Title Redox-Responsive Degradable PEG Cryogels as Potential Cell Scaffolds in Tissue Engineering

Authors Tugba Dispinar, Wim Van Camp, Liesbeth J. De Cock, Gruno G. De Geest, and Filip Du Prez

In: Journal, Volume (Issue), pages, year. Macromolecular Bioscience, 12 (3), 383-394, 2012

Optional: http://onlinelibrary.wiley.com/doi/10.1002/mabi.201100396/pdf

((DOI =10.1002/mabi.201100396))
Article type: Full Paper

Redox-responsive degradable PEG-cryogels as potential cell scaffolds in tissue engineering

Tugba Dispinar, Wim Van Camp, Liesbeth J. De Cock, Bruno G. De Geest, and Filip E. Du Prez*

_____

Tugba Dispinar, Dr. Wim Van Camp, Prof. Filip Du Prez
Department of Organic Chemistry,
Polymer Chemistry Research Group, Ghent University,
Krijgslaan, 281, S4-bis, B-9000 Ghent, Belgium
E-mail: Filip.Duprez@Ugent.be
Liesbeth J. De Cock, Dr. Bruno De Geest
Laboratory of Pharmaceutical Technology,
Department of Pharmaceutics, Ghent University,
Harelbekestraat 72, B-9000 Ghent, Belgium

_____

A Michael addition strategy involving the reaction between a maleimide double bond and amine group has been investigated for the synthesis of cryogels at subzero temperature (-8^{o}C). Low molecular weight PEG-based building blocks with amine end groups on one hand and disulfide containing building blocks with maleimide end groups on the other hand are combined to synthesize redox-responsive PEG cryogels. The cryogels exhibit an interconnected macroporous morphology, a high compressive modulus (> 100 kPa) and gelation yields of around 95%. While the cryogels are stable under physiological conditions, complete dissolution of the cryogels into water soluble products is obtained in the presence of a reducing agent (glutathione) in the medium. Cell seeding experiments and toxicologic analysis demonstrate their potential as scaffolds in tissue engineering.

Introduction

The developments in the area of tissue engineering have highlighted the need for novel degradable scaffolds that are capable to provide a suitable environment for living cells and to allow tissue growth.[1,2] Since these scaffolds are used as a support to grow cells and to regenerate tissue constructs with various shapes and sizes, they have to meet several important requirements.[3] Obviously, the scaffold should consist of a biocompatible material. As it functions as a support, the material should have a proper mechanical strength, preferably with an easily tunable three-dimensional design. The scaffold should allow cellular attachment and provide sufficient space for proliferation inside the three-dimensional scaffold matrix. A porous scaffold structure - preferably with interconnected pores - is required to provide proper oxygen and nutrient transport within the scaffold matrix. Finally, the scaffold should be able to degrade into non-toxic water soluble compounds once it has served its purpose.

From this point of view, macroporous hydrogels have received considerable interest in tissue engineering applications as hydrophilic, cross-linked networks.[4] The hydrophilicity of these materials is tuned by the constructive compound (monomeric unit) and plays a crucial role with regard to swelling properties of the scaffolds. This hydrophilic behaviour leads to water soluble products during the degradation process, therefore providing an easy elimination of the scaffold. The macroporosity of the materials supplies a relatively high surface area for cell attachment and also improves the nutrient and oxygen transport inside the scaffold. Macropores within the hydrogel structure can be created via various techniques including freeze-drying,[5] porogenation,[6] phase separation,[7] gas blowing,[8] and polymerization of a high internal phase emulsion (polyHIPE).[9,10] In addition to these techniques, cryotropic gelation (cryogelation) is considered as a rather new approach to prepare macroporous hydrogels.[11] In this method, the three dimensional gel matrices are produced in a partially frozen medium in which the reactants are concentrated in the unfrozen

regions and then polymerize to a dense polymeric matrix in between the frozen regions (i.e being ice crystals in the case of a hydrogel). These ice crystals template continuous interconnected pores during the gel formation. Upon completion of the gelation process, the actual pores appear after melting of the ice crystals during a defrosting step. Generally, macroporous hydrogels that are synthesized via cryotropic gelation are entitled as cryogels.

Cryogels usually feature large interconnected macropores with sizes ranging from 10 μm up to 100 μm.[12] Furthermore, they show a spongy nature with fast drying and re-swelling capabilities. These specific properties make cryogels potential materials for the use in a wide range of biotechnological applications. (Bio)separation,[13,14] (bio)catalysis,[15] and affinity chromatography[16] are currently the most common application fields of the cryogels. Moreover, cryogels have been recently investigated as potential cell scaffolds. For this purpose, cryogels were prepared from biocompatible precursors in aqueous medium. Recently, our group reported on the synthesis of poly(hydroxyethyl methacrylate) (poly(HEMA)) based cryogels with high potential as cell scaffolds thanks to the excellent biocompatibility and tunable mechanical properties of polyHEMA. In addition, the primary alcohol function of HEMA allows further modification reactions to introduce desired fuctionalities or even biomolecules such as therapeutic agents and growth factors in the final compound. For instance, alkyne functionalities were introduced via esterification with 4-pentynoic acid and then further reacted with various azide-containing model compounds via the copper(I) catalyzed Huisgen 'click' cycloaddition reaction.[17] In another report, we have tuned the cell adhesion properties of the cryogels through grafting of stimuli-responsive poly(*N*-isopropyl acrylamide) from the pore walls by atom transfer radical polymerization (ATRP).[18] Poly(ethylene glycol) (PEG) based cryogels have also been recently reported as potential cell scaffolds.[19] The choice for PEG is motivated by the fact that it is one of the most widely applied synthetic polymers in hydrogel synthesis for tissue engineering because of its biocompatibility.[4] Also in this case, the microstructure of PEG cryogels could be tuned

and it was shown that the manipulation of the microstructure could be used as a tool to fine-tune the swelling behavior and mechanical properties of the cryogels. In general, most of the studies have dealed with an optimization of the physical and structural properties of the cryogels.

