times by normal PBS. To determine the live-dead cells, collected cells were re suspended in binding buffer (followed by company protocol) and stained with Annexin V/ Ethidium Iodide directly after collection. FACS measurement was done after 15 min staining. In order to determine cells proliferation, part of the cell suspension, was washed with PBS and fixed with 2% PF, washed with PBS again and finally stained with Ki67 antibody.

Western Blot

ADSC were cultured in 6 well culture plates either plastic or biomaterial coated, in serum-containing medium. After overnight culture, cells were rinsed twice with 2mL of cold PBS and lysed by 300µl of cold lysis buffer (RIPA with protease inhibitors). Lysed cells were collected in 1.5 ml microcentrifuge tubes. Solution was homogenized by sonication at about 30W for 10 s. The microcentrifuge tube was then centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was transferred to a clean tube. A similar procedure was performed for cells cultured on microspheres (3D). Briefly, cell loaded microspheres were collected in 50 ml tubes and centrifuged at 300g for 5 min at 4°C. The cell-loaded microspheres pellets were rinsed twice with cold PBS and same procedures as monolayer-cultured cells were followed. Protein concentration was measured by BIO-RAD protein assay regent and 20ng of total proteins were loaded in gel.

Membrane was stained with Akt, also known as Protein Kinase B (PKB), and Focal Adhesion Kinase (FAK) as a survival and adhesion markers to investigate the different cross-linkers effect on ADSC survival and adhesion.

Statistical analysis

All data were presented as the mean values plus standard error of the mean (SEM) of three independent studies. Statistical comparisons were performed using One-way ANOVA for chemical cross-linked and student t-test for thermal cross-linked materials. All data analyses were done using GraphPad Prism5.

Evaluation

Non adherent cells were washed away, whereas attached cells were fixed with 2% PF and stained with DAPI (4', 6-diamidino-2-phenylindole). Number of attached cells was assessed by TissueFAXS microscopy. Results are shown in figure 7 and 8. The comparative adhesion on culture plastic was taken as 100%. In Figure 9 RT-PCR evaluation is shown.

Conclusion

The biomaterials modified with RGD to enhance integrin-mediated binding of ADSC, showed faster and higher compared to the scrambled controls. However, only chemically cross-linked (EDC or HMDIC) maintained a difference in selective binding in contrast to physically cross-linked material (DHT). Within the chemically cross-linked biomaterials, high degrees of cross-linking showed the highest binding of ADSC, while the use of HMDIC to cross-link consistently showed higher binding than EDC. In conclusion the highest, specific, binding of ADSC was achieved by cross-linking highly with HMDIC (Fig. 7). Within the time frame of the experiment, proliferation on high cross-linked biomaterial with EDC was lower compared to the other cross-linking conditions. This was caused by an increase in cell death on the EDC-high cross-linked material. The other types of cross-linking did not cause significant cell death. ADSC bound to highly cross-linked biomaterial with HMDIC, proliferated fastest and had occupied the available space and thus withdrew from cell cycle. This appears in the measurements as non-proliferation. In terms of markers for integrin activation (phospho-FAK) and suppression of apoptosis (phospho-Akt), no significant differences were observed, although HMDIC cross-linked biomaterials tended to suppress apoptosis better. In conclusion, highly cross-linked biomaterial with HMDIC, showed the best performance with regard to proliferation, lack of cell death and upregulated survival factors (Fig. 8).

Finally, the degree of cross-linking (High vs Low) and the type of cross-linker (EDC vs HMDIC) had remarkable influence on the expression of 'pro-regenerative' genes of ADSC cultured on the biomaterials. Compared to the Gold Standard (tissue culture polystyrene – TCPS) the potentially adverse pro-inflammatory response of ADSC was suppressed, except on EDC, on highly cross-linked biomaterial. Some genes were upregulated, in particular on highly cross-linked biomaterial with HMDIC, such as HGF and IGF, while other genes were expressed similar to the Gold Standard. Of particular importance is, that none of the cross-linked biomaterials caused upregulation of mesenchymal genes (shown: SM22alpha), which indicates the absence of differentiation of ADSC to potentially adverse myofibroblasts. In conclusion, the gene expression pattern of ADSC cultured on cross-linked biomaterials is not significantly affected compared to TCPS, while on HMDIC, highly cross-linked, the pro-regenerative gene expression is superior to the other types of cross-linking (Fig. 9). To summarize: these analyses indicate SEQ-3 after high cross-linking with HMDIC is the material of choice for adhesion, proliferation, survival and function of ADSC

Example 2: Characterization of Adipose tissue derived stromal cells on HMDIC cross-linked microspheres

With regard to cell loading on the microspheres (MSs), 12.5mg of gelatin SEQ ID3 HMDIC-high crosslinked rough beads were mixed with $1x10^6$ cells and incubated for 16-18h in static condition at 37°C at 5% CO_2 in DMEM medium containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/ streptomycin. Ultra Low Cluster plates (Corning, NY) were used for cell loading on biomaterials. TCPC culture plates and Cultisphers-S (CS)(Sigma-Aldrich, Zwijndrecht, the Netherlands) were used as standard control for microspheres.

Evaluation: To evaluate ADSC characteristics RT-PCR was performed as described above. Results are shown in figure 10.

Conclusions: Virtually all genes tested had comparable expression levels as compared to the control (ADSC cultured on plastic). However, the expression of mesenchymal genes, as represented by SM22alpha, was suppressed. This is advantageous as

mesenchymal transition of ADSC is undesirable. The fact that chemotactic genes, inflammatory genes, as well as matrix-related genes and a plethora of pro-regenerative growth factors were maintained at similar levels as in the control is advantageous too: the formulation of the recombinant gelatin warrants appropriate functioning of administered ADSC.

Example 3: Electrophysiology of neonatal rat ventricular myocytes in contact with bare ADSC's and ADSC's loaded onto recombinant gelatin microspheres

Isolation and culturing of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVM) were isolated as follows:

One day old rat pups were decapitated and the hearts were rapidly explanted. The atrial tissue was removed and the ventricles were dissected into 4-6 pieces and left to rotate overnight at 4°C in HBSS (Gibco, #14170-088) without Ca^{2+} and Mg^{2+} but containing trypsin (1 mg/mL, USB #22720 GM). The following day the enzymatic effect of trypsin was inactivated with culture medium (composition see below) and the ventricles were enzymatically dissociated in HBSS (without Ca^{2+} and Mg^{2+}) containing collagenase (1 mg/mL, Worthington #S8B10315-A, 230 units/mg) at 37°C. Then, dissociation solutions were centrifuged (1000 rpm, 5 minutes) and cells were re-suspended in 10% culture medium. To separate fibroblasts from cardiomyocytes, cells were pre-plated for 2 hours in a polystyrene treated T175 cell culture flask at 37°C in 5% CO^2. After two hours, non-adherent cells were collected and viable cell yield was determined with a cell counting chamber (Zell Zimmer). NRVM were seeded (350,000) onto a microelectrode array (MEA; Multi Channel Systems MCS GmbH, Reutlingen, Germany) with 64 integrated extracellular electrodes arranged in an 8 by 8 matrix at interelectrode distances of 0.7 mm. MEAs were coated with fibronectin (5mg/40ml BD Biosciences 356008) at least 2 hours prior to NRVM seeding. NRVM were cultured at 37°C in 5% CO_2 in medium consisting of M199 (Gibco, #31150) containing (mM) NaCl 117, KCl 5.3, CaCl2 1.8, MgSO4 0.8, NaHCO3 26.2, and Na2HPO4 1.0, supplemented with 10 mM HEPES (Gibco, #15630–080), 5000 U/L penicillin-G (Sigma, #P7794), 2 mg/L vitamin B12 (Sigma, #V2876), 3,5 g/L glucose,1% NEAA non-essential amino acids (Gibco, #11140–050), 2mM L-glutamine (Gibco, #25030–

081), and either 10% or 2% fetal bovine serum (Gibco, #16140071). The day after seeding NRVM were washed twice with HBBS (Gibco, #14025-050) with Ca^{2+} and Mg^{2+} and fresh 10% culture medium was added. Two days after seeding 10% culture medium was replaced by 2% culture medium.

Isolation and culture of adipose-derived stem cells

Adipose tissue was dissected from porcine subcutaneous abdominal fat after resection and stored at 4 °C. Twenty-four hours later, adipose tissue was minced and extensive washed with PBS . The tissue fragments were incubated with 0.1% Collagenase A (Roche Diagnostics, Mannheim, Germany), 1:1 in PBS, containing 1% bovine serum albumin (BSA; Sigma- Aldrich, Boston, MA) at 37°C for 1 hour. Digested tissue was washed with PBS, 1% BSA to remove the adipocytes and lipid content. The cell pellet was suspended in PBS (Phosphate buffered saline) from Biological Industries, 1% BSA and subjected to Lymph prep (Axis-Shield PoC, Oslo, Norway) density gradient centrifugation. The cells from the interface were collected and washed with PBS, 1% BSA and suspended in DMEM (Lonza Biowhittaker, Verviers, Belgium), 10% FBS (Thermo Scientific, Hemel Hempstead, UK), 100 U/mL penicillin, 100 mg/mL streptomycin (Gibco, Invitrogen, Carlsbad, CA) and 2 mM L-glutamine (Lonza Biowhittaker, Verviers, Belgium). Cells were seeded in a T25 culture flasks, cultured till passage 3 and used for experiments.

Medium was refreshed every 2 to 3 days and the ADSC cultures were dissociated and divided (1:2) when 90% confluence was reached. ADSC were washed twice with PBS (Gibco #14190094) and then incubated with accutase (GE Healthcare L11-007) for 5 minutes at 37°C. Accutase was collected from the flask with culture medium and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cell pellet was suspended in culture medium and the cell suspension was divided between appropriate numbers of flaks.

Microsphere preparation and loading

The day prior to making co-cultures, 10 ml ADSC media were loaded onto 10 mg microspheres of HMDIC high cross-linked recombinant gelatin SEQ ID 3.
The ADSC were fluorescently labelled with CDFA-SE (Invitrogen Vybrand® CFDA

SE Cell Tracer Kit) according to manufacturer's protocol, and treated with mitomycin-C (Sigma M4287-2MG) for three hours prior to the co-culture to prevent proliferation. The viable cell yield was determined with a standard cell count chamber.

ADSC were seeded in low-adherent T6 wells (Costar® ultra-low cluster plate #3471) in ADSC medium, together with the microspheres (1 mg microspheres per 250,000 ADSC) and left overnight at 37°C in 5% CO_2. Electrophysiological effects of ADSC were tested using different ratios of ADSC:NRVM. ADSC loaded MS were co-cultured in ratios of 1:4 (25%), 1:2 (50%) or 1:1 (100%) to NRVM. The following day the cell/microsphere solution was collected from the low-adherent plates and centrifuged for 5 minutes at 1000 rpm. Supernatant was removed and the pellet was re-suspended in an equal amount of 2% NRVM culture medium, the cell/microsphere solution (1.4 ml) was then added to the appropriate monolayers. Monolayers containing only ADSC were prepared on the same day by labelling ADSC with CDFA-SE (Invitrogen Vybrand® CFDA SE Cell Tracer Kit) according to manufacturer's protocol, and treating them with mitomycin-C (Sigma M4287-2MG) for three hours prior to the co-culture. Monolayers serving as controls received fresh 2% culture medium on this day. Two days later electrical mapping and microelectrode measurements were performed.

Conditioned medium was collected at day 7, filtered through a 0.22 µm filter (MILLEX®GV SLGV033RS) and stored at -20°C until use.

Electrical mapping and microelectrodes

Electrical mapping were performed on a 7 days old confluent monolayer of NRVM and on co-cultures with NRVM with ADSC (N+A) and NRVM with ADCS loaded microspheres MS (N+A+MS). The cultures were positioned in a temperature controlled MEA holder (Multichannel Systems MCS GmbH) (TC01/02 Multichannel Systems MSC GmbH). All cultures were stimulated from at least three subsequent stimulation sites using a bipolar extracellular stimulus electrode (twice diastolic stimulation threshold, 1 ms or 2 ms rectangular current pulses; see figure 2a). Unipolar electrograms were recorded with a multichannel amplifier (Biosemi, ActiveTwo, Amsterdam, The Netherlands) . The unipolar recordings were made with respect to the integrated reference electrode of the MEA. Conduction velocity (CV) was determined from activation maps constructed using the maximum negative dV/dt as activation time

(AT) with the use of a custom made program based on MATLAB R2006b (The MathWorks, Inc., Natick, MA, USA). Conduction velocity was determined along lines perpendicular to isochronal lines by dividing the distance by the difference in local activation time. The conduction velocity was determined (at a basic cycle length (BCL) of 600 ms) or during spontaneous activity if pacing was not possible during stimulated activation. Effective refractory period (ERP; shortest coupling interval that results in a propagated response) was determined using programmed electrical stimulation consisting of 8 basic stimuli (BCL 600 ms) followed by one premature stimulus with a coupling interval at 400 ms decrementing (steps of 20 ms) until ERP was reached. To accurately determine the ERP the coupling interval of the premature stimulus was increased again with 40 ms and decreased in steps of 5 ms until ERP was reached again. From data obtained during premature stimulation conduction curves were constructed. To quantify the heterogeneity in conduction as a measure of arrhythmia vulnerability we obtained maximum AT differences between each adjacent electrode quartet in the grid and plotted the total range of maximal AT differences in a histogram using the method described by Lammers et all in Am J Physiol (1990) October 59, H1254-H1263.

