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Cell • Environment • Design

3-D Life Hydrogels

User Guide

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1. REAGENTS INCLUDED IN THE *3-D LIFE* HYDROGEL PRODUCTS

Table 1: Reagents provided with the *3-D Life* Hydrogel kits and components

Product name		Maleimide-PVA	Maleimide-Dextran	PEG-Link	CD-Link	Dextranase	10 x CB _{pH 5.5}	10 x CB _{pH 7.2}	Thio-glycerol	Water
Hydrogel kits	<i>3-D Life</i> PVA-PEG Hydrogel Kit, 1 ml	170 μ l	–	200 μ l	–	–	200 μ l	200 μ l	60 μ l	500 μ l
	<i>3-D Life</i> PVA-CD Hydrogel Kit, 1 ml	170 μ l	–	–	200 μ l	–	200 μ l	200 μ l	60 μ l	500 μ l
	<i>3-D Life</i> Dextran-PEG Hydrogel Kit, 1 ml	–	170 μ l	200 μ l	–	500 μ l	200 μ l	200 μ l	60 μ l	500 μ l
	<i>3-D Life</i> Dextran-CD Hydrogel Kit, 1 ml	–	170 μ l	–	200 μ l	500 μ l	200 μ l	200 μ l	60 μ l	500 μ l
Maleimide polymers	<i>3-D Life</i> Maleimide-PVA Set	510 μ l	–	–	–	–	600 μ l	600 μ l	180 μ l	1500 μ l
	<i>3-D Life</i> Maleimide-Dextran Set	–	510 μ l	–	–	1500 μ l	600 μ l	600 μ l	180 μ l	1500 μ l
Thiol crosslinkers	<i>3-D Life</i> PEG-Link	–	–	200 μ l	–	–	–	–	–	–
	<i>3-D Life</i> PEG-Link	–	–	600 μ l	–	–	–	–	–	–
	<i>3-D Life</i> CD-Link	–	–	–	200 μ l	–	–	–	–	–
	<i>3-D Life</i> CD-Link	–	–	–	600 μ l	–	–	–	–	–
Recommended storage temperature		-80°C	-80°C	-20°C n to -80°C	-20°C to -80°C	4°C or -80°C	-20°C to -80°C	-20°C to -80°C	-20°C to -80°C	R/T

2. NOTICE TO PURCHASER

Products of the *3-D Life* Hydrogel system are covered by patents pending of NMI Natural Sciences and Medical Institute at the University of Tübingen. Purchase of these products conveys a license for use for research. For further information please contact Cellendes GmbH. *3-D Life* Hydrogel is for research use only.

3. THE TECHNOLOGY

3.1. Three-dimensional cell culture

Research over the past 15 years has shown that the physiology of cells cultured *in vitro* in three dimensional systems (3-D) resembles that of their counterparts *in vivo* much better than the physiology of cells cultured conventionally on flat surfaces of tissue culture plates. This is reflected in differential gene expression [1] and a difference in cell behaviour and differentiation [2]. As a consequence, 3-D cell culture is often the better choice for investigating cell function [3].

Hydrogels, consisting of synthetic and chemically defined components can be modified to mimic the characteristics of natural extracellular matrices. These biomimetic hydrogels can provide valuable insight into the regulation of cell function and developmental processes in tissue- and organ-specific differentiation and morphogenesis, which depend in many ways on the extracellular environment.

3.2. Three-dimensional biomimetic cell cultures with 3-D Life Hydrogel

The 3-D Life Hydrogel system can be used to culture cells in a biomimetic three-dimensional environment. It is a two-component system consisting of

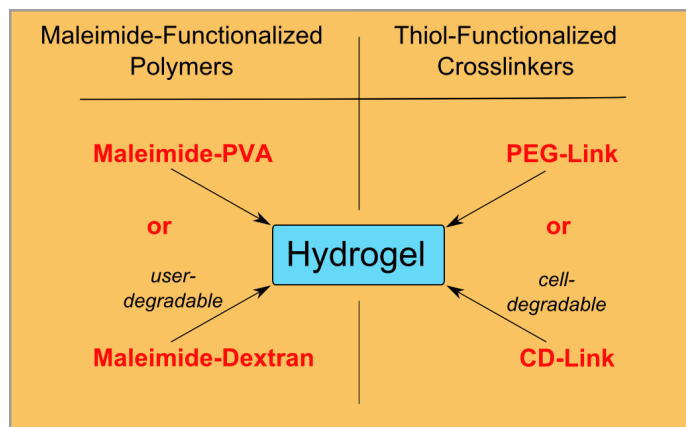
- a maleimide-functionalized polymer and
- a thiol-functionalized crosslinker.