At the same time, some studies have been focusing on the design of degradable cryogels. Most of the reports focus on the use of biodegradable natural compounds such as dextran, lactic acid (LLA), gelatin and chitosan. These natural compounds have either been used as such or combined with other natural or synthetic compounds to prepare degradable cryogels. Examples include HEMA-LLA-dextran,[20] gelatin,[21] gelatin-fibrinogen,[22] and gelatin-chitosan[23] based cryogels. These reported cryogel systems show a hydrolytic degradation behaviour under physiological conditions. As a result, they exhibit gradual degradation kinetics with degradation times ranging from days to months. Degradation rates of these cryogels are adjusted by variation of the molecular weights of the polymeric reactants, incorporation of other polymeric segments, or by adjusting the crosslink density. On the other hand, this continuous hydrolysis process leads to the gradual weakening of the system during the tissue growth. However, as it is often desirable to keep the mechanical support throughout the whole process of tissue growth, systems with relatively fast, on-demand degradation kinetics are desirable. An orthogonal degradation mechanism that allows a rather quick, on-demand degradation upon completion of the tissue growth would be beneficial. Ideally, the degradation of the scaffold should lead to non-toxic, water-soluble compounds as the result of an external trigger. In this respect, disulfide bonds are of particular interest since they are stable against hydrolysis, but can be cleaved orthogonally in the presence of reducing agents such as dithiothreitol (DTT), L-cysteine and glutathione through thiol-disulfide exchange reactions.[24] Therefore, disulfide bonds have been investigated for a variety of applications ranging from drug delivery to soft-tissue engineering.[25,26] Especially in soft-tissue engineering, disulfide containing scaffolds could be more suitable to culture cells in a 3-D

environment or to reconstitute tissues in vitro that might be utilized for the replacement of diseased tissues in vivo.

This redox-responsive cleavage of disulfide bonds also allows the design of cryogels with controllable degradation kinetics, which has already been explored with partial success. Recently, Andac and *et al.* reported partially degradable disulfide containing polyHEMA based cryogels.[27] These cryogels were prepared using a free radical polymerization (FRP) process by employing at the same time a disulfide containing crosslinker as well as a non-degradable crosslinker in the cryogel synthesis. The second non-degradable crosslinker was necessary to improve the gel yields and the stability of the cryogel matrix since the cryogel formation with only disulfide containing crosslinker was not successful.

Until now, the most widely applied synthetic route to prepare cryogels involves free radical polymerization (FRP) using the APS/TEMED (ammonium persulfate/*N,N,N′,N′*-tetramethylethylenediamine) initiation system since this redox system can generate radicals and initiate polymerization, even at low temperatures.[11,28] Since for specific cases, the FRP process has some disadvantages, other synthetic strategies have been explored. Indeed, the disulfide bond might interfere with the FRP process, causing an undesired radical formation in the polymerization medium.[29,30] Moreover, chain transfer reactions might result in additional and permanent crosslinks in the system, and consequently influence the degradation ability of the material.[31,32] We observed ourselves that disulfide containing crosslinkers, namely N,N‘-bis(acryloyl)cystamine and PEG based disulfide containing dimethacrylate, which were employed in the synthesis of cryogels via free radical polymerization, did not result in degradable cryogels.[33] It should be noted that during cryogel synthesis, the amount of undesired chain transfer reactions might be higher as the monomers and crosslinker are concentrated in the non-frozen regions (cryotropic concentration effect).[11,34] In addition, the amount of the crosslinker is generally 10 % of the monomer feed to obtain nice cryogel monoliths. Thus, the rather high concentration of disulfide groups

during the cryogelation process causes an increased probability of chain transfer reactions, which give rise to additional, permanent cross-links in the system. With regard to the above-mentioned discussion, different synthetic strategies - not involving any radical species - should be considered when disulfide containing cryogels are targeted.

Recently, the formation of networks with step-growth reactions such as Michael addition chemistry and 'click' chemistry has drawn significant attention since these techniques give more control over the network structure, and therefore significantly improve the mechanical properties of the materials compared to the chain-growth reactions.[35,36] Especially, Michael-type conjugate addition reactions become attractive to synthesize new biomaterials to meet the demand of improved materials in the field of tissue engineering since these reactions may occur under physiological conditions without the need of any other reagents. In addition, non free-radical, step-growth reactions do not interfere with disulfide moieties, and are therefore an interesting alternative to synthesize network structures that contain disulfide functionalities.

From this viewpoint, the Michael-type conjugate addition reaction between maleimide groups and amine groups was considered in this research as a suitable way to synthesize disulfide containing, step-growth cryogels. The double bonds of maleimide compounds have a low electron density, making them highly reactive towards Diels-Alder reactions and 1,3-dipolar addition reactions. In addition, these double bonds are also accessible to nucleophilic attack *via* Michael-type conjugate addition reaction.[37] For example, the reactions of maleimide double bonds with thiols or thiolate anions are well-known to proceed straightforward and form stable thioether linkages. However, thiol and thiolate nucleophiles might interfere with disulfide groups when the synthesis of redox-responsive cryogels is targeted. Alternatively, primary amines are another class of nucleophiles that react readily with the maleimide double bond, however in a slightly slower fashion compared to the thiols.[38]

In 2001, Hubbell and coworkers were the first to use a conjugate addition strategy reacting an acrylate or vinyl sulfone end functionalized multi-arm PEG macromonomers with thiol end functionalized macromonomers to synthesize PEG-based hydrogels.[39] Since then, Michael-type conjugate addition reactions have become a common reaction to synthesize PEG-based hydrogels.[40,41] While PEG based step-growth hydrogel materials have been explored as a new type of biomaterials, up to our knowledge, step-growth cryogels from PEG-based building blocks have not been reported. The choice of PEG as a building block is not only motivated because of its biocompatible nature but also of its hydrophilic properties, which is crucial in the design of degradable systems when water soluble degradation products are targeted.

In this study, the Michael addition strategy between maleimide double bonds and amine groups has been investigated for the synthesis of redox-responsive PEG cryogels. Amine functionalized, low molecular weight PEG-based building blocks and maleimide functionalized disulfide containing building blocks have been combined in the preparation of the cryogels. Structural characterization, mechanical properties and degradation of the cryogels are demonstrated. The potential use of these cryogels as cell scaffolds in tissue engineering applications has been verified by a toxicology analysis of the degradation products, cell viability analysis and cell seeding experiments.