Microelectrodes were pulled from glass capillaries (Harvard apparatus GC100F-10) and filled with 3M KCl. An AgCl covered silver wire was used as a reference electrode. Following activation mapping, action potentials were recorded during continuous pacing at BCL 600 ms. Resting membrane potential (RMP) and action potential duration (APD) were deduced from the action potentials.

Immunostaining and fluorescence imaging

For immunofluorescence, cultures identical to the MEAs were plated (density 500,000 cells/well) and cultured in a 12-well plate (MP Biomedicals 76-053-05) containing fibronectin-coated (5mg/40ml BD Biosciences 356008) cover-slips in parallel with the MEA based cultures. On the day of electrical stimulation (day 7) these cultures were fixed with ice cold methanol for 2 minutes, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA (Roche BSA fraction V #10735094001) for 20 minutes. Cultures were then stained with mouse anti-sarcomeric-actinin primary antibody (Sigma, 574366A; 1:1000), rabbit anti Connexin 43 primary antibody (Invitrogen 71-0700; 1:200) and mouse anti-human monoclonal CD44 primary antibody (Lifespan

Biosciences, LS-B1862; 1:250) in 1% BSA for 2 hours at RT, washed three times with PBS, and then stained with Alexa Fluor-647 goat anti-mouse IgG (Life Technology, A21235; 1:250), Alexa Fluor-568 goat anti-rabbit IgG (Life Technology, A11011; 1:125), Alexa Fluor-488 goat anti-rabbit IgG (Life Technology, A11008; 1:250), and To-pro (Invitrogen, T3605; 1:1000) for 120 min in 1% BSA. After being stained, the cover-slips were washed an additional three times in PBS before embedded in 50%glycerol/50%PBS. Immunofluorescence imaging was performed using a 10x objective on a Leica SPE confocal laser scanning microscope recorded with a camera using Leica Application Suite Advanced Fluorescene (LAS AF) software.

Statistical analysis

Continuous variables are presented as mean with standard deviation (SD) and compared using one-way ANOVA in case of a normal distribution, and presented as the median with inter-quartile range (IQR) in case of a skewed distribution and tested with the Kruskal-Wallis analysis. Post-hoc analysis was performed using the Bonferroni correction. A P-value of <0.05 was considered statistically significant.

Evaluation

Before electrophysiological characteristics of the different cultures were studied, structural characterization of the cultures was performed. Transmitted light microscopy showed that NRVM formed a confluent monolayer (Figure 1A). Fluorescent microscopy revealed that ADSC are scattered heterogeneously throughout the NRVM monolayer (Figure 1B) and showed that ADSC were loaded on the MS (beads or carriers) in cultures containing MS (Figure 1C).

Next, electrical mapping was performed to determine the conduction characteristics (velocity, conduction restitution) in the various cultures.

Figure 2 shows the layout of the MEA and the organization of the 60 electrode terminals in the core portion of the MEA. A separate reference electrode was positioned in the culture medium in order for recording of unipolar electrograms. From the constructed activation maps conduction velocity was measured along lines perpendicular to isochronal lines by dividing the distance by the difference in local activation time. The control culture shows homogeneous propagation along the MEA.

The culture with the ADSC demonstrates conduction slowing in a heterogeneous manner. The culture with ADSC loaded on MS shows conduction characteristics that resemble those of the control culture.

On average, cultures with ADSC alone showed a lower conduction velocity than monolayers of NRVM (0.12±0.03 m/s vs. 0.21±0.02 p=0.01, respectively figure 3). ADSC loaded MS showed a tendency towards an increase in CV compared to ADSC alone (0.16±0.05 m/s, N=8, p-0.06, figure 3)

The heterogeneity in conduction was quantified according to the method by Lammers in Am J Physiol (1990) October 259 H1254-H1263.

Maximum AT differences between each adjacent electrode quartet in the grid were obtained and the range of maximal AT differences was used.

Cultures with ADSC alone showed an increase in conduction heterogeneity in comparison with NRVM monolayers (10±4 vs. 6±1 ms, p=0.01, respectively, Figure 4). In the presence of ADSC loaded MS conduction this effect showed a trend to be mitigated (8±6 ms, p=0.06, Figure 4).

Fractionation of the initial deflection of the unipolar electrogram represents conduction heterogeneity. We determined the number of deflections in the initial deflection of each electrogram and measured the interval between earliest and latest deflection in the electrogram.

Figure 5A shows an example of a local electrogram recorded from a control culture. The local initial deflection has a single component. This is in contrast to an electrogram recorded from a culture with ADSC (Figure 5B) which demonstrates clear fractionation of the initial deflection (black arrows).

The percentage of electrodes (per MEA) showing fractionation was calculated (Figure 5C). The percentage of electrodes sites with fractionation was significantly increased in cultures with ADSC (alone) compared to controls (84±5% vs. 36±11%, p=0.01). The percentage of fractionation in cultures were ADSC were loaded on MS was not significantly different from controls (37±13% vs. 36±11%, respectively). Compared to ADSC alone ADSC loaded MS significantly decreased fractionation ($p<0.01$ Figure 5C).

Electrotonic coupling between ADSC and ventricular myocytes is suggested to cause depolarization of the resting membrane which may result in changes in APD depending on the membrane potential of the coupled stem cell. The resting membrane depolarization may lead to conduction slowing. Action potentials were recorded following impalement of a single cell in the cell culture during continuous pacing at BCL 600/700 ms. A typical NRVM action potential is illustrated in Figure 6A. RMP was taken as the highest negative membrane potential recorded. Action potential duration was measured at 20%, 50% and 90% of an action potential (Figure 6A). Mean RMP was -71±9 millivolts (mV) for NRVM only, -55±6mV for the co-culture with ADSC and -61±9mV for the co-culture with the ADSC loaded MS (Figure 6B). The largest difference was observed between the RMP of the monolayer and the co-culture with ADSC alone. Action potential duration (APD) and upstroke velocity (maximum positive dV/dT) were measured during different stimulated cycle lengths when possible.

In summary in comparison with a monolayer of NRVM, co-cultures with ADSC alone showed conduction slowing and increased conduction heterogeneity and fractionation (0.21±0.02 vs. 0.12±0.03 m/s, 6±1 vs. 10±4 ms, 36±11 vs. 84±5%, respectively, all $p=0.01$). ADSC loaded MS compared to ADSC alone showed a tendency of an increase in CV and a decrease in conduction heterogeneity (0.16±0.05vs 8±6 ms, respectively, $p=0.06$) and a significant decrease in fractionated EG (37±13%, $p<0.01$). In the presence of ADSC loaded MS conduction heterogeneity and fractionation were not statistically different from NRVM monolayers. RMP (1 MEA per group, 4 cells measured) was -71±9 mV for NRVM only, -55±6 mV for the co-culture with ADSC and -61±9 mV for the co-culture with the ADSC loaded MS.

Conclusions: Application of ADSC to a monolayer of NRVM causes conduction slowing and provokes conduction heterogeneity and fractionation. Stem cell loaded collagen microspheres tended to normalize the conduction velocity and heterogeneity and significantly improved fractionation relative to co-cultures with ADSC only. Biomaterial guided stem cell therapy may prevent potentially arrhythmogenic heterogeneous conduction slowing caused by stem cells.

Example 4: Degree of Crosslinking

Degree of crosslinking of various MS and coatings was determined by using the so-called TNBS method, which is a colorimetric method using colorant trinitrobenzenesulphonic acid (TNBS) to react with non-crosslinked amine groups of the polypeptide (see W.A. Bubnis, C.M. Ofner; The determination of ε-amino groups in soluble and insoluble proteinaceous by a spectrophotometric method using trinitrobenzenesulfonic acid; J. of Ana. Biochem., 1992, 207, 129-133). The degree of crosslinking is defined as the number of crosslinked amine groups being the total number of available amines in the non-crosslinked polypeptide minus the non-crosslinked amine-groups in the cross-linked polypeptide. In table 1 the degree of crosslinking of the MS and 2D coatings is shown. The data suggest that the beneficial effect observed for ADSC on HMDIC high crosslinked coatings and MS as shown in examples 2 and 3 is related to the higher degree of crosslinking of these scaffolds

TABLE 1: degree of crosslinking of MS and 2D coatings

Type of Spheres	Degree of Crosslinking (mmol/g spheres)
Rough MS HMDIC high	0.45
Rough MS HMDIC low	0.30
Rough MS DHT 96 hours	0.19
EDC high 2D coating	0.23
HMDIC high 2D coating	0.38

CLAIMS

1. A recombinant collagen or gelatin micro-carrier crosslinked by hexamethylenediisocyanate (HMDIC) comprising stromal cells for use in the treatment of cardiovascular disease.

2. A micro-carrier according to claim 1 wherein the degree of crosslinking is at least 0.10 mmol/g polypeptide and preferably more than 0.30 mmol/g polypeptide.

3. A micro-carrier according to claim 1 or 2, wherein said stromal cells are stem cells.

4. A micro-carrier according to any one of the claims 1-3, wherein cells are adipose tissue derived cells.

5. A micro-carrier according to any one of the claims 1-4, wherein said cells are human cells.

6. A micro-carrier according to any one of the claims 1-5, wherein said cells are multipotent cells.

7. A micro-carrier according to claim 6, wherein the recombinantly produced gelatin polypeptides comprise at least two amino acid residues, said two amino acid residues being extreme amino acid residues, which independently are selected from an aspartic acid residue and a glutamic acid residue, wherein a first aspartic acid residue or glutamic acid residue is the aspartic acid residue or glutamic acid residue that is closest to the N-terminus of the polypeptide and the second extreme aspartic acid residue or glutamic acid residue is the aspartic acid residue or glutamic acid residue that is closest to the C-terminus of the polypeptide and said extreme aspartic acid residues and/or glutamic acid residues are separated by at least 25 percent of the total number of amino acids in the recombinant gelatin

polypeptide.

8. A micro-carrier according to claim 6 or 7, wherein the recombinantly produced gelatin polypeptides comprise at least one lysine residue between said extreme two lysine residues.

9. A micro-carrier according to any one of the claims 6-8, wherein the recombinantly produced gelatin comprise at least two amino acid residues, said two amino acid residues being extreme amino acid residues, which independently are selected from an aspartic acid residue and a glutamic acid residue, wherein a first aspartic acid residue or glutamic acid residue is the aspartic acid residue or glutamic acid residue that is closest to the N-terminus of the polypeptide and the second extreme aspartic acid residue or glutamic acid residue is the aspartic acid residue or glutamic acid residue that is closest to the C-terminus of the polypeptide and said extreme aspartic acid residues and/or glutamic acid residues are separated by at least 25 percent of the total number of amino acids in the recombinant gelatin polypeptide.

10. A micro-carrier according to any one of the claims 6-9, wherein the recombinantly produced gelatin polypeptides comprise at least one aspartic acid residue or glutamic acid residue between said extreme extreme aspartic acid residues and/or glutamic acid residues.

11. A micro-carrier according to any one of the claims 1-10, wherein the collagen or gelatin comprises at least one RGD motif.

12. A micro-carrier according to any one of the claims 1-11, wherein the percentage of RGD-motifs related to the total number of amino acids in the RGD-motif-containing recombinantly produced gelatin polypeptide is at least 0.4 and if said collagen polypeptide comprises 350 amino acids or more, each stretch of 350 amino acids contains at least one RGD-motif.

13. A micro-carrier according to any one of the claims 1-12, wherein said stromal cells are delivered at myocardial scar tissue sites.

