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

1. Introductory Notes

- 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.
- The *3-D Life* Hydrogel System is a complete set of reagents for the design of extracellular microenvironments of three-dimensional cell cultures. 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 medium to slow gelation rate (SG). For durations of gel solidifications see Table 2 and 3.
- Compared to fast gelling gels (compare General Protocol1), slow gelling gels allow for more time to handle and place the pre-gel solution in culture dishes or other containers (e.g. microchannels, syringes). Slow gelling hydrogels are used when cells must be kept a pH above 7 at all times. Slow gelling hydrogels are also preferred over fast gelling gels when gels of a higher stiffness are needed.

2. Protocol

The following protocol describes the preparation of soft *3-D Life* SG Hydrogels for 3-D cell culture with and without modification with the cell adhesion peptide *3-D Life* RGD Peptide. Please read the full protocol before you start preparing a gel.

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)

3-D Life 10x CB pH 5.5 (Catalog Number B10-3)

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

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

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 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 density 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 100 µl of gel are listed in Table 1[1].

Table 1: Reagent volumes for 100 µl of 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	Volumes for 100 µl gel (µl)	
	w/o peptide	with peptide
Water	55.3	51.2
10x CB, pH 7.2	8	8
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	6.7	8.3
RGD Peptide (20 mmol/L SH groups)	-	2.5
Cell suspension	20	20
PEG-Link or CD-Link (20 mmol/L SH groups)	10	10
Total	100	100

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):

[1] It is advisable to prepare gel solutions with some excess volume to avoid a shortage of gel due to pipetting inaccuracies.

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: After addition of the crosslinker make sure to place the gel at its final location for culture within one minute. After that time, the solution will begin to solidify and will not be pipettable anymore. Incubate the mix for 10 minutes at room temperature or at 37°C in the incubator to allow the gel to solidify.

   When crosslinking with PEG-Link: Crosslinking with PEG-Link takes longer than with CD-Link. After addition of the crosslinker incubate the pre-gel solution for up to 16 minutes at room temperature. Do not incubate longer because the solution will begin to solidify and will not be pipettable anymore. Before you transfer the pre-gel solution in a culture dish resuspend cells to ensure that cells will be uniformly distributed later in the gel. Transfer the pre-gel solution in a culture dish. Incubate for 30 minutes at room temperature or at 37°C in the incubator to allow the gel to solidify.
5. Make sure that the gel has completely formed before adding culture medium in step 6. Optional: test gel formation by carefully touching the gel surface with a pipet tip. The tip should not pull out threads of gel when retracting from the gel surface.
6. Once the gel has solidified, carefully add cell culture medium until the gel is covered.
7. Place culture dish in the incubator for cultivation of cells.
8. Renew medium after 1 hour.
9. Change the medium as needed during cultivation of cells.

3. Variations of Gel Preparations

Reagent volumes for gel variations described below can easily be calculated using the online calculation tool on www.cellendes.com.

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 to 3 mmol/L by dilution with water. This increases the volume to be pipetted. To achieve the correct final gel volume, the volume of the component "Water" has to be reduced accordingly.

Preparation of multiple gels of same composition
To generate multiple gels of same composition, aliquots of the pre-gel solution are placed in the culture dishes. It is recommended to resuspend cells in the pre-gel solution each time before an aliquot is pipetted to obtain an equal number of cells in each gel.

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 (Catalog Number T10-3) 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 calculation tool on www.cellendes.com.

With increasing gel stiffness the time between addition of crosslinker and the beginning of solidification of the gel solution becomes considerably shorter than indicated in the protocol above. In Table 2 and 3 time periods of the fluid state as well as the time points after which gels are solid enough for the addition of medium are indicated for gels with crosslinking strengths of 2 to 4 mmol/L.

Gels of up to 9 mmol/L crosslinking strength can be generated with increasingly shorter gelation times.

IMPORTANT: The times given in table 2 and 3 provide a rough guideline only. It is recommended to perform a test run of gel preparation without cells to confirm times of fluid state and time for the gel to solidify before you start your experiment.

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	Addition of medium after	Fluid up to	Addition of medium after
2 mmol/L	3 min	10 min	20 min	30 min
3 mmol/L	1 min	10 min	10 min	20 min
4 mmol/L	0.5 min	10 min	7 min	15 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	Addition of medium after	Fluid up to	Addition of medium after
2 mmol/L	1 min	10 min	16 min	30 min
3 mmol/L	0.5 min: transfer gel immediately to its final location	7 min	10 min	20 min
4 mmol/L	transfer gel immediately to its final location	5 min	3 min	15 min

Slowing down gelation by pH reduction

The time the pre-gel solution remains fluid and pipettable can be slowed down by reducing the pH. Normally the preparation of slow gelling gels is performed at pH 7.2. If a slower gelation is required, for example when gels of higher stiffnes are generated, the gelation time can be slowed down by the addition of 10x CB of a lower pH. 10x CB of lower pH can be generated by mixing 10x CB pH 7.2 with 10x CB pH 5.5 (Catalog Number B10-3) (Fig. 1).

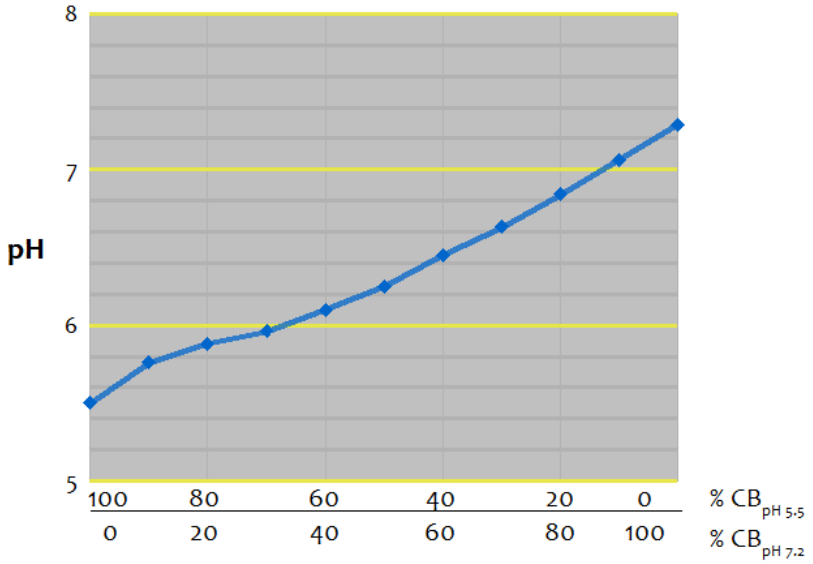

Fig. 1: pH resulting from different mixing ratios of 10x CB $_{pH5.5}$ and 10x CB $_{pH 7.2}$.

4. Dissolving SG-Dextran Hydrogels with Dextranase

Live or chemically fixed cells can be recovered from SG-Dextran Hydrogels by the enzymatic digestion of the gels with dextranase (Catalog Number D10-1). Dextranse is added to the culture medium or buffer at a 1:20 dilution. For example, a 30 µl gel can be dissolved by adding 300 µl of a 1:20 dilution of dextranase in medium followed by an incubation of 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.