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3-D Life Hydrogels

User Guide

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1 NOTICE TO PURCHASER

Products of the *3-D Life* Hydrogel system are covered by patents. Purchase of these products conveys a license for use for research. For further information please contact Cellendes GmbH. *3-D Life* products are for research use only.

2 THE TECHNOLOGY

2.1 *Three-dimensional cell culture*

Research over the past few decades 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 [1,2] and a difference in cell behaviour and differentiation [3-5]. As a consequence, 3-D cell culture is often the better choice for investigating cell function [6]. Moreover, 3-D cell culture models are being developed and increasingly used for drug discovery [7].

Hydrogels, consisting of synthetic and chemically defined components can be modified to mimic the characteristics of natural extracellular matrices [8]. 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 microenvironment.

2.2 *Three-dimensional biomimetic cell cultures with 3-D Life Hydrogels*

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 polymer functionalized with thiol-reactive groups and
- a thiol-containing crosslinker.

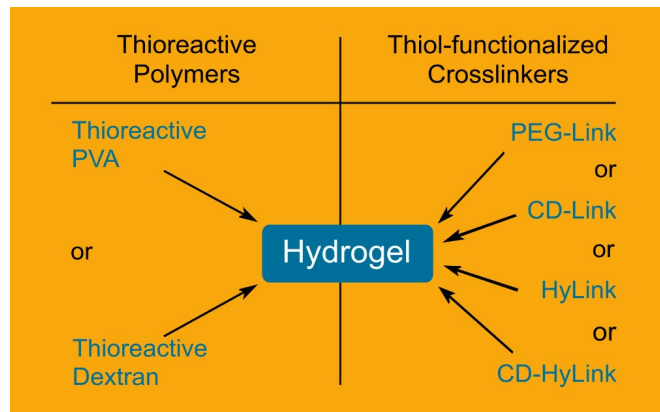
When the two components are mixed in the presence of cells, thiol groups form stable thioether bonds with thiol-reactive groups, which result in the formation of a gel containing embedded cells. Prior to the crosslinking step, peptides (containing *e.g.* cell adhesion motifs) may be covalently attached to a portion of the thiol-reactive groups of the polymer. The hydrogels can also be used to culture cells on top of the gel (see Chapter 4.1.4).

Either polyvinyl alcohol (PVA) or dextran are available as thiol-reactive polymers. 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 to form a hydrogel with either polyethylene glycol (PEG-Link), which is not degradable by cells or a polyethylene glycol peptide conjugate

(CD-Link), which contains a matrix-metalloprotease (MMP) cleavable peptide that allows cells to cleave the crosslinker and spread and migrate within the gel (for further details see also chapter 4.1.2, page 14). Both polymers can also be crosslinked with HyLink or CD-HyLink. HyLink and CD-HyLink are crosslinkers made of hyaluronic acid. While HyLink allows cell spreading and migration to some extent, CD-HyLink contains the MMP cleavage site and significantly enhances cell spreading and migration. Hydrogels crosslinked with HyLink and CD-HyLink are not degradable by dextranase. The thiol-reactive polymers and the thiol-containing crosslinkers can be mixed and matched to set up gels of defined properties (Fig. 1, see also the [Hydrogel Selection Guide](#)). The procedure for setting up biomimetic hydrogels containing embedded cells is very simple and does not require specific equipment or chemical expertise (Fig. 2).

Figure 1: Thiol-reactive polymers (functionalized PVA or dextran) can be combined with thiol-containing crosslinkers (either PEG-Link, CD-Link, HyLink or CD-HyLink) to set up gels with different properties.



PVA, dextran and PEG are water-soluble polymers; they do not bind to cells [9] and are known not to interfere with cellular functions. Thus, *3-D Life* gel 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. The versatility of the hydrogel system is the basis for the development of extracellular microenvironments resembling those of specific tissues.