The two components are mixed in the presence of cells; thiol groups form stable thioether bonds with maleimide groups, which results in the formation of a gel containing embedded cells. Prior to the crosslinking step, a peptide (containing *e.g.* cell adhesion motifs) may be covalently attached to a portion of the maleimide groups of the maleimide polymers. The hydrogels can also be used to culture cells on top of the gel.

Either polyvinyl alcohol (PVA) or dextran is used as a maleimide-functionalized polymer. Hydrogels based on the PVA polymer are stable and cannot be degraded by the user, whereas dextran hydrogels can be degraded by addition of the enzyme dextranase to isolate cells from the hydrogel for further use. Both polymers can be crosslinked with either polyethylene glycol (PEG-Link), which is a stable, non-degradable linker, or a polyethylene glycol peptide conjugate (CD-Link), which contains a Matrix-Metallo-protease cleavable peptide that allows cells to cleave the crosslinker and spread and migrate within the gel (for further details see also chapter 5.1.2, page 13). The maleimide-

functionalized polymers and the thiol-functionalized crosslinkers can be mixed and matched to set up gels of defined properties (Figure 1). The procedure for setting up biomimetic hydrogels containing embedded cells is very simple and does not require specific equipment or chemical expertise (Figure 2).

Figure 1: Maleimide-functionalized polymers (either Maleimide-PVA or Maleimide-Dextran) can be combined with thiol-functionalized crosslinkers (either PEG-Link or CD-Link) to set up gels with undegradable, user-degradable and/or cell-degradable properties, as desired.



The gel components PVA, dextran and PEG are water-soluble; they do not bind to cells [4] and are known not to interfere with cellular function. Thus, the crosslinked components support cells in an inert gel, which provides an ideal basis for analyses of cell phenotype as a function of added bioactive factors, such as adhesion peptides, extracellular matrix proteins or fragments thereof in a three-dimensional environment.

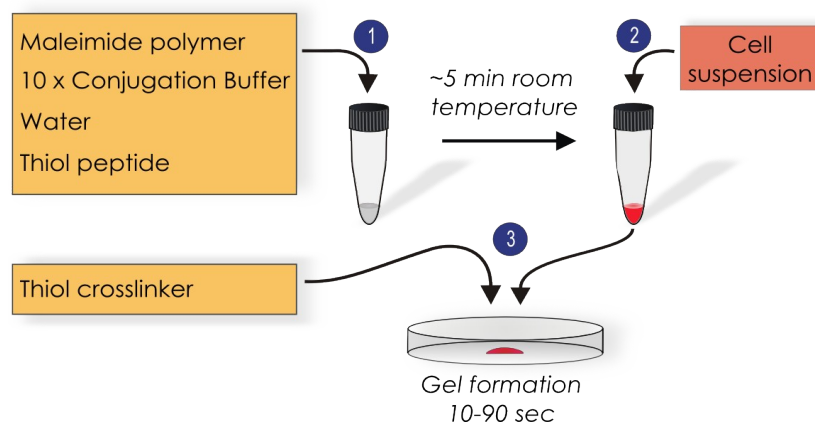


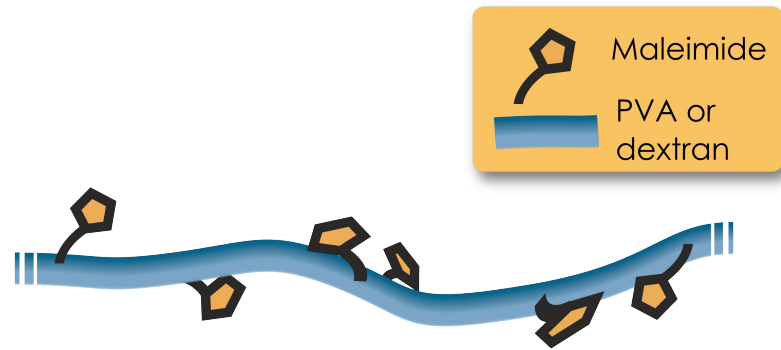
Figure 2: Schematic drawing of the three simple steps required to embed cells in a peptide-modified hydrogel. 1. Polymer, buffer and peptide are mixed and incubated to covalently attach the peptide to the maleimide polymer. 2. A cell suspension is added to the peptide-polymer conjugate solution. 3. Cells and the peptide-conjugated polymer are mixed with crosslinker for gel formation and placed on a culture dish. After the gel is formed, culture medium is added and the culture dish is placed in an incubator for cell cultivation (not shown).