14. A micro-carrier according to any one of the claims 1-13, wherein said microcarrier is injectable.

Fig. 1a

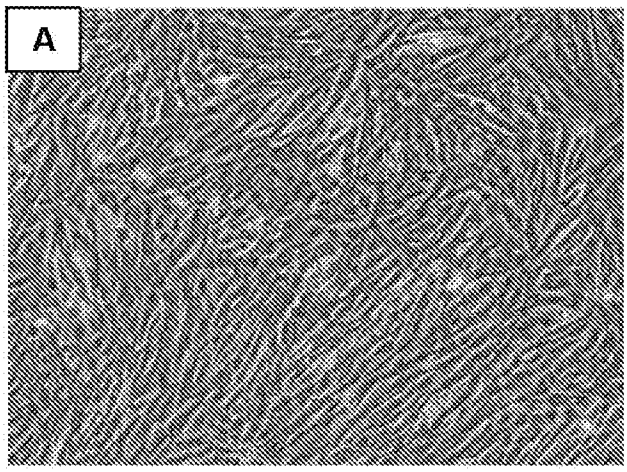


Fig. 1b

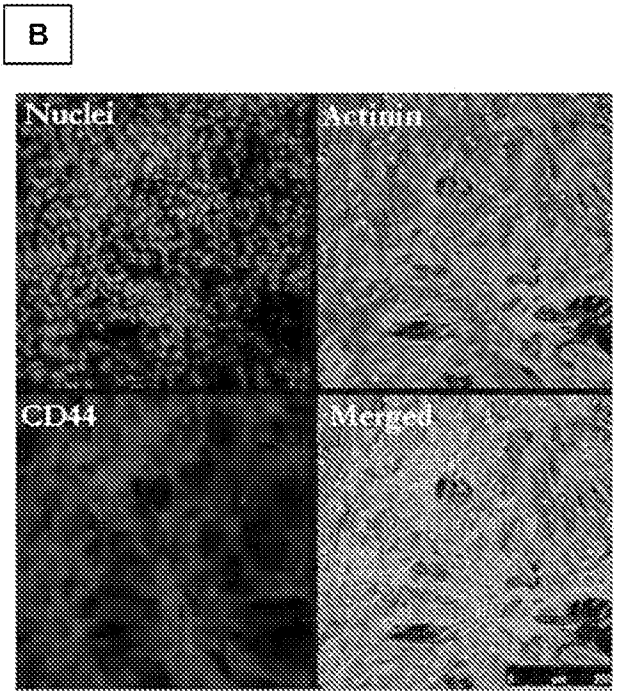


Fig. 1c

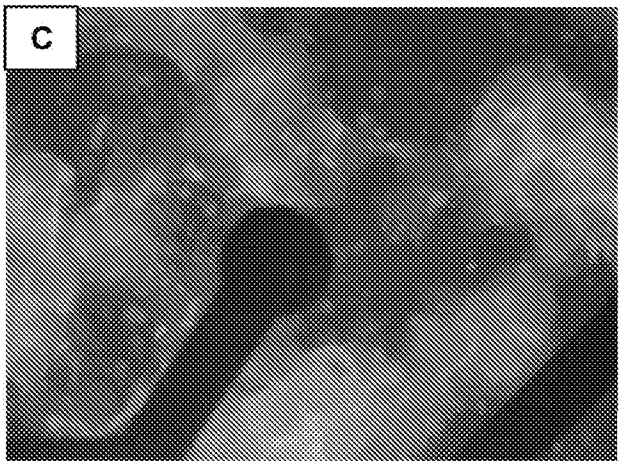


Fig. 2a

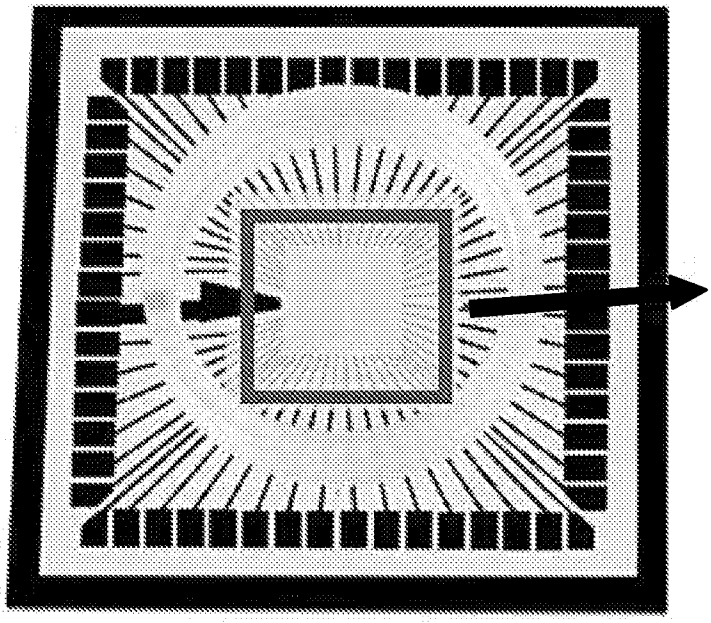


Fig. 2b

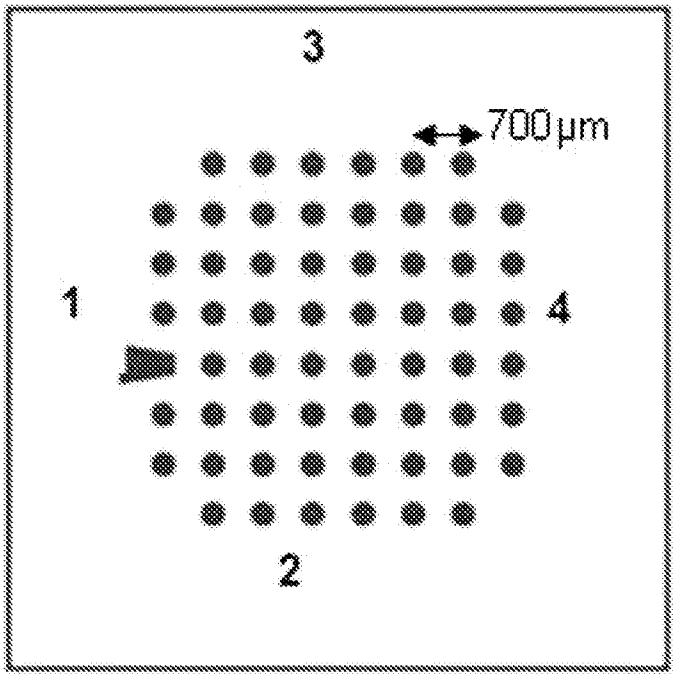


Fig. 3

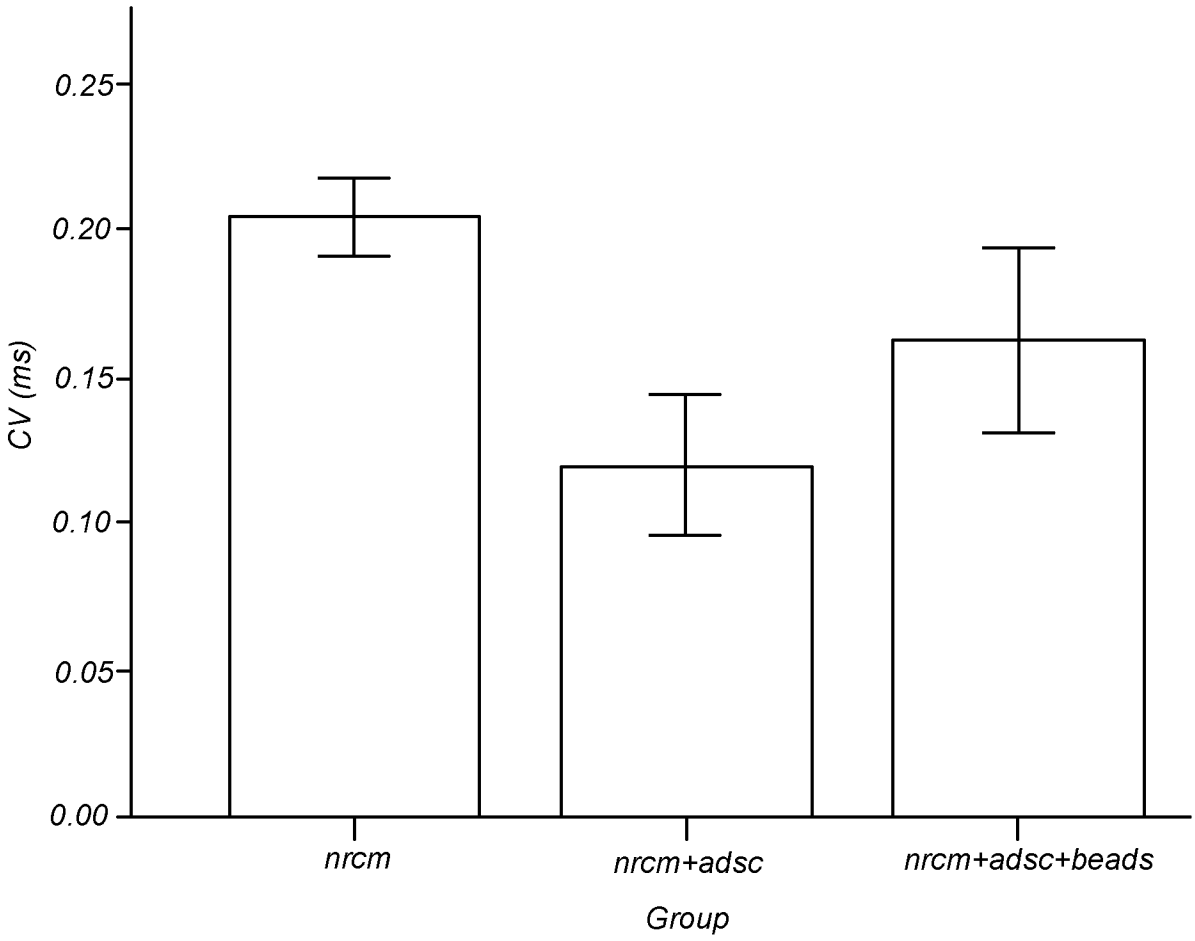

0.25
0.20
0.15
0.10
0.05
0.00
CV (ms)
nrcm
nrcm+adsc
nrcm+adsc+beads
Group

Fig. 4

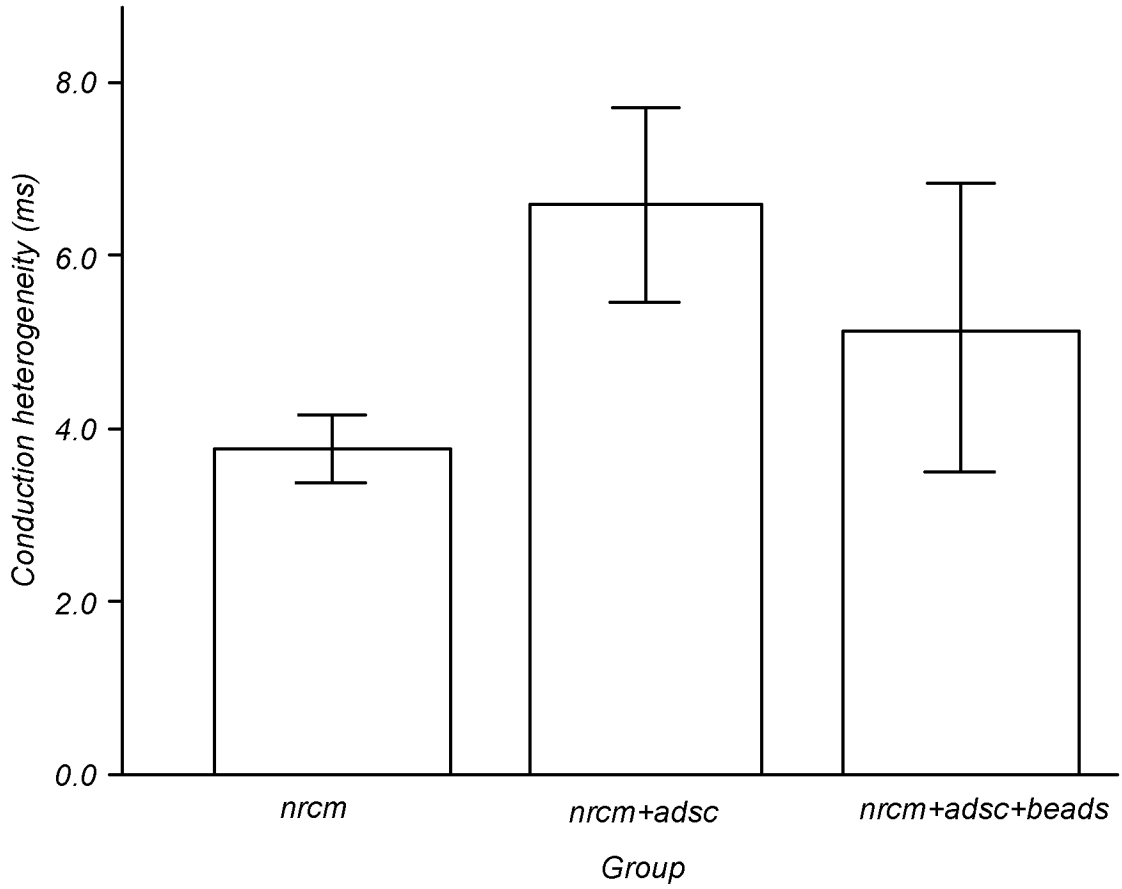

8.0
6.0
4.0
2.0
0.0
Conduction heterogeneity (ms)
nrcm
nrcm+adsc
nrcm+adsc+beads
Group

Fig. 5a

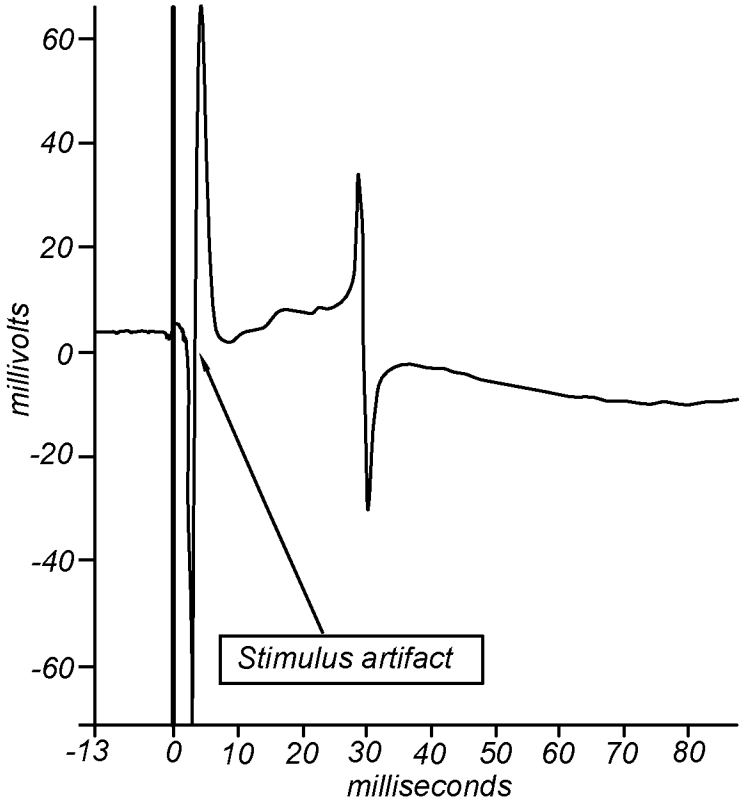

60
40
20
0
-20
-40
-60
millivolts
Stimulus artifact
-13
0
10
20
30
40
50
60
70
80
milliseconds