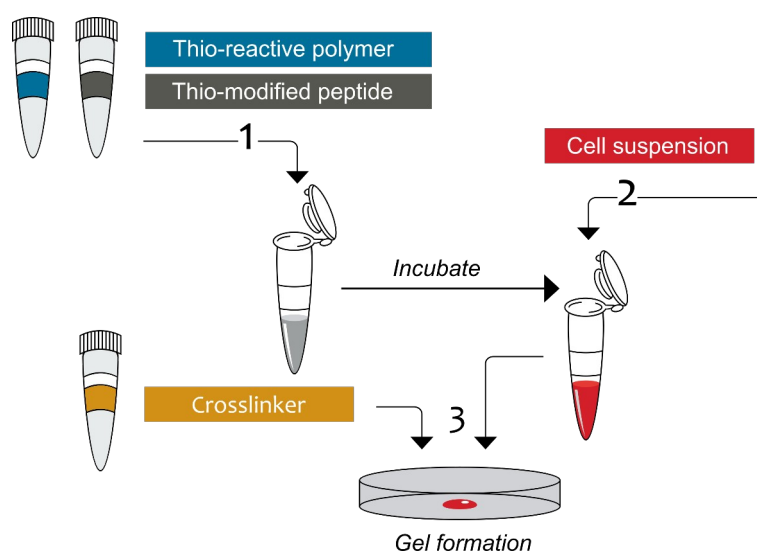


Figure 2: Schematic drawing of the three simple steps required to embed cells in a peptide-modified hydrogel. 1. Polymer, buffer, water and peptide are mixed and incubated to covalently attach the peptide to the thiol-reactive polymer. 2. A cell suspension is added to the peptide-polymer conjugate solution. 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, it is immersed in culture medium and the culture dish is placed in an incubator for cell cultivation (not shown).

2.2.1 Thiol-reactive polymers

Thiol-reactive polymers are PVA and dextran carrying thiol-reactive groups along their backbone. These thiol-reactive groups can, under cell-compatible reaction conditions, form covalent bonds with thiol groups. Depending on the type of the thiol-reactive group, the covalent bond formation proceeds at a fast or a slow rate.

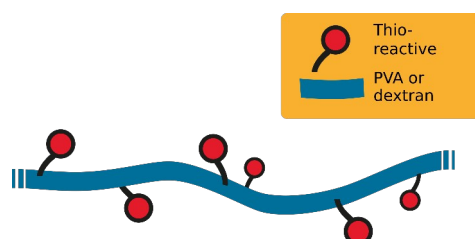
2.2.1.1 Fast-gelling polymers:

For fast gelling hydrogels PVA or dextran are functionalized with many thiol-reactive maleimide groups along their backbone (Fig. 3), sufficient for modification with bioactive factors and for crosslinking. Maleimides react specifically and very rapidly with thiol groups so that hydrogels can form within a few seconds.

2.2.1.2 Slow-gelling polymers:

For hydrogels with a slower gel formation rate, PVA or dextran are functionalized with thiol-reactive groups which react at a slower rate with thiol groups than maleimides, but with a similar reaction mechanism.

Figure 3: PVA or dextran functionalized with thiol-reactive groups.



2.2.2 Thiol-functionalized crosslinkers

PEG-Link consists of linear polyethylene glycol with a thiol (SH) group at each end (α,ω -bis-mercapto polyethylene glycol). CD-Link is derived from PEG-Link. It contains a peptide with a cleavage site for matrix metalloproteases (MMPs; Fig. 4). Crosslinkers made of hyaluronic acid carry several thiol groups along their molecular backbone. In addition to thiols, CD-HyLink contains the MMP cleavable peptide.

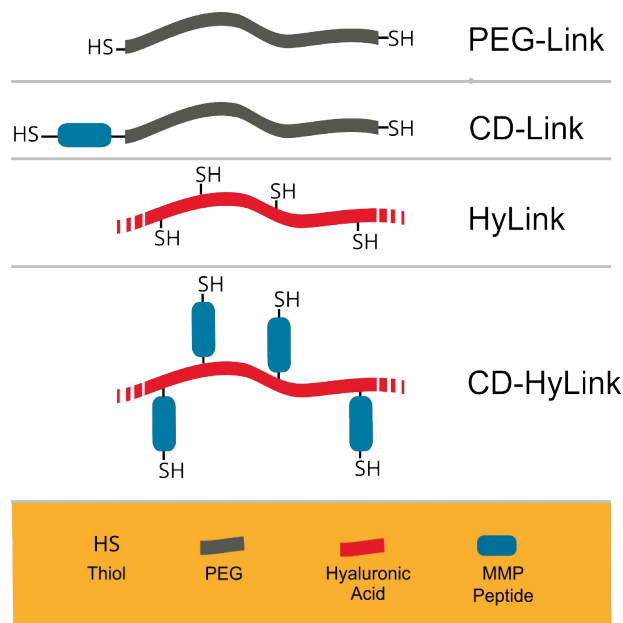


Figure 4: Schematic drawing of crosslinkers depicting their molecular structure.

