Preparation of *3-D Life* Slow Gelling (SG) Hydrogels

1. Introductory Notes

- The *3-D Life* Hydrogel System is a complete set of reagents for the design of extracellular microenvironments of three-dimensional cell cultures and related applications. Ease of use and complete control of biomolecular modifications and gel stiffness allow a great variety of cell culture applications.
- The polymers SG-Dextran and SG-PVA are used for the generation of hydrogels at a slow gelation rate (SG). Incubation times for the attachment of adhesion peptides takes 20 minutes and gel formation takes between 15 and 70 minutes depending on the gel composition and stiffness (compare Table 2 and 3).
- Slow gelling hydrogels are used when the placement of hydrogel requires some handling time, for example the filling of microchannels or syringes. In addition, slow gelling hydrogels are used for the preparation of very stiff gels or when cells must be suspended in a medium with a pH above 7 at all times.
- The *3-D Life* Hydrogel technology and its applications are described in detail in the *3-D Life* Hydrogels User Guide which can be downloaded at www.cellendes.com. For first time users it is recommended to read the User Guide carefully before setting up gels.

2. Protocol

The following protocol describes the preparation of *3-D Life* SG Hydrogels for 3-D cell culture with and without modification with the cell adhesion peptide *3-D Life* RGD Peptide.

Reagents and materials

***3-D Life* products:**

3-D Life Dextran-CD Hydrogel SG (Catalog Number G93-1)

or *3-D Life* Dextran-PEG Hydrogel SG (Catalog Number G92-1)

or *3-D Life* PVA-CD Hydrogel SG (Catalog Number G83-1)

or *3-D Life* PVA-PEG Hydrogel SG (Catalog Number G82-1)

Optional: *3-D Life* RGD Peptide (Catalog Number 09-P-001)

Related products: *3-D Life* Dextranase (Catalog Number D10-1)

Reagents and materials not included in the *3-D Life* products:

Cell culture medium, cell culture plate, reaction tubes, pipet tips, micropipets, serological pipets, cell suspension

Preparations

Hydrogel reagents:

- If hydrogel reagents are provided in lyophilized form, dissolve the lyophilisates according to the instructions in the accompanying Product Data Sheets.
- If hydrogel reagents are frozen, thaw all reagents at room temperature. Make sure that salts in the 10x CB are completely dissolved. Do not put 10x CB on ice, this may cause the salts to crystallize.

Note: Do not expose *3-D Life* thiol-containing reagents (RGD Peptide, CD-Link, PEG-Link) to air and room temperature longer than necessary to avoid oxidation of the thiol-groups. Close cap after each use.

Cell suspension:

Prepare a stock cell suspension or any other biological sample of your choice in culture medium, PBS or in any other physiological solution. When preparing this sample, consider that the volume of this sample will be only 1/5 of the final gel volume. Accordingly, the cell concentration in the gel will be only 1/5 of the stock cell suspension.

Experimental procedure

The following protocol describes the preparation of a soft hydrogel (crosslinking strength of 2 mMol/L) with the option of modification with 0.5 mMol/L RGD Peptide. The volumes of gel reagents required for a 30 µl gel are listed in Table 1.

Note: It is advisable to calculate reagents with some excess volume (e.g. one additional gel volume) to avoid shortage of pre-gel solution due to pipetting inaccuracies.

Table 1: Reagent volumes for a 30 µl gel using SG-Dextran or SG-PVA polymer to be crosslinked with 2 mMol/L SH groups of the crosslinker CD-Link or PEG-Link (2 mMol/L crosslinking strength) with the option of modification with 0.5 mMol/L RGD Peptide.

Reagents	Final concentrations in the gel	Volumes for a 30 µl gel (µl)	
		w/o peptide	with peptide
Water	n.a.	16.6	15.3
10x CB, pH 7.2	n.a.	2.4	2.4
SG-Dextran or SG-PVA (30 mMol/L SH-reactive groups)	2.0 mMol/L (w/o peptide) or 2.5 mMol/L (with peptide) SH-reactive groups	2.0	2.5
RGD Peptide (20 mMol/L SH groups)	0.5 mMol/L SH groups	-	0.8
Cell suspension	user's choice	6.0	6.0
PEG-Link or CD-Link (20 mMol/L SH groups)	2 mMol/L SH groups	3.0	3.0
Total		30.0	30.0

If not indicated otherwise, all steps below are performed in a sterile hood at room temperature:

1. Combine Water, 10x CB (pH 7.2) and the SG-Polymer of your choice (SG-Dextran or SG-PVA) in a reaction tube. Mix well.
2. If RGD Peptide is used (otherwise continue with step 3.):

   Add the RGD Peptide and mix immediately to ensure homogenous modification of the SG-Polymer with the peptide. Incubate sample for 20 min to allow the RGD Peptide to attach to the SG-Polymer.
3. Add the cell suspension.
4. Add the crosslinker (CD-Link or PEG-Link). Mix by pipetting up and down a few times.

   When crosslinking with CD-Link: Incubate 5 minutes at room temperature. Do not incubate the pre-gel solution longer than 5 minutes because the solution will begin to solidify and will not be pipettable anymore. Resuspend cells by pipetting up and down a few times and transfer mix in a culture dish. Incubate the mix for 30 minutes at room temperature or at 37°C in the incubator to allow gel formation.

   When crosslinking with PEG-Link: Crosslinking with PEG-Link takes longer than with CD-Link. Incubate the pre-gel solution for 25 minutes at room temperature. Do not incubate longer than 25 minutes because the solution will begin to solidify and will not be pipettable anymore. Resuspend cells by pipetting up and down a few times to ensure that cells will be uniformly distributed later in the gel and transfer the pre-gel solution in a culture dish. Incubate for 50 minutes (SG-Dextran gels) or 70 minutes (SG-PVA gels) at room temperature or at 37°C in the incubator to allow gel formation.