Experimental Section

Materials

Glycerol ethoxylate (M_n: 1000 g/mol), triethylamine (99 %), maleic anhehydride (99 %), methanesulfonyl chloride (99 %), cystamine dihydrochloride (98 %), glutathione (> 98 %), (PBS) phosphate buffered saline pH:7.4 in foil pounches, potassium hydroxide were purchased from Sigma-Aldrich. The ammonia solution (25 %) and 1,6-hexanediamine (> 99.5 %) were obtained from Acros Organics. The other solvents were all HPLC grade and purchased from Aldrich. All reagents have been used without any further purification.

Synthesis of the trifunctional PEG-amine

Synthesis of the trifunctional PEG-amine was dried over magnesium sulfate and evaporated to dryness. Next, mesylated glycerol ethoxylate (10 g, 8 mmol) was dissolved in 25 % aqueous ammonia solution (150 mL), and the container was tightly closed. The reaction mixture was vigorously stirred for 3 days at room temperature. The ammonia was evaporated and the basic solution was extracted three times with dichloromethane (100 mL). The presence of terminal amine groups was detected by NMR. ^{1}H NMR (300MHz, $CDCl_3$) 3.73 ppm (t, 6H, -CH_2-CH_2-NH_2), 3.63-3.49 ppm (77H, PEG), 2.84 ppm (t, 6H, -CH_2-NH_2).

Synthesis of disulfide containing bismaleimide crosslinker (dithio-bis-maleimidoethane (DTME))

To synthesize the disulfide containing bismaleimide linker, cystamine dihydrochloride salt was first neutralized to cystamine using potassium hydroxide (KOH). Cystamine dihydrochloride (10 g, 44.4 mmol) was suspended in 100 mL of methanol. After the addition of KOH (5.5 g, 97.7 mmol), the white suspension was stirred overnight at room temperature. The day after, a white precipitate was filtered from solution and methanol was evaporated. The residue was dissolved in dichloromethane (200 mL) and washed with a saturated $NaHCO_3$ solution (1 x 50 mL). The dichloromethane phase was dried over magnesium sulfate and pure cystamine was obtained upon evaporation of dichloromethane. The rest of the procedure has been adapted from previously reported reaction conditions.[42] Cystamine (4 g, 26.26 mmol) was dissolved in 100 mL of acetone and maleic anhydride (5.15 g, 52.53 mmol) was added to the mixture. Sudden precipitation of the obtained diacid was observed and the mixture was stirred for one additional hour to complete the reaction. To the reaction mixture, triethylamine (2 mL, 27.2 mmol) and sodium acetate (0.03 g) were added. Then the mixture was heated slowly to reflux with simultaneous addition of acetic anhyride (8 mL, 72.5 mmol). The mixture was allowed to reflux for an additional 3 hours and then acetone was evaporated. The residue was dissolved in dichloromethane (100 mL) and washed with saturated $NaHCO_3$ solution (1 x 50 mL) to get rid of the triethylamine salt. The dichloromethane phase was dried

over magnesium sulfate and evaporated. Residual acetic anhydride was removed via azeotropic distillation with cyclohexane. The crude compound was then purified via column chromatography (40 % ethyl acetate-hexane). ^{1}H NMR (300MHz, $CDCl_3$) 6.69 ppm (s, 4H, -*CH*-*CH*-), 3.83 ppm (t, 4H, N-*CH*$_2$-CH_2), 2.91 ppm (t, 4H, -CH_2-*CH*$_2$-S).

Synthesis of 1,6-bismaleimidohexane

1,6-Bismaleimidohexane was synthesized from maleic anhyride and hexane diamine via adapting the procedure for the synthesis of DTME. 1,6-Hexane diamine (5.7 g, 49 mmol) was dissolved in 100 mL of acetone and maleic anhydride (9.6 g, 98 mmol) was added to the mixture. Sudden precipitation of the obtained diacid was observed and the mixture was stirred for one additional hour to complete the reaction. To the reaction mixture, triethylamine (3.7 mL, 50.7 mmol) and sodium acetate (0.06 g) was added. Then the mixture was heated slowly to reflux with simultaneous addition of acetic anhyride (13.5 mL, 122.3 mmol). The mixture was allowed to reflux for an additional 3 hours and then acetone was evaporated. The residue was dissolved in dichloromethane (100 mL) and washed with a saturated $NaHCO_3$ solution (1 x 50 mL) to get rid of the triethylamine salt. The dichloromethane phase was dried over magnesium sulfate and evaporated. Residual acetic anhydride was removed via azeotropic distillation with cyclohexane and the crude compound was purified via column chromatography (40 % ethyl acetate-hexane). ^{1}H NMR (300MHz, $CDCl_3$) 6.66 ppm (s, 4H, -*CH*-*CH*-), 3.48 ppm (t, 4H, -N-*CH*$_2$-CH_2-), 1.50–1.57 (m, 4 H, -N-*C*H_2-C*H*$_2$-CH_2-), 1.25–1.29 (m, 4 H, -N-*C*H_2-CH_2-C*H*$_2$-).

Synthesis of redox-responsive PEG cryogels via maleimide-conjugate addition

Cryogels were prepared in three different total monomer concentrations (0.36, 0.18 and 0.12 mmol/mL) at -8^{o}C in dioxane. DTME (1.5 eq.) and PEG-$(NH_2)_3$ (1 eq.) were separately dissolved in a calculated amount of dioxane. The monomer solutions were cooled down in an ice bath for a few second and mixed in open-ended plastic tubes (1 mm in diameter). The plastic tubes were placed in a cryostate at -8^{o}C. After 18 hours, the tubes were taken out of the

cryostate and put at room temperature. The gel matrix was first washed with chloroform and then extensively with ethanol, and finally dried at 60^{o}C.