2.2.3 Thiol-functionalized peptides

Cell adhesion peptides can be covalently attached to *3-D Life* polymers by their thiol groups. The RGD peptide carries a cysteine with a free thiol group at its N-terminal end. The GFOGER-3 peptide is designed to form a triple helix and carries thiol groups at each end of the triple helix. Apart from its function as cell adhesion peptide, GFOGER-3 also forms crosslinks between polymers. Peptides available from Cellendes have been tested for biological functionality in the *3-D Life* Hydrogel system.

2.3 The chemical reaction

2.3.1 Parameters affecting the gelation rate: pH and polymer concentration

In the *3-D Life* system thiol groups form stable thioether bonds with thiol-reactive groups under physiological conditions in a reaction called Michael Addition. The reaction usually proceeds at neutral pH. As the pH is lowered, the reaction slows down, since thiols react

as deprotonated thiolate anions with thiol-reactive groups. Thus, the reaction rate can be controlled by altering the pH. The reaction rate also increases with increasing concentrations of reactive groups and gelation starts earlier, which is important to consider when higher polymer concentrations are used for setting up stronger gels.

2.3.2 Cell compatibility

The reaction of thiol-reactive groups with thiol groups is very specific and the reaction proceeds in the presence of cells without any harm on their viability.

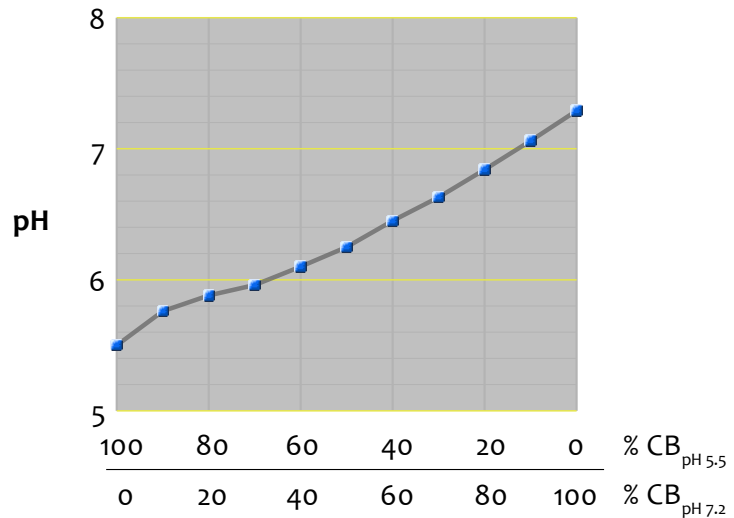
2.4 Molecular basis of gel formation

The thiol groups of the crosslinker molecules react with thiol-reactive groups of different polymer molecules. This leads to large polymer-crosslinker networks, which form a gel. Gel formation occurs best at equimolar concentrations of thiol and thiol-reactive groups. Higher equimolar concentrations of the reactive groups result in stronger gels which form faster.

2.5 The buffer system

Buffers for setting up hydrogels are available at a 10-fold concentration and two different pHs. At a one-fold concentration, the buffers provide an isotonic environment at pH 7.2 or pH 5.5, respectively. 10x CB (pH 7.2) is recommended for slow-gelling hydrogels to ensure an optimal environment for the cells during setup. For fast gelling hydrogels 10x CB (pH 5.5) is recommended, because fast gelation at a higher pH can prevent homogenous mixing of polymer and crosslinker before the gels begin to solidify. Most cells are not affected by a short exposure to pH 5.5.

Figure 5: pH as a result of different ratios of CB (pH 5.5) and CB (pH 7.2).



Alternatively, both buffer systems can be mixed at any ratio to generate intermediate pH values (Figure 5) to adjust gelation times of either gel system, fast or slow gelling. 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. A Phenol Red-free 10x CB (pH 7.2) is available (Cat. No. B21-3) to avoid interference of Phenol Red during light microscopy.

2.6 Covalent attachment of cell adhesion peptides

3-D Life peptides can be covalently attached to a portion of the thiol-reactive groups of a polymer. If comparative studies at different peptide concentrations are to be made, a corresponding amount of a cell neutral compound containing a thiol group, such as thioglycerol, should be added to ensure equal gel strengths (see Table 3 and 4).

We recommend final peptide concentrations in our General Protocols (GP) on www.cellendes.com. The ideal peptide concentration may be dependent on the type of peptide, cell and application. Peptide concentrations outside the recommended range may also be used.

