Preparation of *3-D Life* Hyaluronic Acid Hydrogels

1. Introductory Notes

- *3-D Life* Hyaluronic Acid (HA) Hydrogels are biochemically defined hydrogels that can be applied for three-dimensional cell cultivation, cultivation of cells on top of gels or co-cultivation of many cell types.
- The hydrogel is formed by the crosslinking of thiol-reactive dextran or polyvinyl alcohol (PVA) with thiol-functionalized hyaluronic acid. The presence of hyaluronic acid allows the spreading and migration of cells, if cell adhesion molecules (for example RGD Peptide, Cat. No. 09-P-001 or GFOGER-3 Peptide, Cat. No. P12-1) are present in the gel and cells display the appropriate receptors.
- 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). The gelation speed is 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 *3-D Life* HA Hydrogels for 3-D cell culture with and without modification of the gel matrix with the cell adhesion peptides RGD and/or GFOGER-3. Please carefully read the full protocol before you start preparing a gel.

Reagents and materials

Hydrogel Kits	Catalog Number
3-D Life Dextran-HA Hydrogel SG	G95-1
3-D Life PVA-HA Hydrogel SG	G85-1
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 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, ice.

Preparations

Hydrogel reagents:

- Dissolve HyLink lyophilisates according to the instructions of the Product Data Sheet.
- If hydrogel reagents are frozen, thaw all reagents at room temperature. Make sure that salts in the 10x CB (pH 7.2) buffer are completely dissolved. If necessary, place the buffer vial in a 37°C water bath until all salts are dissolved. Do not place 10x CB (pH 7.2) on ice, this may cause the salts to crystallize.
- If lyophilized peptides are used, reconstitute the peptides according to the instructions of the Product Data Sheets.
- If AgaFloat is used:
  - Prepare AgaFloat according to the instructions of the 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, HyLink) 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 in this protocol the volume of this sample will be 20% of the final gel volume. Accordingly, 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) assists you to find the right volumes of all reagents applied.

Experimental procedure

The following protocol describes the preparation of a soft hydrogel (crosslinking strength of 1.2 mmol/L) with the option of modification with 0.5 mmol/L RGD and/or 1.2 mg/ml GFOGER-3 Peptide. The volumes of hydrogel reagents required for 100 µl of hydrogel are listed in Table 1.

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. Addition of peptides (if no peptides are used continue with step 3):

   Add the peptide(s) and mix immediately to ensure homogeneous modification of the SG-Polymer with the peptide(s). Incubate for 20 min to allow the peptide(s) to attach to the SG-Polymer.
3. Addition of AgaFloat (if AgaFloat is not used 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 or any other biological sample.
5. Add the crosslinker HyLink. Immediately mix by pipetting up and down a few times.

6. a) Without the addition of AgaFloat:

   After addition of the crosslinker incubate the pre-gel solution for up to 11 minutes (SG-Dextran) or 10 minutes (SG-PVA) at room temperature. Do not incubate longer because the solution will begin to solidify and cannot be pipetted anymore. Resuspend cells to ensure that cells will be uniformly distributed later in the gel and transfer the pre-gel solution in a culture dish. Incubate for at least 25 minutes at room temperature or at 37°C in the incubator to allow the gel to solidify (see Table 2).

   b) With the addition of AgaFloat:

   If AgaFloat has been added to the pre-gel 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 11 minutes (SG-Dextran) or 10 minutes (SG-PVA) after the addition of HyLink. Wait for at least 25 minutes after addition of HyLink before you add cell culture medium (see Table 2).

7. Make sure that the gel has solidified and carefully add cell culture medium until the gel is covered.
8. Place the culture dish in the incubator for cultivation of cells.
9. Optional: Renew medium after 1 hour for equilibration of the culture with medium.
10. Change the medium as needed during cultivation of cells.

Table 1: Reagent volumes for 100 µl of hydrogel using SG-Dextran or SG-PVA polymer to be crosslinked with 1.2 mmol/L SH groups of the crosslinker HyLink (1.2 mmol/L crosslinking strength) with the option of modification with 0.5 mmol/L RGD Peptide and/or 1.2 mg/ml GFOGER Peptide. Compositions are given with or without the addition of AgaFloat.