Fig. 5b

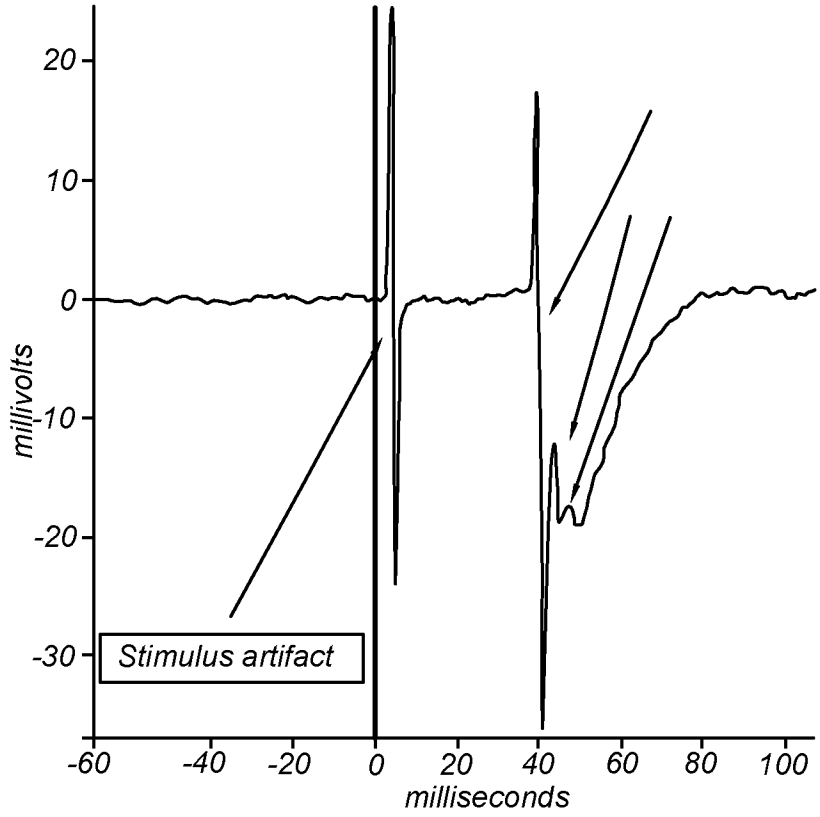

20
10
0
-10
-20
-30
millivolts
Stimulus artifact
-60
-40
-20
0
20
40
60
80
100
milliseconds

Fig. 5c

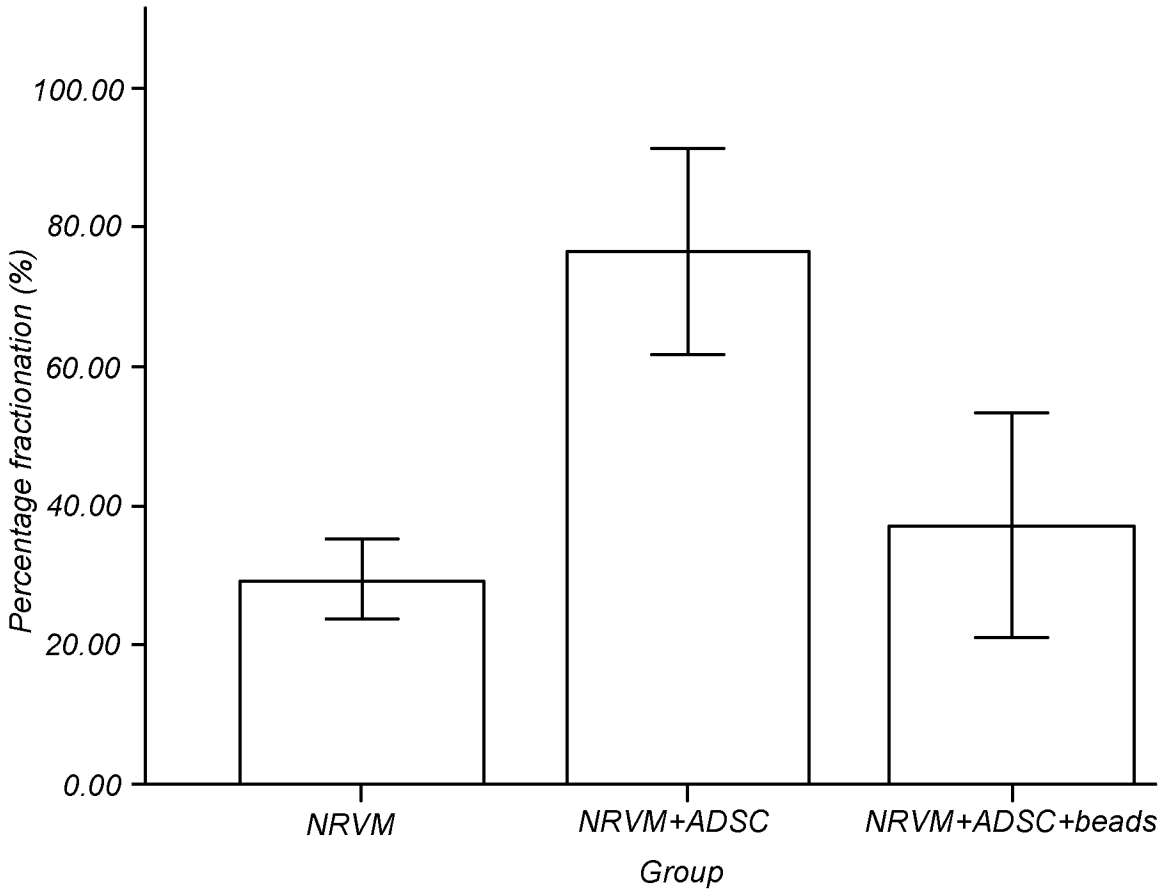

100.00
80.00
60.00
40.00
20.00
0.00
Percentage fractionation (%)
NRVM
NRVM+ADSC
NRVM+ADSC+beads
Group

Fig. 6a

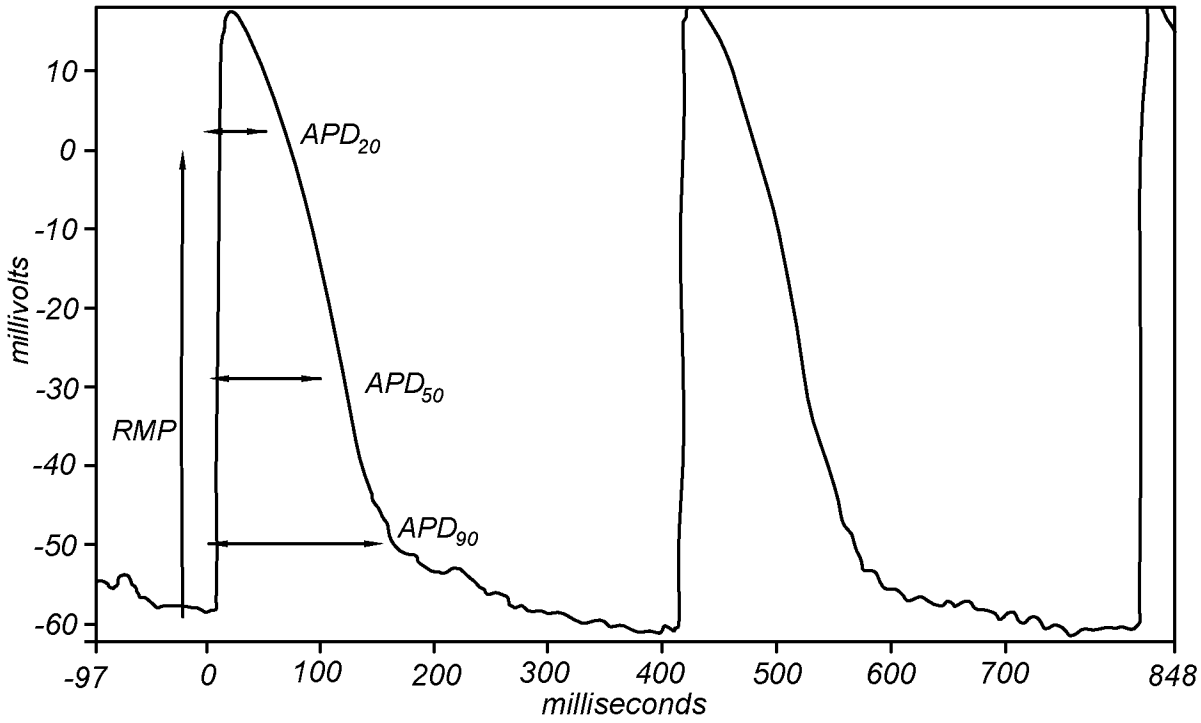

10
0
-10
-20
-30
-40
-50
-60
millivolts
APD20
APD50
APD90
RMP
-97
0
100
200
300
400
500
600
700
848
milliseconds