2.7 Addition of extracellular matrix proteins to hydrogels

Soluble extracellular matrix proteins (e.g. fibronectin, laminin), recombinant ECM proteins or protein fragments can be added to the polymer solution prior to 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. The concentration of the extracellular matrix proteins may be varied. If the presence of extracellular matrix proteins decreases the hydrogel stiffness, an increase of crosslinking strength may be required.

2.8 Preventing cell sedimentation during hydrogel formation with AgaFloat

Hydrogels made with slow gelling polymers (SG) or made with the hyaluronic acid based crosslinker (HyLink) are slow in gelation, especially at low crosslinking strengths. During gelation, cells or other biological specimens can sediment before the gelation process is finished. This prevents an even distribution of cells in 3D. The addition of AgaFloat at 10% of the total gel volume prevents cells from sedimentation during gelation. Gelation speeds are not affected by AgaFloat, nor is cell spreading and migration affected in a noticeable way.

2.9 Hydrogel Calculation Tool

A [Hydrogel Calculation Tool](http://www.cellendes.com) on www.cellendes.com permits easy calculation of volumes of hydrogel components to obtain the hydrogel of your choice. It will guide you to avoid mistakes in calculations of concentrations and volumes.

3 TECHNICAL HINTS

3.1 Reconstitution of lyophilized 3-D Life components

Some 3-D Life product components are stabilized by lyophilization and must be dissolved before use. Carefully follow the procedure for reconstitution as described in the data sheets to make sure that the components dissolve properly.

3.2 Stability and storage of reagents

3.2.1 Mal-PVA, Mal-Dextran and SG-PVA

Maleimide groups are inactivated by hydrolysis in water at a very low rate. This reaction proceeds faster at higher temperatures. Therefore, maleimide-containing reagents should be kept on ice during use.

If stored at -20°C, PVA-based polymers may form insoluble aggregates within weeks to months. Therefore, Mal-PVA and SG-PVA should be stored at -80°C, where it is stable. If necessary, SG-PVA can be stored for up to one week at 4°C.

3.2.2 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.

3.2.3 PEG-Link, CD-Link, HyLink and CD-HyLink

Thiol groups can form inactive disulfides upon exposure to oxygen. As this reaction can be substantially slowed down at acidic pH, the crosslinkers are provided in a mildly acidic environment. Prolonged exposure of these crosslinkers to air should be avoided.

3.2.4 10x CB buffers

The buffers 10x CB (pH 7.2), 10x CB (pH 5.5) and 10x CB (pH 7.2) Phenol Red-free can be stored for up to 1 month at 4°C. 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 at room temperature or incubation at 37° for a few minutes.

3.2.5 Peptides

3-D Life peptides contain thiol groups for attachment to polymers. Thiol groups can form inactive disulfides upon exposure to oxygen. As this reaction can be substantially slowed down at acidic pH, the peptides are provided in a mildly acidic environment. Prolonged exposure of the peptides to air should be avoided.

4 APPLICATIONS

4.1 Types of cell cultures

In general, any cell type can be cultured in 3-D Life Hydrogels as long as the cellular microenvironment provides all factors necessary for cell survival or cell growth. The 3-D Life Hydrogel system is designed to provide full control over the composition of bioactive components in the extracellular microenvironment. Examples of cell cultures are described in Application Notes provided on our website www.cellendes.com. Numerous scientific publications report the use of the 3-D Life Hydrogel system in various types of

applications. [Selected publications](#) are listed on the Cellendes webpage. A general description of the properties of 3-D Life Hydrogels of various compositions is available through the [Hydrogel Selection Guide](#).

4.1.1 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. The requirement of a cell adhesion molecule for specific morphological phenotypes (e.g. epithelial cysts) needs to be considered. Due to a small average pore size of the gel (ca. 20 nm) cells do not spread or migrate in hydrogels crosslinked with PEG-Link.

4.1.2 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 appropriate matrix metalloproteases, the crosslinker can be cleaved in the vicinity of the cells. In the presence of cell adhesion factors, containing *e. g.* the RGD motif, cell spreading and migration can occur. CD-Link contains the peptide sequence Pro-Leu-Gly-Leu-Trp-Ala, which is known to be cleaved by matrix metalloproteases, such as MMP1, MMP2, MMP3, MMP7, MMP9 and MMP13 [10-12].

