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 hydrogels.
- The polymers SG-Dextran and SG-PVA are used for the generation of hydrogels at a medium to slow gelation rate (SG). For gel solidification times see Tables 2 and 3.
- Compared to fast gelling hydrogels (compare General Protocol 1), slow gelling hydrogels allow for more time to handle and place the pregel solution in culture dishes or other containers (e.g. microchannels, syringes). Slow gelling hydrogels are also preferred over fast gelling hydrogels when hydrogels of a higher stiffness are needed.
- AgaFloat (Cat. No. A10-3) can optionally be added at 10% of the total gel volume to prevent cells from sedimentation during gelation. It is recommended to add AgaFloat to hydrogels at low crosslinking strengths (soft hydrogels) and long gelation times, specifically when crosslinking with PEG-Link. The gelation rates are not affected by AgaFloat nor does the addition of AgaFloat change the hydrogel's stiffness significantly.

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 full 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
Accessory	
3-D Life AgaFloat	A10-3
Related Products	
3-D Life Dextranase	D10-1
3-D Life 10x CB pH 5.5	B10-3
3-D Life 10x CB pH 7.2 Phenol Red-free	B21-3

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.
- If AgaFloat is used:
  - Prepare AgaFloat according to the instructions in the accompanying Product Data Sheet. Keep AgaFloat at 37°C until use. Do not keep the molten AgaFloat at room temperature because it will solidify within 30 minutes and will not be pipettable anymore.
  - Prepare a bucket of ice or similar cooling device.

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 20% of the final gel volume. Accordingly, if you prepare a cell suspension, the cell density in the gel will be only 20% of the stock cell suspension. If you chose to use different volumes of biological samples, the online calculation tool (www.cellendes.com) will assist 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 hydrogel 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 for 20 min to allow the peptides to attach to the SG-Polymer.
3. If AgaFloat is used (**otherwise continue with step 4**):

- Add the molten AgaFloat and immediately mix the solution.
- Add the cell suspension and mix gently.
- Incubate the pregel solution for at least 5 min on ice.
- **Continue with step 5** at room temperature.

4. Add the cell suspension.
5. Add the crosslinker (CD-Link or PEG-Link). Immediately mix by pipetting up and down a few times.

   a) 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.

   If cells sediment before the gel has solidified, repeat the hydrogel preparation and perform step 4 to keep cells floating by the addition of **AgaFloat**. This is recommended for a crosslinking strength of up to 3 mmol/L.

   b) When crosslinking with PEG-Link: Crosslinking with PEG-Link takes longer than with CD-Link. After addition of the crosslinker incubate the pregel 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 pregel solution in a culture dish resuspend cells to ensure that cells will be uniformly distributed later in the gel. Transfer the pregel 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.

   If **AgaFloat** has been added to the pregel solution, there is no waiting time and resuspension of cells necessary to keep cells floating. The pregel solution can be placed immediately in the culture dish. The pregel solution will still be pipettable for 20 to 70 minutes after the addition of crosslinker (compare Table 2 and 3). Allow for enough time after addition of crosslinker before you add cell culture medium (compare Table 2 and 3).
6. Make sure that the gel has completely formed. 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.
7. Once the gel has solidified, carefully add cell culture medium until the gel is covered.
8. Place the culture dish in the incubator for cultivation of cells.
9. Renew medium after 1 hour to equilibrate the gel with culture medium.
10. Change the medium as needed during cultivation of cells.

Table 1a: Reagent volumes for 100 µl of hydrogel 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. Compositions are given with or without the use of AgaFloat.

Reagents stock solutions	Volumes for 100 µl gel (µl)							
	w/o peptide		with RGD Peptide		with GFOGER-3 Peptide		with RGD and GFOGER-3 Peptide	
	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float
Water	55.3	45.3	51.2	41.2	47.7	37.7	43.5	33.5
10x CB, pH 7.2	8	8	8	8	8	8	8	8
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	6.7	6.7	8.3	8.3	8.3	8.3	10	10
RGD Peptide (20 mmol/L SH groups)	-	-	2.5	2.5	-	-	2.5	2.5
GFOGER Peptide (20 mg/mL)	-	-	-	-	6	6	6	6
Cell suspension	20	20	20	20	20	20	20	20
AgaFloat	-	10	-	10	-	10	-	10
PEG-Link or CD-Link (20 mmol/L SH groups)	10	10	10	10	10	10	10	10
Total	100	100	100	100	100	100	100	100

Table 1b: Reagent volumes for 100 µl of hydrogel 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. Compositions are given with or without the use of AgaFloat.