3.2.1. Maleimide Polymers

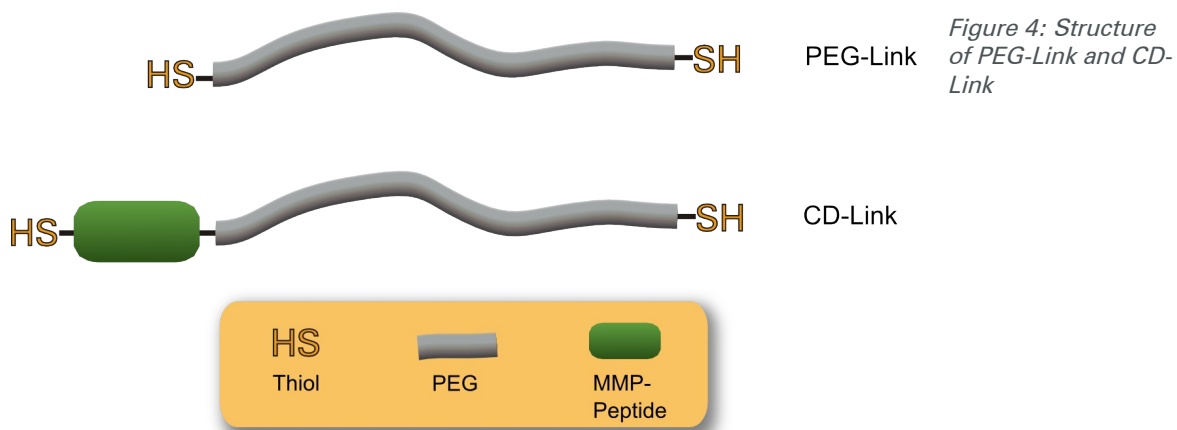
PVA or dextran have been functionalized with many maleimide groups along their backbone (Figure 3), sufficient for modification with bioactive factors and crosslinking.

3.2.2. PEG-Link and CD-Link

Figure 3: PVA or dextran functionalized with maleimide groups



PEG-Link consists of linear polyethylene glycol with a thiol (SH) group at each end (α,ω -bis-mercapto poly(ethylene glycol)). CD-Link is derived from PEG-Link. It contains a peptide with a cleavage site for matrix metalloproteases (MMP; Figure 4):



3.3. The chemical reaction

Thiol groups form a stable thioether bond with maleimide groups under physiological conditions. The reaction proceeds very fast at neutral pH. As the pH is lowered, the reaction slows down, since thiols react as deprotonated thiolate anions with maleimide groups. Thus, the reaction rate can be controlled by altering the pH.

The reaction is very specific. Since the extracellular environment of cells lacks maleimide groups and reactive thiol groups, the reaction proceeds in the presence of cells without any harm on their vitality.

3.4. Gel formation

The two thiol groups of the crosslinker molecules (PEG-Link or CD-Link) react with maleimide groups of different maleimide polymer molecules. This leads to large polymer-crosslinker networks, which, eventually, form a gel. Gel formation occurs best at equimolar concentrations of thiol and maleimide groups starting at approximately 2 mmol/l each. Higher equimolar concentrations of the reactive groups result in stronger gels which form faster.

3.5. The buffer system

3-D Life Hydrogel products come with two 10-fold concentrated buffers (10 x CB_{pH 7.2} and 10 x CB_{pH 5.5}). At one-fold concentration, the buffers provide an isotonic environment at pH 7.2 or pH 5.5. At pH 7.2 the cells are in a pH-neutral solution, but the gel formation reaction proceeds very fast. At higher concentrations of the polymers, gel formation proceeds exceedingly rapid, preventing complete mixing of the polymer solutions before

Figure 5: pH range as a result of different ratios of CB_{pH 5.5} and CB_{pH 7.2}.

the gel forms. Therefore, for setting up stronger gels with higher polymer concentrations, 10 x CB_{pH 5.5} is used as a buffer. The lower pH slows down gel formation allowing sufficient time for mixing before gel formation starts. Most cells are not affected by a short exposure to pH 5.5. For making gels at a pH between 7.2 and 5.5, both buffer systems can be mixed at any ratio to generate intermediate pH values (Figure 5).

Both buffer systems have a strong buffering capacity to ensure a stable pH. They also contain phenol red as a pH indicator, which exhibits a gradual color transition from red to yellow as the pH shifts from alkaline to acidic.