4.1.3 Cell culture in hydrogels crosslinked with HyLink or CD-HyLink

Cells can be embedded as single cells or cell aggregates in gels crosslinked with HyLink or CD-HyLink. Both crosslinkers are derived from hyaluronic acid. Hyaluronic acid is an important component in the extracellular matrix of many tissue types such as skin, cartilage, or connective tissue.

As a component of 3-D cell culture materials it allows cultivation of many different cell or tissue types such as organoids, fibroblasts, skin, cartilage, stem cells, endothelial cells, stromal cells, or cancer cells. Cells expressing hyaluronidase can digest HyLink and thus are able to migrate in the gel, even in the absence of a MMP-cleavable crosslinker. Cell spreading and migration is enhanced when CD-HyLink is used as it contains a MMP cleavable peptide.

Important Note: 3-D Life Hydrogels prepared with HyLink or CD-HyLink as crosslinker cannot be degraded by dextranase.

4.1.4 Cell culture on the surface of gels, sandwich cultures and stroma-epithelia co-cultures

Cells can be cultured on the surface of *3-D Life* Hydrogels. The gel must contain an adhesion factor, for example the *3-D Life* RGD Peptide or the collagen-derived triple-helical peptide *3-D Life* GFOGER-3. Cell attachment to gel surfaces is enhanced when HyLink or CD-HyLink is used as crosslinker.

The culture of cells on top of gels can be of advantage if a soft surface instead of the hard surface of a tissue culture plate is required. Cells can also be cultured between two layers of hydrogels to form a sandwich culture. Stroma-epithelia tissue models can be set up by growing stromal cells (e.g. fibroblasts) in the hydrogel and seeding epithelial cells on top of it.

4.1.5 Organ-on-Chip (OoC) cultures

3-D Life Hydrogels have been used in OoC cultures by applying the hydrogel in microchannels and cavities in chips [13, 14]. *3-D Life* Hydrogels demonstrated good stability without shrinkage and provided a robust 3D matrix for the cells while permitting cell migration.

4.2 Cell fixation and labeling

Chemical fixation (e.g. paraformaldehyde) 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, Life Technologies™], Ca²⁺ indicators etc.) can be performed with standard 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 minutes 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 possible with overnight incubations.

Alternatively, cells grown in *3-D Life* Dextran Hydrogels can be recovered by degrading the gel with dextranase (see Technical Protocol TP-1). 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.

Important Note: Hydrogels crosslinked with HyLink or CD-HyLink, cannot be dissolved by the addition of *3-D Life* Dextranase.

4.3 Choice of hydrogel

4.3.1 PVA- or dextran-based hydrogel

Dextran hydrogels can be dissolved by addition of dextranase (exceptions are dextran-based hydrogels crosslinked with HyLink or CD-HyLink). This allows the isolation of chemically fixed or live 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. Since dextranase degrades specifically the dextran polymer meshwork without protease activity, cells and extracellular matrix are left intact during gel dissolution. After long-term culture a disintegration of dextran gels has been observed.

In contrast, PVA hydrogels are stable against enzymatic digestion and suit many applications if the analysis of the cell culture does not require the isolation of cells from the gel. The gels are stable in culture medium over weeks. If required, PVA hydrogels, which are crosslinked with CD-Link, can be dissolved by trypsin, since the peptide sequence in CD-Link is trypsin-sensitive.

The properties of the dextran and PVA hydrogels are summarized in Table 1.

Table 1: Comparison of dextran and PVA hydrogels. +: possible; -: not possible.

	PVA Hydrogel	Dextran Hydrogel
Covalent attachment of peptides	+	+
Gel formation with stable (PEG-Link), cell-degradable (CD-Link) and hyaluronic acid-based crosslinkers (HyLink and CD-HyLink)	+***	+***
Cell labelling with low molecular weight stains	+	+
Degradable by user	-*	+**
Recovery of live or chemically fixed cells, passaging	-*	+**
Immunostaining	+	+
Stability in long-term culture	+	+/-

*only possible if gel contains CD-Link and is digested by trypsin.

** not possible with dextran hydrogels crosslinked with HyLink or CD-HyLink (based on hyaluronic acid).

*** not possible with Mal-PVA and Mal-Dextran due to a too fast gelation.

4.3.2 Fast or slow gelling hydrogel

Maleimide-modified polymers are used when a fast gel formation is required. For a manual cell culture setup a soft gel composition at pH 5.5 is preferred to slow down the otherwise too rapid reaction kinetics. Gels are prepared by placing the crosslinker (CD-Link or PEG-Link) on the surface of a culture dish, adding the maleimide-containing component to the crosslinker and mixing the two solutions by rapidly pipetting up and down three times. The gel forms within seconds or minutes, depending on the concentration of the polymers and reactive groups. If an extremely fast gel formation is required (e.g. robotic bioprinting) the gels can be set up at pH 7.2, where the gel forms almost instantaneously upon mixing maleimide-polymer with crosslinker.

