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Preparation of *3-D Life* Slow Gelling (SG) Hydrogels with optional peptide modifications

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 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 Tables 2 and 3.
- Compared to fast gelling gels (compare General Protocol 1), 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 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 or without modification with the cell adhesion peptides RGD and GFOGER-3. Please read the entire protocol before you start preparing a gel.

Reagents and materials

Hydrogel Kits	Catalog Number
3-D Life Dextran-CD Hydrogel SG	G93-1
3-D Life Dextran-PEG Hydrogel SG	G92-1
3-D Life PVA-CD Hydrogel SG	G83-1
3-D Life PVA-PEG Hydrogel SG	G82-1
Peptides	
3-D Life RGD Peptide	09-P-001 or P10-3
3-D Life GFOGER-3 Peptide	P12-1 or P12-3
Related Products	
3-D Life Dextranase	D10-1
3-D Life 10x CB pH 5.5	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.

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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, GFOGER-3 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.

Biological sample:
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 no more than 1/5 of the final gel volume. If you chose to use another volume of biological sample, the online calculation tool (www.cellendes.com) is assisting you to find the right volumes of all reagents applied.

Experimental procedure

The following protocol describes the preparation of a soft hydrogel with the option of modification with RGD or GFOGER-3 Peptide, or both peptides in one gel. For most applications a crosslinking strength of 2.0 mmol/L and a RGD Peptide concentration of 0.5 mmol/L and 1.2 mg/ml GFOGER-3 Peptide are sufficient. The volumes of gel reagents required for 100 µl of gel are listed in Table 1a. If a crosslinking strength of 2.0 mmol/L turns out to be too soft for your application, gel compositions for slightly stronger gels of 2.2 mmol/L crosslinking strength are given in Table 1b.

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 Peptides are used (otherwise continue with step 3):

   Add the peptide(s) and mix immediately to ensure homogenous modification of the SG-Polymer with the peptides. Incubate sample for 20 min to allow the peptides 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 between three and seven minutes (compare Table 3 and 4). After that time, the solution will begin to solidify and will not be pipettable anymore. Incubate the mix for 25 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 between 20 and 70 minutes (compare Table 3 and 4) at room temperature. Do not incubate longer because the solution will begin to solidify and cannot be pipetted 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 the culture dish in the incubator for cultivation of cells.
8. Renew medium after 1 hour to equilibrate the gel with culture medium.
9. Change the medium as needed during cultivation of cells.

Table 1a: 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 modifications with 0.5 mmol/L RGD and/or 1.2 mg/ml GFOGER-3 Peptide.

Reagents	Volumes for 100 µl gel (µl)			
	w/o peptide	with RGD Peptide	with GFOGER-3 Peptide	with RGD and GFOGER-3 Peptide
Water	55.3	51.2	47.7	43.5
10x CB, pH 7.2	8	8	8	8
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	6.7	8.3	8.3	10
RGD Peptide (20 mmol/L SH groups)	-	2.5	-	2.5
GFOGER Peptide (20 mg/mL)	-	-	6	6
Cell suspension	20	20	20	20
PEG-Link or CD-Link (20 mmol/L SH groups)	10	10	10	10
Total	100	100	100	100

Table 1b: Reagent volumes for 100 µl of gel using SG-Dextran or SG-PVA polymer to be crosslinked with 2.2 mmol/L SH groups of the crosslinker CD-Link or PEG-Link (2.2 mmol/L crosslinking strength) with the option of modifications with 0.5 mmol/L RGD and/or 1.2 mg/ml GFOGER-3 Peptide.

Reagents	Volumes for 100 µl gel (µl)			
	w/o peptide	with RGD Peptide	with GFOGER-3 Peptide	with RGD and GFOGER-3 Peptide
Water	53.7	49.5	46	41.8
10x CB, pH 7.2	8	8	8	8
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	7.3	9	9	10.7
RGD Peptide (20 mmol/L SH groups)	-	2.5	-	2.5
GFOGER Peptide (20 mg/mL)	-	-	6	6
Cell suspension	20	20	20	20
PEG-Link or CD-Link (20 mmol/L SH groups)	11	11	11	11
Total	100	100	100	100

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 peptides

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.

Note: If greater concentrations of GFOGER-3 peptide are used, be aware that GFOGER-3 has crosslinking properties. It is recommended to test the gelation speed before starting with the experiment. If greater concentrations of GFOGER-3 are used, the crosslinking may interfere with cell spreading and migration because GFOGER-3 does not contain a MMP (matrix metalloprotease) cleavage site.

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 „Biological sample“ 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). Currently, we do not offer a scrambled version of GFOGER-3 Peptide.

Preparation of gels of higher 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 approximations of 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 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. Specifically, the addition of GFOGER-3 can increase gelation speed because GFOGER-3 has crosslinking properties.

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	
	w/o GFOGER-3	With GFOGER-3	w/o GFOGER-3	With GFOGER-3	w/o GFOGER-3	With GFOGER-3	w/o GFOGER-3	With GFOGER-3
2 mmol/L	7 min	4 min	25 min	25 min	70 min	45 min	30 min	30 min
3 mmol/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4 mmol/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

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	
	w/o GFOGER-3	With GFOGER-3	w/o GFOGER-3	With GFOGER-3	w/o GFOGER-3	With GFOGER-3	w/o GFOGER-3	With GFOGER-3
2 mmol/L	5 min	3 min	25 min	25 min	35 min	20 min	30 min	30 min
3 mmol/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4 mmol/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

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 prepared, 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).

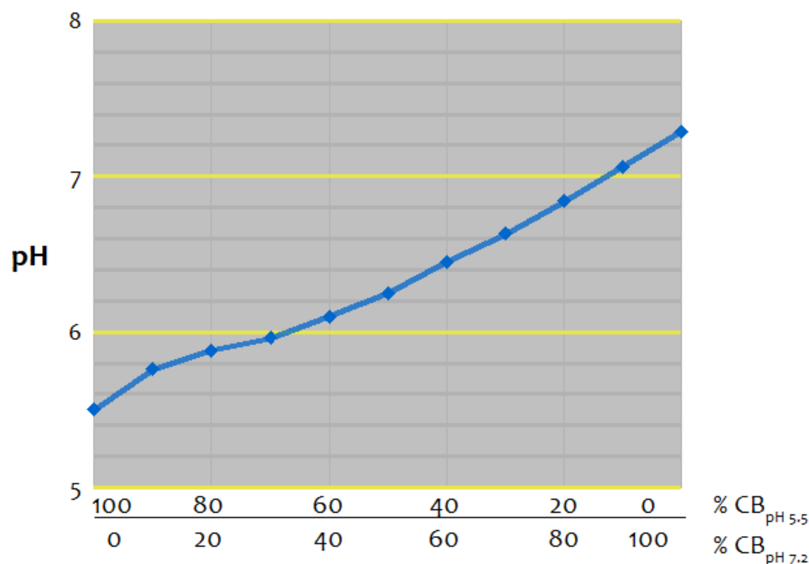

Fig. 1: pH resulting from different mixing ratios of 10x CB $_{pH5.5}$ and 10x CB $_{pH 7.2}$.

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 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.

For a detailed protocol see TP-1 on www.cellendes.com.