3.6. Covalent attachment of cell adhesion peptides

Peptides can be covalently attached to a portion of the maleimide groups of the maleimide polymers. The peptides must contain a thiol group, which can be easily inserted by including a cysteine residue during peptide synthesis. If comparative studies at different peptide concentrations are to be made, a corresponding amount of a cell neutral compound containing a thiol group should be added to ensure equal gel strengths. For this purpose 1-thioglycerol is included in the *3-D Life* Hydrogel kits (see also Table 1).

We recommend final peptide concentrations of up to 1 mmol/l, if only one kind of peptide is used in a single hydrogel. Higher peptide concentrations up to 5 mmol/l may be used. This is especially convenient when different kinds of peptides are to be immobilized in the hydrogel.

3.7. Addition of extracellular matrix proteins to hydrogels

Soluble extracellular matrix proteins (e.g. fibronectin, laminin) or protein fragments can be added to the polymer solution before crosslinking. The protein solutions should contain no reducing agents, such as dithiothreitol or 2-mercaptoethanol, which would inhibit gel formation. We recommend to start with a concentration of 30 $\mu\text{g/ml}$ protein in a hydrogel that is crosslinked at 3-4 mmol/l maleimide groups. The concentration of the extracellular matrix proteins may be varied.

An example of mixing a fibronectin-modified Hydrogel (30 $\mu\text{g/ml}$ fibronectin in PVA-Hydrogel) is shown in Table 2.

Table 2: Example of composing a gel¹ containing extracellular matrix protein

Reagent	[μl]
Water	8.0
10 x CB _{pH 5.5} ²	2.5
Maleimide polymer (30 mmol/l maleimide groups)	4.0
Fibronectin (200 $\mu\text{g/ml}$)	4.5
Cell suspension (2-4 x 10 ⁶ cells/ml)	5.0
PEG-Link (20 mmol/l thiol groups)	6.0
Total	30.0

¹ Concentration of maleimide and thiol groups for crosslinking: 4 mmol/l

² Volume of 10 x CB_{pH 5.5} is calculated for the total volume minus the volume of the cell suspension to ensure isoosmotic conditions.

4. TECHNICAL HINTS

4.1. Maleimide-PVA and Maleimide-Dextran

Maleimide groups are hydrolyzed by water at a very low rate. This reaction proceeds faster at higher temperatures, therefore, the reagents should be kept on ice during use. If stored at -20°C , Maleimide-PVA forms insoluble aggregates within days to weeks. Therefore, Maleimide-PVA should be stored at -80°C , at which temperature it is stable. It is recommended that it be stored in small portions to avoid frequent freeze-thaw cycles. If necessary, it can be stored for up to one week at 4°C .

4.2. Dextranase

3-D Life Dextranase is based on a dextranase preparation from *Chaetomium gracile* (5). This enzyme can be stored for several months at 4°C , where its activity decreases by approximately 30% per year. Prolonged storage should be at -80°C .

4.3. PEG-Link and CD-Link

Thiol groups can form inactive disulfides upon exposure to oxygen. As this reaction can be substantially slowed down at acidic pH, PEG-Link and CD-Link are provided in mildly acidic solutions. Prolonged exposure of PEG-Link and CD-Link to air should be avoided.

4.4. $\text{CB}_{\text{pH } 7.2}$ and $\text{CB}_{\text{pH } 5.5}$

The buffers $10 \times \text{CB}_{\text{pH } 7.2}$ and $10 \times \text{CB}_{\text{pH } 5.5}$ can be stored for up to 1 month at room temperature or at 4°C . Prolonged exposure to light, however, should be avoided. Long term storage should be at -20°C or at -80°C . During refrigeration, some of the buffer components may precipitate. Prior to use, it is important to ensure complete dissolution of the buffer. Precipitates can be solubilized by vigorous agitation (Vortex) at room temperature or incubation at 37° for a few minutes.

4.5. Guidelines for peptide design

Celldes offers peptides for use with 3-D Life Hydrogels. However peptides can also be readily acquired from companies specializing in peptide synthesis.

A thiol group can be easily inserted by including a cysteine residue at the N-terminal end of a peptide. The adjacent amino terminus of the peptide should be protected (*e.g.* by an acetyl group) to remove the positive charge of the free amino terminus, which might otherwise enhance the rate of oxidation of the thiol group. In general, positively charged amino acids in the immediate vicinity of the thiol should be avoided, *e.g.* by employing a spacer. Only purified peptides (*e.g.* by HPLC) in the Cl⁻-salt form should be used. If possible, peptides should be stored in small portions under inert gas to avoid repeated exposure to air.