Fig. 6b

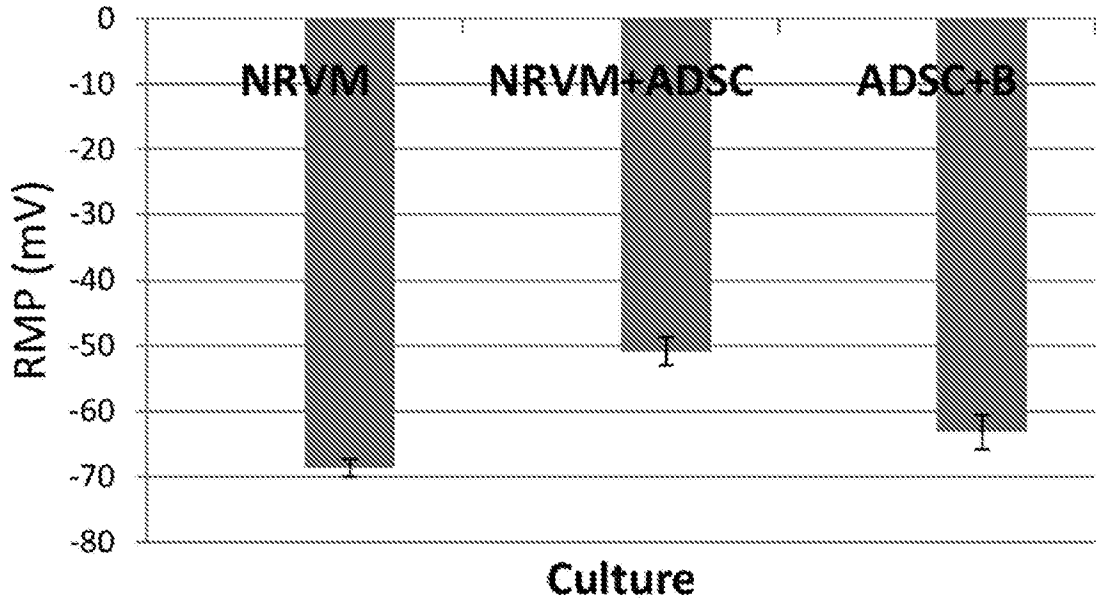

0
-10
-20
-30
-40
-50
-60
-70
-80
RMP (mV)
NRVM
NRVM+ADSC
ADSC+B
Culture

Fig. 7a

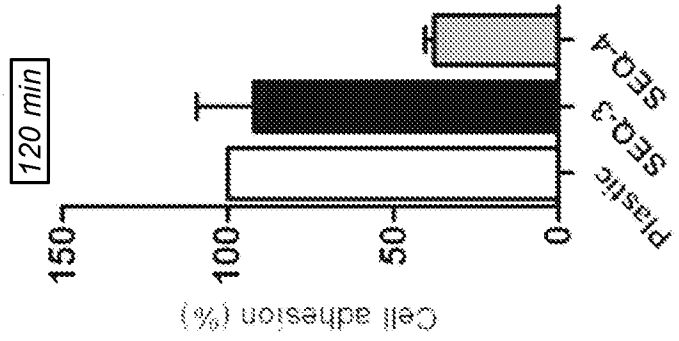


Fig. 7b

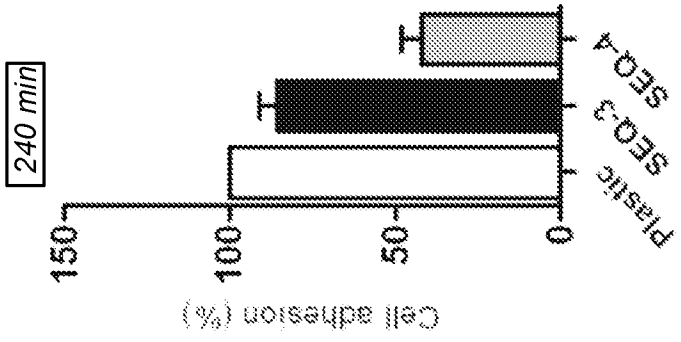


Fig. 7c

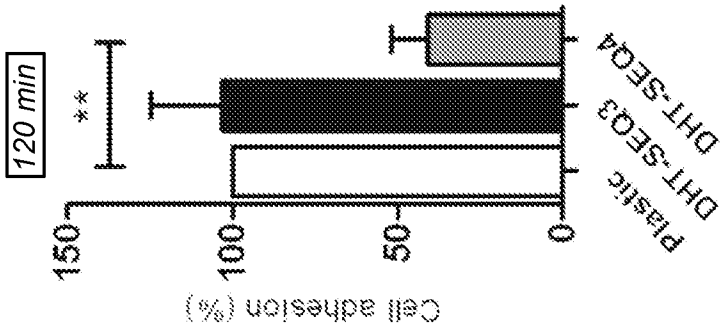


Fig. 7d

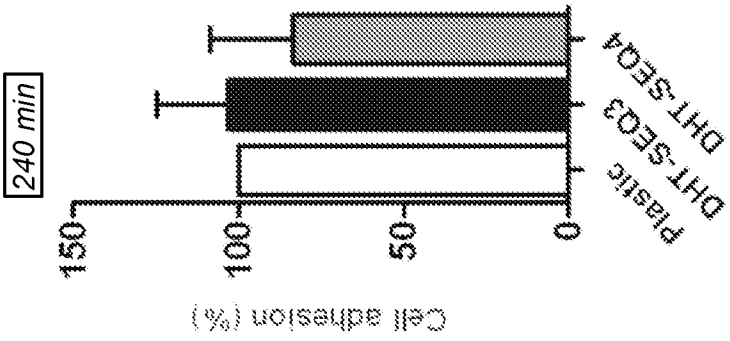


Fig. 7f

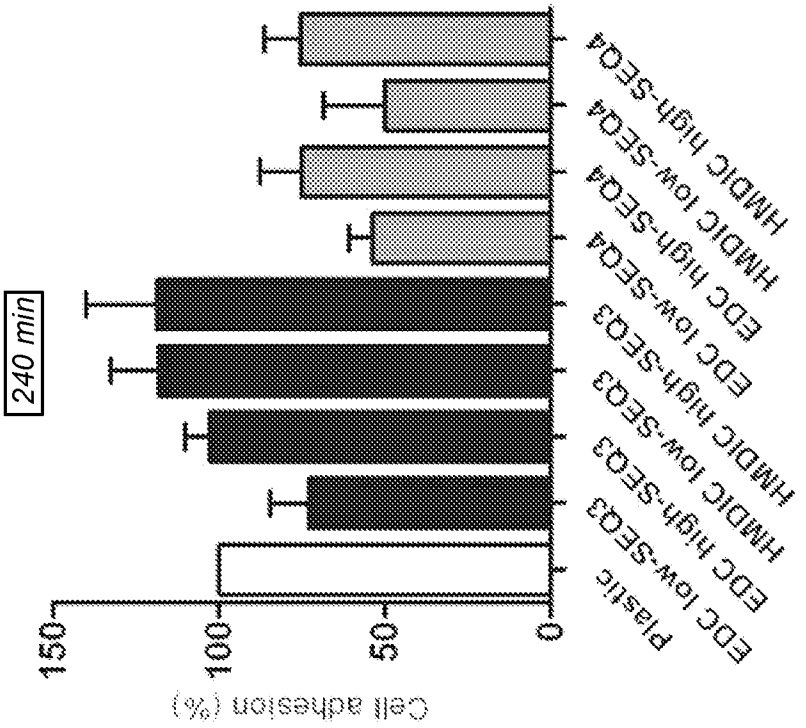

240 min
Cell adhesion (%)
150
100
50
0
Plastic
EDC low-SEC3
EDC high-SEC3
HMDIC low-SEC3
HMDIC high-SEC3
EDC low-SEC4
EDC high-SEC4
HMDIC low-SEC4
HMDIC high-SEC4

Fig. 7e

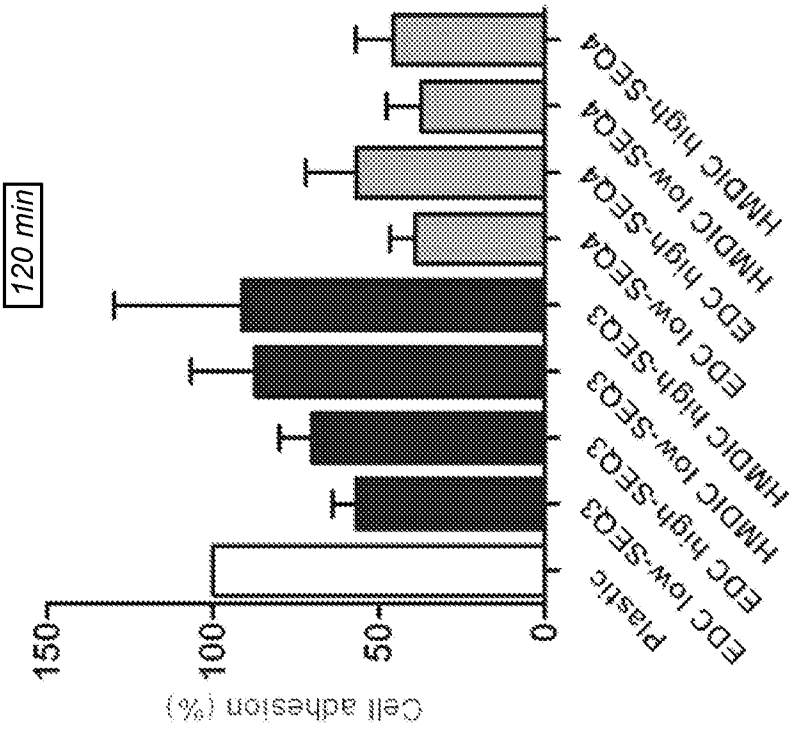

120 min
Cell adhesion (%)
150
100
50
0
Plastic
EDC low-SEC3
EDC high-SEC3
HMDIC low-SEC3
HMDIC high-SEC3
EDC low-SEC4
EDC high-SEC4
HMDIC low-SEC4
HMDIC high-SEC4

Fig. 8b

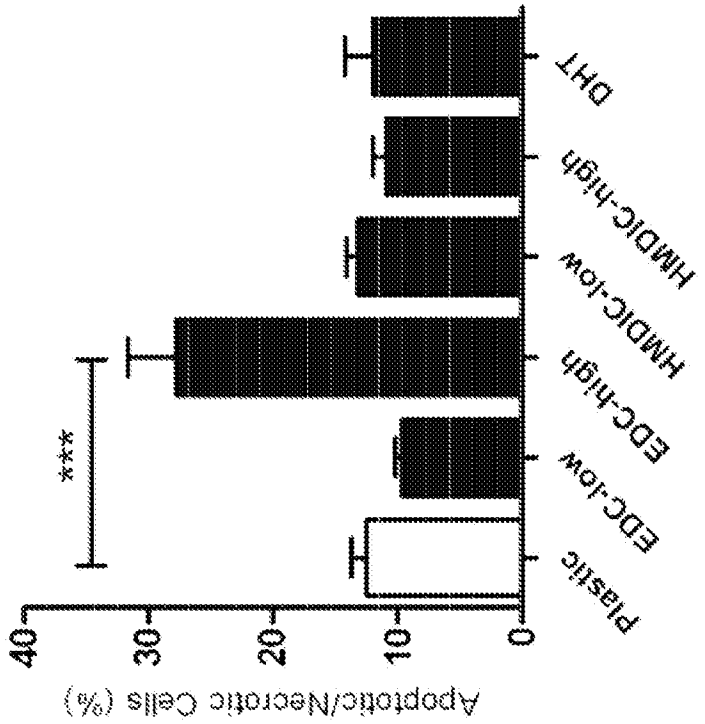

Apoptotic/Necrotic Cells (%)
0
10
20
30
40
***
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high
DHT

Fig. 8a

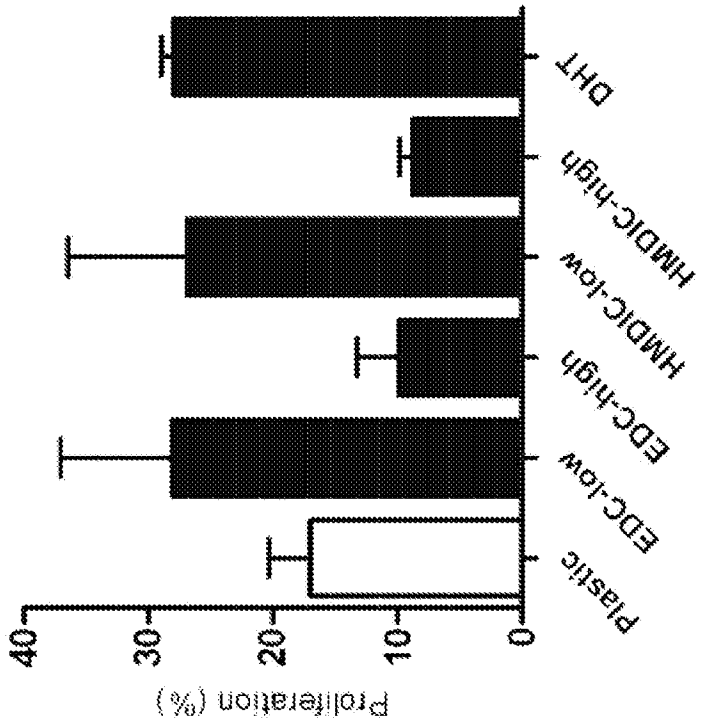

Proliferation (%)
0
10
20
30
40
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high
DHT

Fig. 8d

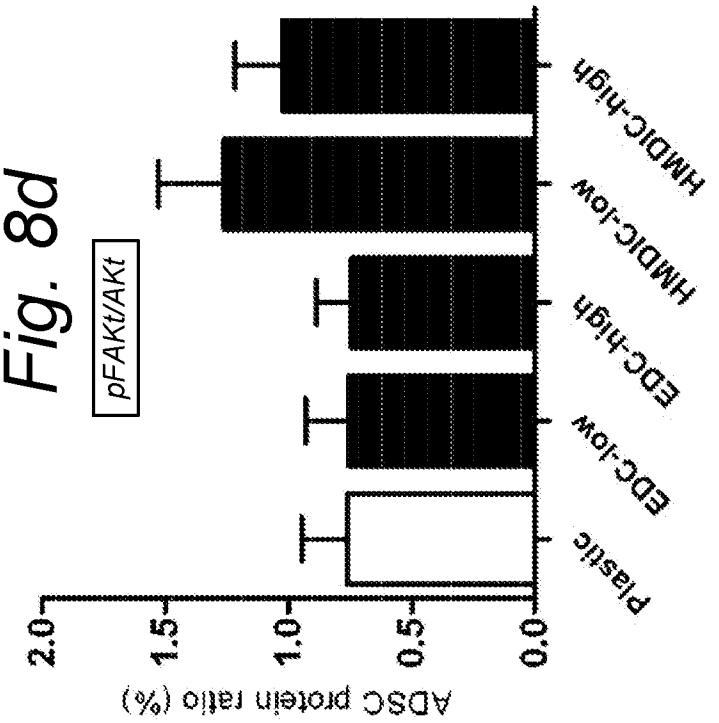

pFAKt/AKt
ADSC protein ratio (%)
2.0
1.5
1.0
0.5
0.0
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high

Fig. 8c

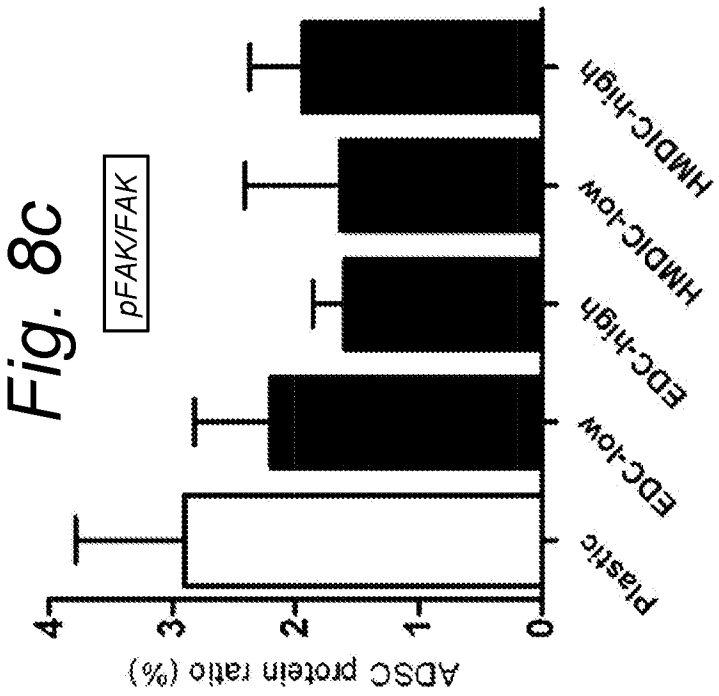

pFAK/FAK
ADSC protein ratio (%)
4
3
2
1
0
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high