In contrast, slow gelling components allow more time for the placement of the pre-gel solution in a culture dish. Depending on the composition and concentration of the gel components the fluid condition after mixing SH-reactive polymer with crosslinker can last several minutes before the beginning of gelation is noticeable. Gelation times are listed in the General Protocol „Preparation of 3-Life Slow-Gelling (SG) Hydrogels“ (GP-2).

Slow gelling gels are used

- when more time is required for placing the hydrogels (for example filling microchannels or syringes),
- when gels of higher stiffness are required,
- when cells need to be above pH 7.0 at all times.

4.3.3 Cultures using a combination of PVA- and dextran-based hydrogels

Using the two types of gels placed next to each other, it is possible to construct co-cultures that can be sub-sequently taken apart by dissolving the dextran hydrogel* and leaving the PVA gel intact. This may be convenient for the separate analysis of two cell populations after co-culture.

***Important Note:** Hydrogels crosslinked with HyLink or CD-HyLink, cannot be dissolved by the addition of 3-D Life Dextranase.

4.4 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, or laser scanning confocal microscopy.

5 COMPOSITION OF REAGENTS

All specifications below apply to solutions supplied in the products or after reconstitution of lyophilisates as indicated in the Product Data Sheet.

Mal-PVA:	30 mmol/L maleimide groups on polyvinyl alcohol, 5 mmol/L phosphate buffer
Mal-Dextran:	30 mmol/L ¹ maleimide groups on dextran, 5 mmol/L phosphate buffer
SG-PVA:	30 mmol/L thiol-reactive groups on polyvinyl alcohol, 5 mmol/L phosphate buffer
SG-Dextran:	30 mmol/L thiol-reactive groups on dextran, 5 mmol/L phosphate buffer
PEG-Link:	20 mmol/L ¹ thiol groups on polyethylene glycol (PEG)
CD-Link:	20 mmol/L ¹ thiol groups on a PEG-peptide conjugate
HyLink:	10 mmol/L ¹ thiol groups attached to hyaluronic acid
CD-HyLink:	10 mmol/L ¹ thiol groups attached to peptide-modified hyaluronic acid
Dextranase:	Aqueous solution of dextranase from <i>Chaetomium gracile</i>
Thioglycerol:	20 mmol/L 1-thioglycerol; 2 mmol/L HCl (20 mmol/L thiol groups)
10x 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
10x CB (pH 7.2)	

Phenol Red-free:	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 ; pH-adjusted with NaOH
10x 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 culture grade)
RGD Peptide:	Peptide with attached terminal cysteine (20 mmol/L ¹ thiol groups)
Scrambled RGD:	Peptide with attached terminal cysteine (20 mmol/L ¹ thiol groups)
GFOGER-3:	Peptide with attached terminal cysteines. The peptides form triple helices. (20 mg/mL ¹ ; 1.2 mg/mL correspond to approximately 0.5 mmol/L thiol groups)
AgaFloat:	Modified Agarose in water

¹: Concentration after reconstitution of lyophilized material

6 CALCULATION OF REAGENT VOLUMES FOR HYDROGEL SETUP

Reagent volumes for each composition of gel have to be calculated to ensure proper gel formation.

6.1 General guidelines

1. As a general rule, thiol-reactive groups and thiol groups should always be kept at equimolar ratios.

For forming stable hydrogels the concentration of reactive groups of polymer (SH-reactive groups) should match the concentration of the SH-groups of the crosslinker. If peptides are included in the gel, the concentration of reactive groups of polymer should match the sum of the SH-groups of crosslinker and peptide.

2. The concentration of the reactive groups should be at least 1.8 mmol/L for gels formed with PEG- and CD-Link. Gel formation with HyLink and CD-HyLink starts at 1 mmol/L reactive groups.
3. If cells or other specimens are to be embedded in the gel, they are prepared in physiological salt solution (e.g. PBS) or medium. Since these solutions contain physiological salt concentrations, the volume of 10x CB has to be calculated as one tenth of the gel solution remaining after subtracting the volume of cell suspension from the total gel volume.

- The volume of water is calculated by subtracting all volumes of hydrogel components (including the cell suspension) from the final volume of the gel.