Peptides available from Cellendes have been tested in the 3-D Life Hydrogel system.

5. APPLICATIONS

5.1. Types of cell cultures

In general, any cell type can be cultured in 3-D Life Hydrogels as long as the cellular environment provides all factors necessary for cultivation. The 3-D Life Hydrogel system is designed to provide full control over the composition of bioactive components in the extracellular environment. Examples of cell cultures can be found in Application Notes at www.cellendes.com.

5.1.1. Cell culture in hydrogels crosslinked with PEG-Link

Cells can be embedded as single cells or cell aggregates in gels crosslinked with PEG-Link. Depending on the type of cells, formation of epithelial cysts, spheroids or colonies can be obtained. However, due to a small average pore size (ca 8 nm) cells do not spread or migrate.

5.1.2. Cell culture in hydrogels crosslinked with CD-Link

Cells can be embedded as single cells or cell aggregates in hydrogels crosslinked with MMP-cleavable CD-Link. If the embedded cells produce Matrix Metalloproteases, the linker can be cleaved in the vicinity of the cells. In the presence of adhesion factors containing *e. g.* the RGD motif, cell spreading and migration can occur. For the specificity of the MMP cleavage site in CD-Link please refer to the CD-Link product data sheet (www.cellendes.com).

5.1.3. Cell culture on the surface of gels

Cells can be cultured on *3-D Life* Hydrogels when gels are cast with a flat surface. The gel must contain an adhesion factor, for example a peptide containing the RGD motif (e.g. *3-D Life* RGD Peptide), that can be recognized by the cells and promote adhesion. The culture of cells on gels can be of advantage if a soft surface instead of the hard surface of a tissue culture plate is required for cell culture.

5.2. Cell fixation and labeling

Chemical fixation of cells and labeling of live or fixed cells within the gel with small molecules (e.g. fluorescently labeled phalloidin, nucleic acid stains, viability and cytotoxicity assay reagents, proliferation assay reagents based on small molecule components [e.g. Click-iT™ EdU, Invitrogen], Ca²⁺ indicators etc.) can be performed with protocols used for 2-D cell cultures.

The incubation with fixation solution and labeling reagents, as well as washing buffer may be prolonged to allow for longer diffusion times in the hydrogel. For a cell culture within a 30 μl gel, an incubation time of 30 min to 1 hour is recommended for labeling reagents. Cells containing genetically encoded reporters (e.g. green fluorescent protein) can easily be observed, since *3-D Life* hydrogels are fully transparent. Labeling with reagents with a larger molecular mass (e.g. antibodies) within the gel is not possible due to its small pore size. However, cells grown in *3-D Life* Dextran Hydrogels can be recovered by degrading the gel with dextranase. The recovered cells can subsequently be treated in suspension with high molecular weight reagents. Before the isolation from dextran gels cells may be chemically fixed to preserve the 3-D cell culture phenotype.

5.3. Choice of PVA or dextran hydrogel

5.3.1. PVA hydrogel

PVA hydrogels are stable against enzymatic digestion and suit many applications in cell culture when analysis of the cell culture does not require the isolation of cells from the gel. The gels can be made from a soft to a firm consistence and they are stable in culture medium over weeks.

5.3.2. Dextran hydrogel

Dextran hydrogels can be dissolved by addition of dextranase. This allows the isolation of chemically fixed or living cells after 3-D cell culture for downstream processing of

cells. For example, cells can be passaged for further growth or analyzed by immunostaining, RT-PCR, biochemical analyses or flow cytometry, similar to cells from 2-D cell cultures.

Dextran gels are slightly softer than PVA-Hydrogels and can deteriorate to some degree after prolonged culture over weeks. The properties of the dextran and PVA hydrogels are summarized in Table 3.

Table 3: Comparison of dextran and PVA hydrogels

	PVA Hydrogel	Dextran Hydrogel
Covalent attachment of peptides	+	+
Gel formation with stable (PEG-Link) and cell-degradable (CD-Link) crosslinkers	+	+
Cell labelling with low molecular weight stains	+	+
Degradable by user	–	+
Biochemical analyses of cells	+/-	+
Recovery of living cells, passaging	–	+
Immunostaining	–	+ ¹

¹ after recovery of cells from gel by dextranase treatment

Cultures using both types of hydrogels

Using the two types of gels, it is possible to construct co-cultures that can be subsequently taken apart by dissolving the dextran hydrogel and leaving the PVA gel intact. This may be convenient for developing tissue models or organotypic cell cultures.