Synthesis of the control cryogels

Cryogels without disulfide groups have been prepared under the same conditions as mentioned for the synthesis of disulfide containing cryogel, using 1,6-bismaleimidohexane as the bismaleimide compound. The cold solutions of 1,6-bismaleimidohexane (1.5 eq.) and PEG-$(NH_2)_3$ (1 eq.) in dioxane were mixed in plastic tubes and kept at -8^{o}C for 18 hrs. The gel matrix was first washed with chloroform and then extensively with ethanol, and finally dried at 60^{o}C.

Characterization of cryogels

Instrumentation

1D ^{1}H-NMR, 2D ^{1}H-^{1}H COSY and 2D ^{1}H-^{13}C HSQC spectra were recorded on a Bruker Avance II 700 spectrometer (700 MHz) using a hr-MAS probe equipped with a ^{1}H, ^{13}C, ^{119}Sn and gradient channel. Samples for high resolution magic angle spinning NMR were prepared as follows: a dry cryogel sample was cut in small pieces and put in a 4 mm hr-MAS rotor (50 µL). Next, solvent (DMF-d7) was added to allow the cryogel to swell. *Scanning electron microscopy (SEM) images* were recorded with a Quanta 200 FEG FEI scanning electron microscope operated at an acceleration voltage of 5 kV. Samples were sputtered with gold prior to SEM analysis. *Compression measurements* were performed on swollen cryogels using a Tinius Olsen-Instrument (H10KT) with a cell load of 100 N. Samples were compressed between two parallel plates at the maximum loading of 20 N with a a compression rate of 1 mm/min^{-1}. Triple experiments have been done for each sample. *Raman spectra* were collected using Bruker FRA 106 FT-Raman spectrometer. Samples were placed on a glass slide to perform the analysis.

Gelation yield of cryogels

Cryogels were first washed with chloroform and then extensively with ethanol to remove the

unreacted gel precursors. Afterwards, they were dried in an oven at 60°C until a constant weight was reached (m_{dried}). The gelation yields of the cryogels were calculated as (m_{dried} / m_t) x 100 %, where m_t is the total mass of the gel precursors in the reaction mixture.

Swelling ratio measurement

After drying the cryogel samples until a constant weight, they were immersed in PBS for 24 hours to reach the equilibrium swelling. The samples were taken out and the excess of water was removed from the cryogel surface with a tissue paper. The swelling degrees of the cryogels were calculated from the ratio of weights of swollen cryogel to dried cryogel. Triple experiments have been done for each sample.

Volume of Macropores

The water inside the swollen cryogel is found in three different states: polymer bound water, water inside small pores and water inside large pores.[43] The water present inside large pores is more free compared to the other types and can be mechanically removed by simply squeezing the cryogel. This squeezing method can be used for an approximative estimation of the total volume of macropores in a cryogel sample. In this study, the volume of macropores in the cryogel matrix was calculated as follows;

$$(m_{swollen\ gel} - m_{squeezed\ gel}) / m_{swollen\ gel} \times 100\ \%$$

Visual determination of residual primary amine groups

The TNBS test was used for visual detection of primary amine groups. A cryogel sample of 10 mg was placed in a small glass vial. 3 drops of n,n-diisopropylethylamine (DIPEA) solution (10 % in DMF) and 3 drops of 1 M aqueous 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution were added successively. The orange to red color change on the cryogel matrix was visually inspected after 30 min, which demonstrated qualitatively that residual primary amine groups are present on the cryogels.

Cell culture

Human dermal foreskin fibroblasts (ATCC; CRL-2522) were cultured in EMEM supplemented with antibiotics (streptomycin/penicillin) and 20 % heat inactivated fetal bovine serum (FBS; Gibco). Cells were grown at 37°C in a humidified atmosphere containing 5 % CO_2.

Degradation of cryogel for cell viability analysis

The cryogels were sterilized by 2h soaking in 70% ethanol followed by extensive soaking in sterile PBS. 4 mg/ml of the cryogel were dissolubilized in BPS buffer solution containing 5 mM glutathione at 37^{o}C. This solution was further used for cell viability analysis.

Cell viability

Fibroblasts were seeded in a 96-well plate (450 cells/well) and cultured in the presence of degradation products. After 24 hours, the cells were quantified using a p-nitrophenyl phosphate (pNPP; Sigma-Aldrich) cell viability assay. *p*-nitrophenyl phosphate was hydrolyzed by acid phosphatase resulting in the release of *p*-nitrophenol and phosphate by incubating the cells with a pNPP solution under acidic condition during 2 hours at 37 °C and 5 % CO_2. The reaction was stopped with NaOH resulting in the formation of a yellow color, measured spectrophotometrically at 405 nm. All experiments were performed in triplicate.

Cell seeding on gel slab and immunohistochemistry

The gel slabs were sterilized by 2h soaking in 70% ethanol followed by extensive soaking in sterile PBS. Fibroblasts were seeded on a gel slab and cell adhesion and spreading was evaluated after a 24 hour incubation period. Cells were fixed using 3.7 % formaldehyde in PBS and permeabilized with triton X-100 (0.1 %). After blocking the samples with Tris buffercontaining 5 % BSA, actin was stained fluorescently using Alexa Fluor®546 Phalloidin (1/40) (Invitrogen, Molecular Probes). Cell nuclei were stained with HOECST 33258 (Sigma-

Aldrich, 2 μg/ml in PBS). Microscopy images were recorded on a Nikon EZ-C1 confocal microscope.

Cell seeding in cryogel

The cryogels were sterilized by 2h soaking in 70% ethanol followed by extensive soaking in sterile PBS. 300 000 fibroblasts were seeded on a cryogel (1 x 1 x 1 cm) by drop seeding. After one week of culturing period, fibroblasts were stained using CellTracker™ Green (1/5000) (Invitrogen, Molecular Probes) to evaluate the population of cryogel. Microscopy images were recorded on a Nikon EZ-C1 confocal microscope.

Reduction triggered degradation

A 6 mm in diameter round shaped cryogel was sliced to obtain a 1 mm thick sample (20 mg) and put at room temperature in aqueous medium (phosphate buffered saline – 10 mL) containing 25 mM glutathione. After several time intervals the cryogel was transferred to a glass cover slip and optical microscopy images were recorded with an Olympus SZX9 stereomicroscope equipped with a digital camera. Afterwards, the cryogel was again transferred back to the aqueous glutathione solution.