   Note: Make sure that the gel has completely formed before adding culture medium in step 5. Optional: Test gel formation by careful inspection with a pipet tip. The tip should not pull out threads of gel when touching and retracting from the gel surface.
5. Once the gel has formed, add cell culture medium until the gel is covered.
6. Place culture dish in the incubator for cultivation of cells.
7. Renew medium after 1 hour.
8. Change the medium as needed during cultivation of cells.

Variations of gel preparation

Reagent volumes for gel variations described below can easily be calculated using the

online calculation tool on www.cellendes.com/calculation_form.php.

Preparation of small gel volumes:
If small volumes of gel are prepared (less than 100 µl) only very small volumes of the RGD Peptide stock solution are required. To avoid the pipetting of such small volumes, it is recommended to reduce the concentration of the RGD Peptide stock solution (e.g. 3 mMol/L) by dilution with water to increase the volume to be pipetted. In this case the volume of the component "Water" has to be adjusted accordingly.

Preparation of multiple gels of same composition:
If several gels with the same composition are generated, corresponding multiples of the indicated reagent volumes are used. To generate multiple gels, adequate volumes of aliquots of the pre-gel solution are then placed in the culture dishes. It is recommended to resuspend cells in the pre-gel

solution each time before an aliquot is pipetted to ensure an equal number of cells in each gel.

Preparation of gels of larger volumes:
If larger volumes of gel are prepared, reagents and cell suspension are prepared in multiples of the indicated volumes or adapted proportionally in volume.

Preparation of gels with different concentrations of RGD Peptide:
If gels of different concentrations of adhesion peptide are to be prepared, please consult the User Guide or the online calculator for calculating volumes of reagents.

Preparation of plain gels (without cells) or embedding other specimens:
If no cells are included in the gel, e.g. for encapsulation of tissues or preparation of plain gels, replace the volume of cell suspension with PBS or other physiologically compatible solution of your choice. Alternatively, use the online calculator and keep the component „cell suspension“ blank or enter "0".

RGD Peptide replacements for control experiments:
Instead of the RGD-Peptide, Thioglycerol can be added to a gel. In this case the gel does not provide cell attachment sites and can be used as a control to RGD Peptide-modified gels. Cellendes also offers a scrambled version of RGD Peptide for control experiments (*3-D Life* Scrambled Peptide, Catalog Number 09-P-003).

Preparation of gels of different stiffness:
Gels of higher stiffness than the gels described in Table 1 can be made by increasing the concentrations of the SG-Polymer (SG-Dextran or SG-PVA) and crosslinker (CD-Link or PEG-Link). For calculating reagent volumes, please consult the User Guide or the online calculator.

With increasing gel stiffness, however, the time between addition of crosslinker and beginning of solidification of the gel solution becomes considerably shorter than indicated in the protocol above. A resuspension of cells may not be necessary and the gel mix may be transferred immediately to the incubator after the addition of crosslinker. In Table 2 and 3 time periods of fluid state as well as the time points after which gels are solid enough for the addition of medium after mixing SG-Polymer and crosslinker are indicated for gels with crosslinking strengths of 2 to 4 mMol/L. Gels of up to 10 mMol/L crosslinking strength can be generated with increasingly shorter gelation times.

Table 2: Gelation times of SG-Dextran hydrogels at different grades of stiffness (crosslinking strength) at room temperature.

Crosslinking strength	Time after mixing polymer and crosslinker			
	SG-Dextran + CD-Link		SG-Dextran + PEG-Link	
	Fluid up to	Solid gel after	Fluid up to	Solid gel after
2.0 mMol/L	6.0 min	30 min	25 min	50 min
2.5 mMol/L	5.0 min	25 min	15 min	40 min
3.0 mMol/L	3.5 min	20 min	13 min	30 min
3.5 mMol/L	3.0 min	18 min	10 min	25 min
4.0 mMol/L	2.5 min	15 min	8 min	20 min

Table 3: Gelation times of SG-PVA hydrogels at different grades of stiffness (crosslinking strength) at room temperature.

Crosslinking strength	Time after mixing polymer and crosslinker			
	SG-PVA + CD-Link		SG-PVA + PEG-Link	
	Fluid up to	Solid gel after	Fluid up to	Solid gel after
2.0 mMol/L	4.0 min	30 min	25 min	70 min
2.5 mMol/L	3.5 min	25 min	18 min	60 min
3.0 mMol/L	2.5 min	20 min	10 min	40 min
3.5 mMol/L	2.0 min	10 min	8 min	30 min
4.0 mMol/L	1.5 min	8 min	6 min	25 min

Dissolving SG-Dextran Hydrogels with Dextranase

SG-Dextran Hydrogels containing live or chemically fixed cells can be dissolved by adding dextranase to the culture medium or buffer. For example, a 30 µl gel can be dissolved with 300 µl of a 1:20 dilution of dextranase in medium incubated for 30-60 minutes at 37°C. Gels can be dissolved faster, if they are cut in pieces.

After dissolution of the gel, centrifuge the cell suspension and resuspend the pelleted cells in fresh medium or physiological buffer as required. Repeat this washing procedure once or twice to more effectively remove remains of dextranase and dissolved gel components. The removal of dextranase is important when cells are being embedded again in dextran hydrogels to continue culture. If dextranase is not removed completely, it can destabilize the newly set up hydrogel.