Reagents	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	56	46	51.8	41.8	48.3	38.3	44.2	34.2
10x CB, pH 7.2	8	8	8	8	8	8	8	8
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	4	4	5.7	5.7	5.7	5.7	7.3	7.3
RGD Peptide (20 mmol/L SH groups)	-	-	2.5	2.5	-	-	2.5	2.5
GFOGER-3 Peptide (20 mg/mL)	-	-	-	-	6	6	6	6
Cell suspension	20	20	20	20	20	20	20	20
AgaFloat	-	10	-	10	-	10	-	10
HyLink (10 mmol/L SH groups)	12	12	12	12	12	12	12	12
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 correspondingly.

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 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 peptides are to be prepared, please consult the User Guide or the online Calculation Tool 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 higher concentrations of GFOGER-3 are used, the crosslinking may interfere with cell spreading and migration because GFOGER-3 does not contain a matrix metalloproteinase (MMP) 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 Calculation Tool 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 for 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 1 can be made by increasing the concentrations of the SG-Polymer (SG-Dextran or SG-PVA) and the crosslinker HyLink. 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 solidification of the gel solution is considerably shorter when compared to the indicated time in the protocol above. In Table 2 approximate time periods of the fluid state as well as the time after which gels are solid enough for the addition of medium are indicated for gels with crosslinking strengths of 1.2 mmol/L.

> **IMPORTANT:** The times given in Table 2 provide a rough guideline only. It is recommended to perform a test run of gel preparations 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 hydrogels crosslinked with HyLink at 1.2 mmol/L crosslinking strength at room temperature. GFOGER-3 Peptide concentration: 1.2 mg/ml.

Crosslinking strength	Time after mixing polymer and crosslinker							
	SG-Dextran + HyLink				SG-PVA + HyLink			
	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
1.2 mmol/L	15 min	11 min	40 min	36 min	11 min	10 min	36 min	35 min

Slowing down gelation by pH reduction for highly crosslinked hydrogels

If hydrogels are prepared that have a higher stiffness than indicated in Table 1, gelation speed is higher and the pregel solution may solidify too fast to be placed. The time to keep the pregel solution fluid and pipettable can be prolonged by reducing the pH. Usually, the preparation of *3-D Life* HA 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 10x CB (pH 7.2) to slow down gel formation.

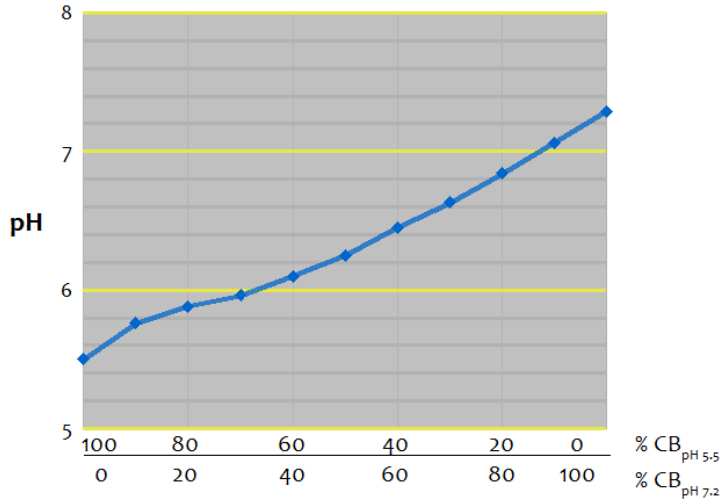

Figure 1: pH resulting from different mixing ratios of 10x CB pH 5.5 and 10x CB pH 7.2.

Addition of AgaFloat

AgaFloat keeps cells suspended in the pregel solution of gels of low crosslinking strength (soft stiffness). For hydrogels crosslinked with HyLink at a crosslinking strength of up to 2 mmol/L the addition of AgaFloat is recommended to prevent the sedimentation of cells before the gel is formed. Higher crosslinking rates decrease the gelation time and, therefore, AgaFloat is not necessary to keep cells floating.

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 Dextran HA Hydrogels with Dextranase

Dextran hydrogels crosslinked with HyLink cannot be dissolved by dextranase.