Results and Discussion

Synthesis of redox-responsive cryogels

In a first step, amine and maleimide functionalized building blocks were prepared for the synthesis of cryogels. For the amine containing building block, an amine terminated tri-arm polyethylene glycol (PEG-$(NH_2)_3$) was synthesized from commercially available glycerol ethoxylate via mesylation of hydroxyl groups with methanesulfonyl chloride and subsequent amination reaction with ammonia aqueous solution **(Scheme 1A)**. Next, a redox-responsive disulfide bond was incorporated in the structure of the bismaleimide crosslinker (dithio-bis-maleimidoethane, DTME), which was synthesized by reaction of cystamine with maleic

anhydride (Scheme 1B). The disulfide containing cryogels were then synthesized via Michael addition reaction of the electron poor double bond of the maleimide compound with the amine groups of the tri-arm (PEG-$(NH_2)_3$) moiety (Scheme 1C).

Because of the limited solubility of DTME in water, dioxane was used as the solvent for the cryogel synthesis. The conjugate addition between the amine and maleimide compounds is a straightforward reaction, which even at subzero temperatures does not require additional reagents or a catalyst. The reagents are dissolved separately in dioxane and pre-cooled in an ice bath for a few seconds before mixing in plastic tubes (1 mm in diameter). Finally, the mixture is frozen by immersing the plastic tubes in a cryostate thermostated at -8^{o}C.

Influence of polymerization conditions on the properties of redox-responsive cryogels

In the synthesis of cryogels, both the crosslink density and the total monomer concentration are key parameters to obtain stable monoliths.[11] In our case, cryogels monoliths are formed via step growth polymerization of tri-functional PEG macromonomer and di-functional DTME (Scheme 1). For the synthesis of step-growth networks, both the molar ratios and the molecular weights of precursors can be considered to adjust the crosslink density.[35] However, in this stage of the research, only the molar ratio of the gel precursors was considered. Ideally, a molar ratio of 1.5 (DTME to PEG-$(NH_2)_3$) was used to synthesize step-growth cryogels and gave the most suitable cryogel monoliths. A further increase (to 1.75) or decrease (to 1.25) in the molar ratio resulted in a loosely crosslinked system and resulted in unstable cryogel monoliths. Furthermore, to see the effect of the total monomer concentration on the properties of the cryogels such as the degree of swelling, pore sizes and mechanical properties, cryogels were synthesized starting from three different total monomer concentrations (0.36, 0.18 and 0.12 mmol/mL) at -8^{o}C while keeping the molar ratio of the precursor DTME to PEG-$(NH_2)_3$) at 1.5.

The influence of the total monomer concentration on the swelling degrees of the cryogels is displayed in **Table 1**. With a decreasing initial concentration of the gel precursors, the

cryogels' degree of swelling increases. Moreover, the volume of the macropores decreases with increasing initial total monomer concentration. This result is consistent with the previously reported assumption that a higher total monomer concentration causes more dense cryogel structure with smaller pores and thicker walls.[43]

The differences in the cryogel structure and their macroporous nature can also be observed by SEM pictures (**Figure 1**). The SEM picture of cryogel 1 (Table 1), with a relatively high initial monomer concentration, shows a more oriented structure with smaller pores. As the system gets more diluted, the pore sizes of the cryogels increase and more random pore structures are observed, leading to a more elastic cryogel structure. On the other hand, it is also observed that a further decrease in initial precursor concentration (<0.18 mmol) might have a negative effect on the stability of monoliths causing the collapse of the pore walls.

As a specific characteristic related to the cryogelation procedure, all cryogels show an interconnected macroporous structure (Figure 1). This interconnected nature is reflected in their extremely fast swelling kinetics. These cryogels reach their equilibrium swelling ratio within 1-2 min in phosphate buffer saline (Figure 6a-6b).

Literature studies have demonstrated that the temperature during cryogel preparation has an important effect on the pore size of the cryogels.[11] Lower synthesis temperatures give rise to a fast freezing process of the solvent, therefore smaller solvent crystals are formed during the polymerization and correspondingly, cryogels with smaller pore sizes are obtained. In our study, cryogels have been synthesized at -8^{o}C and further increase in the synthesis temperature to obtain larger pore sizes has not been considered since the formation of ordinary gel matrices has been observed in the system during longer freezing times.

Structural analysis of redox-responsive cryogels

The structural investigation of cryogels is important since the maleimide-amine system might react in different ways depending on the reaction conditions. Other than the maleimide-amine conjugate reaction, there are two reactions that could occur in this system: crosslinking

between two maleimide groups and a ring opening aminolysis reaction between maleimide and amine groups. The conditions of these reactions have been studied extensively.[44-47] These studies show that reactions usually require elevated temperatures (>100^{o}C) while the solvent choice is also an important parameter to determine the reaction pathway. Even though these two reactions are not being expected to occur in our system, we performed a detailed analysis to elucidate the exact chemistry of our approach.

High-resolution Magic Angle Spinning (hr-MAS) NMR spectroscopy is a valuable tool to characterize the chemical structure of swollen cryogel samples. This technique was previously used in our group to monitor and quantify modification reactions of polyHEMA cryogels using the copper(I)-catalyzed Huisgen azide-alkyne coupling strategy.[17]

Figure 2 shows the ^{1}H hr-MAS NMR spectrum of a redox-responsive cryogel sample (cryogel 3 in table 1) that was swollen in deuterated DMF. This spectrum clearly shows that the reaction that took place is solely the conjugate addition between the amine and the maleimide double bond. The protons of the maleimide double bond give rise to a singlet at 6.9 ppm, which completely disappears, demonstrating the success of the conjugate addition. As a result, three signals appearing at 2.54 ppm (a), 3.04 ppm (b), and 3.91 ppm (c) are assigned to the protons of the new succinimide ring that is conjugated to the PEG-amine. This conjugation is confirmed by the presence of the signal at 2.83 ppm (f), which arises from the CH_2 group vicinal to the secondary amine of conjugated PEG. The signals at 3.78 ppm (d) and 2.98 ppm (e) belong to the CH_2 groups between the succinimide ring and the disulfide group, while the major signal at 3.60 ppm represents the CH_2 groups of the PEG moeity.