Fig. 9a

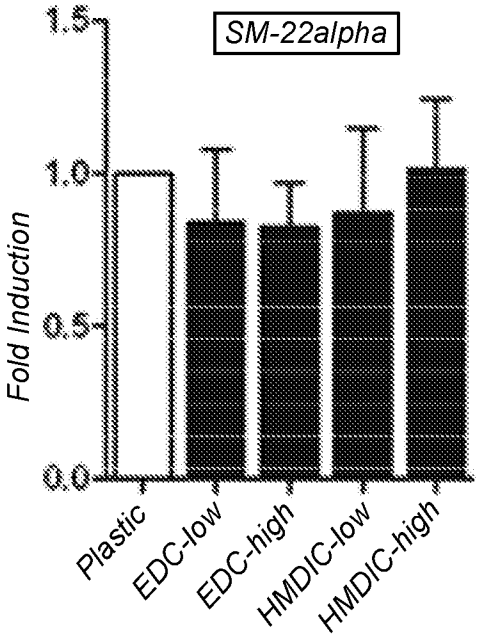


Fig. 9b

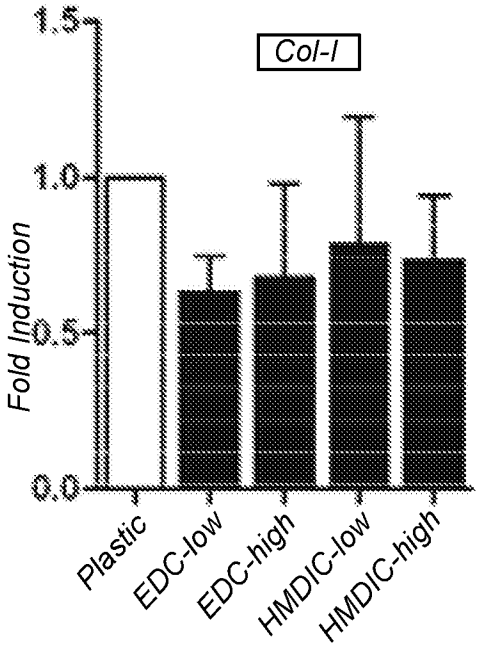


Fig. 9c

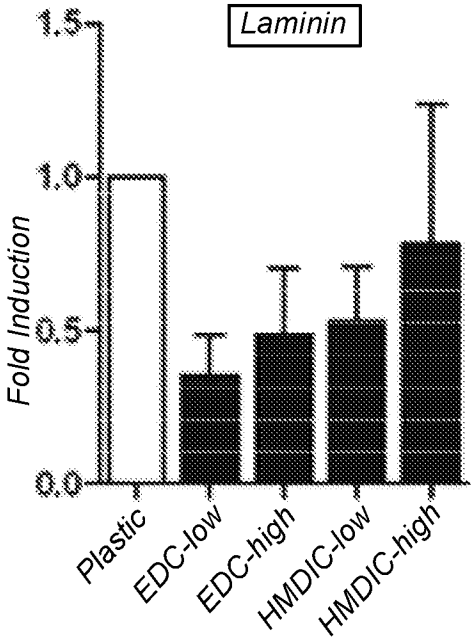
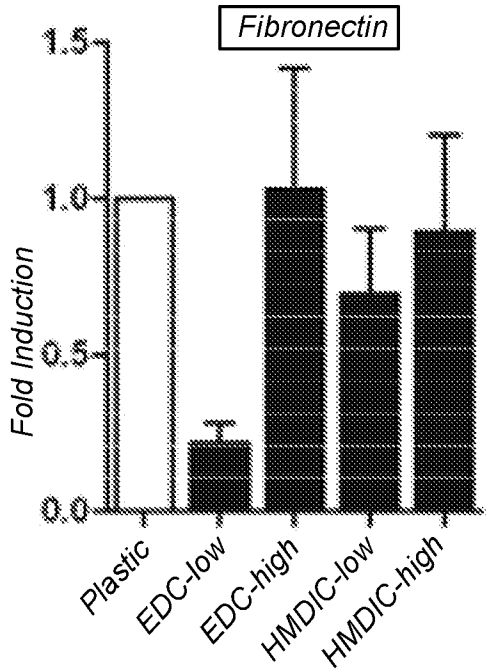
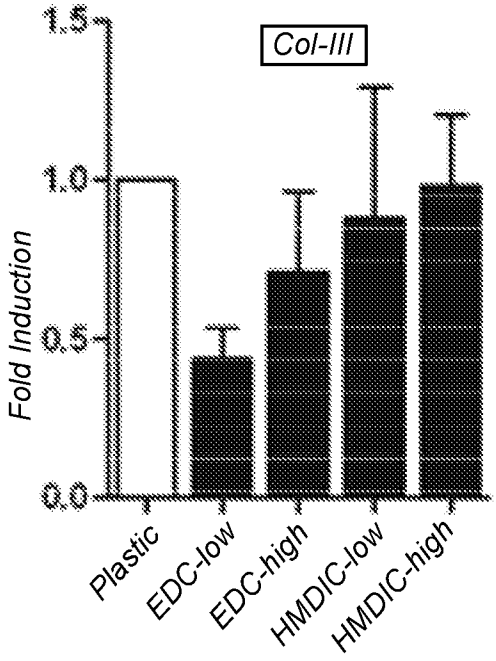
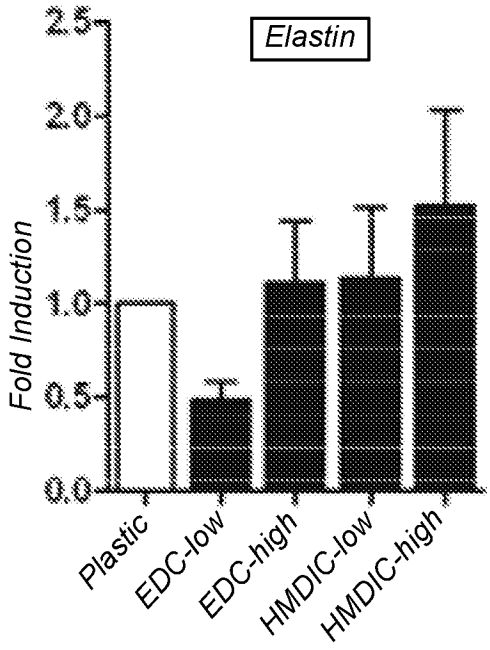
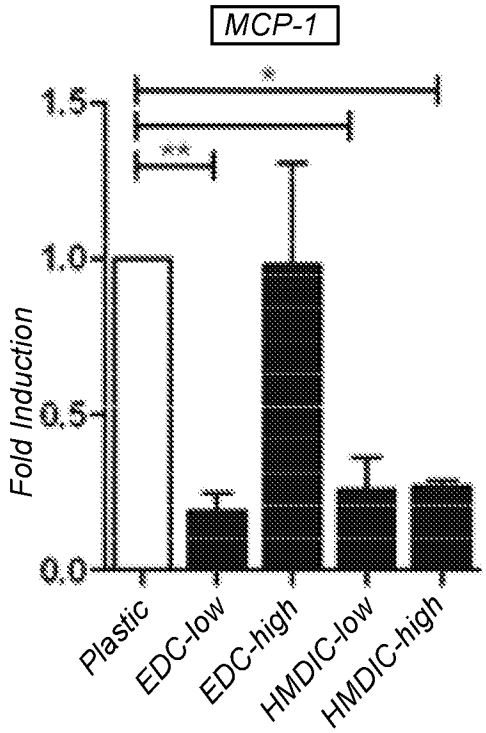


Fig. 9d




Col-III
Fold Induction
1.5
1.0
0.5
0.0
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high


Elastin
Fold Induction
2.5
2.0
1.5
1.0
0.5
0.0
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high


MCP-1
Fold Induction
1.5
1.0
0.5
0.0
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high

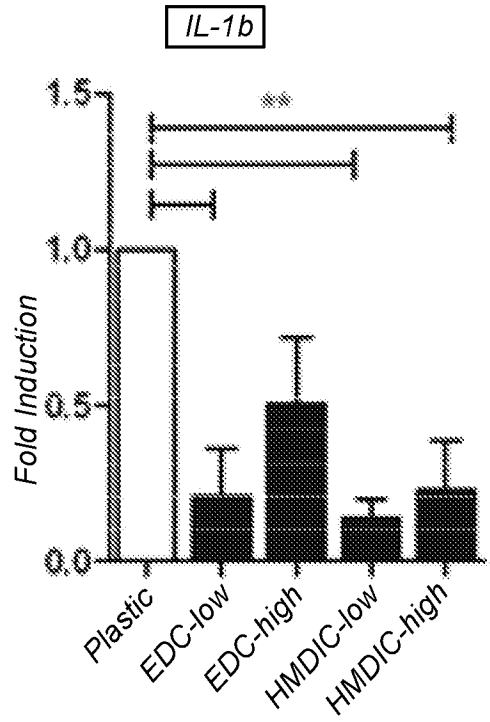

IL-1b
Fold Induction
1.5
1.0
0.5
0.0
Plastic
EDC-low
EDC-high
HMDIC-low
HMDIC-high