5.4. Microscopy

3-D Life hydrogels are fully transparent and cells can be observed by any form of light microscopy, e.g. bright field and phase contrast microscopy, fluorescence microscopy, laser scanning confocal microscopy.

6. COMPOSITION OF REAGENTS

Maleimide-PVA:	30 mmol/l maleimide groups on polyvinyl alcohol, 5 mmol/l phosphate buffer
Maleimide-Dextran:	30 mmol/l maleimide groups on dextran
PEG-Link:	20 mmol/l thiol groups on PEG-Link; 2 mmol/l HCl
CD-Link:	20 mmol/l thiol groups on CD-Link; 2 mmol/l HCl
Dextranase:	Aqueous solution of dextranase from <i>Chaetomium gracile</i>

Thioglycerol:	20 mmol/l 1-thioglycerol; 2 mmol/l HCl
10 x CB _{pH 7.2} :	10 g/l glucose; 0.5 mol/l HEPES; 0.05 mol/l KCl; 1.1 mol/l NaCl; 0.2 mol/l NaH ₂ PO ₄ ; 0.2 g/l phenol red; pH-adjusted with NaOH
10 x CB _{pH 5.5} :	10 g/l glucose; 0.5 mol/l MES; 0.05 mol/l KCl; 1.1 mol/l NaCl; 0.2 mol/l NaH ₂ PO ₄ ; 0.2 g/l phenol red; pH-adjusted with Hcl.
Water:	H ₂ O (cell biology grade)

7. STEP BY STEP-PROTOCOLS

All steps for setting up a *3-D Life* Hydrogel cell culture are performed under a sterile hood. In the following protocols 10 x CB_{pH 5.5} is used. 10 x CB_{pH 7.2} should only be used if the cells need to be protected from exposure to pH 5.5. If 10 x CB_{pH 7.2} is employed, gel formation occurs very rapidly, preventing complete mixing at higher polymer concentrations. Intermediate pH values can be used to slow down the reaction speed and can be achieved by mixing the two buffers. Mixing ratios and resulting pH values are shown in Figure 5. Cell suspensions can be prepared in PBS or medium or as required for each specific cell type.

7.1. Preparations

- Thaw *3-D Life* Hydrogel reagents; make sure that reagents are completely dissolved.
- If gels are to be modified with peptides:
 - Dissolve peptide and dilute to a concentration of 3.0 mmol/l with Water.
 - Dilute Thioglycerol 1:6.7 with Water (final concentration: 3.0 mmol/l).
- Keep Maleimide-PVA or Maleimide-Dextran on ice.
- Prepare a cell suspension in PBS or medium (we recommend a cell suspension of 2-4 x 10⁶ cells per ml to reach a final cell density of 10,000-20,000 cells per 30 μ l gel according to the protocol below). The cell density in the gel can be varied as desired.

7.2. Setup of 3-D cultures without peptide modification

Gel strength increases with increasing concentrations of maleimide polymers and corresponding crosslinker concentrations in the gels. Maleimide groups and thiol-groups should always be kept at equimolar ratios.

Table 4 depicts examples for the preparation of 3-D cultures at different gel strengths.

Table 4: Pipetting schemes for generation of gels at different strengths (volume: 30 μ l).

Gel strength						
Final concentration of maleimide groups and thiol groups (mmol/l)	2.5	3.0	4.0	5.0	7.0	9.0
Reagent	Volumes in μ l					
Water	16.25	15.0	12.5	10.0	5.0	-
10 x CB _{pH 5.5} ¹	2.50	2.5	2.5	2.5	2.5	2.5
Maleimide-PVA or Maleimide-Dextran (30 mmol/l)	2.50	3.0	4.0	5.0	7.0	9.0
Cell suspension	5.00	5.0	5.0	5.0	5.0	5.0
PEG-Link or CD-Link (20 mmol/l)	3.75	4.5	6.0	7.5	10.5	13.5
Total	30.0	30.0	30.0	30.0	30.0	30.0

¹ Volume of 10 x CB_{pH 5.5} is calculated for the total volume minus the volume of the cell suspension to ensure iso-osmotic conditions.