The assignments of these signals are also confirmed via 2D ^{1}H-^{1}H COSY (correlation spectroscopy) and ^{1}H-^{13}C HSQC (heteronuclear single quantum coherence) NMR spectra recorded under hr-MAS conditions (**Figure 3**). In Figure 3A, the ^{1}H-^{1}H COSY spectrum also reveals the correlation between the protons of the new succinimide ring with three signals appearing at 2.54 ppm (a), 3.04 ppm (b), and 3.91 ppm (c).

Apart from NMR analysis, visual analysis on the cryogel structures by the TNBS test, which is able to detect primary amine groups, demonstrated that residual amine groups are present in the cryogel system. In fact, the presence of primary amine groups on the cryogel structure could be beneficial for further functionalization studies of the cryogel matrix.

Raman spectroscopy of redox-responsive cryogels

Raman spectroscopy has been used to verify the presence of the disulfide groups.[48] The raman spectra of both blank cryogels (without disulfide groups) and redox-responsive degradable cryogels (containing disulfide groups) are shown in **Figure 4**, traces (a) and (b), respectively. In the spectrum of the redox-responsive cryogel (Figure 4b), the ν_{CS} band at 644 cm^{-1} and ν_{SS} band at 508 cm^{-1} indicate the presence of disulfides in the system. In the case of the blank cryogel (Figure 4a), these two bands are not detected. The ν_{CS} band at 644 cm^{-1} is surrounded by two other bands at around 694 cm^{-1} and 613 cm^{-1}, which are assigned to the $\nu_{C=O}$ stretching of the succinimide ring.

Mechanical properties of redox-responsive cryogels

Compression tests were performed on swollen cryogel samples to determine the stress-strain curves and to calculate the compressive moduli. Samples were compressed under a maximum loading of 20 N and the moduli were calculated from the initial linear region of the stress-strain curve (0-10 % strain). All cryogels exhibited a similar compressive modulus around 114 kPa (**Table 2**), which is likely due to their equal crosslink density.

Moreover, the studies on the mechanical properties of the redox-responsive degradable cryogels also showed that the initial precursor concentrations have an influence on the mechanical properties of cryogels. Related to its dense microstructure, cryogel 1 was not able to unload 20 N and broke at a maximum loading of 7.6 N at 59 % compression (**Figure 5**). On the other hand, cryogel 2 and 3 were able to manage a maximum loading of 20 N at 75 % compression and regained their shape gradually upon removal of the applied force. This

elastic nature of cryogels is further illustrated in **Figure 6**. In this figure, it is demonstrated that a swollen cryogel sample is compressed to a maximum limit while the cryogel regained its original shape upon removal of the applied force without any damage on the cryogel structure. This elastic nature and relatively high compressive modulus of the cryogels can be attributed to the step-growth formation of cryogels, leading to a more ideal network structure in comparison to the radical chain growth network formation as was observed for hydrogels before.[36]

Reduction triggered degradation of cryogels

The redox-responsive degradable cryogels synthesized via Michael-type conjugate addition were observed to be stable under physiological conditions (PBS, pH: 7.4). However, they got solubilized in a few hours in the presence of a reducing agent in the medium. Blank cryogels that were prepared from the same PEG based precursor with a non-cleavable bismaleimide, namely 1,4-di(maleimido)butane, did not show any degradation, proving that the disulfide groups are responsible for cryogel degradation.

As a nice demonstration of reduction triggered degradation of cryogel, a 1 mm thick slice was cut from a cryogel and incubated at room temperature in glutathione (25 mM) containing physiological buffer (i.e. PBS; pH 7.4; 150 mM NaCl). After different time intervals the cryogel was transferred to a glass cover slip and optical microscopy images were recorded. As shown in **Figure 7**, the cryogel gradually swells and decomposes after 12h of incubation before turning into a clear solution.

Potential of the cryogels as scaffold in tissue engineering

In first instance we evaluated whether the degradation products of the cryogels did affect cell viability. Therefore we incubated fibroblasts *in vitro* with solutions ranging from 0.1 mg/ml until to 1 mg/ml of the cryogels decomposed in glutathione containing phosphate buffered saline. None of these solutions did affect cell viability as shown in **Figure 8A**. In a second series of experiments we investigated cell adhesion and spreading on the cryogels as this is

essential to serve as tissue engineering scaffold. For visualisation purposes we first investigated planar gel slabs, which were prepared at room temperature via film casting. These gels slabs were then seeded with fibroblasts. As shown in Figure 8B cell adhesion did take place, however the cells exhibited a round morphology and did not spread out as observed on common tissue culture polystyrene substrates. To improve cellular behaviour, the gel slabs were coated with fibronectin through simple adsorption, which had a dramatic effect on cell spreading as shown in the lower row of Figure 8B where the elongated morphology of the actin filaments – stained with phalloidin – clearly indicate good cell spreading.

As a final experiment, fibronectin coated cryogels were drop-seeded with fluorescently labelled fibronectin cells. For fluorescent labelling, Cell-Tracker Green (CFSE-green fluorescent) was used to stain the fibronectin cells. Cell-Tracker Green only stains the living cells, thus it gives the advantage of tracing living cells inside the cryogel matrix. After one week of culturing period, z-stack confocal images were taken inside the cryogel matrix at different depths. In **Figure 9**, the elongated green structures are fluorescently labelled living cells, which spread themselves inside the cryogel matrix, which therefore clearly demonstrates the successful population of the cells at different depths of cryogel matrix. This experiment confirms that the pore sizes of the cryogel are sufficiently large for cell penetration when using the cryogels as scaffolds.