Fig. 9i

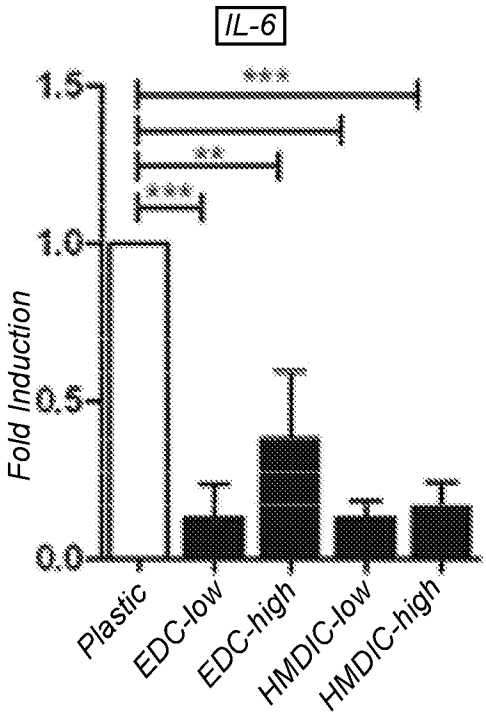


Fig. 9j

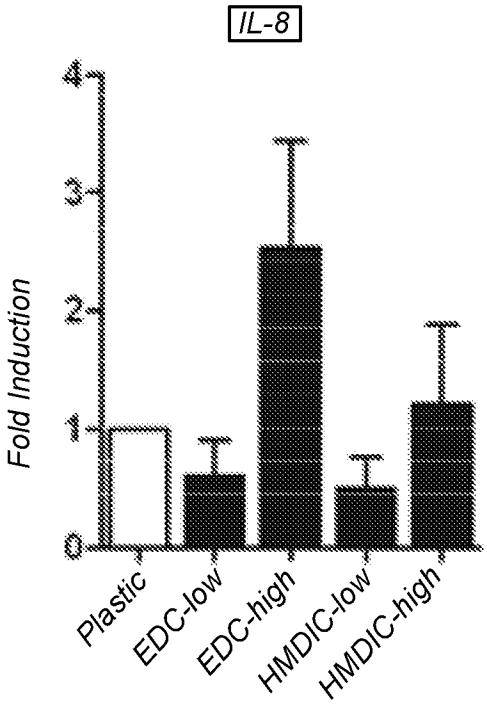


Fig. 9k

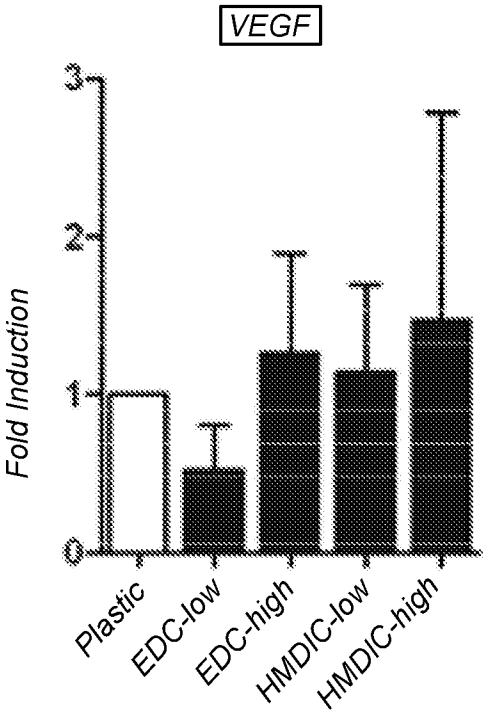


Fig. 9l

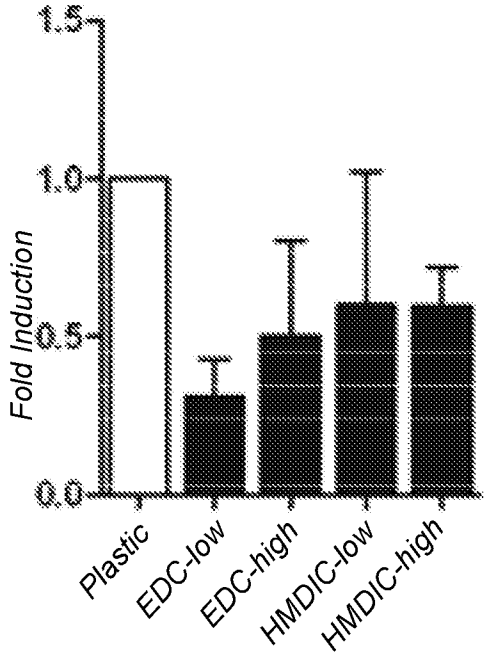


Fig. 9m

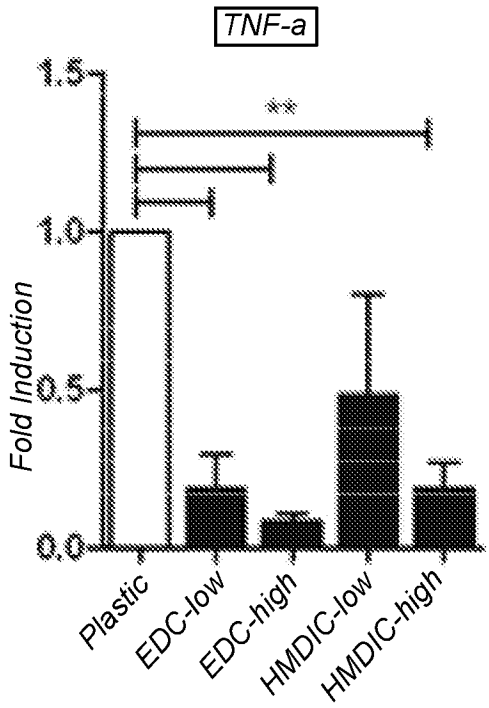


Fig. 9n

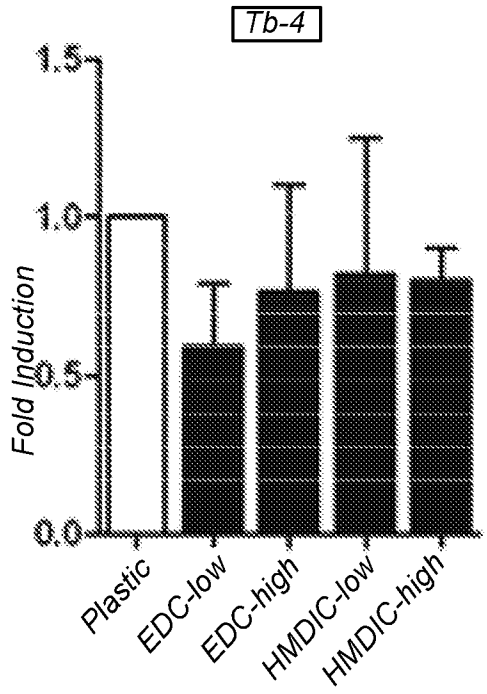


Fig. 9o

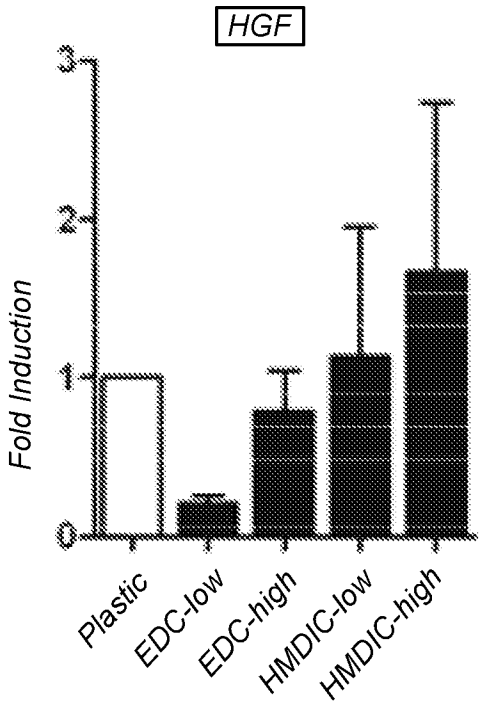


Fig. 9p

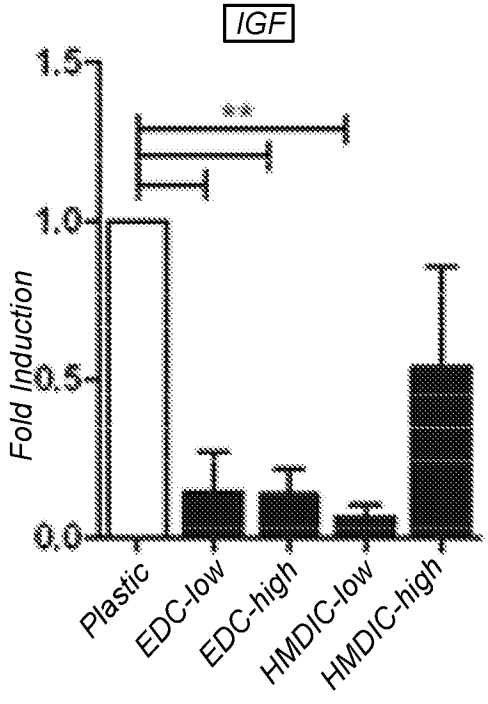


Fig. 10a

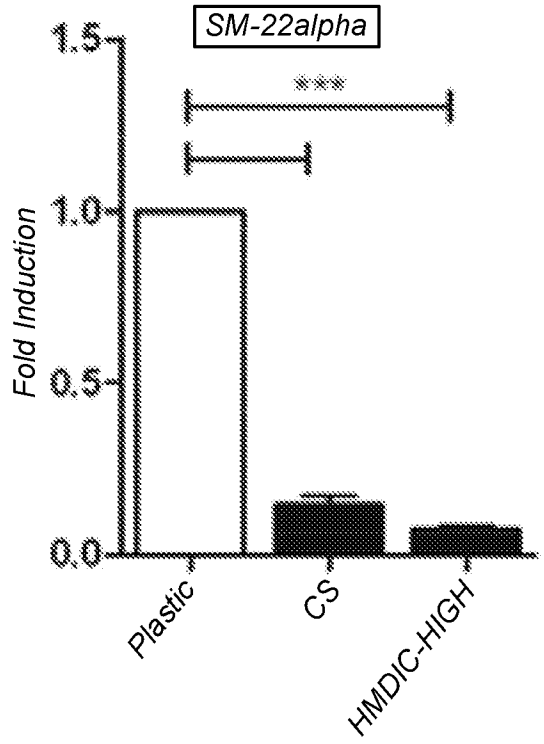


Fig. 10b

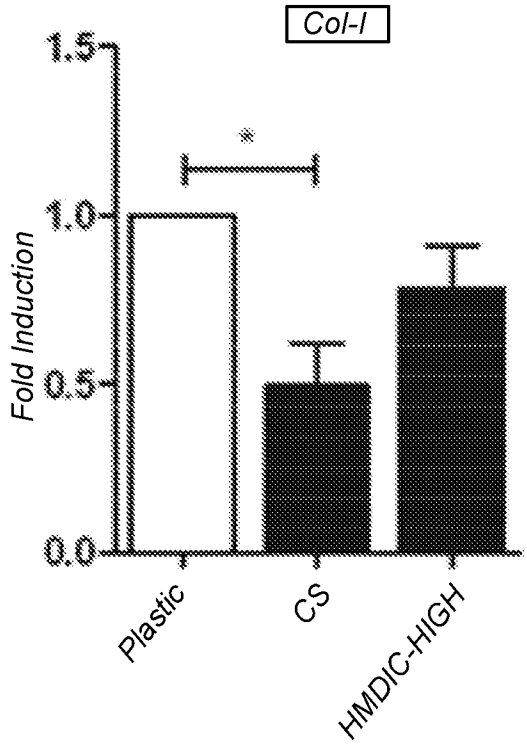


Fig. 10c

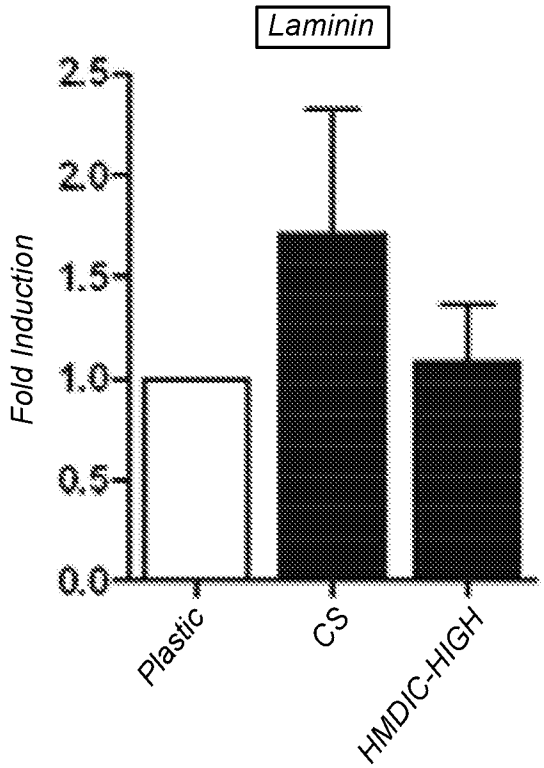


Fig. 10d

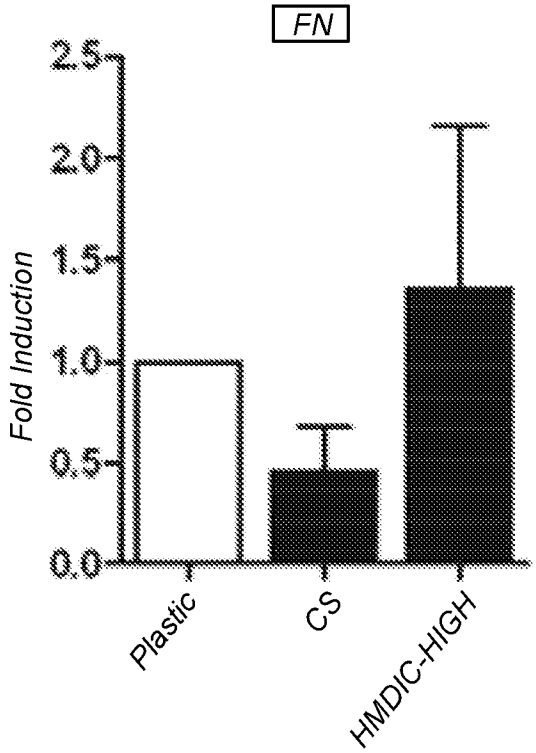
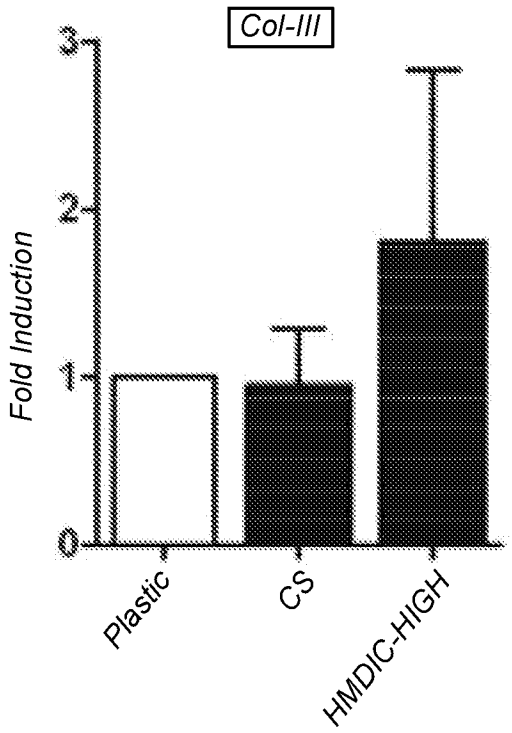


Fig. 10e



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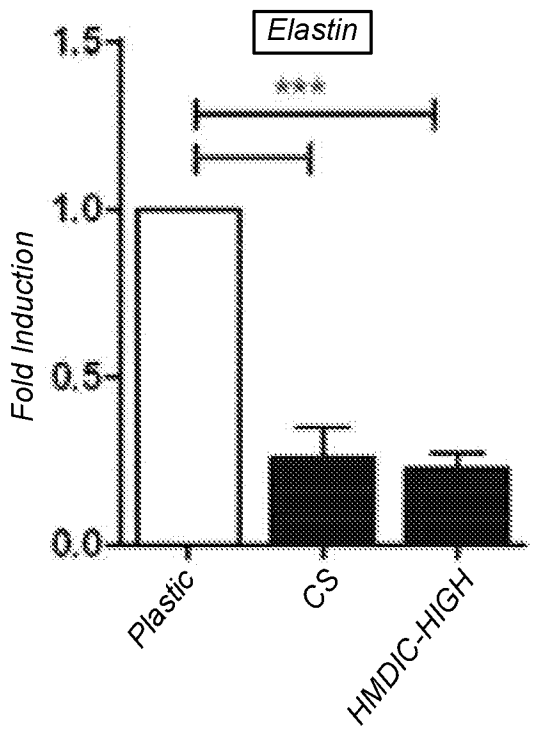
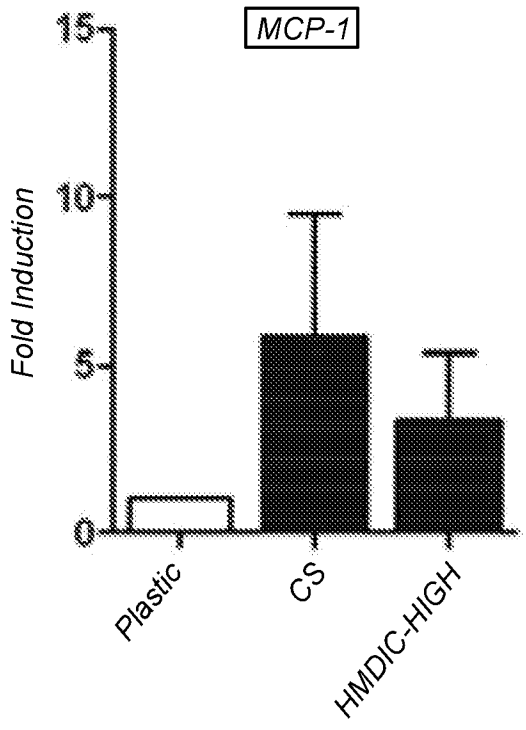