Reagents stock solutions	Volumes for 100 µl gel (µl)							
	w/o peptide		with RGD Peptide		with GFOGER-3 Peptide		with RGD and GFOGER-3 Peptide	
	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float
Water	53.7	43.7	49.5	39.5	46	36	41.8	31.8
10x CB, pH 7.2	8	8	8	8	8	8	8	8
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	7.3	7.3	9	9	9	9	10.7	10.7
RGD Peptide (20 mmol/L SH groups)	-	-	2.5	2.5	-	-	2.5	2.5
GFOGER Peptide (20 mg/mL)	-	-	-	-	6	6	6	6
Cell suspension	20	20	20	20	20	20	20	20
AgaFloat	-	10	-	10	-	10	-	10
PEG-Link or CD-Link (20 mmol/L SH groups)	11	11	11	11	11	11	11	11
Total	100	100	100	100	100	100	100	100

3. Variations of Gel Preparations

Online calculation tool
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 and thus improves pipetting accuracy. To obtain 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 pregel solution are placed in the culture dishes. It is recommended to resuspend cells in the pregel 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 higher 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 higher 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 on www.cellendes.com 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 peptide-modified gels. Cellendes also offers a scrambled version of RGD Peptide (Cat. No. 09-P-003) for control gels to be compared with gels containing RGD Peptide.

Preparation of gels of higher stiffness
Gels of higher stiffness than the gels described in Table 1a and 1b 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 solidification of the gel solution is considerably shorter when compared to the indicated time 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 - 2.2 mmol/L. Gels of up to 9 mmol/L crosslinking strength can be generated with increasingly shorter gelation times.

> **IMPORTANT:** The lengths of time 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 the indicated crosslinking strength at room temperature.

Crosslinking strength	Time after mixing polymer and crosslinker							
	SG-Dextran + CD-Link				SG-Dextran + PEG-Link			
	Pipettable up to		Addition of medium after		Pipettable 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 - 2.2 mmol/L	7 min	4 min	32 min	29 min	70 min	45 min	100 min	75 min

Table 3: Gelation times of SG-PVA hydrogels at the indicated crosslinking strength at room temperature.

Crosslinking strength	Time after mixing polymer and crosslinker							
	SG-PVA + CD-Link				SG-PVA + PEG-Link			
	Pipettable up to		Addition of medium after		Pipettable 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 - 2.2 mmol/L	5 min	3 min	30 min	28 min	35 min	20 min	65 min	50 min

Slowing down gelation by pH reduction

With increasing crosslinking strength gelation of hydrogels starts earlier and there may be not enough time to place the gel. The time to keep the pregel solution fluid and pipettable can be extended by reducing the pH. Usually, the preparation of slow-gelling hydrogels is performed at pH 7.2. Fig. 1 shows how 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). Such a mixture of 10x CB can be used instead of 10xCB (pH 7.2) to slow down gel formation.

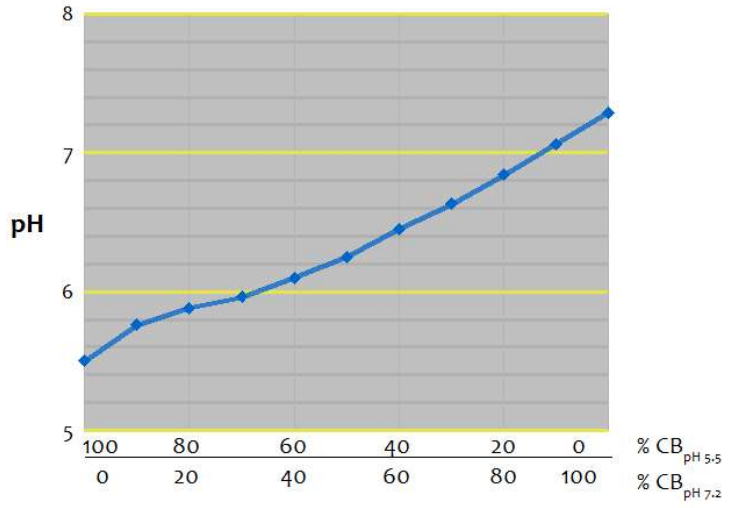

Fig. 1: pH resulting from different mixing ratios of 10x CB $_{pH5.5}$ and 10x CB $_{pH 7.2}$.

Addition of AgaFloat

AgaFloat keeps cells suspended in the pregel solution of gels with a slow gelation rate (soft stiffness). For hydrogels crosslinked with CD-Link at a crosslinking strength of up to 3 mmol/L the addition of AgaFloat is recommended to prevent the sedimentation of cells before the gel is formed. At Higher

crosslinking strengths the gelation time is shorter and, therefore, AgaFloat is not necessary to keep cells floating. Hydrogels crosslinked with PEG-Link require AgaFloat at all crosslinking strengths due to a much slower gelation time.

If bigger biological specimens than single cells are used, the AgaFloat concentration can be increased up to 20%. AgaFloat concentrations above 20% are not recommended as the pregel solution will not be pipettable anymore.

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, resuspend cells, 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.

The addition of AgaFloat (10% of total gel volume) to hydrogels does not interfere with hydrogel degradation by dextranase. If a higher percentage of AgaFloat is added to the hydrogel (>15%), the digestion of hydrogel by Dextranase will be impaired.

For a detailed protocol see Technical Protocol 1 (TP-1) on www.cellendes.com.