Alternatively, for calculation of reagent volumes for gel preparation the online [Calculation Tool](#) is available on our website www.cellendes.com.

The experimental procedure of setting up cultures with all *3-D Life* products is described in detail in the [General Protocols \(GP\)](#) on www.cellendes.com.

6.2 Preparing basic gels of different stiffness

It is recommended to use the slow gelling (SG) series of hydrogels to set up gels of medium and higher stiffnesses. The slower reaction kinetics of these gels allows for more time to place gels with higher concentrations of polymer and crosslinker in the respective culture vessel. Gel stiffness increases with increasing concentrations of thiol-reactive polymers and corresponding concentrations of crosslinker. Table 2 depicts examples for the preparation of 3-D cultures at different gel stiffness.

Table 2: Reagent volumes for the setup of gels at different stiffness (gel volume: 30 μ l).

Gel stiffness 						
Final concentration of thiol-reactive groups and thiol groups (mmol/L)	2.0	3.0	4.0	5.0	7.0	9.0
Reagent	Volumes in μ l					
Water	17.5	15.0	12.5	10.0	5.0	-
10x CB ¹	2.5	2.5	2.5	2.5	2.5	2.5
Thiol-reactive polymer (30 mmol/L thiol-reactive groups)	2.0	3.0	4.0	5.0	7.0	9.0
Cell suspension	5.0	5.0	5.0	5.0	5.0	5.0
PEG-Link or CD-Link (20 mmol/L thiol groups)	3.0	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 10x CB is calculated for the total gel volume minus the volume of the cell suspension to ensure iso-osmotic conditions. Use 10xCB (pH 5.5) for fast gelling gels and 10xCB (pH 7.2) for slow gelling gels.

6.3 Preparing gels with peptide modifications

6.3.1 Hydrogels with RGD Peptide modification

A final peptide concentration of 0.5 mmol/L or less is usually sufficient to provide enough attachment sites for cells. Table 3 illustrates the ratios of the final concentrations of reactive groups of peptide and thioglycerol (see Chapter 2.6) in gels with a constant crosslinking strength. In this example, a total of 2.5 mmol/L polymer-bound thiol-reactive groups are used:

- 0.5 mmol/L thiol-reactive groups are used to provide sufficient attachment sites for peptide **and** thioglycerol modification;
- 2 mmol/L thiol-reactive groups are used for crosslinking with 2 mmol/L thiols from PEG- or CD-Link) to provide a constant gel stiffness.

Table 3: 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	0.3	0.5
Thiol groups of thioglycerol	0.5	0.4	0.2	0.0
Thiol groups of PEG- or CD-Link	2.0	2.0	2.0	2.0
Thiol-reactive groups of polymers	2.5	2.5	2.5	2.5

The corresponding reagent volumes for setting up these gels in volumes of 30 μ L are shown in Table 4.

Table 4: Reagent volumes for the generation of gels (crosslinking strength: 2 mmol/L) with different RGD peptide concentrations.

Final peptide concentration (mmol/L):	0.0	0.1	0.2	0.5
Volumes (μ L)				
Water	12.0	12.0	12.0	12.0
10x CB ¹	2.5	2.5	2.5	2.5
Thiol-reactive polymer (30 mmol/L thiol-reactive groups)	2.5	2.5	2.5	2.5

SH-peptide (3.0 mmol/L) ²	0.0	1.0	2.0	5.0
Thioglycerol (3.0 mmol/L) ²	5.0	4.0	3.0	0.0
Cell suspension	5.0	5.0	5.0	5.0
PEG-Link or CD-Link (20 mmol/L SH-groups)	3.0	3.0	3.0	3.0
Total	30.0	30.0	30.0	30.0

¹ Volume of 10 x CB is calculated for the total volume minus the volume of the cell suspension to ensure iso-osmotic conditions.

² SH-peptide and Thioglycerol stock solutions (20 mmol/L) have been diluted to 3 mmol/L to increase the pipetting volumes.

6.3.2 Hydrogels with GFOGER-3 Peptide modification

The GFOGER-3 Peptide is modified with SH-groups at each end which bind to SH-reactive groups of 3-D Life polymers (e.g. SG-Dextran or SG-PVA). GFOGER-3 spontaneously forms triple helices similar to those found in collagen fibers [15-17]. As a result, each triple helix contains several thiol-groups at each end. A concentration of 1.2 to 2.4 mg/mL of GFOGER-3 Peptide is sufficient to mediate its biological effect on cells. The average thiol group concentration of 1.2 mg/mL peptide solution is approximately 0.5 mmol/L. If higher concentrations of GFOGER-3 peptide are used, be aware of its crosslinking properties. It is recommended to test the gelation before setting up cell cultures. At higher GFOGER-3 concentrations, the crosslinking may interfere with cell spreading and migration because GFOGER-3 does not contain a MMP (matrix metalloprotease) cleavage site.