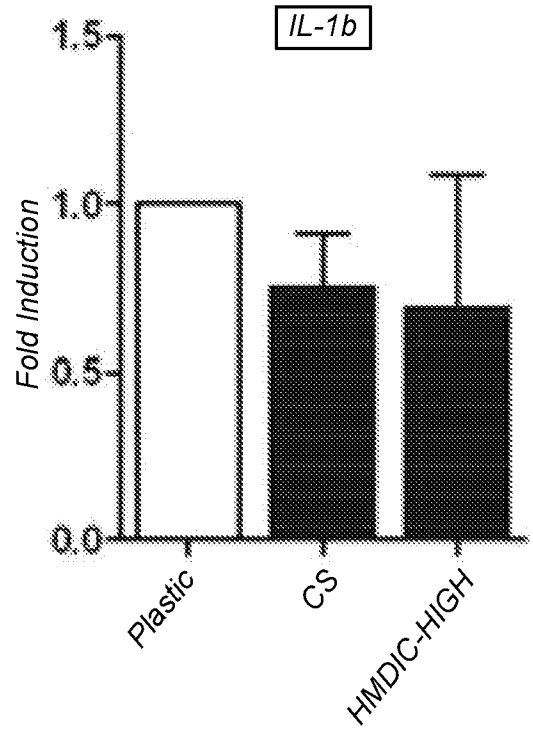


Fig. 10i

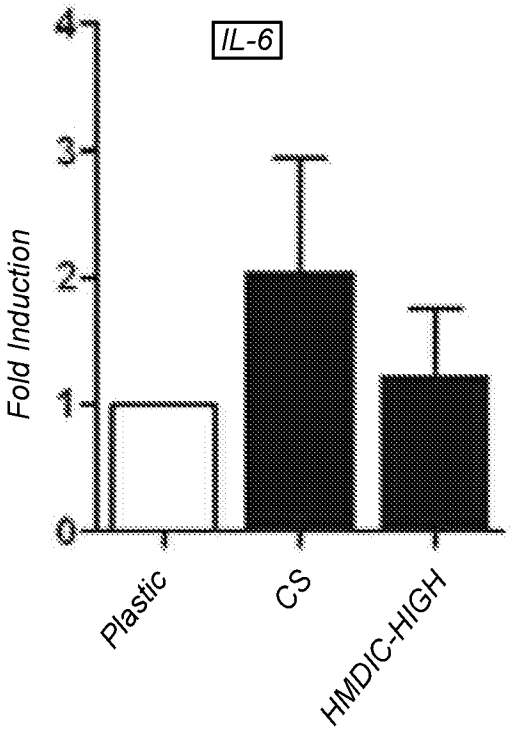


Fig. 10j

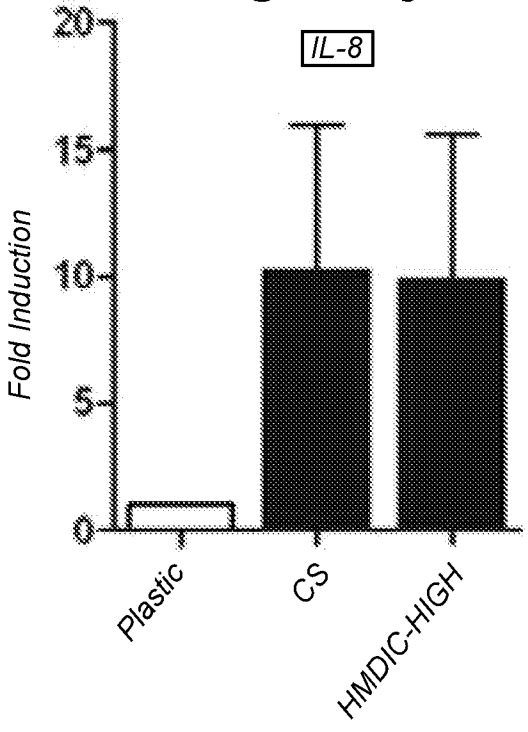


Fig. 10k

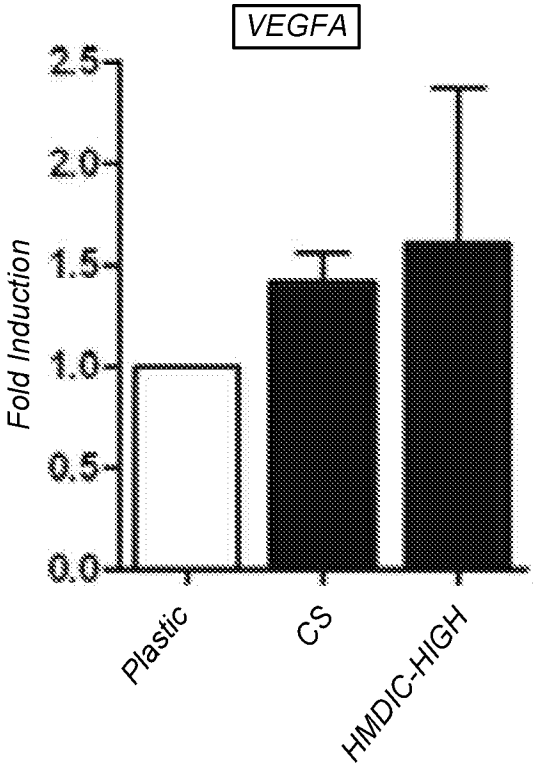


Fig. 10l

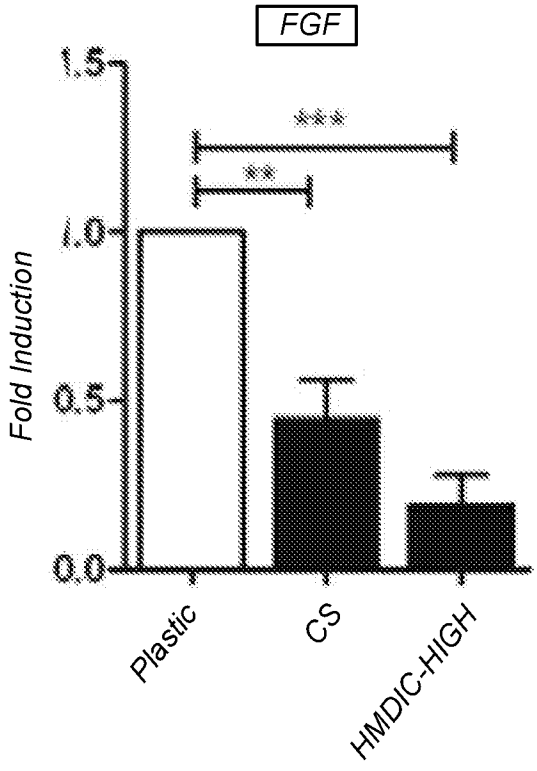


Fig. 10m

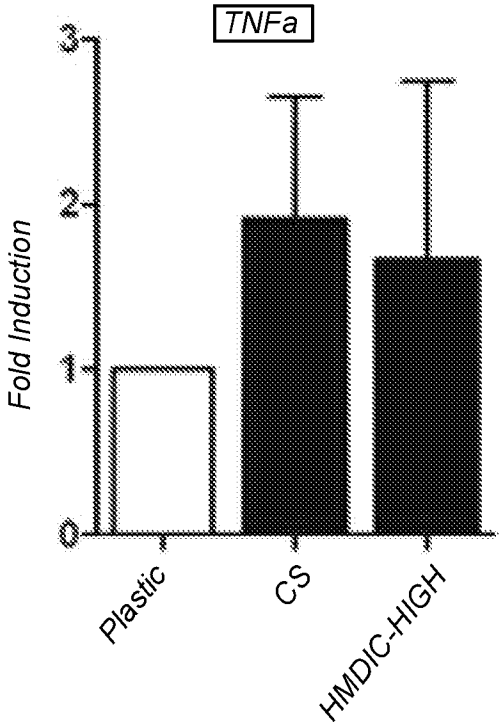


Fig. 10n

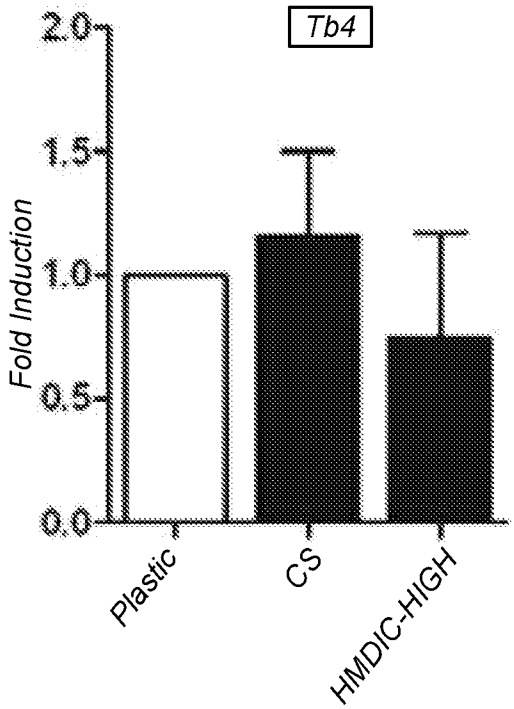


Fig. 10o

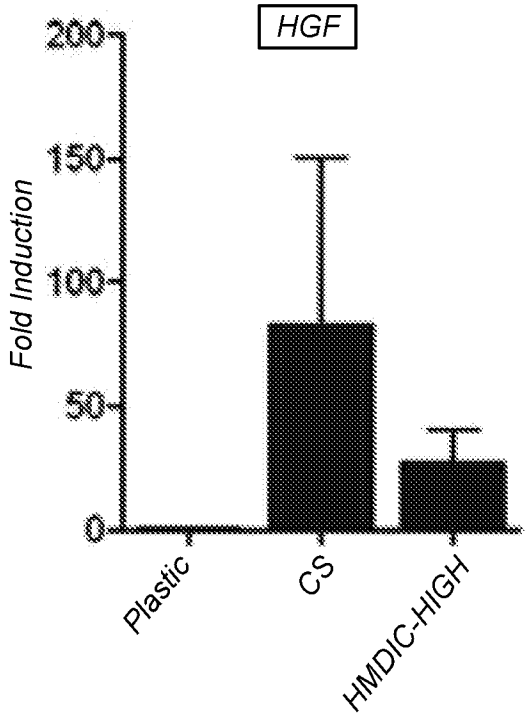
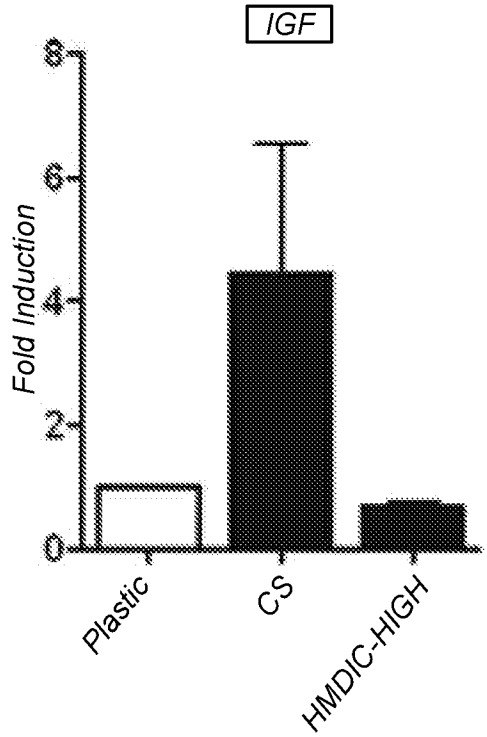


Fig. 10p



INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2015/050093

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/28 A61L27/14 A61L27/38
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNEMARIE TUIN ET AL: "Recombinant Gelatin Microspheres: Novel Formulations for Tissue Repair?", TISSUE ENGINEERING PART A, vol. 16, no. 6, 1 June 2010 (2010-06-01), pages 1811-1821, XP055184532, ISSN: 1937-3341, DOI: 10.1089/ten.tea.2009.0592 abstract page 1819 - page 1820 ----- -/--	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
21 April 2015	30/04/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vandenbogaerde, Ann

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2015/050093

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YALEI ZHOU ET AL: "Expansion and Delivery of Adipose-Derived Mesenchymal Stem Cells on Three Microcarriers for Soft Tissue Regeneration", TISSUE ENGINEERING PART A, vol. 17, no. 23-24, 1 December 2011 (2011-12-01), pages 2981-2997, XP055184558, ISSN: 1937-3341, DOI: 10.1089/ten.tea.2010.0707 abstract ----- ARAÑA MIRIAM ET AL: "Epicardial delivery of collagen patches with adipose-derived stem cells in rat and minipig models of chronic myocardial infarction", BIOMATERIALS, vol. 35, no. 1, 9 October 2013 (2013-10-09), pages 143-151, XP028762498, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2013.09.083 abstract -----	1-14
A		1-14