Conclusion

Disulfide containing degradable PEG-cryogels were successfully synthesized thanks to a radical-free conjugate addition strategy. These step-growth PEG-cryogels exhibit the important requirements to function as potential cell scaffolds in tissue engineering. For example, they have an interconnected macroporous nature with good mechanical properties and show good swelling behaviour in physiological conditions. While these PEG-cryogels are stable under physiological condition, thanks to the presence of disulfide bridges in the cryogel structure, they are able to degrade to water soluble components in the presence of an external

stimulus, such as the presence of glutathione as a reducing agent in this case. Moreover, cell viability experiments clearly showed that neither the degradation products nor the gel structure itself are toxic to the cells. Cell seeding experiments also demonstrated the excellent ability of the PEG-cryogel matrix to serve as scaffolds in tissue engineering, since the PEG-cryogel successfully sustained the cell population at different depths within the entire cryogel matrix.

Acknowledgements: T.D. thanks Ghent University for a PhD scholarship. W.V.C. and B.G.D.G. thank the Research Foundation – Flanders (FWO) for a postdoctoral research fellowship. L.J.D.C. thanks the IWT for a PhD scholarship. The NMR and Structural Analysis research group of UGent is acknowledged for the help with the hr-MAS NMR analysis of cryogels.

Received: ((will be filled in by the editorial staff)); Revised: ((will be filled in by the editorial staff)); Published online: ((DOI =10.1002/mabi.201100396))

Keywords: cryogels; Michael addition; PEG; stimuli-sensitive polymers; tissue engineering

[1] R. Langer, J. P. Vacanti, *Science* **1993**, *260*, 920.

[2] L. G. Griffith, G. Naughton, *Science* **2002**, *295*, 1009.

[3] L. E. Freed, G. Vunjaknovakovic, R. J. Biron, D. B. Eagles, D. C. Lesnoy, S. K. Barlow, R. Langer, *Bio-Technology* **1994**, *12*, 689.

[4] J. L. Drury, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337.

[5] V. R. Patel, M. M. Amiji, *Pharm. Res.* **1996**, *13*, 588.

[6] C. D. Wood, A. I. Cooper, *Macromolecules* **2001**, *34*, 5.

[7] Y. S. Nam, T. G. Park, *Biomaterials* **1999**, *20*, 1783.

[8] K. Kabiri, H. Omidian, M. J. Zohuriaan-Mehr, *Polym. Int.* **2003**, *52*, 1158.

[9] D. Cummins, P. Wyman, C. J. Duxbury, J. Thies, C. E. Koning, A. Heise, *Chem. Mater.* **2007**, *19*, 5285.

[10] E. M. Christenson, W. Soofi, J. L. Holm, N. R. Cameron, A. G. Mikos, *Biomacromolecules* **2007**, *8*, 3806.

[11] V. I. Lozinsky, *Russ. Chem. Rev.* **2002**, *71*, 489.

[12] V. I. Lozinsky, I. Y. Galaev, F. M. Plieva, I. N. Savina, H. Jungvid, B. Mattiasson, *Trends in Biotechnol.* **2003**, *21*, 445.

[13] V. I. Lozinsky, F. M. Plieva, I. Y. Galaev, B. Mattiasson, *Bioseparation* **2001**, *10*, 163.

[14] A. Jungbauer, R. Hahn, *J. Sep. Sci.* **2004**, *27*, 767.

[15] M. Hedstrom, F. Plieva, I. Yu, G. B. Mattiasson, *Anal. Bioanal. Chem.* **2008**, *390*, 907.

[16] D. Josic, A. Buchacher, *J. Biochem. Biophys. Methods* **2001**, *49*, 153.

[17] W. Van Camp, T. Dispinar, B. Dervaux, F. E. Du Prez, J. C. Martins, B. Fritzinger, *Macromol. Rapid Commun.* **2009**, *30*, 1328.

[18] I. N. Savina, V. Cnudde, S. D'Hollander, L. Van Hoorebeke, B. Mattiasson, I. Y. Galaev, F. Du Prez, *Soft Matter* **2007**, *3*, 1176.

[19] Y. S. Hwang, C. Zhang, S. Varghese, *J. Mater. Chem.* **2010**, *20*, 345.

[20] N. Bolgen, F. Plieva, I. Y. Galaev, B. Mattiasson, E. Piskin, *Journal of Biomaterials Science, Polymer Edition* **2007**, *18*, 1165.

[21] S. Van Vlierberghe, V. Cnudde, P. Dubruel, B. Masschaele, A. Cosijns, I. De Paepe, P. J. S. Jacobs, L. Van Hoorebeke, J. P. Remon, E. Schacht, *Biomacromolecules* **2007**, *8*, 331.

[22] M. B. Dainiak, I. U. Allan, I. N. Savina, L. Cornelio, E. S. James, S. L. James, S. V. Mikhalovsky, H. Jungvid, I. Y. Galaev, *Biomaterials* **2010**, *31*, 67.

[23] N. Kathuria, A. Tripathi, K. K. Kar, A. Kumar, *Acta Biomaterialia* **2009**, *5*, 406.

[24] H. F. Gilbert, *Biothiols, Pt A* **1995**, *251*, 8.

[25] F. H. Meng, W. E. Hennink, Z. Zhong, *Biomaterials* **2009**, *30*, 2180.

[26] Z. A. A. Hamid, A. Blencowe, B. Ozcelik, J. A. Palmer, G. W. Stevens, K. M. Abberton, W. A. Morrison, A. J. Penington, G. G. Qiao, *Biomaterials* **2010**, *31*, 6454.

[27] M. Andac, F. M. Plieva, A. Denizli, I. Y. Galaev, B. Mattiasson, *Macromol. Chem. Phys.* **2008**, *209*, 577.

[28] P. G. Righetti, B. C. W. Brost, R. S. Snyder, *J. Biochem. Biophys. Methods* **1981**, *4*, 347.

[29] K. Endo, K. Murata, T. Otsu, *Macromolecules* **1992**, *25*, 5554.

[30] K. Endo, T. Shiroi, K. Murata, *J. Polym. Sci.: Part A: Polym. Chem.* **2001**, *39*, 145.

[31] J. K. Oh, C. Tang, H. Gao, N. V. Tsarevsky, K. Matyjaszewski, *J. Am. Chem. Soc.* **2006**, *128*, 5578.