The cultures are set up as follows:

- Combine Water, 10 x CB_{pH 5.5}, and Maleimide-Dextran (or Maleimide-PVA) in a reaction tube .
- Place PEG-Link or CD-Link on the surface of a culture dish.
- Add cell suspension to the reaction tube containing the maleimide polymer.
- Transfer the content of the reaction tube into a pipet tip and mix it with the PEG-Link (or CD-Link) on the dish by pipetting the mixture three times up and down. Avoid the formation of air bubbles.
- Place the sample on the surface of the culture dish and leave it for gel formation. Wait for at least one minute.
- Optional: test gel formation by careful inspection with *e.g.* a pipet tip.
- Once the gel has formed, carefully add growth medium to the dish to cover the gel.
- Add the lid to the culture dish and place in incubator.
- Renew medium after approximately 2 hours of incubation.

7.3. Setup of 3-D cultures with peptide modification

A final peptide concentration of 1 mmol/l is usually sufficient to provide enough attachment sites for cells. Table 5 illustrates the ratios of reagents and their reactive groups at

different final concentrations of peptide in the gel. In this example, a total of 5 mmol/l maleimide groups on Maleimide-Dextran or Maleimide-PVA is used:

- 1 mmol/l maleimide is used to provide sufficient attachment sites for peptide modification (1 mmol/l thiols from peptide(s) and Thioglycerol);
- 4 mmol/l maleimide is used for crosslinking with 4 mmol/l thiols from PEG- or CD-Link) to provide a constant gel strength.

Table 5: Ratios and final concentrations of reactive groups when setting up gels at different peptide concentrations.

Peptide concentration					
	Final concentration (mmol/l)				
Thiol groups of Peptide	0.0	0.1	0.2	0.5	1.0
Thiol groups of Thioglycerol	1.0	0.9	0.8	0.5	0.0
Thiol groups of PEG- or CD-Link	4.0	4.0	4.0	4.0	4.0
Maleimide groups of Maleimide-PVA or Maleimide-Dextran	5.0	5.0	5.0	5.0	5.0

The corresponding pipetting schemes for setting up these gels in volumes of 30 μ l are shown in Table 6.

Table 6: Pipetting schemes for the generation of gels with different peptide concentrations.

Final peptide concentration (mmol/l):	0.0	0.1	0.2	0.5	1.0
	Volumes in μ l				
Water	1.5	1.5	1.5	1.5	1.5
10 x CB _{pH 5.5} ¹	2.5	2.5	2.5	2.5	2.5
Maleimide-PVA or Maleimide-Dextran (30 mmol/l maleimide groups)	5.0	5.0	5.0	5.0	5.0
SH-peptide (3.0 mmol/l)	0.0	1.0	2.0	5.0	10.0
Thioglycerol (3.0 mmol/l)	10.0	9.0	8.0	5.0	0.0
Cell suspension	5.0	5.0	5.0	5.0	5.0
PEG-Link or CD-Link (20 mmol/l SH-groups)	6.0	6.0	6.0	6.0	6.0
Total	30.0	30.0	30.0	30.0	30.0

¹ Volume of 10 x CB_{pH 5.5} is calculated for the total volume minus the volume of the cell suspension to ensure iso-osmotic conditions

The cultures are set up using the following steps:

- Combine Water, 10 x CB_{pH 5.5}, and Maleimide-Dextran or Maleimide-PVA in a reaction tube.
- Add the peptide and Thioglycerol and mix immediately to ensure homogenous modification of the maleimide polymer with the peptide/Thioglycerol.
- Incubate samples for 5-10 min at room temperature to allow for enough time for the completion of the attachment of peptides and thioglycerol to the maleimide polymers.
- Place crosslinker (PEG-Link or CD-Link) on the surface of a culture dish.
- Add cells to the reaction tube containing the peptide-polymer conjugate.
- Transfer the content of the reaction tube into a pipet tip and mix it with the crosslinker on the dish by pipetting up and down three times. Avoid the formation of air bubbles.
- Place the mix on the surface of the culture dish. Wait for at least one minute.
- Optional: test gel formation by careful inspection with *e.g.* a pipet tip.
- Once the gel has formed, carefully add growth medium to cover the gel.
- Add the lid to the culture dish and place the dish in an incubator.
- Renew medium after approximately 2 hours incubation.

7.4. Dissolving 3-D Life Dextran Hydrogels

Dextran hydrogels containing intact cells can be dissolved by adding dextranase to the culture medium of the 3-D hydrogel culture. Cells can also be chemically fixed (*e.g.* with 4% formaldehyde in PBS) prior to the dextranase treatment.

As a general guideline, a 30- μ l gel can be dissolved at a 1:20 dilution of dextranase in 300 μ l medium incubated for 60 min at 37°C. Highly crosslinked gels (5-8 mmol/l Maleimide/SH) may take longer for dissolution than gels with lower degrees of crosslinking (3-4 mmol/l Maleimide/SH).