For the calculation of hydrogel compositions, for example in combination with RGD peptide or with HyLink and CD-HyLink, please consult the [Hydrogel Calculation Tool](https://www.cellendes.com) on www.cellendes.com. For a detailed guidance to set up hydrogels with GFOGER-3 Peptide please consult protocols GP2 and GP4.

6.4 Dissolving 3-D Life Dextran Hydrogels

Except for dextran-based hydrogels crosslinked with HyLink or CD-HyLink, dextran hydrogels containing intact cells can be dissolved by adding dextranase to the culture medium of the 3-D hydrogel culture. If needed, cells can be chemically fixed (e.g. with 4% formaldehyde in PBS) prior to the dextranase treatment.

For example, a 30 μ l gel can be dissolved by adding 300 μ l of a 1:20 dilution of dextranase in medium and incubation for 30-60 minutes at 37°C. Gels can be dissolved

faster, if they are cut in pieces. For a detailed protocol, please see www.cellendes.com (Technical Protocol 1).

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8 TROUBLESHOOTING

Observation	Possible causes	Possible remedies
No or weak gel formation.	Thiol-reactive groups and thiol groups used for crosslinking are not near equimolar concentrations.	Check the balance of thiol-reactive groups and thiol groups of your components.
	Too much peptide or thioglycerol used, resulting in too few thiol-reactive groups for crosslinking.	Check the calculations for gel setup.
	Concentration of reactive groups for crosslinking are too low to form a polymer network (FG and SG Hydrogels: below 1.8 mmol/L. HA-Hydrogels: below 1 mmol/L).	Increase the concentration of both types of reactive groups for crosslinking to >1.8 mmol/L each or >1 mmol/L, respectively.
	Reaction pH too low, caused by acid in peptide sample.	Check the pH of the sample with pH-paper; when slow gelling system used: try to increase the portion of CB buffer by reducing the portion of cell suspension or equivalent. To maintain concentration of cells or equivalent increase their concentration in the stock solution accordingly. When fast gelling system used: (partially) replace CB (pH 5.5) with CB (pH 7.2).
	Slow gelling gel system used at too low pH.	Use 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.	When fast gelling system used: pH is too high.	Use lower pH (CB (pH 5.5)) for performing the crosslinking reaction or use slow gelling gel system.
Gel forms too slowly.	When slow gelling gel system is used: pH is too low.	Use CB (pH 7.2).

Viability of cells decreases rapidly.	Cell type requires presence of an adhesion factor or of a different adhesion factor.	Add appropriate cell adhesion peptides or extracellular adhesion proteins.
	When low pH is used: Cells may be sensitive to low pH during gel setup.	Use neutral pH during gel setup, preferably with the slow gelling system.
	Cells require other biomimetic modifications in the gel.	Investigate which biomimetic factors may be needed and add to gel if possible.
Dissolution of dextran-based hydrogel is not complete after incubation of 1 hr at 37°C with Dextranase.	High gel strength.	Incubate up to 2 hr; add more volume of dextranase solution; mechanically disrupt gel prior to digestion.
	Dextranase activity reduced due to long storage at 4°C or higher.	Use new aliquot of Dextranase and adhere to storage conditions indicated in the Product Data Sheet.
	The dextran-based hydrogel was crosslinked with HyLink or CD-HyLink.	No remedy available at the moment. The hydrogel may be degradable by adding a hyaluronidase.
Thiol-reactive polymer solution becomes turbid after addition of custom peptide.	Some peptides may cause precipitation of Mal-PVA after covalent attachment.	Try to use a lower peptide concentration.
Cells sediment before gelation is finished. Uneven three-dimensional distribution of cells in the gel.	In slow gelling hydrogels or hydrogels crosslinked with HyLink at low crosslinking strengths cells may sediment before the hydrogel is solidified.	Add AgaFloat to the pregel solution. AgaFloat keeps cells evenly distributed in the gel during the gelation process.

9 PRODUCTS

See www.cellendes.com for a full list of 3-D Life Hydrogel products.