[32] L. Zhang, W. Liu, L. Lin, D. Chen, M. H. Stenzel, *Biomacromolecules* **2008**, *9*, 3321.

[33] T. Dispinar, F. E. Du Prez, *unpublished results*.

[34] P. Perez, F. Plieva, A. Gallardo, J. S. Roman, M. R. Aguilar, I. Morfin, F. Ehrburger-Dolle, F. Bley, S. Mikhalovsky, I. Y. Galaev, B. Mattiasson, *Biomacromolecules* **2008**, *9*, 66.

[35] A. E. Rydholm, S. K. Reddy, K. S. Anseth, C. N. Bowman, *Polymer* **2007**, *48*, 4589.

[36] M. Malkoch, R. Vestberg, N. Gupta, L. Mespouille, P. Dubois, A. F. Mason, J. L. Hedrick, Q. Liao, C. W. Frank, K. Kingsbury, C. J. Hawker, *Chem. Commun* **2006**, *26*, 2774.

[37] B. D. Mather, K. Viswanathan, K. M. Miller, T. E. Long, *Prog. Polym. Sci.* **2006**, *31*, 487.

[38] Sharples.Ne, M. Flavin, *Biochemistry* **1966**, *5*, 2963.

[39] D. L. Elbert, J. A. Hubbell, *Biomacromolecules* **2001**, *2*, 430.

[40] M. P. Lutolf, J. A. Hubbell, *Biomacromolecules* **2003**, *4*, 713.

[41] P. van de Wetering, A. T. Metters, R. G. Schoenmakers, J. A. Hubbell, *J. Controll. Release* **2005**, *102*, 619.

[42] J. V. Crivello, *J. Polym. Sci.: Part A: Polym. Chem.* **1976**, 14, 159.

[43] F. M. Plieva, M. Karlsson, M. R. Aguilar, D. Gomez, S. Mikhalovsky, I. Y. Galaev, *Soft Matter* **2005**, *1*, 303.

[44] L. R. Dix, J. R. Ebdon, N. J. Flint, P. Hodge, R. Odell, *Eur. Polym. J.* **1995**, *31*, 647.

[45] L. R. Dix, J. R. Ebdon, P. Hodge, *Eur. Polym. J.* **1995**, *31*, 653.

[46] J. E. White, M. D. Scaia, D. A. Snider, *J. Appl. Polym. Sci.* **1984**, *29*, 891.

[47] D. B. Curliss, B. A. Cowans, J. M. Caruthers, *Macromolecules* **1998**, *31*, 6776.

[48] H. Okabayashi, K. Izawa, T. Yamamoto, H. Masuda, E. Nishio, C. J. O'Connor, *Colloid Polym. Sci.* **2002**, *280*, 135.

Scheme 1. A) Synthesis of amine terminated poly(ethylene) glycol based building block, PEG-$(NH_2)_3$. B) Synthesis of disulfide containing bismaleimide crosslinker, DTME. C) Schematic representation of Michael addition reaction in the synthesis of cryogels.

Figure 1. SEM images of redox-responsive degradable cryogels prepared with different monomer concentrations. A) cryogel 1 (0.36 mmol/mL), B) cryogel 2 (0.18 mmol/mL), C) cryogel 3 (0.12 mmol/mL).

Figure 2. ^{1}H NMR spectrum of a redox-responsive cryogel (cryogel 3).

Figure 3. A) ^{1}H-^{1}H COSY spectrum and B) ^{1}H-^{13}C HSQC spectrum of a redox-responsive cryogel in DMF-d7 (cryogel 3).

Figure 4. Raman spectra of (a) blank cryogel (without disulfide groups), (b) disulfide containing cryogel (cryogel 3).

Figure 5. Stress-strain slopes of compression test on redox-responsive degradable cryogels under the maximum loading of 20 N with a displacement control rate of 1 mm/min^{-1}.

Figure 6. Redox-responsive cryogel (a) in dry state, (b) in swollen state, (c-d) under compression.

Figure 7. Optical microscopy images of a round sliced cryogel in dry state after incubation in glutathione during different time intervals.

Figure 8. (A) Cell viability of fibroblasts in media containing different concentrations of a decomposed cryogel. Circles-cryogel 1, triangles- cryogel 2, rectangles- cryogel 3. (B) Confocal microscopy images of fibroblasts seeded onto blank gel slabs and fibronectin coated gel slabs. Left panels show the overlay of blue and red fluorescence while the right panels show the DIC channel. Cell nuclei were stained blue fluorescent with HOECHST and the actin skeleton was stained red fluorescent with alexa546 conjugated phalloidin.

Figure 9. Z-stack depicting confocal microscopy sections at different depths of a fibronectin coated cryogel drop-seeded with fibroblasts. For visualisation purposes the fibroblasts were stained green fluorescent with CellTracker Green before drop-seeding.

Table 1. Properties of redox-responsive cryogels produced at -8^{o}C with different concentrations of gel precursors.

Entry	**Concentration of gel precursors**	**Yield (%)**	**Swelling degree**	**Volume of macropores (%)**	**Cryogel**
Cryogel 1	0.36 mmol/mL	96	6.5	62	dense
Cryogel 2	0.22 mmol/mL	94	7.3	66	elastic
Cryogel 3	0.18 mmol/mL	93	8.1	70	elastic

Table 2. Mechanical properties of redox-responsive degradable cryogels

Entry	**Max strain**	**Compressive Modulus [kPa]**	**Max Load [N]**	**Max Stress [MPa]**
Cryogel 1	59%	114	7.6	0.08
Cryogel 2	76%	92	20	0.315
Cryogel 3	75%	116	20	0.399

Michael addition reaction between a maleimide double bond and amine group is utilized for the synthesis of redox-responsive (PEG)-cryogels. The cryogels exhibit an interconnected macroporous morphology, good mechanical properties and responsive degradation behaviour without any negative effect on the cell viability. Biological analysis shows that the cryogels provide a suitable environment for cell growth.

Tugba Dispinar, Wim Van Camp, Liesbeth J. De Cock, Bruno G. De Geest, and Filip E. Du Prez*

Redox-responsive degradable PEG-cryogels as potential cell scaffolds in tissue engineering