After dissolution of the gel, the cell suspension can be centrifuged and pelleted cells can be resuspended in fresh medium or other solutions as required. This washing procedure can be repeated to more effectively remove remains of dextranase and dissolved gel components.

8. REFERENCES

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9. TROUBLESHOOTING

Observation	Possible causes	Possible Remedies
Maleimide-PVA solution becomes turbid after addition of peptide.	Some peptides may cause precipitation of Maleimide-PVA after covalent attachment.	Try to use a lower peptide concentration.
No gel formation	Maleimide groups and thiol groups used for crosslinking are not close to equimolar concentrations of 2,5-9 mmol/l.	Check the balance of maleimide groups and thiol groups of your components.
	Too much peptide or thioglycerol used, resulting in too few maleimide groups for crosslinking	Check the calculations for the setup of the experiment.
	Reaction pH too low, caused by too much acid in peptide sample	Check the pH of the sample with pH-paper; (partially) replace $CB_{pH\ 5.5}$ with $CB_{pH\ 7.2}$.
	Deteriorated reactive groups	Use a fresh sample of reaction components; adhere to storage conditions as recommended in Table 1. Consider technical hints on page 12.
Gel forms too fast.	Concentration of reactive components are too high (>9 mmol/L).	Use lower equimolar concentration of maleimide and thiol groups for crosslinking.
	pH too high	Use lower pH ($CB_{pH\ 5.5}$) for performing the crosslinking reaction.
Vitality of cells decreases rapidly.	Cell type requires presence of an adhesion factor or of a different adhesion factor; cell may be sensitive to low pH during gel setup.	Add appropriate cell adhesion peptides or extracellular adhesion proteins; use neutral pH during gel setup.
Dissolution of dextran-based hydrogel is not complete after 1 hr at 37°C.	Gel strength too high; dextranase activity reduced due to long storage at 4°C or higher	Prolong digestion up to 2 hr; add more volume of dextranase-medium solution; use higher dextranase concentration.

10. PRODUCTS

	Product Name	Unit Size	3-D Life	
			Hydrogel Volume ¹	Catalog Number
Hydrogel Kits ²	3-D Life PVA-PEG Hydrogel Kit, 1 ml [†]	1 kit	1 ml	09-G-001
	3-D Life PVA-CD Hydrogel Kit, 1 ml [†]	1 kit	1 ml	G81-1
	3-D Life Dextran-PEG Hydrogel Kit, 1 ml	1 kit	1 ml	G90-1
	3-D Life Dextran-CD Hydrogel Kit, 1 ml	1 kit	1 ml	G91-1
Hydrogel components ³	3-D Life PEG-Link	200 μ l		L50-1
		3x 200 μ l		L50-3
	3-D Life CD-Link	200 μ l		L60-1
		3x 200 μ l		L60-3
	3-D Life Maleimide-PVA Set [†]	1 set		M80-3
	3-D Life Maleimide-Dextran Set	1 set		M90-3
Adhesion Peptides ⁴	3-D Life RGD Peptide	1 mg		09-P-001
		3x 1 mg		P10-3
	3-D Life Scrambled RGD Peptide	1 mg		09-P-003
		3x 1 mg		P11-3

¹ Sufficient for indicated volume of 3-D Life Hydrogel, when modified with 1 mmol/l adhesion factor (*e.g.* peptides) and crosslinked with 4 mmol/l thiol groups.

² Contains all reagents for formation of 3-D Life Hydrogels.

³ The 3-D Life Maleimide-PVA Set or the 3-D Life Maleimide-Dextran Set must be combined with either the 3-D Life PEG-Link and/or the 3-D Life CD-Link for formation of 3-D Life Hydrogels. 200 μ l of 3-D Life PEG-Link or 3-D Life CD-Link is sufficient for formation of 1 ml 3-D Life Hydrogel crosslinked by reacting 4 mmol/l maleimide groups with 4 mmol/l thiol groups. One set of 3-D Life Maleimide-PVA or 3-D Life Maleimide-Dextran contains sufficient reagent for formation of 3 ml 3-D Life Hydrogel crosslinked by reacting 4 mmol/l maleimide groups with 4 mmol/l thiol groups.

⁴ 1 mg peptide is sufficient for modification of 1 ml of 3-D Life Hydrogel at a final peptide concentration of 1 mmol/l.

[†]Not available in the USA

